Trabajo 11

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Journal of Neurotrauma, En prensa, 2000

JOURNAL OF NEUROTRAUMA

The Zinc or copper deficiency-inducen impaired inflammatory response to brain trauma may be caused by the concomitant metallothionein changes

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ABSTRACT

The role of zinc and copper deficient diets on the inflammatory response to traumatic brain injury (TBI) has been evaluated in adult rats. As expected, zinc deficiency decreased food intake and body weight gain, and the latter effect was higher than that observed in pair-fed rats. In non injured brains, zinc deficiency only affected significantly lectin (increasing) and glial fibrillary acidic protein (GFAP) and Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (decreasing) immunoreactivities (irs). In injured brains, a profound gliosis was observed in the area surrounding the lesion, along with severe damage to neurons as indicated by neuron specific enolase (NSE) ir, and the number of cells undergoing apoptosis (measured by TUNEL) was dramatically increased. Zinc deficiency significantly altered brain response to TBI, potentiating the microgliosis and reducing the astrogliosis, while increasing the number of apoptotic cells. Metallothioneins (MTs) are important zinc and copper binding proteins in the CNS which could influence significantly the brain response to TBS because of their putative roles in metal homeostasis and antioxidant defenses. MT-I+II expression was dramatically increased by TBI, and this response was significantly blunted by zinc deficiency. The MT-III isoform was moderately increased by both TBI and zinc deficiency. TBI strongly increased oxidative stress levels, as demonstrated by malondialdehyde (MDA), protein tyrosine nitration (NITT) and nuclear factor κB (NF-κB) levels irs, all of which were potentiated by zinc deficiency. Further analysis revealed unbalanced expression of prooxidant and antioxidant proteins besides MT, since the levels of inducible nitric oxide synthase (iNOS) and Cu,Zn-SOD were increased and decreased, respectively, by zinc deficiency. All these effects were attributable to zinc deficiency, since pair-fed rats did not differ from normally fed rats. In general, copper deficiency caused a similar pattern of responses, albeit more moderate. Results obtained in mice with a null mutation for the MT-I+II isoforms strongly suggest that most of the effects observed in the rat brain after zinc and copper deficiencies are attributable to the concomitant changes in the MT expression.

Key words: Gliosis; apoptosis; TUNEL; oxidative stress; MDA; NITT; NF-κB; iNOS; Cu,Zn-SOD; MT-KO

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INTRODUCTION

Zinc and copper are essential metals for normal development and function of biological systems, including those of humans (Vallee, 1988; Cousins, 1985; Uauy *et al*., 1998; Keen *et al*., 1998; Vallee, 1993). These metals are essential for the normal function of the central nervous system, and have been related to several human neurodegenerative diseases, but much remains to be understood about their metabolism, roles and association with metal binding proteins (Frederickson *et al*., 1984; Nalbandyan, 1983; Cuajungco and Lees, 1997).

Metallothioneins (MTs) are important zinc and copper binding proteins (Hamer, 1986; Kagi and Schaffer, 1988; Bremner, 1987; Sewell *et al*., 1995) that could have an essential role in the metabolism of both metals in the normal and injured CNS. In rodents, there are four MT isoforms, MT-I to MT-IV (Palmiter *et al*., 1992; Quaife *et al*., 1994). In the CNS, MTs occur in the isoforms MT-I, MT-II and MT-III. MT-I+II are regulated coordinately by metals and hormones (Yagle and Palmiter, 1985), and are localized mainly in astrocytes, microglia, leptomeningeal cells, ependyma and choroid plexus epithelium (Penkowa and Moos, 1995; Penkowa *et al*., 1997; Masters *et al*., 1994b; Young *et al*., 1991). The regulation of MT-III differs substantially from that of MT-I+II and is poorly understood (Naruse *et al*., 1994; Imagawa *et al*., 1995; Palmiter *et al*., 1992).

The intracerebral expression of MT-I+II is clearly upregulated during pathological conditions induced by trauma (Penkowa and Moos, 1995; Penkowa *et al*., 1999a; Penkowa *et al*., 1999c), immobilization stress (Hidalgo *et al*., 1990), kainic acid-induced seizures (Zheng *et al*., 1995; Dalton *et al*., 1995), excitotoxic NMDA cortex damage (Hidalgo *et al*., 1997; Acarin *et al*., 1999b), and administration of 6-aminonicotinamide (Penkowa *et al*., 1997; Penkowa *et al*., 1999b). Furthermore, MT-I+II expression is increased in the myelin-deficient jimpy mouse (Vela *et al*., 1997) and in several human adult neurodegenerative disorders such as Alzheimer´s disease (AD) and Pick´s disease (Duguid et al., 1989; Nakajima and Suzuki, 1995), and amyotrophic lateral sclerosis (ALS) (Sillevis Smitt *et al*., 1992), as well as in aging (Suzuki *et al*., 1992) and after brain ischaemia (Neal *et al*., 1996). MT-III was discovered unexpectedly as a factor decreased in AD and initially was named growth inhibitory factor due to its inhibitory effect upon nerve cell growth/survival in vitro (Uchida *et al*., 1991). MT-III is also decreased in ALS, which is characterized by the loss of motorneurons (Uchida, 1994). However, the down-regulation of MT-III during AD has not been thoroughly confirmed (Erickson *et al*., 1994; Carrasco *et al*., 1999). A number of animal models have shown that MT-III mRNA and/or protein levels are significantly altered during CNS damage (Acarin *et al*., 1999a; Carrasco *et al*., 1999; Penkowa *et al*., 1999c; Penkowa *et al*., 1999b; Anezaki *et al*., 1995; Yuguchi *et al*., 1995a; Yuguchi *et al*., 1995b; Hozumi *et al*., 1995; Hozumi *et al*., 1996; Inuzuka *et al*., 1996). Taken together, these studies strongly suggest that MTs are important proteins in the brain for coping with the tissue damage caused by a wide array of factors and diseases. In a recent study using MT-I+II deficient mice (Penkowa *et al*., 1999a), we have indeed demonstrated that MT-I+II are essential for a normal wound healing and neuronal survival after traumatic brain injury (TBI). In accordance, overexpression of MT-I reduces the extent of tissue loss and vascular edema and improve functional outcome following focal cerebral ischemia (van Lookeren Campagne *et al*., 1999). It has also been demonstrated that MT-III is important for coping with kainic acid-induced neuronal damage in the CA3 hippocampal field (Erickson *et al*., 1997).

