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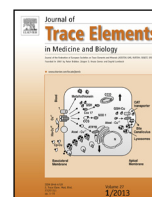

Review about the manganese speciation project related to neurodegeneration: An analytical chemistry approach to increase the knowledge about manganese related parkinsonian symptoms

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ABSTRACT

Neurodegenerative diseases get a growing relevance for societies. But yet the complex multi-factorial mechanisms of these diseases are not fully understood, although it is well accepted that metal ions may play a crucial role. Manganese (Mn) is a transition metal which has essential biochemical functions but from occupational exposure scenarios it appeared that Mn can cause severe neurological damage. This "two-faces"-nature of manganese initiated us to start a project on Mn-speciation, since different element species are known to exhibit different impacts on health. A summary about the step-wise developments and findings from our working group was presented during the annual conference of the German trace element society in 2015.

This paper summarizes now the contribution to this conference. It is intended to provide a complete picture of the so far evolved puzzle from our studies regarding manganese, manganese speciation and metabolomics as well as Mn-related mechanisms of neural damage. Doing so, the results of the single studies are now summarized in a connected way and thus their interrelationships are demonstrated. In short terms, we found that Mn-exposure leads to an increase of low molecular weight Mn compounds, above all Mn-citrate complex, which gets even enriched across neural barriers (NB). At a Mn serum concentration between 1.5 and 1.9 μg/L a carrier switch from Mn-transferrin to Mn-citrate was observed. We concluded that the Mn-citrate complex is *that* important Mn-carrier to NB which can be found also beyond NB in human cerebrospinal fluid (CSF) or brain of exposed rats. In brain of Mn-exposed rats manganese leads to a decreased iron (Fe) concentration, to a shift from Fe(III) to Fe(II) after long term exposure and thus to a shift toward oxidative stress. This was additionally supported by an increase of markers for oxidative stress, inflammation or lipid peroxidation at increased Mn concentration in brain extracts. Furthermore, glutamate and acetylcholineesterase were elevated and many metabolite concentrations were significantly changed.

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Abbreviations: AD, Alzheimer disease; CSF, cerebrospinal fluid; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; DMT-1, divalent metal transporter 1; DRC, dynamic reaction cell; ESI-FT-ICR-MS, electrospray ionization—Fourier transform ion cyclotron resonance—mass spectrometry; Fe(S), total Fe in serum; Fe-Tf(S), Fe-transferrin in serum; Fe-Cit(S), Fe-citrate in serum; GPx, glutathione peroxidase; IC, ion chromatography; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectrometry; IEC, ion exchange chromatography; MMT, methylcyclopentadienyl-manganese-tricarbonyle; HMM, high molecular mass; LMM, low molecular mass; MCT, monocarboxylate transporter; Mn(C), total Mn concentration in cerebrospinal fluid; Mn-Cit(S), Mn-citrate in serum; MRI, magnetic resonance imaging; Mn(S), total Mn concentration in serum; Mn-Tf(S), Mn-transferrin in serum; NB, neural barriers; PD, Parkinson disease; ROS, reactive oxygen species; SEC, size exclusion chromatography; SOD, superoxide dismutase; Tf, transferrin; Tf-R, transferrin-receptor.

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1. Introduction—background of the Mn speciation project

Today, neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) are gaining increasing relevance in our aging society. However, the complex multi-factorial mechanisms of these diseases are yet not sufficiently understood [1–3]. Metal ions, specifically chronic or excessive Manganese (Mn) exposure is a cause of severe neurological dysfunction although Mn is an element of janiform nature: It is an essential micronutrient required for normal physiological processes in mammals. Particularly, it plays important roles as a cofactor for a series of enzymes including Mn-dependent superoxide dismutase [2], which are crucial for antioxidant defence, energy metabolism, immune function, reproduction, brain function, or DNA repair [4–6]. However, already since 1837 toxic neurological health effects of Mn are known. The most severe one is called manganism and was first described by James Couper in 1837. He observed paraplegia in the lower extremities of Scottish MnO_2-ore grinding workers [7]. Since then it has been explored that elevated levels of Mn may cause damage of the central nervous system with symptoms such as adynamia/fatigability, sialorrhea, cephalalgia, sleep disturbances, muscular pain and hypertonia, masklike face, gait changes, reduced coordination, hallucinations, and mental irritability [8]. These detrimental health effects are presumably induced by oxidative stress, with inhalation being the primary route of concern for occupational health effects. Several of the above mentioned health effects are similar to symptoms of Parkinsonism (PD).

Exposure scenarios from occupational health are known for miners, industrial steel workers or welders. Symptoms of manganism were found for *some* of the exposed individuals, but even more alarming, for the *whole group* an increased prevalence for PD was reported. First concern about additional manifestation of Mn neurotoxicity other than manganism was raised by a study from Racette et al. [9], reporting that in 953 cases the age at PD diagnosis was 17 years earlier in career welders than in non-welders. Nowadays, Mn neurotoxicity is becoming of great public health concern due to diverse factors affecting even a broader range of population [10].

For example it is reported that population living in the surroundings of big industrial vicinities or living close to highly-frequented traffic routes with Mn containing car exhaust from methylcyclopentadienyl-manganese-tricarbonyle (MMT) charged fuel probably is at risk [11,12]. The group around Lucchini had extensively studied the population and PD prevalence around the big ferroalloy plant in the province of Brescia, Italy, which were operating until 2001 [13]. Their results showed that an environmental exposure to Mn was associated with an increased prevalence of PD disturbances. Such chronic exposures may progressively extend the site of Mn deposition and toxicity from the globus pallidus to the entire area of the basal ganglia, including the substantia nigra pars compacta, involved in PD [14].

Based on these recent epidemiologic studies, Lucchini et al. developed the concept of lifetime Mn exposure with the hypothesis of an increased risk of Parkinsonian disturbances, where lifetime exposure to low Mn levels, starting from prenatal to older age, may be a risk factor for Parkinsonism.

Another epidemiological study investigated associations between PD and exposure to industrial emissions of Mn as well as vehicle exhaust due to the use of MMT added to gasoline since 1976 [11]. From these findings Finkelstein et al. [11] concluded that ambient Mn exposure lowers the age of PD diagnosis, suggesting that Mn exposure amplifies the natural loss of neurons caused by aging process. Overall, Finkelstein et al. [11] conclusions were in line with Lucchiniís hypothesis [14] of an increased risk of Parkinsonian disturbances after lifetime Mn exposure.

The janiform nature of manganese—its' essential or potentially detrimental role—prompted us to investigate Mn speciation in human serum, CSF and rat brain extracts after exposure, since different Mn compounds might be metabolized or transported by different pathways, and by that way causing opposing health effects.

The absorption of inhaled Mn is high and it is well accepted that Mn is transported to the liver, where it is metabolized and the newly formed Mn species get transported with blood to brain. In literature transferrin (Tf) was supposed to be a plasma Mn-carrier for physiological condition, and a well-controlled transferrin-receptor (Tf-R) mediated Mn transport across the NB is known. However, under Mn exposure, different Mn species seem to cross the NB independently from Tf-R. These different Mn compounds appear in CSF. The first question in our project therefore related to Mn-speciation at NB, because these Mn-compounds are key-metabolites which had to be addressed according to differences in transport of Mn to the brain under varying conditions [15].

