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Time-course expression of metallothioneins and tissue metals in chronic relapsing form of experimental autoimmune encephalomyelitis

Hrvoje Jakovac[1*], Damir Grebić[2*], Marin Tota[3], Vesna Barac-Latas[1], Ines Mrakovčić-Šutić[1], Čedomila Milin[3] and Biserka Radošević-Stašić[1]

Departments of [1]Physiology and Immunology, [2]Surgery and [3]Chemistry and Biochemistry, Medical Faculty, University of Rijeka, Rijeka, Croatia

*These authors equally contributed to this work

Summary. To elucidate the role of metallothioneins (MTs) in the pathomechanisms of autoimmune CNS disorders we estimated the expression of MTs I+II and the tissue concentrations of Zn^{2+} and Cu^{2+} in the brain, spinal cord (SC) and in the liver during the periods of attacks and remissions in chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE). Disease was induced in the genetically susceptible Dark Agouti (DA) rats by subcutaneous injection of bovine brain homogenate in CFA. Control rats were treated with CFA. The data, obtained by clinical assessment, immunohistochemistry and inductivity coupled plasma spectrometry, have shown that during the first attack (on the 12th day) MTs I+II were markedly upregulated in subarachnoid regions and perivascular space on astrocytes, microglia and on spinal neurons. Simultaneously, the concentrations of zinc in the SC and zinc and copper in the liver have found to be increased. During the second attack (on the 22nd day) a new overexpression of MTs was found in the cerebellum, in sulcus hippocampi, in spinal neurons and particularly in hepatocytes around the central vein. Concomitantly, in the brain and SC the concentration of copper increased.

The data point to a neuroprotective role of MTs and to an important regulatory role of essential metals and hepatic MTs in the pathogenesis of CR-EAE

Key words: Chronic relapsing experimental autoimmune encephalomyelitis, Metallothioneins I+II, Zinc, Copper, CNS, Liver

Offprint requests to: Biserka Radosevic-Stasic, Medical School, University of Rijeka, B. Branchetta 22, 51000 Rijeka, Croatia. e-mail: biserr@medri.hr

Introduction

Chronic relapsing form of experimental autoimmune encephalomyelitis (CR-EAE), which may be induced in genetically susceptible Dark Agouti (DA) rats, mimics many, though not all, of the clinical and immunopathological features of multiple sclerosis (MS) in humans. It is characterized by periods of attacks and remissions, based on the processes of inflammation and demyelination in the central nervous system (CNS) and those related to recovery, in which different players participate. Currently, strong evidence shows that antiapoptotic, antioxidant and regenerative effects in neurological diseases also have metallothioneins (MTs), a highly conserved family of low-molecular-weight, cystein-rich proteins (Aschner, 1996; Penkowa et al., 2006; West et al., 2008; Asmussen et al., 2009; Pedersen et al., 2009a). As zinc and copper binding proteins they are involved in metalloregulatory processes, such as cell growth, multiplication and apoptosis, which are ubiquitous in eukaryotes and are expressed particularly in fast growing normal and malignant tissues (Cherian and Kang, 2006; Jakovac et al., 2006; Thirumoorthy et al., 2007; Pedersen et al., 2009b). However, MT I+II isoforms are also critically involved in several pathophysiological states, since after tissue injuries or exposure to heavy metals and other stressful conditions, the transcription of these MTs could be upregulated by glucocorticoid responsive elements (GREs), by the antioxidant (or electrophile) response element (ARE), by the elements activated by STAT-3 (signal transducers and activators of transcription) proteins through cytokine signaling, as well as by metal response elements (MREs), indicating that they are multifunctional proteins that participate in a variety of cellular functions (Moffatt

and Denizeau, 1997; Coyle et al., 2002; Sato and Kondoh, 2002; Thirumoorthy et al., 2007; Pedersen et al., 2009a). As presented in excellent recent reviews (Aschner, 1996; Aschner et al., 1998; Mocchegiani et al., 2005; Penkowa et al., 2006; West et al., 2008; Asmussen et al., 2009; Pedersen et al., 2009a), these proteins confer a protective effect also within the mammalian CNS, where MT expression dramatically increases in response to many types of CNS and spinal cord (SC) injury or ischemia, as well as in senescence and in several neurodegenerative diseases such as Alzheimer's or Pick's disease and amyotrophic lateral sclerosis (Aschner, 1996; Hidalgo et al., 2001; Giralt et al., 2002; Mocchegiani et al., 2002, 2005; Penkowa et al., 2006; West et al., 2008).

The expression of MT-I+II was also found to be upregulated in brain lesions of MS patients (Penkowa et al., 2003b), as well as in EAE (Penkowa and Hidalgo, 2000). Moreover, it was shown that oxidative stress and demyelination might be diminished by treatment with Zn-MT-II (Penkowa and Hidalgo, 2000, 2001, 2003), and that axonal damage might significantly increase in MT-I+II deficient mice during EAE, emphasizing that MT might have anti-inflammatory properties, and that they might also participate in the clinical recovery of EAE (Penkowa et al., 2003a; Espejo et al., 2005). It is generally accepted that they are produced primarily by astrocytes, where they are involved in metal ion control and scavenging of reactive oxygen species (ROS) (Chung et al., 2008; West et al., 2008; Pedersen et al., 2009a). It is, however, also suggested that neuronal survival can be induced by extracellular MT I+II, which might be internalized to the cytoplasm and to the nucleus of injured neurons after binding to low-density lipoprotein receptors-lipoprotein receptor-1 (LRP1) and megalin (LRP2) (Ambjfirn et al., 2008; Chung et al., 2008; Pedersen et al., 2009a). Regulatory mechanisms are still being investigated, but postulated functions include the control of zinc and copper availability to zinc or copper-dependent enzymes, such as the zinc finger transcription factors, and superoxide dismutase that regulate the transcription of anti-oxidative, anti-inflammatory and cytoprotective factors (Zeng et al., 1991; Rothstein et al., 1999; Prohaska and Gybina, 2004; Kozlowski et al., 2009).

In an attempt to elucidate some aspects of these events in this study we simultaneously analyzed MT I+II expression and tissue concentrations of Zn^{2+} and Cu^{2+} in the brain and spinal cord during the phases of attacks and remissions of CR-EAE, induced in genetically susceptible DA rats. Additionally, since particularly the liver is reacting on oxidant damage, inflammatory mediators and metabolic dysbalance by acute phase reaction (Moffatt and Denizeau, 1997; Coyle et al., 2002) and by generation of autoreactive NKT and regulatory T cells (Wu and Van Kaer, 2009), in this study we analyzed also the kinetics of hepatic MTs expression and tissue metals during different clinical phases of CR-EAE. The data showed that during CR-EAE MT I+II were markedly upregulated firstly in periventricular and perivascular spaces, and then not only in parenchymal astroglial cells of the brain and SC, but also in several neurons in SC and in the liver. Simultaneously, during the attacks of EAE the cyclic changes in the concentrations of zinc and copper were noticed in the spinal cord and in the liver, implying that these metals might have a regulatory role in the pathogenesis of autoimmune disease.

Materials and methods

Animals

For the experiments male, Dark Agouti (DA) rats, aged 2-3 months were used. They were bred and maintained according to the guide for Institutional Animal Care and used with the approval of the local Ethical committee.