In the present report, we have characterized the CNS response to TBI in rats fed with zinc or copper deficient diets, and in normal and MT-I+II knock out mice. The results in rats strongly suggest that the altered glial responses and neuronal survival caused by the metal deficient diets are related to the concomitant changes of the MT levels. In support of this possibility were the results obtained in MT-I+II knock out mice. Thus, the present results indicate that zinc and copper deficient diets significantly compromise the capacity of the CNS to cope with injury, likely because of an unbalanced antioxidant status where MTs appear to have a major role.

MATERIALS AND METHODS

Rats.

Sprague-dawley male rats, weighing about 60 g and being 22 days old at the beginning of the experiment, were used. The rats were divided randomly in 5 groups, which were given a semisynthetic diet (Panlab SL, Barcelona, Spain) for 14 days consisting of either: a normal diet containing 43.3 mg Zn/kg and 6.5 mg Cu/kg (n=6); a zinc-deficient diet containing 1.9 mg Zn/kg (n=6); or a copper-deficient diet containing 0.8 mg Cu/kg (n=6). These rats were allowed to eat ad libitum. The timing and metal dosages have been shown previously to cause significant metal deficiencies (Gasull *et al*., 1994; Bremner *et al*., 1987; Cousins, 1985). The amount of food of each diet ingested by all rats was controlled every day. Additional pair-fed rats (eating normal diet) were also used for the rats fed with either the zinc- or the copper-deficient diets to

differentiate between the effects caused by the metal deficiency from those of the voluntary food restriction.

At the end of the feeding period, 3 rats from each group were lesioned as follows under tribromethanol anesthesia, while the rest served as controls. The skull over the right fronto-parietal cortex was exposed, and a focal cryo injury on the surface of the skull was produced during 60 sec with dry ice pellets (-78°C). This method is highly reproducible in both rats and mice (Penkowa and Moos, 1995; Carrasco *et al.*, 1999; Penkowa *et al.*, 1999c; Penkowa *et al.*, 1999a). The animals were housed in cages with free access to food (except otherwise stated) and water. Some of the rats were used for MT-III in situ hybridization analysis and were handled differently (see below).

Mice

129/SvJ and metallothionein-I+II knock-out (Masters *et al.*, 1994a) adult mice (n=3) were subjected to a cryolesion for 30 sec. These animals were fed only the normal diet.

Fixation

Lesioned rats and mice were killed 3 and 2 days post lesion (3 and 2dpl) respectively, along with the unlesioned animals. Rats and mice were deeply anesthetized with Brietal and cardially perfused with isotone saline plus heparine (0.9% NaCl added 3ml/l heparine 5000 IU/ml), followed by perfusion with Zamboni´s fixative for 5-10 min, pH 7.4. Afterwards the brains were dissected and immersions fixed in Zamboni´s fixative for 4h, pH 7.4, followed by dehydration in graded alcohols and xylol, before the brains were embedded in paraffine and cut in 10 µm coronal sections for immunohistochemistry, histochemistry and in situ detection of DNA fragmentation/TUNEL labeling.

Cellular counts

In addition to morphological analysis, cellular counts of all the variables analyzed were carried out from a 1 mm^2 area for statistical evaluation of the results. To this end, positively stained cells, defined as cells with staining of the soma, or in the case of NF-kB and TUNEL, cells with nuclear staining, were counted in the border of the lesion, where gliosis is prominent (see boxes in Fig. 1A,B). In unlesioned animals, countings were carried out in the corresponding cortex area.

For routine histological evaluation, toluidine blue stainings were made of all the used rats.

Histochemistry

Biotinylated tomato lectin from the *Lycopersicon esculentum* (Sigma, USA, code L9389) 1:500, was used as a marker for cells of the myelo-monocytic cell lineages, such as macrophages/microglia, as well as a marker for vessels. The lectin was developed using streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dakopatts, DK; code K377) prepared according to the manufacturer´s recommendations with further dilution 1:4 for 30 min at room temperature. The reaction product was visualized using 0.015% H_2O_2 in DAB/TBS, with DAB as a chromogen.