The second question relates to the neurotoxic mechanism of Mn on the cellular level, which is still unclear. Common explanations comprise reactive oxygen species (ROS) generation: Reasons for ROS production were discussed regarding the various oxidation states of Mn, or its affinity to mitochondria connected to energy supply depletion in neurons, or by disturbance of cellular iron and calcium homeostasis [16]. But also Mn-related DNA damage in neurons may cause neurodegenerative effects, since Mn(II) has been reported to substantially decrease the fidelity of DNA replication and it has been shown that in intact human cells Mn(II) reduces the extent of poly(ADP-ribosyl) ation stimulated by H_2O_2 at low, non-cytotoxic concentrations. It is important to know that poly(ADP-ribosyl) ation is considered as a DNA-repair system which occurs as one of the first nuclear events shortly after

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DNA strand break induction and is mediated by members of the "Poly(ADP-ribose) polymerase (PARP)-family" [17].

2. The Mn-speciation project

2.1. The concept

According to the above mentioned questions our scientific aims were 1.) to identify the mechanisms of less controlled and increased Mn-transport into brain (Mn-carrier species) and 2.) to clarify the mechanism of neuronal damage by Mn.

The concept was realized by employing element speciation for identification of the changed Mn-species in relevant human body fluids before (serum) and behind (cerebrospinal fluid) NB.

Consequently the strategy for our studies was focused on

2.1.1. Mn transport into brain by

a) Mn-speciation in serum (before NB) and in CSF (behind NB),

b) investigating the permeability of the NB for e.g., Mn applying element speciation in paired serum and CSF samples to reveal disturbed metal species homeostasis,

c) evaluating the results from human samples by comparing to speciation results of animal experiments after well-defined Mn-exposure.

2.1.2. Clarification of the mechanism of neuronal damage by **Mn** and cellular defence by investigating the molecular mechanism behind Mn-induced neurotoxicity on the cellular level,

a) after low-dose singe i.v. injection, or

b) after low-dose 7 weeks feeding.

2.2. The methodical approach

Classical speciation methods were used, comprising of hyphenations of chromatography with element selective detectors, mainly inductively coupled plasma mass spectrometry (ICP-MS) for Mn and Fe speciation, but also inductively coupled plasma optical emission spectrometry (ICP-OES) for Fe(II)/(III) speciation. For differentiation of Fe(II) from Fe (III) a hyphenation of IC with ICP-OES was employed. This combination of methods was useful with regard to non-interfered element lines, short analysis time (~5 min), applicability in biological samples, low sample amount (~25 μl) as well as good LoDís (Fe(II): 9.1 μg/l, Fe(II): 6.3 μg/l) and column recoveries (Fe(II): 83–105%, Fe(III): 43–66%).

Element determinations were checked by analisis of reference materials and participation in GEQUAS quality control system. Serial and day-to-day precision were 2.5% or 2.8%, accuracy was determined at 100.7%. Mn-speciation results comprised of samples which had been analyzed twice, with storage between first and second measurement. Uncertainties of Mn-speciation measurement thus referred to storage stability and measurement variance of the hyphenated system. For Mn-species concentrations below 300 ng/L an average RSD $18 \pm 4\%$ was found, whereas for Mn-species higher than 300 ng/L an average RSD $7 \pm 3\%$ was seen.

Size exclusion chromatography (SEC) coupled to ICP-mass spectrometry with dynamic reaction technology (ICP-DRC-MS) and—for clear identification—capillary zone electrophoresis (CZE)-ICP-DRC-MS were used in a 2D approach for speciation of Mn. SEC offers separation of even labile metal species together with size characterizations of compounds, however, it suffers from sufficient resolution and baseline separation. As a consequence doubtless peak identification is impossible. Therefore, ion exchange (IEC) and reversed phase chromatography (RPC) were tested. However, these methods hampered Mn-species stability. When using RPC, standards like Mn-citrate showed only one peak co-eluting with the inorganic Mn standard, indicating a separation of Mn from the citrate-standard complex. Other standards appeared as multiple peaks for only one compound and additionally sample sticking was observed. IEC resulted in irreproducible Mn-chromatograms. These limitations redirecting the experiments back to SEC combined with CZE for a 2D identification approach. Capillary electrophoresis (CE) is one of the most powerful separation techniques. It provides very efficient species separation, typically without altering species stability. This is because no stationary phase with active sites and high surface is involved in separation. Furthermore, CE offers different modes of separation, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), isoelectric focusing (IEF), isotachophoresis (ITP), and capillary electrochromatography (CEC). This is important for multidimensional strategies. For additional species verification SEC fractions from serum and CSF were subject also to electrospray ionization—Fourier transform ion cyclotron resonance—mass spectrometry (ESI-FT-ICR-MS) [18], a molecular mass spectrometric technique with ultra-high resolution.

Fig. 1 summarizes the applied techniques for metal speciation and non-targeted metabolomics investigations.

3. Manganese-speciation studies

3.1. Manganese-speciation in human samples

Investigations started with species characterization in human serum and CSF. Pooled serum samples from the Munich region showed mean Mn concentration of $1.7 \pm 0.8\,\mu g\,L^{-1}$. Mn was predominantly associated with the transferrin/albumin fraction and to lesser amounts to the α-2-macroglobulin fraction, each of these carriers known as physiological ligands. However, an additionally Mn peak was monitored at Mn-citrate standard retention time which was higher in those sera having slightly elevated total Mn concentration up to 2.3 μg/L [19]. This finding was discussed as a first indication that excess Mn might be transported in blood by a low molecular mass (LMM) carrier like Mn-citrate.

Fifteen pooled CSF samples showed Mn concentrations in the range 1.8–6.7 $\mu g\,L^{-1}$ (mean 2.66 $\mu g\,L^{-1}$, ≙ mean 48 nM). Other than in serum, 90% of Mn in CSF was bound to LMM Mn carriers. The major fraction co-eluted with the Mn-citrate standard. We confirmed this also by standard addition of Mn-citrate standard to CSF (refer to Fig. 1 in Ref. [20]). Therefore, citrate was additionally determined in CSF using enzymatic reaction with UV spectrophotometric detection [19]. The mean concentration was 573 μM (range 420–713 μM), proving citrate in huge excess compared to Mn (ratio $Mn_{\,(meanvalue)}$: $citrate_{\,(meanvalue)}$ = 1:12000). This surplus of citrate made a Mn-citrate complex (aside from other metal citrate complexes) in CSF likely. Fig. 2 shows a typical chromatogram of CSF (Mn and UV trace) and Mn-HSA or citrate standards for comparison.

For clear identification of Mn-species capillary electrophoresis (CE) was used to analyze CSF samples and SEC fractions from serum and CSF, here as a second device for a 2D approach. Compounds being supposed in literature to act as potential Mn carriers were analyzed. The respective standard mixture comprised of albumin, histidine, tyrosine, cystine, fumarate, malate, inorganic Mn, oxal-acetate, a-keto-glutarate, nicotinamide-dinucleotide (NAD), citrate, adenosin, glutathione, and glutamine. At the end, 13 Mn-species were monitored in pooled CSF samples, most of them being identified: Mn-histidine, Mn-fumarate, Mn-malate, inorganic Mn, Mn-oxal-acetate, Mn-α-keto-glutarate, Mn carrying NAD, Mn-citrate and Mn-adenosin. By far the most abundant Mn-species was Mn-citrate showing a concentration of $0.7 \pm 0.13\,\mu g$ Mn L^{-1}. Interestingly, most other Mn-species can be related to the citric acid cycle, too.