EAE induction and evaluation

Immunization was performed by bovine brain white matter homogenate emulsion (BBH) in the complete Freund's adjuvant (CFA) (Sigma, St. Louis, Mo., USA). Each animal received 2x0.1 ml of the emulsion, which was injected subcutaneously, in each hind footpad. Rats in the control groups were injected with the same dose of CFA. The severity of disease was clinically assessed according to the following criteria: 0-no symptoms; 1-flaccid paralysis of tail; 2-hind legs paresis; 3-hind legs paralysis with incontinence and 4-death of the animal.

Tissue preparation

The brain, spinal cord and liver were rapidly removed from three rats in each group and all tissue samples were fixed in 10% buffered formalin solution, for a minimum of 24 h. Tissue was then embedded in paraffin wax and sections were cut at 4 μm using HM 340E microtome, Microtom, Germany. Heat induced epitope retrival was done prior to staining procedures by heating tissue slides in boiled citrate buffer pH 6.0 four times, each 5 minutes, using a microwave steamer.

Immunohistochemistry

Immunohistochemical studies were performed by DAKO EnVision+System, Peroxidase (DAB) kit, as previously described (Jakovac et al., 2006; Grebic et al., 2007), on sections of the brain, the cervical and lumbal spinal cord and the liver tissue embedded in paraffin wax, according to the manufacturer's instructions (DAKO Corporation, USA). Monoclonal anti-MT I+II antibody (clone E9; Dako Cytomation, USA) and anti-glial fibrillary acidic protein (anti-GFAP; Becton Dickinson, USA) were used. The specificity of the reaction was confirmed by substitution of anti-MT and anti-GFAP antibodies with mouse irrelevant IgG1 kappa

immunoglobulin.

Double immunohistochemical labeling was performed employing DakoCytomation EnVision Doublestain System according to the manufacturer's instruction (DakoCytomation, USA). Binding of the first primary antibody (anti-GFAP, Becton Dickinson, USA) was visualized by peroxidase labelled polymer conjugated with secondary antibodies, using DAB as a tracer, resulting in brown staining at the first antigen site. Upon completion of the first reaction, slides were incubated with double stain block to remove any potential cross-reactivity between the reactions, along with blocking any endogenous alkaline phosphatase that may be present. Afterwards, the second primary antibody (anti-MT I+II) was added, followed by incubation with alkaline phosphatase labelled polymer containing secondary antibodies. The second antigen staining was finalized by incubation with the Fast Red substrate-chromogen, which results in a red-colored precipitate at the sites of the second immunoreaction, while reddish brown coloration indicates the presence of both labeled antigens. Control slides were identically treated, but with the omission of primary antibodies. The slides were examined on an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Tissue sample preparation for mineralization and inductively coupled plasma spectrometry

The brain, spinal cord and liver were carefully removed using plastic instruments, following a technique previously described (Jakovac et al., 2006). Liquid tissue samples were introduced into the apparatus by pneumatic nebulisation. The measurements of zinc and copper were performed using a PHILIPS PU 7000-ICP Spectrometer, by the method ASTM D 19756-91 (power 1 kW, coolant 12 l/min, nebulizer 38 psi), at a fixed wavelength of 213.856 nm for zinc, and 324.754 nm for copper.

Statistical Analysis

The statistical analysis was performed using a Sigma Plot Scientific Graphing System, v.8.0. Differences between groups were assessed by Friedman one-way analysis of variance (ANOVA) and by Mann-Whitney U test (clinical scores), and by two-tailed Student's t-test for unpaired samples (metal tissue concentrations). The level of significance was set at $p<0.05$.

Results

Clinical course of EAE in DA rats

Genetically susceptible DA rats, immunized by bovine brain homogenate in CFA developed a typical chronic-relapsing form of disease (Fig. 1). The first clinical symptoms appeared on 10th post-immunization day. Two peaks of disease were noticed on the 12th and on the 22nd post-immunization day, while the remissions of symptoms were most prominent on the 18th and the 28th day. Rats in the control group, which were treated by CFA, did not develop clinical symptoms of EAE (Fig. 1; $p<0.001$).

Expression of MT I+II during CR-EAE

MT-I+II expression during the CR-EAE was evaluated in the tissue samples of the brain, cervical and lumbal spinal cord and in the liver taken at the time of first and second attack (on the 12th and the 22nd post-immunization day) and in the periods of remissions (on the 18th and the 28th day), respectively. The data were compared with findings obtained in rats treated only with CFA on the same post-inoculation day.

As shown on Figure 2, in the samples of the brain (a), the cervical spinal cord (e), the lumbal spinal cord (i) and the liver (m), obtained from non-immunized rats (on the 12th day after injection of CFA), the expression of MT I+II proteins was not found. On the contrary, in rats immunized with BBH+CFA during the first attack of CR-EAE, a high upregulation of MT I+II was found in subarachnoid regions (Fig. 2b), in choroid plexus epithelial cells (embedded picture in Fig. 2b), in the molecular (Fig. 2c) and granular layer of cerebellum (Fig. 2d), in subarachnoid and perivascular regions of the cervical spinal cord (Fig. 2f,g), as well as on cells lining the central canal in the lumbal spinal cord (Fig. 2j), implying that MTs were upregulated, particularly on

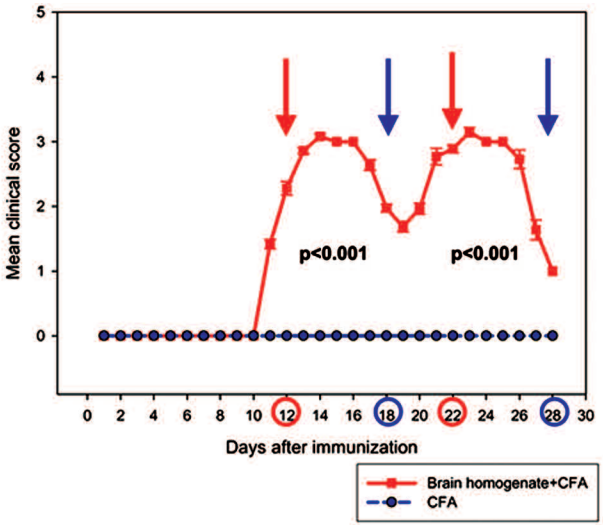

Fig. 1. Clinical score of CR-EAE induced in Dark Agouti (DA) rats (N=14) by injection of bovine brain homogenate in complete Freund's adjuvant (CFA). Rats in control group (N=14) were injected with CFA alone. Arrows point to the time when tissue samples for detection of metallothioneins and tissue metals were taken (i.e. on the first and second attack, and on the first and second remission). Data are mean ± SE. Significance between the experimental and control group was assessed by Mann-Whitney U test.

sites which during EAE convey inflammatory immune signals into the brain and enable immigration of autoreactive T cells into CNS. MTs were, however, also found to be upregulated on several neurons in lumbal spinal cord (Fig. 2l) and on numerous cells in the parenchyma that morphologically looked like astrocytes (Fig. 2h,k). Simultaneously, in the liver moderate upregulation of MTs was noticed in hepatocytes, located in periportal regions (Fig. 2n,o,p).