Immunohistochemistry

Sections were rehydrated in graded alcohols and incubated in 1.5% H_2O_2 (3 ml H_2O_2 in 200 destilled H_2O) to quench endogenous peroxidase, followed by incubation with pronase E (protease type XIV, Sigma No. P5147, 0.025 g dissolved in 50 ml TBS) for 10 min, pH 7.4, at 37°C. Afterwards, sections were boiled in citrate buffer, pH 9.1 or pH 6.0 for 10 min, followed by incubation in 10% goat serum in TBS/Nonidet (TBS: 0.05 M TRIS, pH 7.4, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet) for 20 min at room temperature. Afterwards, sections were incubated overnight with one of the following primary antibodies: polyclonal rabbit anti-cow GFAP 1:250 (Dakopatts, DK code Z 334) (as a marker for astrocytes); polyclonal rabbit anti-human NSE, 1:1000 (Callbiochem, USA, code PC237) (as a marker for neurons); polyclonal rabbit anti-rat MT-I+II 1:500 (Gasull *et al.*, 1993; Gasull *et al.*, 1994); polyclonal rabbit anti-rat MT-III 1:1000 (Carrasco *et al.*, 1999); polyclonal rabbit anti-nitrotyrosine (NITT) (as a marker for peroxynitrite-induced nitration of tyrosine residues) 1:100 (Alpha Diagnostic Int., USA, code NITT 12-A); polyclonal rabbit anti-malondialdehyde (MDA) (marking a byproduct of fatty acid peroxidation) 1:100 (Alpha Diagnostic Int., USA, code MDA 11-S); monoclonal mouse anti-human NF-κB 1:100 (Boehringer Mannheim, code 1697838); polyclonal rabbit anti-mouse iNOS 1:100 (Biomol Res. Lab., USA, code SA200); monoclonal mouse anti-human Cu/Zn-

SOD 1:50 (Sigma, USA, code S2147); The primary antibodies were detected using biotin-conjugated mouse anti-rabbit IgG (Sigma, USA, code B3275) 1:400 or biotin-conjugated goat anti-mouse IgG (Sigma, USA, code B8774) 1:200 for 30 min at room temperature. These secondary antibodies were detected by StreptABComplex/HRP and visualized by using DAB.

In order to evaluate the extent of non-specific binding of the antisera in the immunohistochemical experiments, 1:100 – 1:1000 of normal rabbit or mouse serum or rat serum or just the preincubation agent was substituted for the primary antibody step described above. Results were considered only if these controls were negative.

In situ detection of DNA fragmentation

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labelling (TUNEL) staining was performed after tissue processing as mentioned above. Sections were deparaffinized and incubated with 20 mg/ml proteinase K (Sigma, St. Louis, MO) for 5 min to strip off nuclear proteins. TUNEL was accomplished using the Apoptag Plus, In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD, Code S7101-KIT). After immersion in equilibration buffer for 10 min, sections were incubated with TdT and dUTP-digoxigenin in a humified chamber at 37°C for 1 hr and then incubated in the stop/wash buffer at 37°C for 30 min to stop the reaction. After washing in PBS buffer, the sections were incubated in antidigoxigenin-peroxidase solution for 30 min. Afterwards, DAB was used as chromogen, and the sections were counterstained with methyl-green. Negative control sections were treated similarly but incubated in the absence of TdT enzyme, dUTP-digoxigenin, or anti-digoxigenin antibody. We also compared our sections with positive control slides from Oncor (code S7115). Furthermore, we evaluated morphologic criteria for apoptosis too, since the TUNEL is known to be able to stain necrotic cells also.

Double and triple TUNEL-immunofluorescence histochemistry

To detect the type of cells undergoing apoptosis, sections were first incubated with ApopTag In Situ Apoptosis Detection Kit (TUNEL) linked with fluorescein (Oncor, USA, code S7110-KIT) prepared following manufacturer´s recommendations. Afterwards, sections were incubated overnight with either monoclonal mouse anti-human neurofilament protein (NF) 1:250 (Dakopatts, DK, code M762) or with both Texas Red labeled tomato lectin from the *Lycopersicon esculentum* 1:50 (Sigma, USA, code L-9139) and polyclonal rabbit anti-cow GFAP 1:250 (Dakopatts, DK, code Z 334) simultaneously. Anti-NF antibodies were detected by using goat anti-mouse IgG linked with Texas Red 1:50 (Southern Biotechnology Ass., Inc., USA, code 1030-07), and anti-GFAP antibodies were detected by using goat anti-rabbit IgG linked with aminomethylcoumarin (AMCA) 1:20 (Dakopatts, DK, code W0478) for 30 min at room temperature. The sections were embedded in 20 ml fluorescent mounting (Dakopatts, DK, code S3023) and kept in darkness at 4°C.

MT-I+II radioimmunoassay

Liver MT-I+II levels were measured by radioimmunoassay as previously described (Gasull *et al.*, 1993). Briefly, livers were homogenized in a Potter-Elvehjem with ice-cold 10 mM Tris-HCl, pH 8.2, containing 250 mM sucrose, 10 mM sodium azide, 10 mM 2-MSH and 0.1 mM phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 50000g for 20 min at 4°C and the supernatant was stored at -20°C until assay. All samples to be directly compared were processed simultaneoulsy. The antibody cross-reacts with MT-I and MT-II but not with MT-III (Gasull *et al.*, 1994).

MT-III in situ hybridization

In situ hybridization for MT-III mRNA was performed on additional, unlesioned rats (n=3-4) which were killed by cervical dislocation and the brains immediately frozen in liquid nitrogen and stored at -80°C. In order to avoid cross-hybridization with MT-I and MT-II mRNAs, we have used a specific DNA fragment of 153 bp that contains the coding regions from the terminal 15 amino acids and the 3' untranslated regions until the poly G stretch of MT-III mRNA (generously provided by Dr. G.K. Andrews, Dept. Biochemistry, Kansas City, KS, USA). The MT-III cDNA was labeled with (^{35}S) α–UTP by in vitro transcription.