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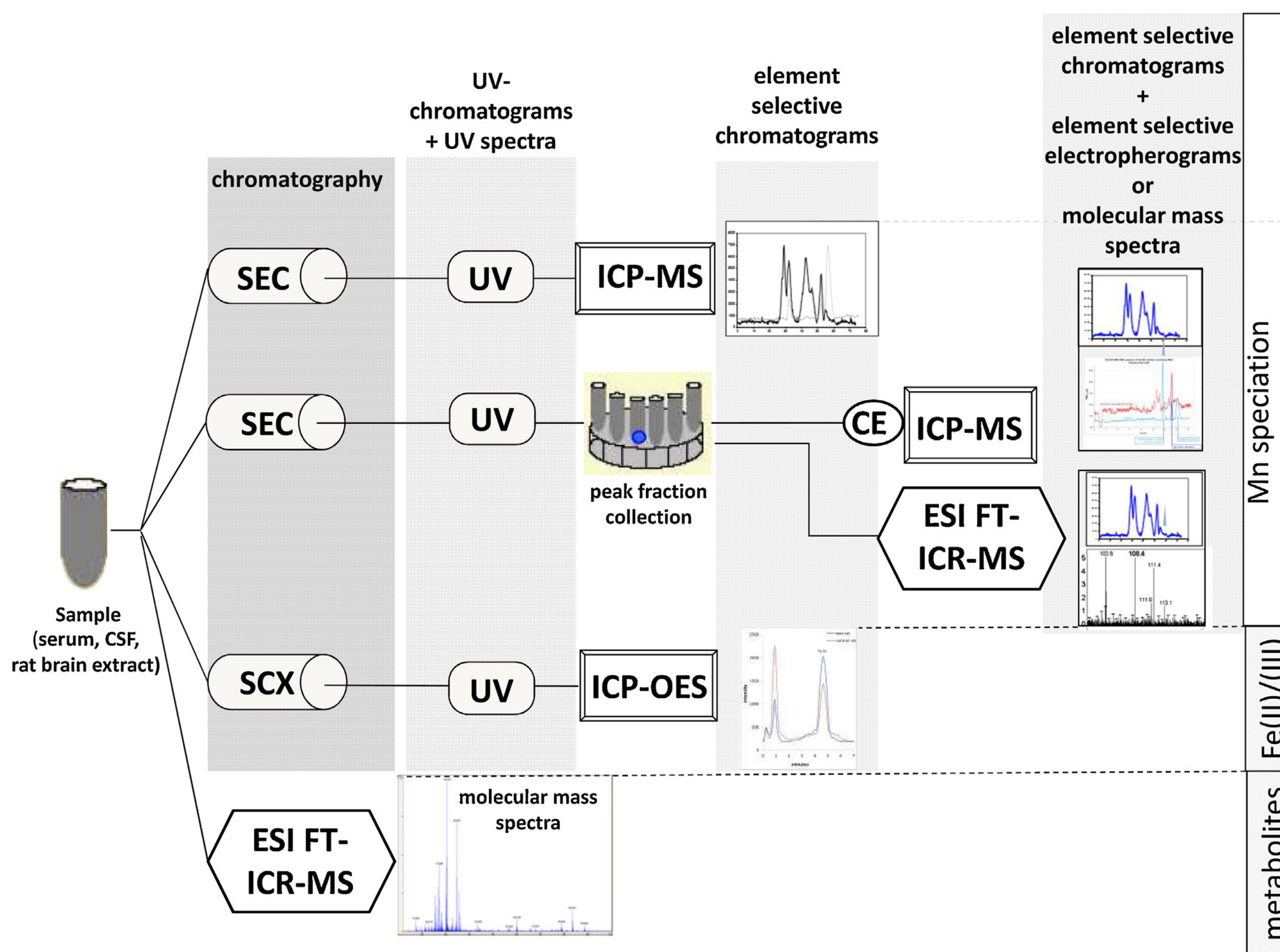

Fig. 1. Schematic overview about the used methods in the Mn-speciation project. Mn-speciation was performed by SEC-ICP-DRC-MS and for clear species identification by two 2D approaches SEC/CE-ICP-DRC-MS or SEC/ESI-FT-ICR-MS. Fe(II and Fe(III) were analyzed by IC-ICP-OES whereas metabolomics studies used methanolic extracts and ESI-FT-ICR-MS measurements.

The above findings motivated our group to investigate Mn-species on an individual basis, with serum and CSF samples corresponding to the same individual and time of sampling. Such samples are considered as "paired samples" and allow Mn speciation before (serum/plasma) and behind (CSF) the NB: Consequently, Nischwitz et al. [21] investigated the permeability of the human blood-CSF-barrier for selected metals including Mn. Manganese peak fractions in serum and CSF were quantified and ratios of CSF vs. serum were calculated. The main results of this study were significant differences in the molecular size distribution of the species of investigated metals for CSF and serum samples. Supposedly these differences were due to selective permeation of metal species from serum across the NB. With respect to Mn nearly all species were lower concentrated in CSF than in serum except the Mn-citrate fraction, which was significantly enriched by a factor of 5. This was consistent with other findings from serum and cerebrospinal fluid. The summarized key-messages of the above studies were the low amounts of LMM Mn-species and the higher amounts of high molecular mass (HMM) Mn-compounds in human serum, the latter being assigned to α-2-macroglobulin and transferrin/albumin [21–23]. Contrary, in CSF only little amounts of Mn-HMM-compounds were detected but predominantly LMM Mn-species, with Mn-citrate above others being even five-fold enriched, were identified [21].

The importance of Mn-citrate is supported from others, too: Yokel [8] found Mn-citrate present in plasma and suggested it as a candidate for uptake into brain since the K_{in} for Mn-citrate exceeded the calculated K-value for simple diffusion in six brain regions, including the caudate [24]. The caudate, being part of the basal ganglia, is involved in manganism and a site of Mn accumulation after exposure. With such a high uptake rate, Mn-citrate brain influx compares to the Mn^{2+} ion influx.

In a follow-up study using paired samples, we investigated correlations between Mn-citrate or other Mn-species and total Mn concentration in paired samples of human serum and CSF. The Mn-species concentrations from the different sample types (serum vs. CSF) were interrelated and correlation coefficients were calculated. In a first pilot study from 2013 [18], a limited sample size from Munich was investigated (n = 24) employing two 2-D approaches based on SEC/CE-ICP-DRC-MS and SEC/ESI- FT ICR–MS technology.

In this pilot study the relevant $[Mn(C_6H_5O_7)_2]^{4-}$ complex was detected as the predominant Mn-citrate species existing around neutral pH in a native sample. The concentrations ranged from ca. 0.2–0.55 μg/L for the citrate complex, related to Mn, determined in CSF samples at total Mn concentrations of 0.21–1 μg/L. This was an improvement compared to our former report with a limit of detection for Mn-citrate of 250 μg Mn/L [25], which however was produced with an old ESI–MS/MS instrument.