In an attempt to better characterize the cells expressing the MTs, the single and double labeling with anti-GFAP and anti-MT-I+II antibodies were additionally made on serial, paraffin-embedded tissue sections of the same organs (Fig. 3). The data revealed that most ependymal cells lining the brain's ventricles were MT positive cells (Fig. 3a), and among them were GFAP-positive astrocytes, which seem to form a dense network of glia limitans in the BBB (Fig. 3d). In the cerebellum many MT-positive cells (Fig. 3e) were also GFAP-positive cells (Fig. 3f) that extended their processes into the molecular layer of the cerebellum (Fig. 3g). Some of these cells resembled the radial Bergmann glias that form the pia-glial membrane (Fig. 3h), but this speculation needs to be proven. In the lumbal spinal cord it was confirmed that during the first attack of EAE many neurons are MT-positive (Fig. 3i) and GFAP-negative cells (Fig. 3j), which expressed particularly perinuclear MT-immunoreactivity (Fig. 3k). In the parenchyma numerous single and double- positive astrocytes for GFAP and for MTs were also found (Fig.

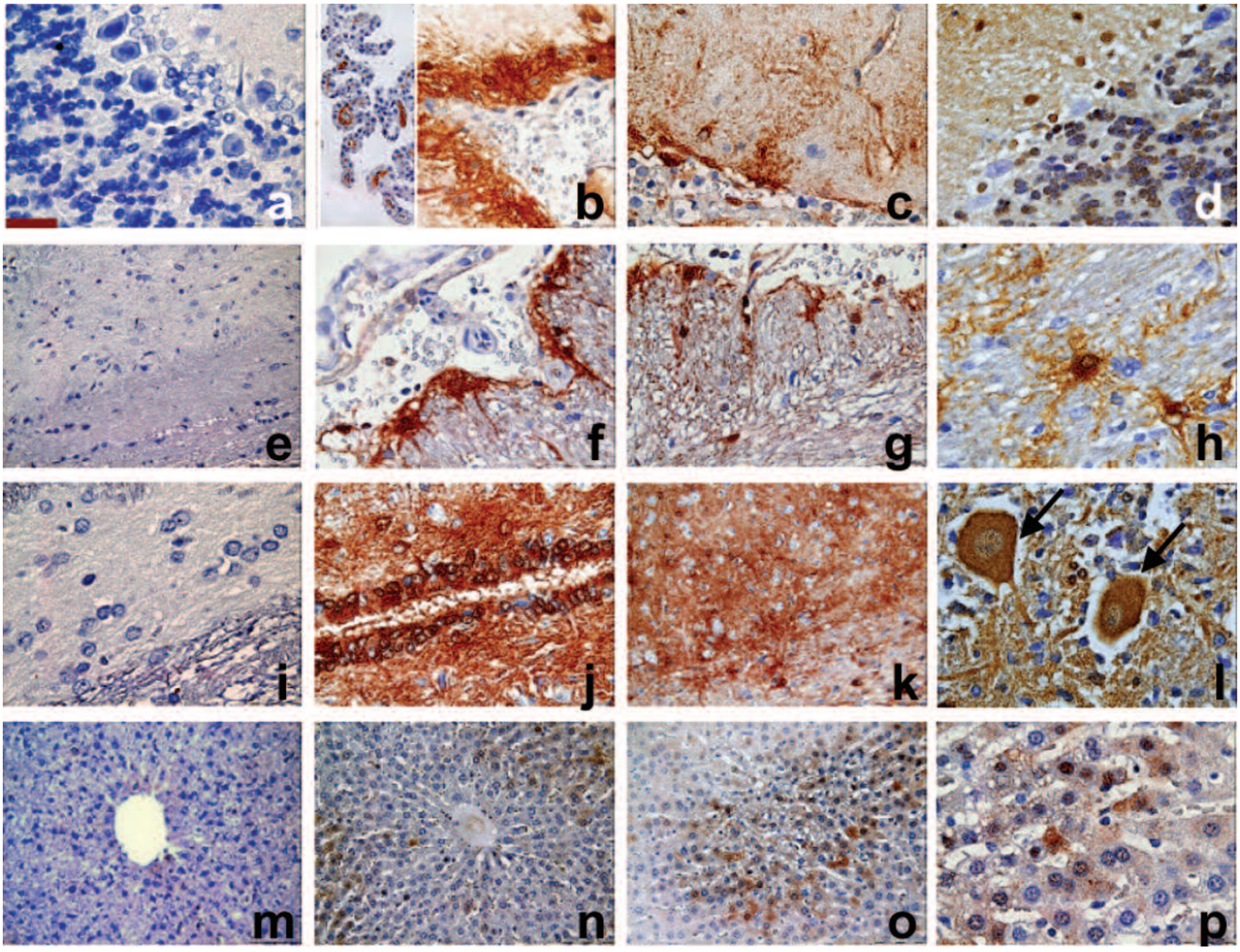

Fig. 2. Expression of MT I+II proteins during the first attack of CR-EAE (on the 12th day after immunization with bovine brain white matter homogenate in the CFA) in the brain **(b-d)**, cervical spinal cord **(f-h)**, lumbal spinal cord **(j-l)** and liver **(n-p)**. First column **(a, e, i, m)** shows the MT I+II expression in the control tissue samples, obtained from rats injected with CFA alone. Embedded picture on **(b)** shows expression of MT-I+II on choroid plexus. Arrows in picture **(l)** point to MT-positive neurons in lumbal spinal cord. The results are representative findings of 3 rats. Scale bars: a-m, p, 20 μm; n, 200 μm; o, 50 μm.

3k,l).

During the first remission (on the 18th post-inoculation day) in experimental group, MTs expression was visible on cells in the periventricular area and hippocampal sulcus (Fig. 4b), in the white matter of cerebellum (Fig. 4c,d), as well as in multiple localized areas of the cervical (Fig. 4f,g) and lumbal spinal cord (Fig. 4j,k). Some MT-positive cells were also seen inside a blood vessel and in perivascular spaces (Fig. 4h). Most neurons in the lumbal spinal cord were MT-negative (Fig. 4l). In the liver MT expression was not observed (Fig. 4n-p).

During the second attack of CR-EAE (on the 22nd post-immunization day) a new, high upregulation of MT proteins was observed on ependymal lining of ventricle and endothelial cells (Fig. 5b), as well as in the molecular layer of the cerebellum (Fig. 5c,d). In cervical and lumbal spinal cord MTs were present in the subarachnoid area (Fig. 5f,j), and on multiple glial cells in white matter (Fig. 5g,j,k). Additionally, in the lumbal spinal cord the numerous MT-positive neurons were found (Fig. 5l). Simultaneously, in the liver a very high cytoplasmic expression of MT I+II was seen in hepatocytes around the central vein (Fig. 5n-p). In the control samples of brain, spinal cord and liver (obtained from DA rats, injected with CFA 22 days previously), the expression of MTs was not seen in any of these organs (Fig. 5a,e,i,m).

In the second remission MT I+II expression was found mostly in white matter of the cerebellum on several glial cells (Fig. 6b-d). Additionally, high MT immunoreactivity was found in white matter of the cervical (Fig. 6f-h) and lumbal spinal cord on numerous glial cells and myelinated fibers (Fig. 6j-l). Neurons in lumbal spinal cord did not express MTs (Fig. 6l). Similarly, in the liver the expression of MT I+II proteins was not seen (Fig. 6m-p).