Preparation of sense and antisense probes and the in situ hybridization procedure were performed as previously described (Carrasco *et al.*, 1998; Hernández *et al.*, 1997). Autoradiography was performed exposing the film (Hyperfilm-MP, Amersham, UK) to the slides for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film.

Cytosolic zinc and copper levels

Zinc and copper levels measured by atomic absorption spectrophotometry. Brains were homogenized with PBS using a Politron, and the homogenate was centrifuged at 50000g for 20 min at 4°C and the supernatant was stored at -20°C until assay. All samples to be directly compared were processed simultaneoulsy.

Statistical analysis

Results were evaluated by two-way analysis of variance (ANOVA), with lesion and type of diet as main factors. When the effect of the lesion was significant, one-way ANOVA followed by post-hoc comparisons of the means were carried out for both unlesioned and lesioned rats. In the mice experiment, the Student "t" test was used.

RESULTS

Effect of the diets on food consumption and body weight gain

The rats fed either Zn or Cu deficient diets were clinically unaffected, as determined from their ability to walk and run. However, the rats fed with the Zn deficient diet showed a significant and continuous decrease in their food consumption from day 5 on the diet and throughout the whole dietary period, while the Cu deficient diet did not affect food consumption (Table 1). Consequently, a pair-fed group for the zinc deficient diet group was established which was given the equivalent amount of food eaten by the zinc deficient rats but with normal zinc levels.

As could be expected from the food consumption, the rats fed with the zinc deficient diet showed a decreased body weight gain (Table 1). This decrease was more severe than that of the pair-fed rats, indicating that zinc deficiency causes specific effects on growth that can not be explained by the reduced food consumption it causes. The copper deficient diet did not affect body weight gain significantly. Brain zinc levels were significantly decreased by the zinc-deficient diet, whereas copper levels tended to be decreased by the copper-deficient diet (Table 1).

Following the cryolesion, rats were able to walk and eat as the unlesioned control rats. By gross examination of the brain lesion, a focal hemorrhagic injury was seen on the right cerebral hemisphere. In toluidine blue stained sections the freeze lesion was seen as a necrotic area without neuronal cells. Instead, numerous small mononuclear cells were observed inside of the lesion. On the contralateral side, the parenchyma was unaffected.

Effect of the diets on glial responses and neuronal affectation

The histological examination of the brains of the zinc pair-fed rats revealed that their responses were not different from those of normally fed rats. Thus, the results of the zinc pair-fed rats in the brains are not shown. In contrast, these rats did differ from control rats regarding the liver results and those results are therefore shown (see below).

In unlesioned rats the observed microglial cells were ramified, while round monocytic macrophages were virtually absent as determined from histochemical lectin stainings (not shown). Following the lesion, a dramatic increase in the number of lectin+ round and amoeboid macrophages was seen at the lesion site of all the examined rats (Figs. 1 and 3). In normally fed rats, the lesion zone was filled with round hypertrophic macrophages, while in the parenchyme surrounding the lesion both round and amoeboid macrophages were seen (Fig. 1A,C). In zinc deficient rats, the number of round hypertrophic macrophages encircling the lesion was further increased (Figs. 1B,D and 3). However, macrophages were almost absent from the lesion center and thus remained at the circumference of the lesion. Additionally, in the parenchyme below the injured area, numerous lectin+ macrophages were observed. Thus, both the number and distribution of activated macrophages in lesioned zinc deficient rats were different from those of normally fed rats. In copper deficient rats, the macrophage response was comparable to that of normally fed rats (Fig. 3).

In unlesioned rats stellate astrocytes of grey and white matter were equally distributed in normally fed and dietary zinc and copper restricted rats, as determined by using GFAP immunoreactivity (not shown). However, a small decrease of the GFAP+ cells was observed in the cortex of the zinc deficient rats (Fig. 3). Following the lesion, all rats exhibited reactive astrocytosis (Figs. 1 and 3). The reactive astrocytes showed hypertrophy with thickening and retraction of cell processes in normally fed rats (Fig.1E). Zinc deficiency resulted in a significantly decreased astrogliosis around the lesion site, and the majority of cells had long thin processes (Figs. 1F and 3). Again, the response of the rats fed a