The evaluation of speciation results revealed that Mn-species concentrations correlated differently to total Mn concentration dividing the samples into two groups:

Below serum Mn (total) concentration ≤1.55 μg/L the Mn-transferrin/albumin fraction was positively correlated with total Mn in serum and with total Mn in CSF, whilst above a Mn total

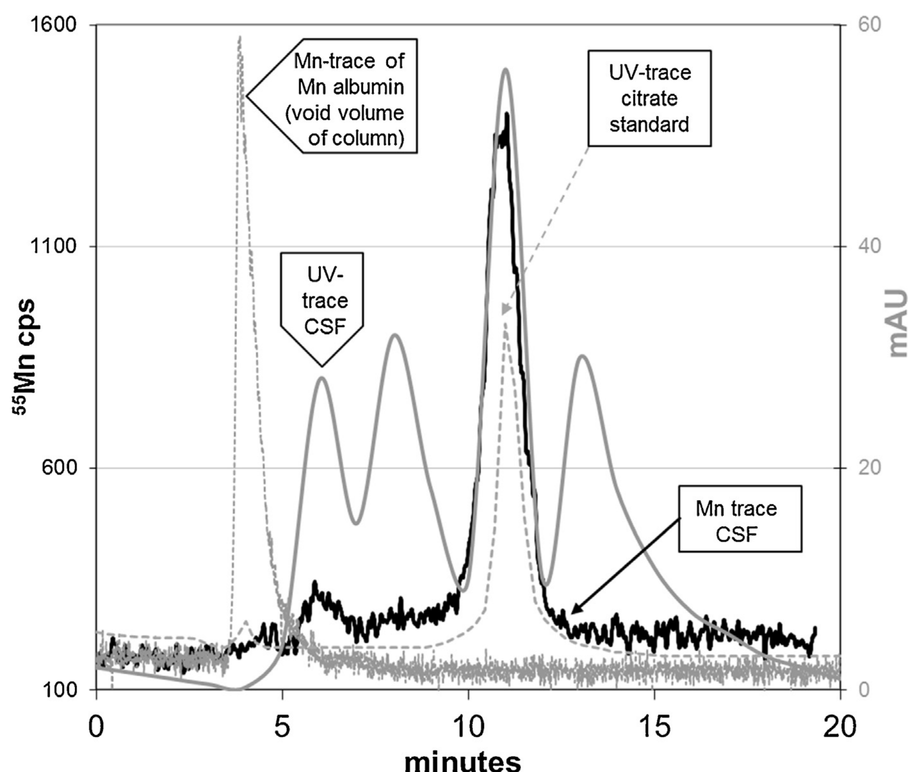

Fig. 2. Size screening of Mn species in a CSF sample using a mass calibrated TSK-40 SW SEC column (separation range <2000 Da). Most of Mn is co-eluting with a citrate standard. The Mn-albumin trace indicated the void volume where no Mn from CSF was eluting (modified from [19] with permission from Royal Society of Chemistry).

concentration of ca. 1.9 μg/L, the total Mn concentrations of serum and CSF were positively correlated with Mn-citrate in serum. For increasing the statistical power of these results a follow-up study with a sample size of 141 serum and 39 serum/CSF sample pairs (total 180 serum samples) were used and additionally regional differences were explored. The whole sample set in this second study consisted of three subsets from Munich, Emilia Romagna region in Italy and from Sweden.

Total manganese serum concentrations were similar for the Munich and Swedish samples but differed for the Italian subset. The majority of Swedish samples were in the range of 0.4–1.5 μg/L total Mn, the Munich samples ranged mainly from 0.5–1.9 μg/L, while Italian samples tended to be higher in the range of 0.9–ca. 4 μg/L. From all subsets some samples reached even higher values. These concentration ranges are in line with literature data. Total manganese serum concentrations are reported e.g., from USA on a daily, weekly and monthly basis to be stable between 0.84–0.98 μg/L [26]. Versiek et al. report a range from 0.4–1.05 μg/L for Belgium [27]. Heitland reports for whole blood a range of 4–18 μg/L with a mean at 9 μg/L [28]. Taking into account that serum contains 10% of blood Mn (e.g., Baker et al. [26]) the serum concentration in those samples would be again ca. 0.4–1.8 μg/L, with a mean at 0.9 μg/L. This is covering the majority of values reported for the samples in this paper. This shows that Mn is strictly regulated but long-term, nutritional, environmental or occupational exposure can results in rather stable, but somewhat elevated values compared to non-exposed persons.

However, independent on their origin for all samples it was shown that at low total Mn concentration in serum (Mn(S)) a change in major Mn carriers in serum took place from Mn-transferrin (Mn-Tf(S)) toward Mn-citrate (Mn-Cit(S)) with high statistical significance.

This carrier switch from Mn-Tf(S) to Mn-Cit(S) was observed between Mn(S) concentrations of 1.5 μg/L to ca. 1.7 μg/L. For the Munich sample donors both, serum and cerebrospinal fluid were available. For these samples additionally the concentration of Mn in cerebrospinal fluid (Mn(C)) positively correlated to MnCit(S) when Mn(S) concentration was above 1.7 μg/L. (Fig. 3).

It should be taken into mind that the findings regarding the switch of the Mn-carrier in serum samples were related to serum samples from different regions, i.e. from Munich ($n = 39$), the Emilia Romagna region in Italy ($n = 52$) and from Sweden ($n = 89$). This change in Mn-carrier was observed in each of these sample subsets but nevertheless regional differences were elucidated by application of a multiple regression model. With respect to the same level of total Mn(S), in average the level of Mn-Cit(S) was lower by −0.921 μg/L in Emilia-Romagna-Region compared to Munich, whereas in Sweden it was higher by 0.289 μg/L compared to Munich. These differences were partly significant: The variables "Emilia Romagna Region" and "Munich" were significant contributions to the multiple regression model ($p = 4.8 \times 10^{-120}$ and $p = 0.000649$, respectively), whereas the variable "Sweden" appeared to be not significant: $p < 0.145$). As no paired samples were available from Italy or Sweden, it was not possible to study the consequences of these regional/occupational differences on Mn(C). Switch points from Mn-Tf(S) to Mn-Cit(S) were calculated for each subset additionally and were found at Mn(S) concentrations of 1.31 μg/L (Swedish samples), 1.26 μg/L (Munich samples) and 2.14 μg/L (Emilia Romagna samples). The reason for the higher value of the switch point for the "Emilia Romagna" samples is still unclear [29].

Iron and manganese are known as competitors for binding sites, e.g., at transferrin or citrate [15,30]. Therefore, measurements of total Fe in serum (Fe(S)), Fe-transferrin in serum (Fe-Tf(S)) and Fe-citrate in serum (Fe-Cit(S)) were included in this study. These measurements showed that the Fe-species had the same mean values for serum samples, irrespective of the Mn serum concentrations. No correlation between Mn(S) and Fe(S) or Fe-species was

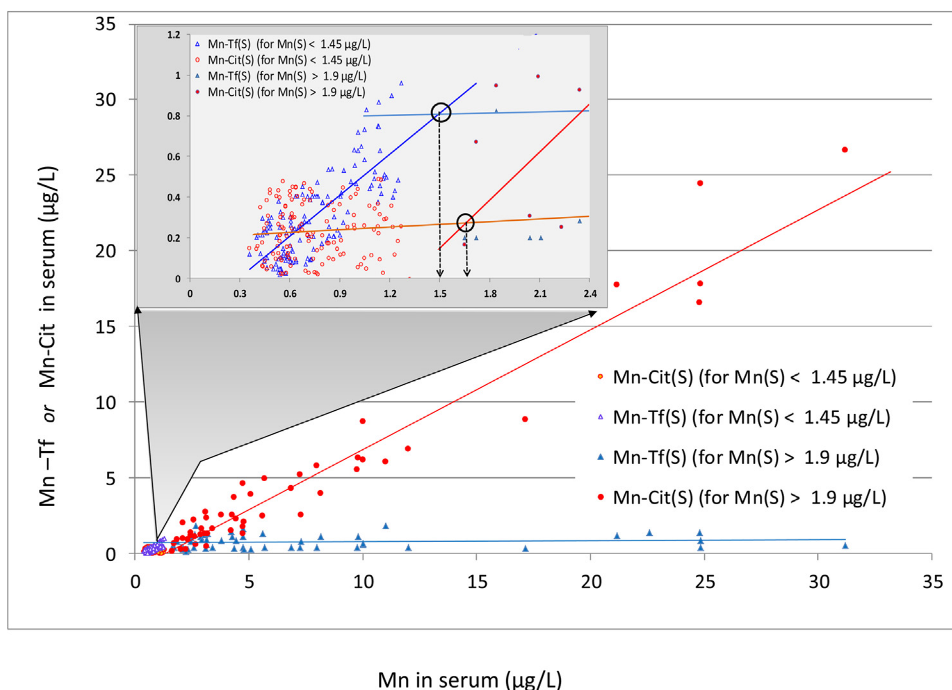

Fig. 3. Correlation of Mn-Tf(S) and Mn-Cit(S) versus Mn(S) are plotted. The zoomed window shows the Mn(S) concentration range below 2.4 µg/L where the Mn-carrier switch can be observed. The arrows mark the switch points at 1.51 µg/L, calculated from Mn-Tf(S) intercepts, or at 1.66 µg/L, calculated from Mn-Cit(S) intercepts. Modified from [29], with permission from Elsevier.