Metal tissue kinetics during CR-EAE

Since MT-I+II are major factors controlling zinc and copper metabolism, in the same time intervals during CR-EAE, we estimated the tissue concentrations of these metals in brain, spinal cord and liver, using inductively coupled plasma spectrometry. Control samples were obtained, in the same time intervals, from rats treated

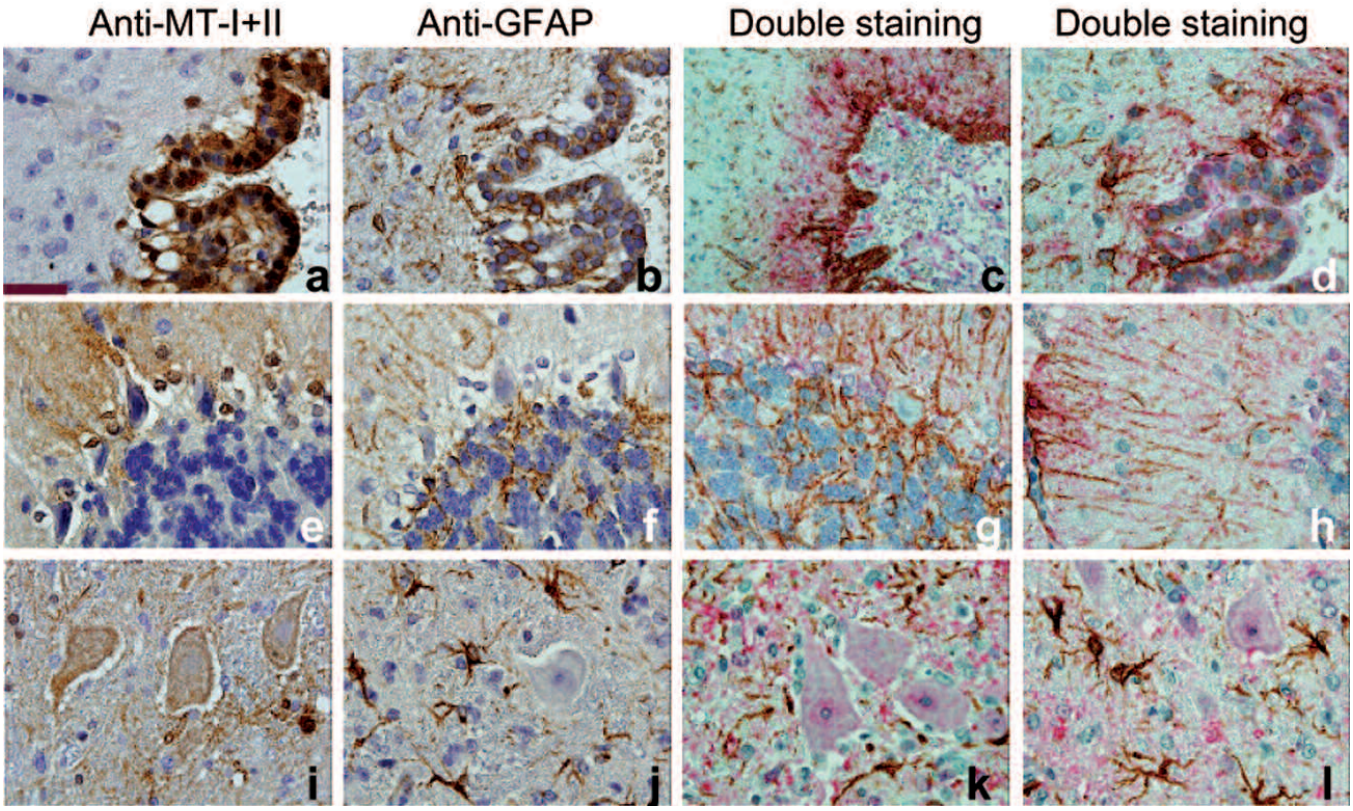

Fig. 3. Single and double immunohistochemical labeling of MT-I+II proteins and GFAP in the brain **(a-h)** and lumbal spinal cord **(i-l)** during the first attack of CR-EAE (on the 12th day after immunization with bovine brain white matter homogenate in the CFA). Brown staining indicates a single positivity for MTs **(a, e and i)** or for GFAP **(b, f and j)**. In slides with double staining a red-colored precipitate indicates positivity for MTs, while reddish brown (russet) coloration indicates the presence of both MTs and GFAP. The results are representative findings of 3 rats. Scale bars: 20 μm.

with CFA alone.

As shown on Figure 7, in the a experimental group, we found a marked, statistically highly significant, increase of Zn^{++} in the cervical spinal cord on the 12th, 18th and 22nd post-immunization day (for 59%, 107% and 60%, respectively), and marked early accumulation of Zn^{++} in the liver on the 12th day (for 61%; $p<0.0003$). In the brain Zn^{++} levels changed only in the second remission (on the 28th day), when a decrease of 27% ($p<0.002$) was found. In the lumbal spinal cord no significant changes were noticed (Fig. 7).

Similar changes were found in kinetics of copper, whose content increased at the cervical spinal cord in all intervals (for 100%, 49%, 33% and 66%, respectively) and in the liver during the first attack (for 36.7%; $p<0.0003$) (Fig. 8). A tendency to accumulate Cu^{++} was also noticed in the lumbal spinal cord, but these data were on the border of statistical significance ($p<0.06$).

Discussion

The present study points to time-related changes in the expression of MT-I-II proteins and tissue metals during CR-EAE in rats, confirming several essential factors, related with the role of MT-I+II isoforms in the pathogenesis of demyelinating diseases. Thus, as previously well documented (Ebadi et al., 1995; Penkowa et al., 1997; Penkowa and Hidalgo, 2000), we show herein that during the attacks of disease their expression may be strongly upregulated in multiple astrocytes (Figs. 2f-h, 3c,d,g,h,k,l), which are considered to be the primary cellular source of MT-I+II within the