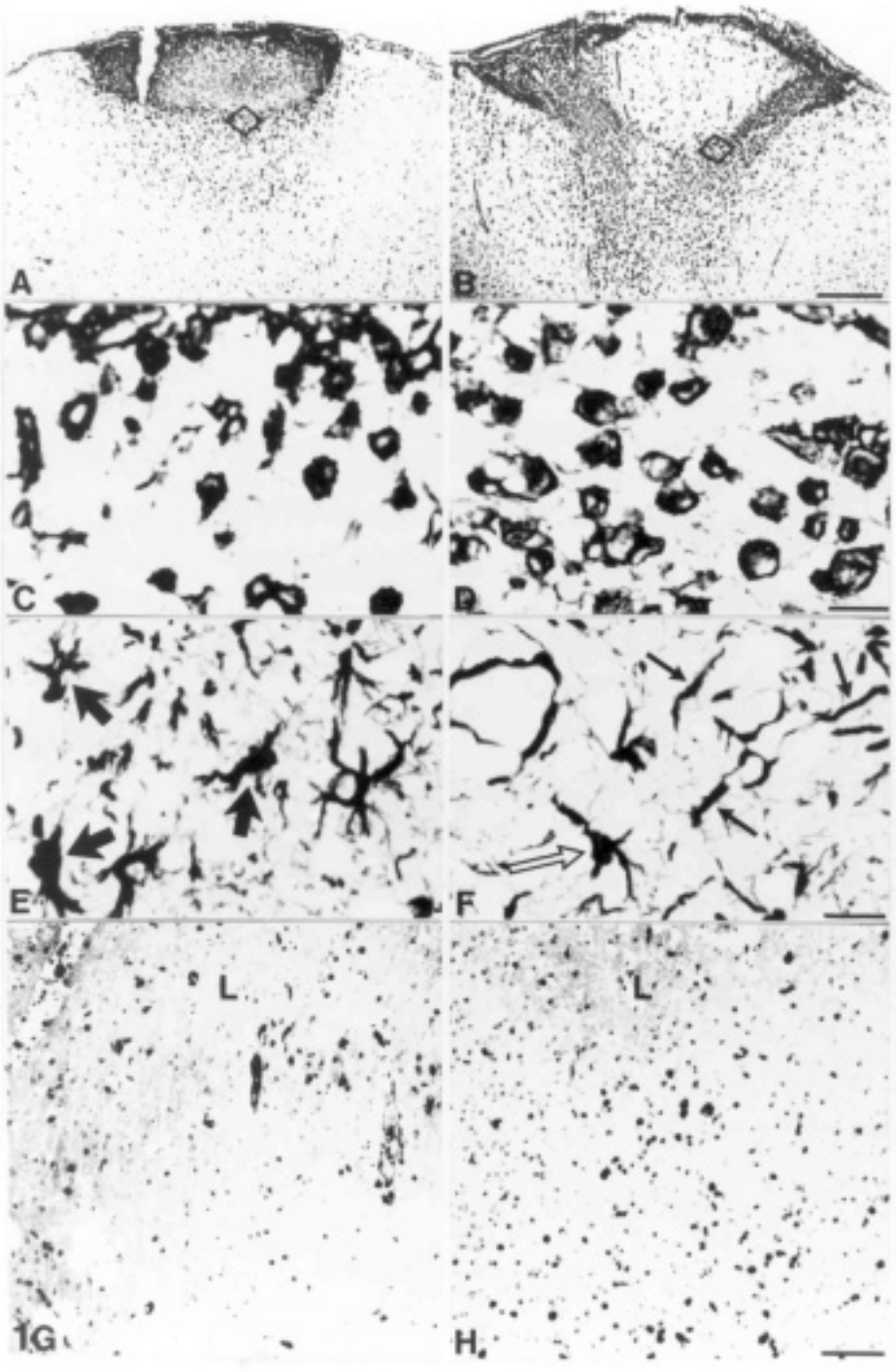


Fig. 1. Lectin, GFAP and TUNEL stainings following the lesion. **A:** In lesioned normally fed rats, activated microglia and brain macrophages appear at the lesion site. **B**: In lesioned zinc deficient rats, activated microglia and brain macrophages were seen around the lesion. Inside of the lesion some brain macrophages also appeared. However, macrophages remained at the borderline of the injured area in all sections examined. **C:** Higher magnification of the rectangle in A, showing many round and amoeboid brain macrophages at the border of the lesioned necrotic area. **D:** Higher magnification of the rectangle in B, showing numerous round or amoeboid macrophages. **E:** Normal rats display GFAP+ reactive astrocytes around the lesion (arrows). **F:** Zinc deficient rats also show GFAP+ reactive astrocytes following the lesion (arrow). However, the number and size of the astrocytes are decreased compared to that of normal rats (small arrows). **G:** TUNEL staining of normally fed rats showing apoptotic cells at the lesion site (L). **H:** TUNEL staining of zinc deficient rats showing an increased number of apoptotic cells compared to that of normal rats. Cellular counts were carried out in several animals for statistical purposes (see Fig. 3).
Scale bars: A,B: 355 μm; C-H: 22 μm.

Table 1: Effect of zinc and copper deficient diets on some physiological variables.

	Body weight gain (g/12 days)	Food consumption (g/rat/day 12)	Liver MT (µg/g)	Brain Zinc (µg/g)	Brain Copper (µg/g)
Control	72.0 ± 5.9	28.4 ± 1.4	2.10 ± 0.13	9.20 ± 0.25	2.92 ± 0.66
Zn-deficient	26.5 ± 3.0*°∂	15.9 ± 1.1*	1.40 ± 0.24*°∂	7.65 ± 0.15*	1.72 ± 0.18
Cu-deficient	68.3 ± 9.8	29.7 ± 1.1	2.14 ± 0.10	8.70 ± 0.17	1.44 ± 0.21
Zn pair-fed	54.1 ± 2.9*	(15.9)	5.88 ± 1.09*	8.60 ± 0.53	2.52 ± 1.46

Results are mean ± SEM. Zn pair-fed rats were always given the amount of food consumed by the zinc deficient rats, but of a normal diet. * $p<0.05$ vs control rats. °∂$p<0.05$ vs pair-fed rats