detected. The explanation was given by the thousand fold Fe concentration compared to Mn concentration and the Fe/transferrin saturation rate being only at 25–30% [31,32], which means that approximately 70% of the binding sites are still available for Mn binding. As an example, Aschner quantified the available binding sites for Mn as 50 µM, corresponding to 2750 µg Mn/L [15]. Thus, Mn displacement from transferrin to citrate by iron seems not likely and the independency of Fe from Mn concentration in our serum samples was in line with this unlikeliness.

So far the reason why Mn switches from Tf(S) to citrate(S) is not explained. May be it is related to the similar switch for iron from Fe-Tf(S) to Fe-Cit(S) [33]. This reference gives a comparative overview about the non-transferrin-bound iron. Nevertheless the authors state that the source of plasma non-transferrin-iron is not fully understood. It remains to be clarified whether it appears after cellular iron defluxion or whether it is following the equilibrium between transferrin-iron and the potential carriers (mainly citrate).

3.2. Mn-Transporters across NB discussed in literature

It should be underlined that the positive correlation between Mn-Cit(S) and Mn(C) does not justify yet the conclusion of a facilitated Mn transport through NB by means of Mn-citrate. Actually, the exact mechanism of Mn uptake across the NB into brain has not been investigated within the Mn-speciation project of our group. It is still under debate in literature and has to be revealed: An *in vitro* study in a porcine model compared the effect of $MnCl_2$ on blood-cerebrospinal fluid barrier (BCB) and blood-brain barrier (BBB) where Mn affected the BCB more than the BBB [34]. Bornhorst et al. [34] concluded that the passage across BCB into the brain is the preferred route for Mn over BBB. Whether this conclusion may be transferred also to mechanisms of *in vivo* Mn intake in humans presently remains to be shown.

In general it is accepted that Mn can cross the neural barriers (NB) by means of transporters and in different oxidation states, since the Mn absorption, transport, and tissue levels are stringently regulated [35]. Although Mn transporter systems across the NB have been vigorously investigated, a probative result is presently not available [35,36]. Referring to references [37,38] Aschner et al. [6] favour transferrin receptor (TfR) and divalent-metal transporter 1 (DMT-1) for Mn transporters across NB whereas Yokel [8] ranked the role of DMT-1 as questionable. He assessed the evidence to be stringently against a role of DMT-1 in Mn transport, but found no direct evidence for its role. Yokels appraisal is close to Refs. [3], [34] and [39] who accordingly report DMT1 to be not the major transporter for Mn uptake into the brain via the NB.

Apart from the controversially debated DMT-1, several more transporter systems were investigated and are summarized in [40].

In connection with our findings of a switch of Mn-carriers from transferrin to citrate at elevated Mn concentration the paper from Bowman is of interest: They implied a citrate transporter across NB [35] with a Mn-citrate tridentate complex (Mn-CTC) having a non-coordinated central carboxylate recognition moiety. They hypothesized, that the Mn-CTC might be a substrate for the organic anion transporter or a monocarboxylate transporter (MCT). Various cellular processes could regulate the activity of the above mentioned transporters in response to Mn deficiency or overload.

3.3. Mn-speciation in animal studies

Although, the debate about Mn transporters across NB is not finished yet, it is clear from various studies in humans including MRI and from animal studies that manganese enters the brain. There it is able to deploy its detrimental neurotoxic potential.

That fact necessitated the implementation of further animal studies for additional information about Mn-species formation within a complete biological system (=organism) and related metabolic changes in brain.

As summarized by Gwiazda et al. [41] previous animal studies may be considered as inappropriate with respect to chosen exposure concentrations, which considerably exceeded the doses where neurological symptoms appeared in occupationally exposed

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humans (10–15 mg/kg b.w., cumulative). Such studies were inapplicable for risk assessment of chronically, low-level Mn exposure for humans. Therefore, our experimental set-up was oriented on concentrations relevant for single-low-dose occupational exposure or for exposure by diffuse sources like environmental pollution (as suggested by Lucchini and Zimmermann [42]).

In a first trial Diederich et al. [43] exposed rats by a single i.v. Mn-injection (1 mg/kg b.w., Mn-species: inorganic $MnCl_2$) and monitored Mn-speciation in serum one hour after exposure (serum "1h") and after sacrifice of animals four days later (serum "4d") together with brain extracts (brain "4d"). This four-days period corresponded to Takeda et al. [44] who reported maximum Mn distribution from serum to brain after 4 days.

Serum "1h" showed a 100-fold total Mn concentration increase, the major Mn-species still being the injected inorganic Mn, while Mn-Tf exhibited ca. 33 fold and Mn-Cit ca. 4–5 fold increase, each compared to controls. In serum "4d" Mn species were nearly planished again with Mn-Tf and Mn-Cit both having less than 1.3 fold increase (vs. controls) [43]. This supported the fast turnover of Mn described in literature [3].

In brain "4d", however, Mn-quantitation (as μg Mn/g brain "4d") showed LMM-Mn species to be the absolute dominating Mn compounds with inorganic Mn to be the major Mn species, followed by a considerable increase of Mn—citrate. Interestingly, the level of Mn-Tf in brain "4d" (behind NB) was increased only 1.1 fold, remained nearly as low as in controls, although in serum "1h" (before NB) it had been significantly increased compared to controls. Summarizing Diederichís findings we concluded that i.v. injected inorganic Mn complexed quickly with physiological Mn-carriers like Mn-Tf in serum, but for distribution across NB into brain LMM-Mn-species were the more important carriers. These results confirmed our previous findings on Mn distribution and our conclusions from human serum and CSF samples. They were also in accordance with others who suggested a LMM Mn-carrier to brain, which was independent from transferrin and Tf-R [24,45–47].

The starting animal study conducted by Diederich et al. [43] did not include metabolomics aspects. For a complete simulation of single-low-dose occupational exposure including both, metabolomics investigation and Mn-speciation, we repeated this injection study and furthermore compared it with a simulation of long-term-low dose (environmental) exposure by application of a 53 days lasting sub-chronic, low-dose feeding (500 mg Mn/kg feed) of rats followed from Mn-speciation and metabolomics studies in brain [48,49].

Samples from both exposure scenarios were analyzed for total Mn in serum and brain, Mn species as well as Fe(II)/(III) in brain extracts. Besides, glutamate, acetylcholinesterase, oxidative stress- and inflammation markers were measured and non-targeted metabolomics was performed [48–50].