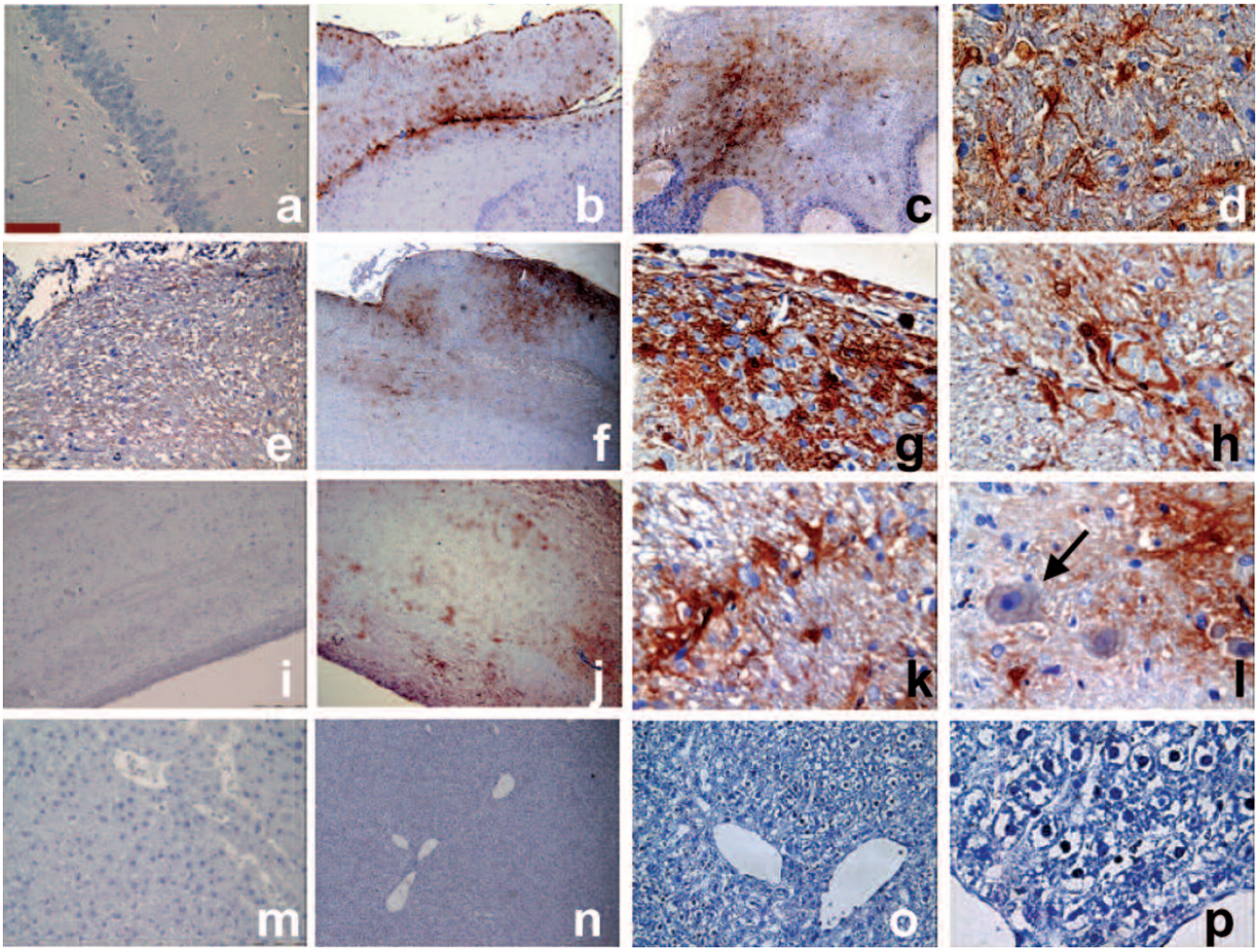

Fig. 4. Expression of MT I+II proteins during the first remission of CR-EAE (on the 18th day after immunization with bovine brain white matter homogenate in the CFA) in the brain **(b-d)**, cervical spinal cord **(f-h)**, lumbal spinal cord **(j-l)** and liver **(n-p)**. First column **(a, e, i, m)** shows the MT I+II expression in the control tissue samples, obtained from rats injected with CFA alone. Arrows on **(l)** point to neurons in lumbal spinal cord. The results are representative findings of 3 rats. Scale bars: a, e, m, o, 50 μm; b, c, f, i, j, 200 μm; d, g, h, k, l, p, 20 μm.

mammalian brain. Moreover, MT expression was found also on vascular endothelium, ependymal and choroid plexus epithelial cells in the brain (Fig. 2b, 3a) and in the spinal cord (Fig. 2fg,j), pointing to specific function of MT-I+II in the Virchow–Robin space which forms the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) and provide stimulatory and antigen presenting signals for T cells invasion in cases of CNS inflammation (Ballabh et al., 2004; Becher et al., 2006; Engelhardt and Sorokin, 2009).

Furthermore, supporting the proposal that in immunized animals a high inflammation and a loss of BBB integrity occurs particularly in the cerebella (Tonra, 2002; Spitsin et al., 2008), during both attacks of EAE we found a high MT-I+II immunoreactivity on neuroglial cells and their fibrils in the molecular layer in this region (Figs. 2c,d, 3e,g,h, 4,c,d).

These data underline that during attacks of EAE the expression of MT I-II proteins might be induced by pro-inflammatory signals, oxidative and/or nitrosative stress at the sites of intense inflammation that permit a large influx of peripherally activated T cells, B cells and macrophages into the CNS. In this context, it was suggested that antigen recognition and re-stimulation of encephalitogenic T cells occurs primarily in the perivascular, Virchow–Robin space connected to the subarachnoid space, where perivascular macrophages /microglia and dendritic cells (DCs) present to encephalitogenic T cells a cognate Ag, in the context of major histocompatibility complex (MHC) class II