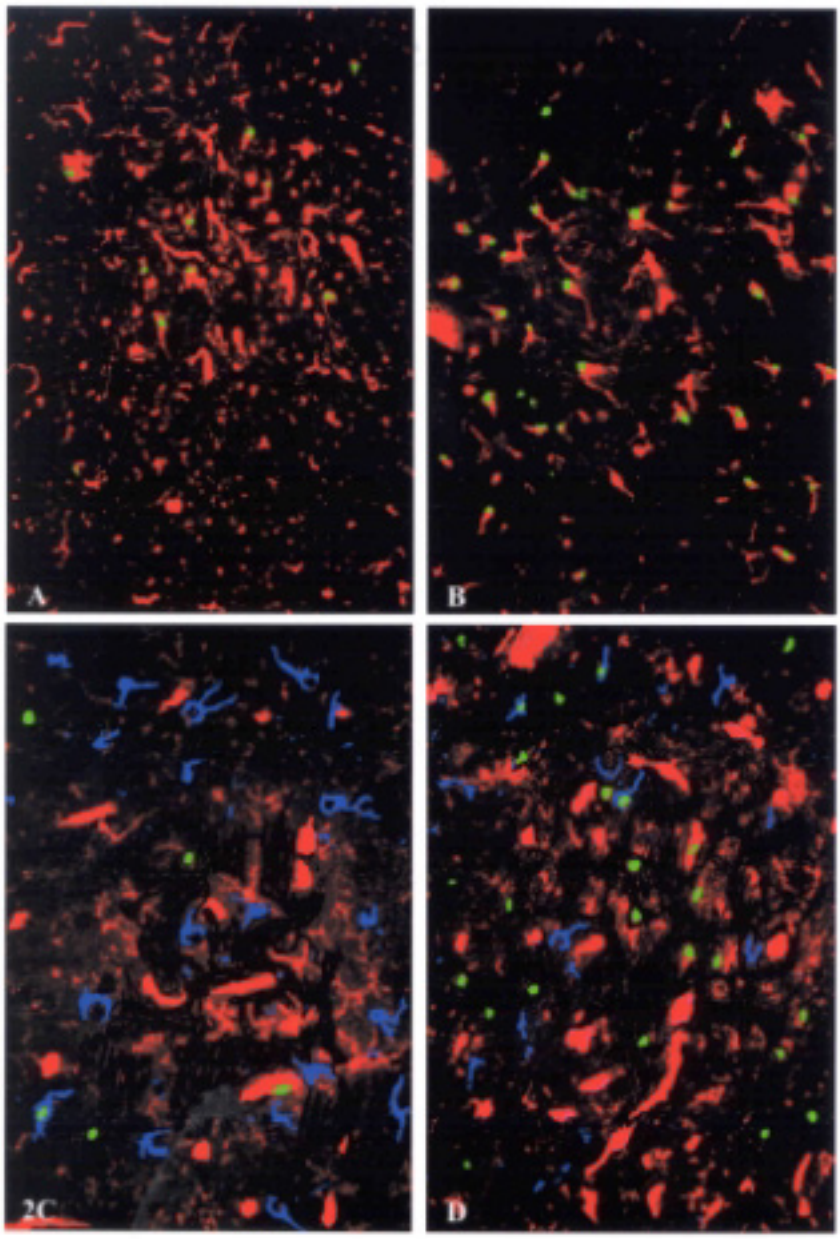


Fig. 2. Double immunofluorescent labeling of TUNEL (green) and NSE (red) in normally fed (A) and zinc deficient rats (B) after lesioning. Triple immunofluorescent labeling of TUNEL (green), lectin+ macrophages (red) and GFAP+ astrocytes (blue) in normally fed (C) and zinc deficient (D) rats. Results shown correspond to the borderline of the injured area.

copper deficient diet was comparable to that of normally fed rats (Fig. 3).

Neurons were detected thoroughly in the parenchyme by NSE immunostaining, and no apparent differences in shape and general morphology between the different groups of unlesioned rats were observed (not shown). In lesioned rats and in line with previous studies (Penkowa *et al*., 1999c; Penkowa *et al*., 1999a), a dramatic decrease of the number of NSE-positive cells was observed in the border of the lesion (Fig. 3). Neither zinc nor copper deficiencies changed that effect at 3 days post-lesion.

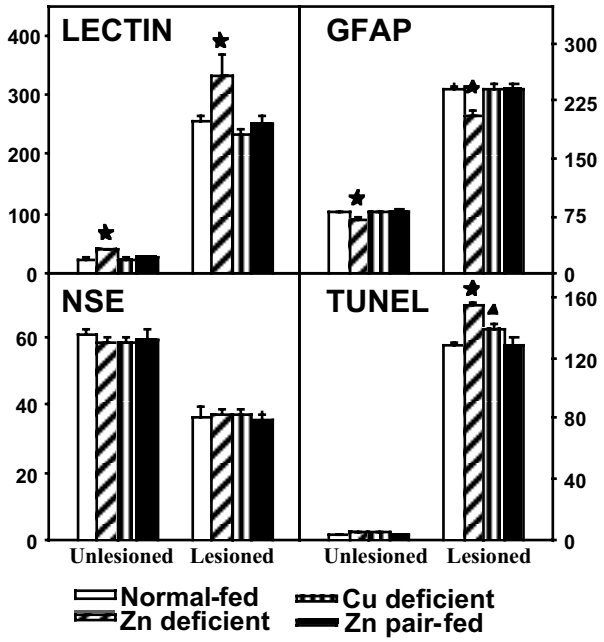


Fig. 3. Immunohistochemical cell countings of lectin, GFAP, NSE and TUNEL in the brain cortex of normally fed rats and rats fed zinc and copper deficient diets (cells/mm^2). Counting was carried out in the border of the lesion where prominent glial response occurs (see boxes of Fig. 1A,B for localization) or in the normal cortex in unlesioned rats. Cellular countings shown are mean ±SE (n=3 independent rats per group). The lesion affected significantly ($p<0.001$) all the variables analyzed. Separate one-way ANOVA followed by post-hoc comparison of the means were the carried out for unlesioned and lesioned rats. * denotes a significant effect ($p<0.05$) of the zinc deficient diet versus the corresponding normally fed rats. °∂ denotes a significant effect ($p<0.05$) of the copper deficient versus the corresponding normally fed rats.