Total Mn was increased in serum "1h" from 7 ± 2 μg/L to 194 ± 49 μg/L ($p < 0.001$), however, four days later, in serum "4d" the concentration was re-balanced and practically the same as in controls (controls: 6.0 ± 1.5 μg/L, exposed: 6.7 ± 1.3 μg/L). This is according with serum from the feeding trial where the concentrations were about the same as in controls (controls: 4.5 ± 0.8 μg/L, exposed: 4.7 ± 0.7 μg/L) [48,51]. This unchanged Mn-concentration in serum after long-term-low-dose Mn-exposure was surprising when looking just on serum but might be explained by distribution to organs in parallel as monitored by the Mn increase e.g., in brain (see below: +19%; and supposedly increased in kidney or liver, as detected in another experiment by Diederich et al. [43]).

In Mn-exposed groups of both exposure conditions the concentrations of HMM compounds were lower and LMM Mn-species such as Mn-citrate or Mn-amino acids higher than in controls. Interestingly, this increase of LMM Mn-species was even strongest after the sub-chronic Mn feeding. In contrast, four days after Mn injection, these LMM fractions were only about 2-fold increased whereas one hour after injection Mn was still present predominantly as inorganic Mn. Summarizing the above results, we concluded that a short Mn exposure already started the formation of LMM Mn-species within serum. This formation rapidly developed under longer exposure time, as imitated by the feeding study. From our previous studies we learnt that there is an enhanced influx specifically of Mn-LMM-species across NB to brain [21,43] which was found by elevated Mn values in brain, too. This "drain" from serum to other organs, i.e., brain, explains the unchanged serum Mn values.

After Mn exposure, total Fe in serum was decreased, significantly by 80% in the feeding trial but only 16% after injection [51]. These effects seem not to be in line with the findings from humans, where no correlations between Mn and Fe concentrations were seen (see above). A final explanation is not at hand, yet, but, on the one hand, there might be differences in the physiological behavior between rats and humans. On the other hand an explanation could be that the unchanged Mn serum concentrations after the feeding experiment result from an efficient transport from serum into organ (including brain) which appears parallel to the continuous, daily exposure by feed. This had been observed previously by Diederich et al. [43] and in Neth's paper [48] it is seen by an increase of Mn in brain. The question why iron is changed by −80% in the feeding trial, while no changes were seen in humans could be explained by the fact, that the overall Mn-mass exposed to rats during seven weeks feeding is much higher than Mn being typically present in unexposed humans. Changes in Fe concentration supposedly appear only when at least a certain Mn-exposure has been exceeded. This explanation seems to be supported by the much smaller reduction of Fe concentration after the injection experiment.

In brain total Mn was increased by around 19% to ca. 429 ± 17 μg Mn/kg in both trials, whereas Fe concentrations decreased in the feeding trial 1.44 fold down to 9 μg Fe/kg or remained practically constant at 13 μg/kg after injection.

Mn-speciation in brain extracts of the feeding trial showed LMM-Mn-carriers to be the major Mn-compounds with 38% increase compared to controls. This increase was accompanied by a remarkable decrease in HMM-Mn molecules by 57%. Aside from Mn-citrate and Mn-amino acids, inorganic Mn was present in higher amounts than in control samples (~32%). Four days after the i.v. injection of $MnCl_2$, LMM Mn-species were increased in exposed rats in similar manner as in the feeding study by 27%. The percentaged amount of Mn bound to HMM or inorganic Mn was comparable between control and exposed animals.

In summary, the findings from these animal studies can be seen as concordant with respect to formation of LMM Mn-species during the applied exposure conditions. Dependent on the kind of exposure the preferential formation of Mn-citrate or Mn-amino acids in serum turned out to be stronger for example in the case of a subchronic, low-dose increased feeding of Mn. These LMM-species might then be involved in the transport of Mn across NB, what was substantiated by the Mn-speciation in brain extracts. One major prerequisite for this is that control mechanisms, such as it is the case for transport via the Tf-receptor, might not be facilitated any more under exposure situations.

3.4. *Mn exposure and mechanisms of detrimental effects*

In brain, parallel to changes of Mn-species also changes of total iron and Fe-species were observed related to Mn-exposure scenarios.

It is known that Fe deficiency can enhance Mn accumulation while Mn treatment can likewise alter Fe homeostasis [52–55]. Despite the knowledge of altered Fe concentrations in the brain of Mn-exposed individuals or animals, little is known about the

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oxidation state of Fe during such conditions. Therefore, within our project Fernsebner et al. [50] developed a method for measuring Fe(II) and Fe(III) within the animal studies of subchronic and acute Mn exposure.

Regarding the sufficiently high Fe concentrations in brain extracts and the non-interferred iron elemental lines, Fe was determined by ICP-OES without complications. For separation of the two Fe species, ion chromatography (IC) was applied.

The most important finding of the analysis of brain extracts was a shift from Fe (III) toward Fe(II) by a factor of around 2.5 in the samples from the subchronic feeding of Mn in rats when compared to controls. This clear shift was not observed after the single i.v. injection of $MnCl_2$ in rats, suggesting that longer incubation times of Mn are required for such changes in the neural Fe(II)/(III) ratio. Fe(II) was positively correlated with Mn concentrations in the brain extracts. This positive correlation was even more pronounced under subchronic Mn-exposure.

It is generally accepted that free Fe(II) is oxidized to Fe(III) by superoxide dismutase (SOD) or glutathione peroxidases (GPx) leading to neutralization of this toxic Fe species [56]. Since Mn might inhibit anti-oxidative enzymes such as SOD, this neutralization may not be maintained, leading to an accumulation of Fe(II) as observed in the feeding study [57]. By reaction of Fe(II) with H_2O_2 via the Fenton reaction, reactive oxygen species (ROS) are formed, which can contribute to inflammation or destruction of neural tissue. Concluding, Mn low level long term exposure (feeding) shifted Fe(III) toward Fe(II), i.e. by this means toward generation of oxidative stress in brain.

The hints about ROS generation were further verified by the subsequent metabolomics studies using ESI-FT-ICR-MS, specifically in the feeding trial. In brain extracts, the oxidative stress marker glutathione-disulfide (GSSG, oxidized glutathione (GSH)) was increased about two-fold, inflammation markers prostaglandine B1 (PGB1) or prostaglandine H2 (PGH2) were increased two or six-fold and the marker for lipid peroxidation 15-hydroxyeicosaeonic acid (15(S)-HETE) was elevated even 28-fold [50].

The Mn-caused disturbance in oxidative defence mechanisms, shown by the increase of the oxidized form of glutathione, is relevant for understanding brain damage mechanisms. Generation of ROS and—as a consequence—the unbalanced anti-oxidative defence system has been reported for several neurological disorders. During PD progression an increased GSSG/GSH ratio indicates oxidative stress [58]. The observed shift toward GSSG was further supported by the reduction of the GSH precursor molecule γ-glutamylcysteine, indicating that the synthesis of GSH seemed to be interfered at an early stage. Interestingly, ophthalmic acid was elevated in the brain of Mn-treated rats. This GSH analogous peptide is considered as biomarker of oxidative stress [59].

Our finding of increased lipid peroxidation, demonstrated by the increase of 15 (S)-HETE or ResolvinD2, has parallels in literature, too: Kapich et al. observed an effective lipid peroxidation of fungal manganese peroxidase on C18 unsaturated fatty acids [60]. Furthermore, the pool of the major poly-unsaturated fatty acids docosahexanoic acid (DHA) and arachidonic acid appeared depleted whereas their lipid mediators PGB1, 15(S)-HETE and ResolvinD2 were found to be increased after Mn injection. This points to an activation of inflammatory response using cyclooxygenase (COX)- or lipoxygenase (LOX)—pathways [61], where the latter was found to be involved also in other neurodegenerative diseases such as Alzheimer's Disease [62].