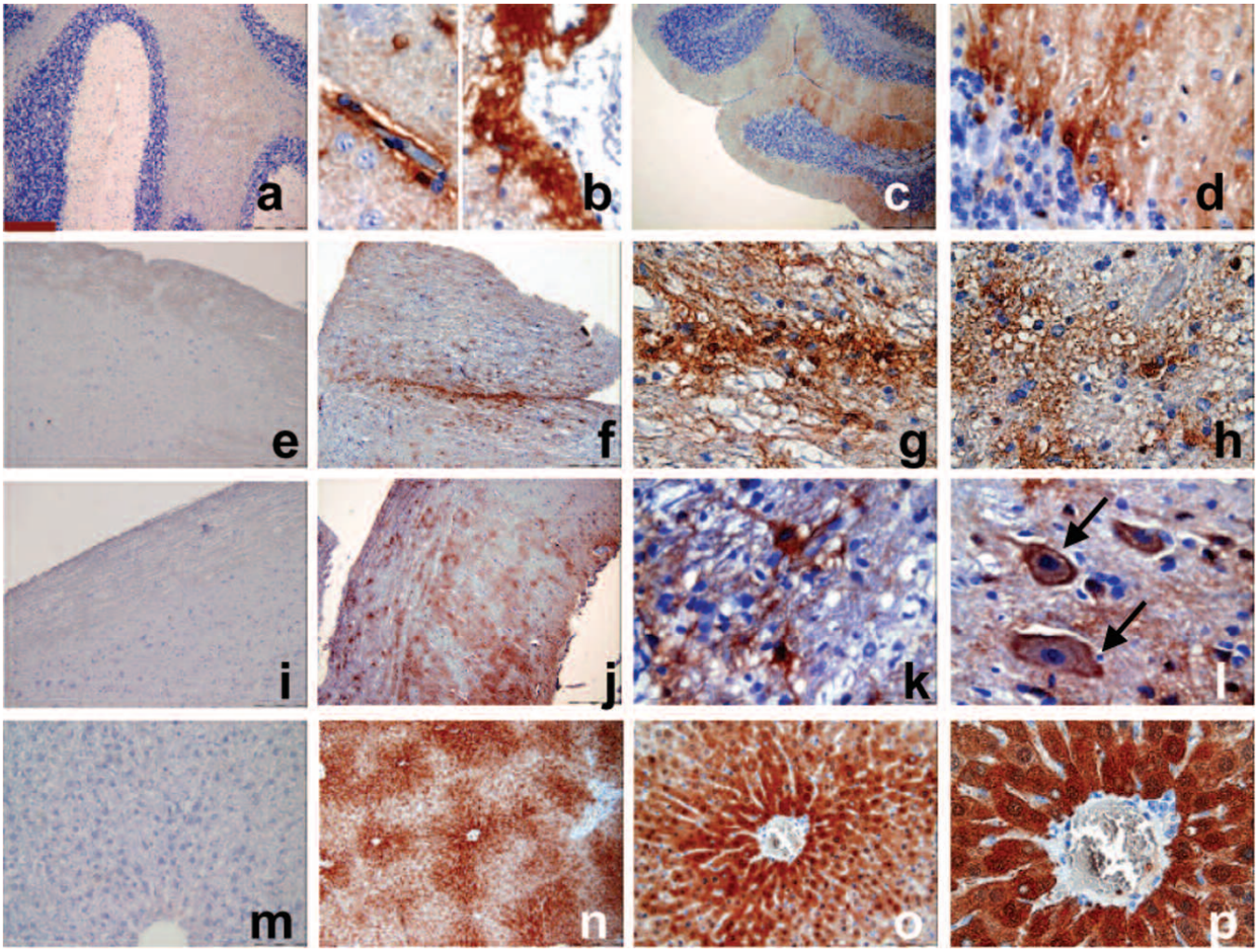

Fig. 5. Expression of MT I+II proteins during the second attack of CR-EAE (on the 22nd day after immunization with bovine brain white matter homogenate in the CFA) in the brain **(b-d)**, cervical spinal cord **(f-h)**, lumbal spinal cord **(j-l)** and liver **(n-p)**. First column **(a, e, i, m)** shows MT I+II expression in the control tissue samples, obtained from rats injected with CFA alone. Embedded picture on **(b)** shows expression of MT-I+II on vascular endothelium. Arrows on **(l)** point to neurons in lumbal spinal cord. The results are representative findings of 3 rats. Scale bars: a, c, e, f, i, j, m, n, 200 μm; g, h, k, o, 50 μm; b, d, l, p, 20 μm.

molecules (Becher et al., 2006). This enables T lymphocytes to pass the glia limitans and to enter the brain parenchyma, where they interact with microglial cells that drive the inflammatory cascade, leading to tissue damage and an amplification of the initial innate immune reaction (Becher et al., 2006). Subsequently, inflammatory cytokines released from infiltrating T cells and from activated microglia trigger synaptic alteration and degeneration resulting in irreversible dendritic pathology (Centonze et al., 2009). However, as previously reported (Ebadi et al., 1995; Penkowa et al., 1997; Penkowa and Hidalgo, 2000) and confirmed herein, it is likely that the anti-inflammatory and neuroprotective mechanisms are activated on these strategic places, resulting in upregulation of MT I+II proteins on several endothelial, epithelial and astroglial cells. Moreover, since, during both attacks of EAE we found numerous MT I+II positive motoneurons in the spinal cord (Figs. 2j, 3i,j, 5j) it could be speculated that injured neurons have also activated their own protective pathways, or that they had internalized MTs, which have been synthesized in astrocytes and released into the extracellular environment. The latter speculation is in high agreement with the recent hypothesis implying that extracellular MT I+II interact with endocytosis receptors, such as LRP1 and LRP2 (megalin), which after internalization of MT I+II and their ligands enable the neuroprotective and neurite outgrowth function of MTs (reviewed by (Ambjfirn et al., 2008; Chung et al., 2008; Pedersen et al., 2009a). Our findings of perinuclear localization of MTs in neurons (Fig. 3k,l), point to their nucleoprotective activities, but this data

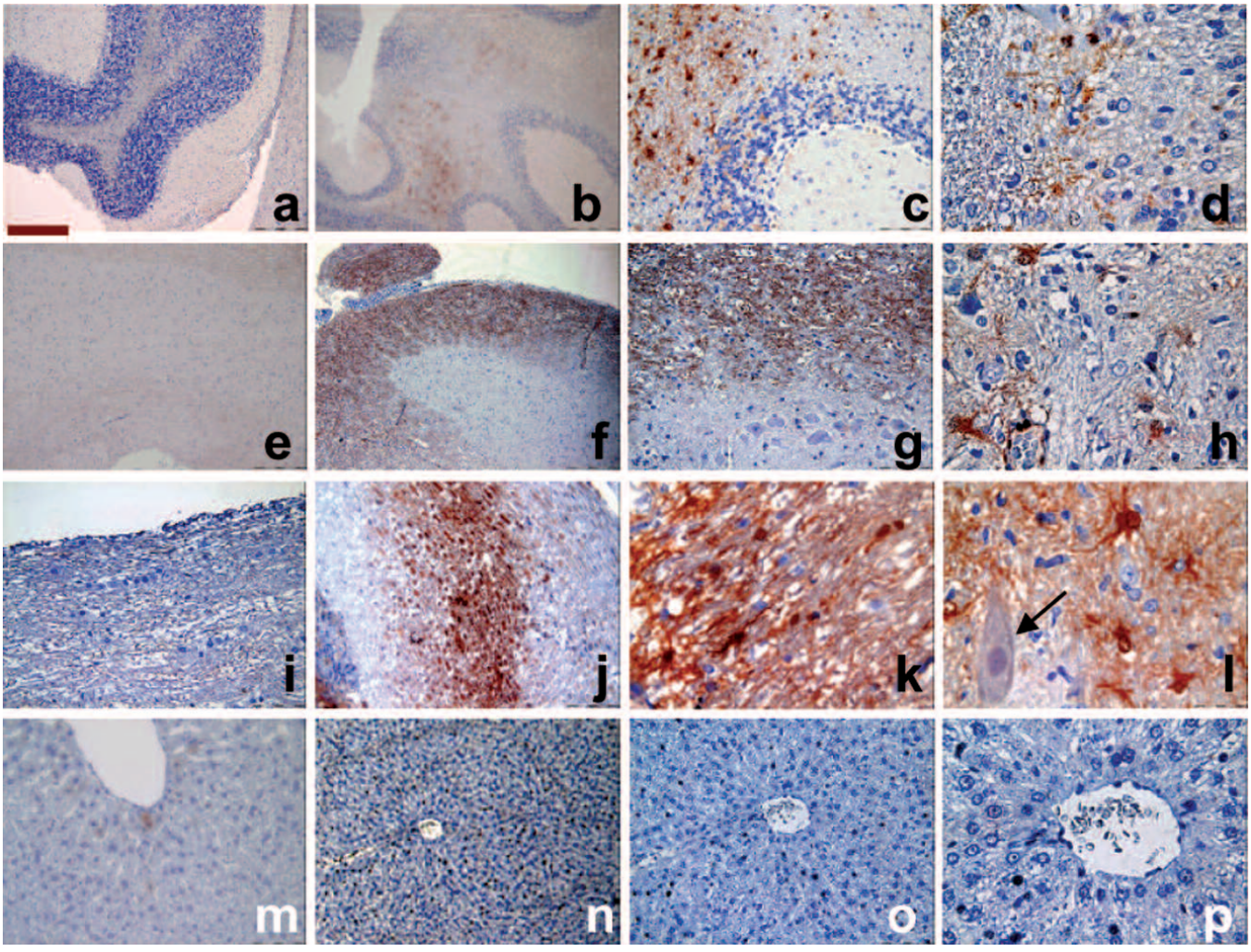

Fig. 6. Expression of MT I+II proteins during the second remission of CR-EAE (on the 28th day after immunization with bovine brain white matter homogenate in the CFA) in the brain **(b-d)**, cervical spinal cord **(f-h)**, lumbal spinal cord **(j-l)** and liver **(n-p)**. First column **(a, e, i, m)** shows MT I+II expression in the control tissue samples, obtained from rats injected with CFA alone. Arrow on "l" points to neuron in lumbal spinal cord. The results are representative findings of 3 rats. Scale bars: a, b, e, f, i, n, 200 μm; c, g, j, m, o, 50 μm; d, h, k, l, p, 20 μm.

needs additional investigations.