The putative additional damage caused by the metal deficient diets during TBI was further examined by analyzing the number of cells undergoing apoptosis (as determined by using in situ nick end labeling (TUNEL)). While no differences were observed between the unlesioned rats, a dramatic increase of the number of cells engaged in apoptosis was observed after TBI, and both zinc and copper (to a lower extent) deficiencies potentiated that effect (Figs. 1G,H and 2 and 3). Since TUNEL+ cells may be apoptotic as well as necrotic, care was taken to count TUNEL+ cells which also fulfilled the morphologic criteria for apoptosis, namely compaction of chromatin into uniformly dense masses, cell shrinkage and formation of apoptotic bodies. TUNEL results were confirmed with other apoptosis-related variables such as ssDNA, ICE and caspase-3 (not shown).

By using double and triple immunofluorescence histochemistry, we found that the TUNEL+ cells were neurons, astrocytes and microglia/macrophages situated around the lesion (Fig. 2). In zinc deficient rats, the number of TUNEL+ cells was increased for neurons (Fig. 2A,B), but astrocytes and microglia/macrophages also displayed a higher apoptotic signal (Fig. 2C,D).

The metal deficient diets alter the response of the metallothionein isoforms to TBI

In the brain of unlesioned rats, the expression of MT-I+II was confined to ependymal cells, meninges, glia limitans and some grey matter astrocytes, and no significant effect of the diets was noticed, although the zinc deficient diet tended to decrease the number of MT-I+II positive cells. Following the lesion, normal fed rats increased MT-I+II expression in activated macrophages and reactive astrocytes widely distributed at the basis of the lesion (Figs. 4 and 5). In lesioned zinc deficient rats, the expression of MT-I+II was mildly upregulated compared to normally fed rats (Figs 4B and 5). MT-I+II expression of macrophages and reactive astrocytes situated around the injury in copper deficient rats was only slightly lower than that of normally fed rats (Fig. 5). As expected (Gasull *et al*., 1994; Bremner *et al*., 1987), liver MT-I+II levels were decreased by zinc deficiency, especially when compared with the pair-fed rats, which show a clear upregulation compared to normally fed rats (Table 1). This is in contrast to the brain, since no effect of food restriction on MT-I+II levels was observed (not shown).

In the brain of unlesioned rats, the expression of MT-III was confined to ependymal cells, meninges, glia limitans, perivascular cells and some astrocytes scattered in cortex. Following the lesion the immunoreactivity of MT-III was increased in all rats examined (Figures. 4

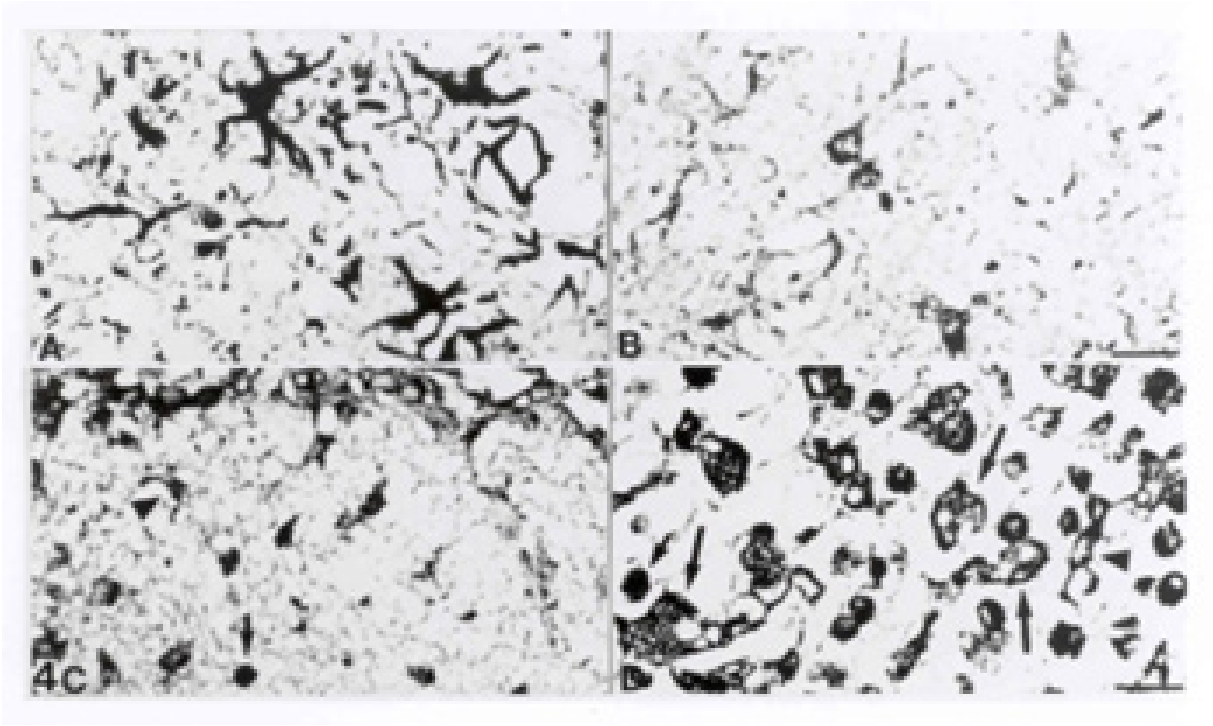


Fig. 4. MT-I+II and MT-III immunostainings of freeze-lesioned normally fed and zinc deficient rats. **A:** MT-I+II immunoreactivity in the parenchyma surrounding the lesion site of normal rats. **B:** MT-I+II immunoreactivity in the parenchyma surrounding the lesion site of zinc deficient rats, showing a decreased number of MT-I+II expressing cells, which are also decreased in size compared to those of normally fed rats. **C:** MT-III expression was mildly increased around and inside of the lesion of normally fed rats. **D:** Zinc deficient rats increased the MT-III immunoreactivity post-lesional when compared to that of normally fed rats. Numerous round macrophages and astrocytes surrounding the injury were expressing MT-III (arrows), and the distribution of MT-III positive cells matched that of lectin positive cells. Also inside of the lesion, some round macrophages were expressing MT-III.
Scale bars: A,B: 25 μm. C,D: 22 μm