To complete our puzzle, it was further of interest whether neurotransmitters were influenced in our exposure trials. The influence of Mn on glutamate and gamma-aminobutyric acid (GABA) is known from literature [63]. In this review the summarized data were still conflicting, but it was beyond question, that the GABAergic systems of the basal ganglia were affected and excitatory neurotransmitters such as glutamate or inhibitory neurotransmitters like GABA or the activity of acetylcholinesterase (AchE) were shown to be affected in Mn-induced neuroinflammation [63,64]. Based on these reports we measured glutamate and acetylcholinesterase activity in brain extracts. Glutamate showed no change in the brain "4d" extracts after Mn injection but its concentration was significantly elevated (+16%, $p < 0.05$) in brain extracts from the feeding trial. There we observed actually a clear positive correlation between Mn and glutamate concentration. Other than glutamate, AchE activity was affected in both trials, i.e. increased by 9% ($p < 0.05$) in brain extracts of the feeding trial and by 15% ($p < 0.05$) in brain "4d" extracts of the injection trial. This difference might be explained by previous reports where AchE activity rapidly increases after short term exposure [65] but decreases during long term (e.g., 1–2 years) exposure [66,67]. While four days is considered to be still a short period, seven weeks may be already in an early stage of the declining period from an initial increase to the decrease after several months.

Both, the increase of glutamate concentration as well as the increase of AchE activity in brain are accompanied by changes of neuronal excitability in basal ganglia and are linked to neurodegenerative diseases like amyotrophic lateral sclerosis, Alzheimerís and Parkinson disease [68–70]. Thus, with the above findings a molecular mechanism how Mn exposure leads to symptoms similar to PD was observed.

Finally non-targeted metabolomics studies completed our puzzle so far.

ESI-FT-ICR-MS analysis was applied in methanolic brain extracts. In sum, 9865 masses were detected in all extracts, of which ca. 13.5% could be annotated by MassTRIX webserver [71] and thus were regarded as "known" metabolites. Statistical analysis with OPLS-DA of the entire dataset revealed a good separation between the control and Mn-treated group.

"Known" metabolites were subsequently analyzed by "KEGG color pathway database" [72] to find changes in involved metabolic pathways. Major changes could be observed for purine and pyrimidine metabolism, amino acid metabolism, glucose, and glutathione metabolism as well as for biosynthesis of fatty acids.

Purine nucleosides are involved in a variety of intracellular neuronal processes and provide precursors of RNA and DNA [73]. In our studies nearly all detected purines or pyrimidines were significantly decreased in rat brain extracts after Mn exposure, except for hypoxanthine, AMP (adenosine monophosphate), GMP (guanosine monophosphate) and GDP (guanosine diphosphate)-3,6-dideoxygalactose. Their concentration changes are mediated by adenosine receptors which can complex with dopamine receptors, enabling purines to fine-tune neurotransmission in basal ganglia [74,75]. This includes inverse regulation of glutamate, serotonin and acetylcholine excretion [74,76–78]. In short terms: The more purines are interacting with adenosine receptors, the less neurotransmitters mentioned are excreted.

Another interesting finding of OPLS data analysis revealed that Mn-species in brain extracts correlated group-wise with pyridines or pyrimidines: LMM-Mn species, including Mn-citrate, correlated exclusively positive with AMP, UDP, UMP, GMP but mostly negative with all the other metabolites, whereas Mn-proteins showed inverse correlation (negative to AMP, UDP, UMP, GMP, but positive to all others; except for one protein fraction which correlated only negative to all compounds). (Fig. 4).

For perfecting our Mn-speciation project we investigated the spatial distribution of Mn, Fe, Zn and Cu in frozen microtome slices of rat brains (Mn-exposed vs. controls) using laser ablation-ICP-MS. For quantification (wet-) frozen layers of spiked brain homogenates were employed [51] resulting in values given in [μg (element)/g wet brain mass]. Fig. 5 shows photographs of the brain slices (exposed/control) in the ablation chamber snapshotted by the inte-

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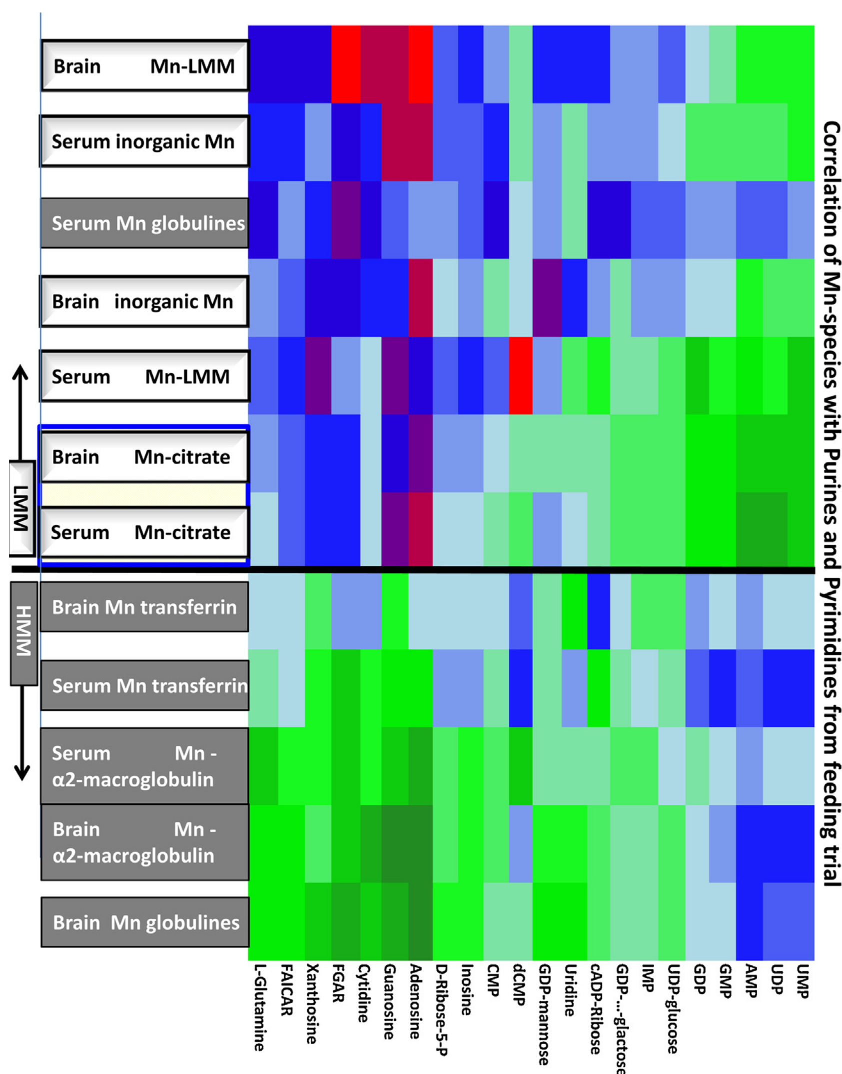

Fig. 4. Heatmap of the OPLS data analysis of the purin/pyrimidin analysis and its interrelation to Mn-species: LMM-Mn species positively correlate with UMP, UDP, AMP and GMP and correlate mainly negative with all other compounds. In contrast, Mn-proteins positively correlate with all other but negatively with UMP, UDP, AMP and GMP. Modified from [51], with permission.