In the spinal cord the primary cellular source of extracellular MT seems to be the neighboring astrocytes, around which we found diffuse MT I+II immunoreactivity (Figs. 2k, 3k,l, 5k), but owing to the findings of very high upregulation of MT I+II immunoreactivity in the liver (particularly during the second attack of EAE; Fig. 5n-p) we cannot exclude the possibility that circulating MTs contributed to these events, since they might be transported across the disrupted BBB. Moreover, as clearly shown by Penkowa and Hidalgo (Penkowa and Hidalgo, 2001) the significant amelioration of EAE in Lewis rats after intraperitoneal application of Zn-MT-II, which reduced the expression of proinflammatory cytokines and the number of apoptotic neurons and oligodendrocytes, seems to be obtained by the similar route.

As it is generally accepted, MT-+II inducers are proinflammatory cytokines and bacterial lipopolysaccharide, ROS, hormones, such as glucocorticoids, catecholamins, progesterone, vitamin D3, and metal cations, such as Zn, Cu, Cd, Hg, Pb, which may induce the transcriptional regulation of MT-1/MT-2 genes by activation of STAT-3, ARE, GREs and MREs, respectively (Coyle et al., 2002; Haq et al., 2003; Pedersen et al., 2009a). In the tissues damaged by immune attack, it is likely that they were induced by pro-inflammatory cytokines and oxidative stress, while in the liver, the MT induction might be driven not only by cytokines, such as TNFα, IL-1α, IL-1ß and IL-6, but also by glucocorticoid hormones (GCs), which might induce MT independently of other regulatory sequences, as a part of acute phases response (Hidalgo et al., 1988; Coyle et al., 2002).The same initial stimuli probably

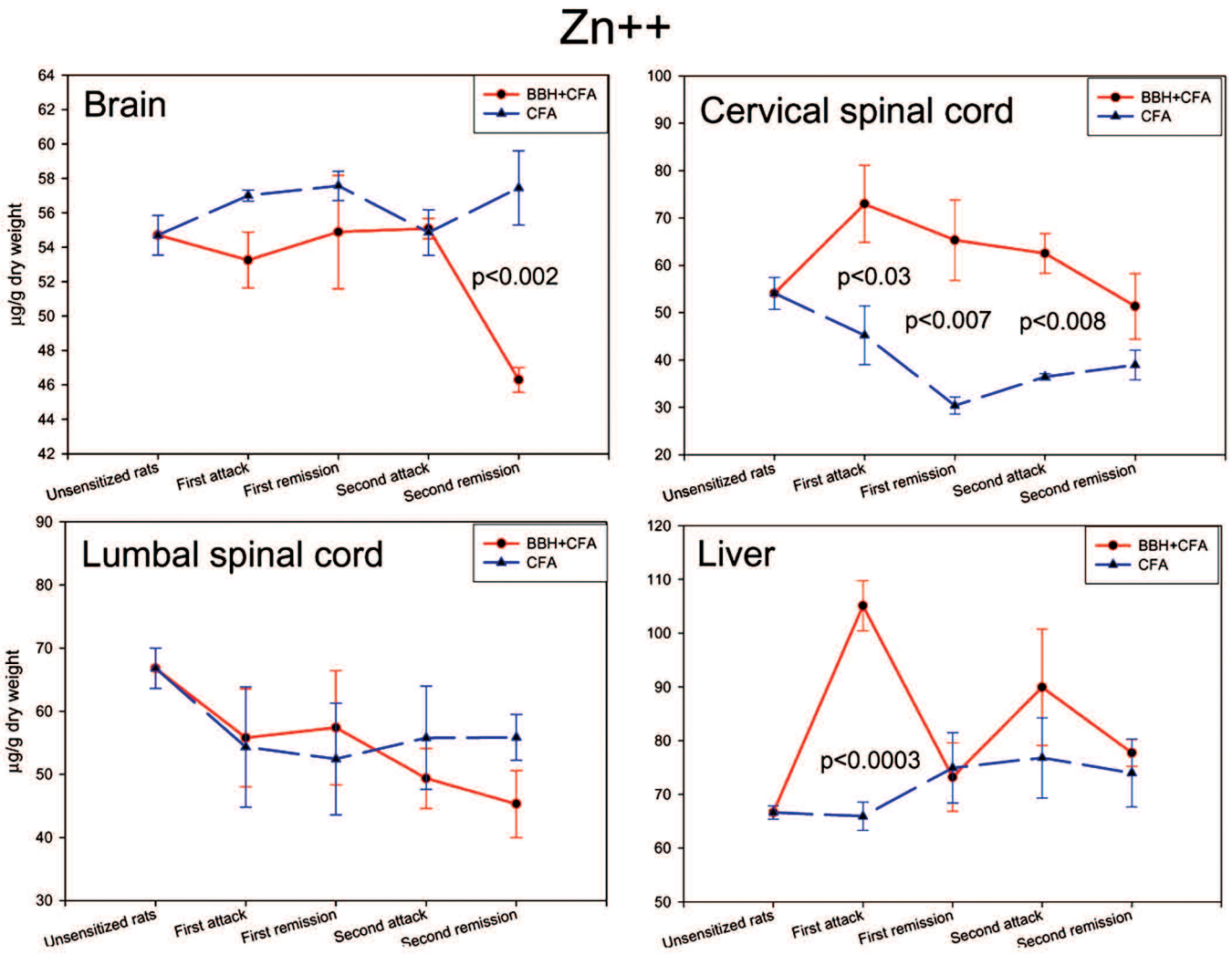

Fig. 7. Zinc content in the brain, cervical and lumbal spinal cord at various time points during the CR-EAE. The data are expressed as *µ*g/g of dry tissue weight. The values represent the mean ± SE of 3 rats.

contributed also to zinc and copper dyshomeostasis (Figs. 7, 8), since exposure of cells to oxidative stress or ROS might enhance dissociation of zinc from MTs, due to oxidation of thiols responsible for co-ordinate binding of zinc (Maret, 2006). Moreover, since copper can directly catalyze the production of hydroxyl radicals from hydrogen peroxide by Fenton or Haber–Weiss type reactions increasing the oxidative damage of macromolecules (Kozlowski et al., 2009), it could be speculated that during EAE the initial sequestration of copper in the liver and in the spinal cord (Fig. 8) contributed to a local release of zinc from MTs in these organs, enabling its use for numerous metalloproteins and enzymes that control cell growth and death (Kozlowski et al., 2009; Pedersen et al., 2009a; Sensi et al., 2009).

In this regard it could be speculated that zinc accumulated in the liver (Fig. 7) also affected the structure of α2-macroglobulin and its interaction with cytokines and proteases, the function of zinc-finger proteins that are required for DNA replication of immune cells and for the clonal expansion of lymphocytes in response to antigenic stimulation, as well as the numerous other aspects of innate and adaptive immunity (Prasad, 2007; Rink and Haase, 2007), which in the liver create a distinctive local immune environment responsible for the generation of an effective immune response and/or for the development of tolerance (Crispe, 2009). In this context, for the pathogenetic mechanisms of MS and EAE it might be particularly relevant that in the liver are unusually abundant MHC class-I-like molecule CD1 restricted NKT cells (Swain, 2008), which are able to react on immunogenic features of lipids released after