Color code for correlation:

strong positive correlation | positive correlation | no correlation | negative correlation | strong negative correlation

grated camera and the elemental maps of the brains scanned by LA-ICP-MS. Most notably, the Mn-map of the exposed brain shows yellow color areas with even red spots in the basal ganglia, indicating high Mn concentration. This is opposite to the same brain region of the control rat showing mainly green color (low Mn concentration). The same brain regions show even more elevated concentration for zinc, as indicated by the red color, whereas iron decreased considerably compared to controls, shown by blue color for respective brain regions. The well known competition of Mn and Fe explains the invers situation in basal ganglia for both elements. The findings also confirm the above reported results about total element concentrations in brain.

4. Summary

The manganese speciation project in our laboratory was aimed first to clarify in which chemical form Mn is reaching neural barriers. Therefore, Mn-speciation in serum (before NB) and in CSF (behind NB), notably in paired serum and CSF samples was investigated. Most important findings from these experiments were that under Mn exposure HMM-Mn species did not increase in serum or were even lowered, in contrast to LMM-Mn species, e.g., Mn-citrate, which were increased. A Mn-citrate complex could be identified by 2-D approaches including ESI-FT-ICR-MS. It was further seen,that

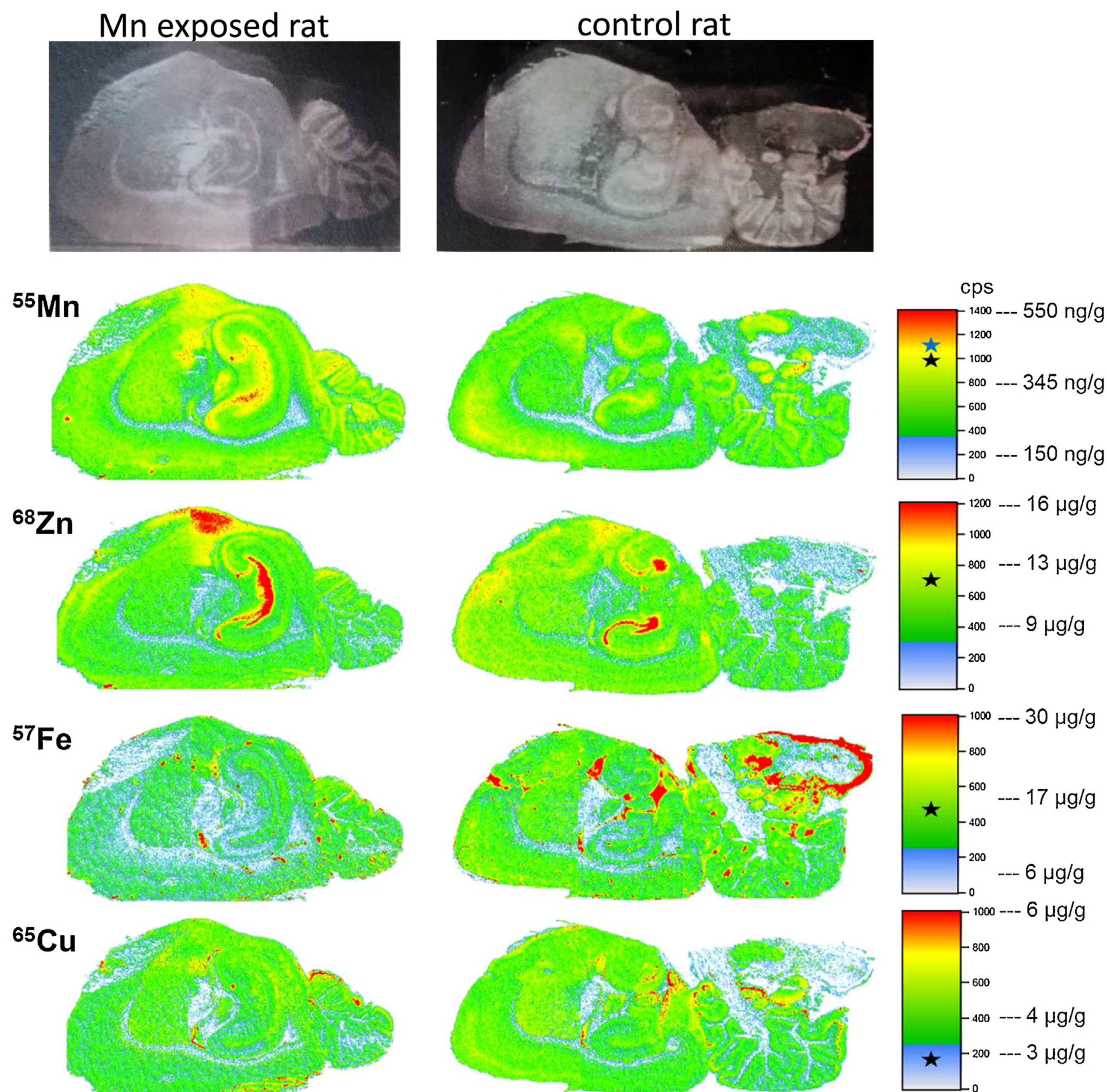

Fig. 5. Photographs (top) of the brain slices (exposed/control) are plotted snapshotted by the integrated camera of the LA system. Below the elemental maps of the brains are seen, scanned by LA-ICP-MS. The asterisks in the concentration-color code bare on the right side shows the (wet-) concentration determined for the bulk concentrations of respective elements determined in rat brain. For manganese, the black asterisk indicates the bulk concentration for control animals, the blue one for exposed rats. Modified from [51], with permission.

Mn-citrate was enriched up to five-fold across NB. This was contradictory to all other Mn-species investigated. We showed with an increased sample size that at slightly elevated total Mn(S) concentration a change in Mn carriers toward Mn-citrate takes place. Parallel to this carrier change, for an especially studied subset we have shown that Mn beyond the neural barrier, i.e., Mn(C), correlates to this second carrier Mn-citrate(S) at higher Mn(S) concentration, reflecting the facilitated transport of Mn into the brain. The latter has been confirmed also in rat brain after single low dose exposure [43] and also after low-dose 7 weeks feeding. Comparing short term vs. long term exposure (both low levelled) we found that the short Mn exposure already started the formation of LMM Mn-species within serum. This formation rapidly developed under longer exposure time, as imitated by the feeding study. Therefore, Mn-Citrate appears to be *that* important Mn metabolite reaching the brain after Mn exposure.

The second aim of the manganese project considers the clarification of the mechanism of neuronal damage, caused by Mn. Therefore, iron, being a competitor to Mn, was investigated.

Mn exposure altered the Fe metabolism in brain. The total Fe concentration was reduced and the important homeostasis of the Fe(III)/Fe(II) ratio was disturbed by a shift from Fe(III) to Fe(II) observed after a low dose (long-term) exposure. This shift indicated a severe metabolic change toward oxidative stress in brain. This finding was supported by results from our metabolomics studies: Oxidative stress- and inflammation markers as well as lipid peroxidation markers were significantly elevated after Mn exposure.

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Additionally, many metabolic pathways (of which several have to be still identified) were severely altered.

Finally, changes in the GABAergic system were monitored with significant elevation of glutamate concentration (only in feeding trial) and AchE activity (in both trials). Both effects cause changes of neuronal excitability in basal ganglia. This relationship between neuronal excitability and increase of AchE or glutamate explains the similarity of Mn-dependent symptoms to Parkinson disease. Although, many pieces of this puzzle were clarified, a couple of questions remain open so far, such as probably a definitive answer on the Mn-transported across NB or the balance/imbalance of metal species ratios of different metals like Zn-Cu-Mn-Fe which might interact in a network.

Conflict of interest

The author does not have any conflict of interest.

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