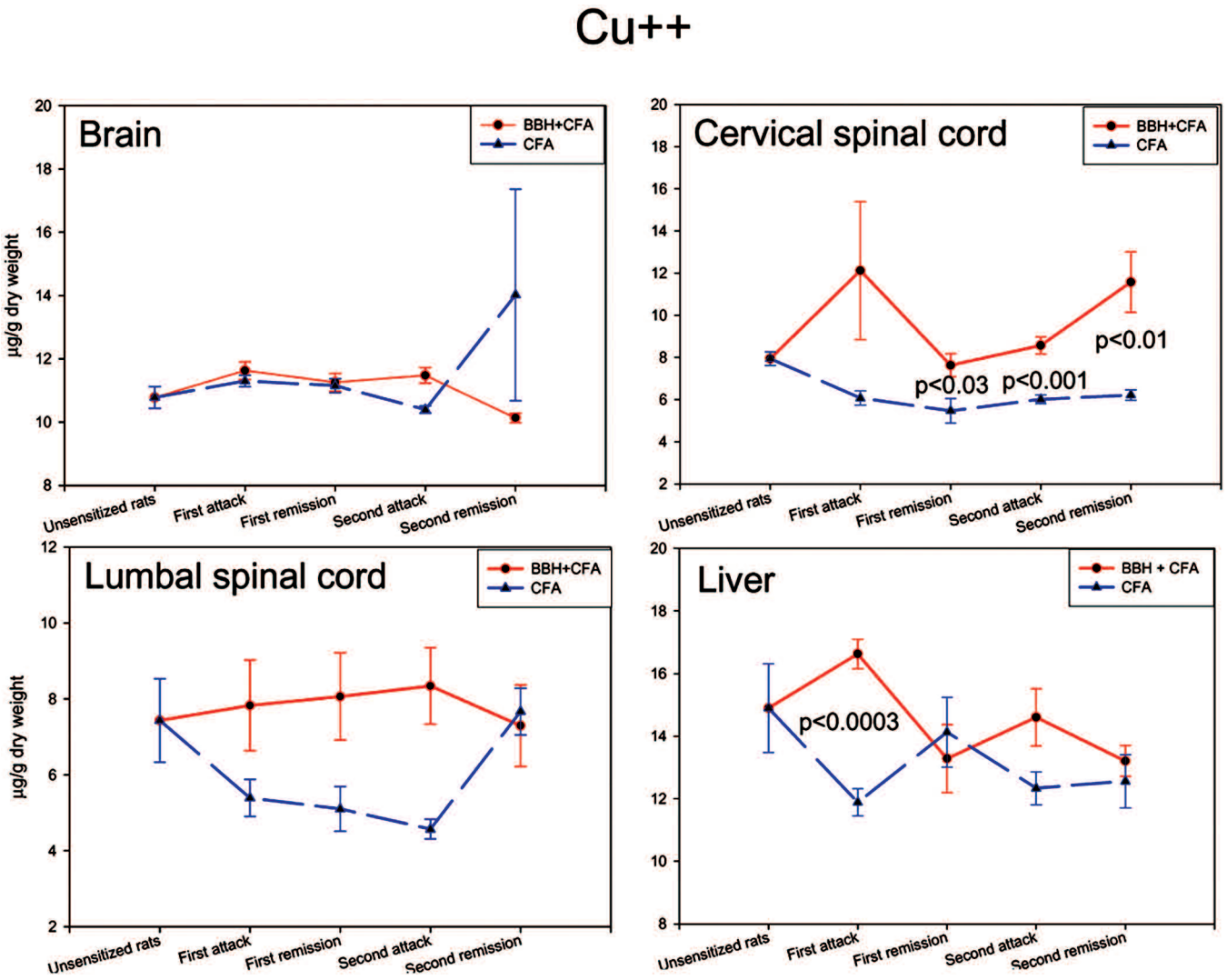

Fig. 8. Copper content in the brain, cervical and lumbal spinal cord at various time points during the CR-EAE. The data are expressed as µg/g of dry tissue weight. The values represent the mean ± SE of 3 rats.

demyelinization. Among them are glycolipids from myelin, such as a sphingolipid self-antigen isoglobotrihexosyl ceramide (iGB3) (Speak, 2007), as well as 3 sulfogalactosyl ceramide (sulfatide), which is one of the most abundant galactolipids in the axon-insulating myelin sheet of the CNS (Zajonc et al., 2005). Moreover, as cells which rapidly secrete large amounts of cytokines, NKT cells participate in regulation of Th1 or Th2 differentiation (Bendelac et al., 2007), in NK activation and Treg cells recruitment to the liver (Santodomingo-Garzon et al., 2009), having a critical role in a wide variety of immune responses, including those against autoantigens (Swain, 2008; Wu and Van Kaer, 2009). Furthermore, supporting the role of the liver in the development of EAE, recently it was shown that agonists of liver X receptors (Gabbi et al., 2009) were able to suppress the activation of primary glial cells and block the development of EAE, suppressing the induction of IL-21 and IL-22 mRNA in splenocyte and suppressing the activation of CD4+ Th17 cells (Xu et al., 2009). Moreover, the hypothesis about the close relationship of hepatic MTs and Zn with the NKT and NK cells is in high agreement with published evidence and a proposal that hepatic Zn sequestration might induce low zinc ion bioavailability for thymic and extrathymic immune efficiency (Mocchegiani et al., 2004, 2009; Jakovac et al., 2006), pointing to the importance of metallothionein/thionein balance for maintenance of the neuroendocrine–immune network (Mocchegiani and Malavolta, 2004).

The data are in high agreement with recent hypotheses, showing that MT I+II, as metallochaperons, participate in housekeeping processes, such as protection against ROS and energy production (regulating the catalytic activity of numerous metalloenzymes or stabilizing the conformation of zinc-dependent protein domains, such as zinc fingers, zinc clusters, and RING fingers, which are commonly found in transcriptional regulatory proteins. As described in recent reviews (Aschner, 1996; Aschner et al., 1998; Mocchegiani et al., 2005; Penkowa et al., 2006; Chung et al. 2008; West et al., 2008; Asmussen et al., 2009; Pedersen et al., 2009a) neuroprotective effects of MTs are based on a reduction of apoptotic cell death, activation of several astroglial, vascular and hematopoetic growth/trophic factors, as well as on their stem/progenitor and neurite growth promoting properties.

In conclusion, our data point to a link between the expression of MT I+II proteins and zinc and copper homeostasis, but also to the complexity of these interactions, pointing to the regional distribution of MT-immunoreactivity and significant time-related interplay of oxido-reductive mechanisms during CR-EAE, which through the processes of release and binding of free metals to MT-I+II might affect the local and systemic immune response in the brain and in the liver, as well as the neuronal synaptic transmission and plasticity. It is likely that during the attacks of CR-EAE the pro-inflammatory cytokines and nitro-oxidative stress initially trigger Zn^{2+} release from MT-I+II, and vice-versa that during the remissions the metals are more bound to MTs, since in normal conditions through thiolate bonds MT-I+II usually bind 7 divalent ions (Zn(II), Cd(II) and up to 12 monovalent copper ions (Hidalgo et al., 2001; Pedersen et al., 2009a), but complex interaction of zinc and copper with other buffering proteins, multiple Zn^{2+} transporters, Zn^{2+} importing proteins and other metals should be taken into account (Kozlowski et al., 2009; Sensi et al., 2009).

Taken together, we would like to emphasize that the expression of MT-I+II proteins in the brain and spinal cord and in the liver during CR-EAE is time related and followed by marked changes in the zinc and copper homeostasis, confirming that these stress and metal-binding proteins have an important regulatory role in the pathogenesis of demyelinating autoimmune diseases, and that they might be of diagnostic and therapeutic value in human diseases.

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