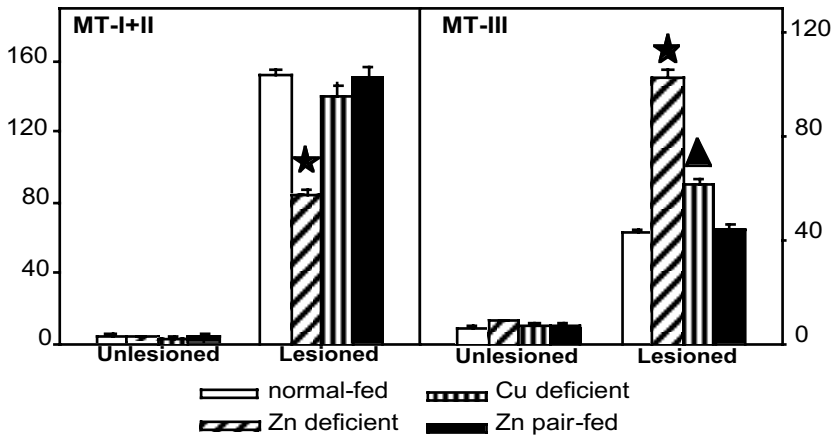


Fig. 5. Immunohistochemical cell counts of MT-I+II and MT-III. Counting was carried out as in Fig. 3. Results are mean ± SE (n=3 independent rats per group). The lesion affected significantly ($p<0.001$) all the variables analyzed. Separate one-way ANOVA followed by post-hoc comparison of the means were the carried out for unlesioned and lesioned rats. * denotes a significant effect ($p<0.05$) of the zinc deficient diet versus the corresponding normally fed rats. °∂denotes a significant effect ($p<0.05$) of the copper deficient versus the corresponding normally fed rats.

and 5). Normal fed rats upregulated their MT-III expression in round and amoeboid macrophages around and inside of the lesion (Fig. 4C). Some reactive astrocytes were also MT-III+. In lesioned zinc deficient rats, the MT-III expression was significantly increased compared to that of normally fed rats (Figs. 4D and 5). Copper deficient rats displayed a similar albeit more moderate response to TBI

An in situ hybridization analysis for MT-III mRNA was carried out in unlesioned rats (see Fig. 6 for a representative result). Quantitative measurements were carried out in specific areas of the autoradiographies, and no major effects were observed of the zinc or copper deficient diets albeit both tended to increase MT-III mRNA levels in the cortex (3295 ± 273, 3858 ± 553, 4127 ± 503 and 3191 ± 263, mean ± SE, arbitrary units, for normally fed, zinc deficient, copper deficient and zinc pair-fed rats, respectively).

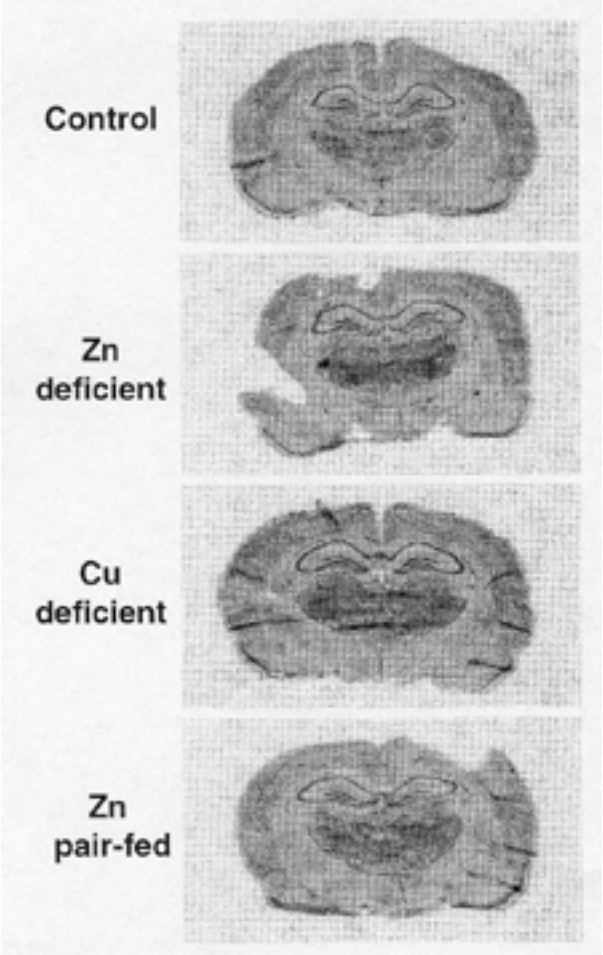


Fig. 6. Representative in situ hybridization of the MT-III isoform. The MT-III signal was prominent in the CA1-CA3 hippocampal areas, as expected. Semiquantitative measurements of specific brain areas carried out in several animals per group (n=3-4) did not reveal significant effects of the zinc and copper deficient diets on MT-III mRNA levels.

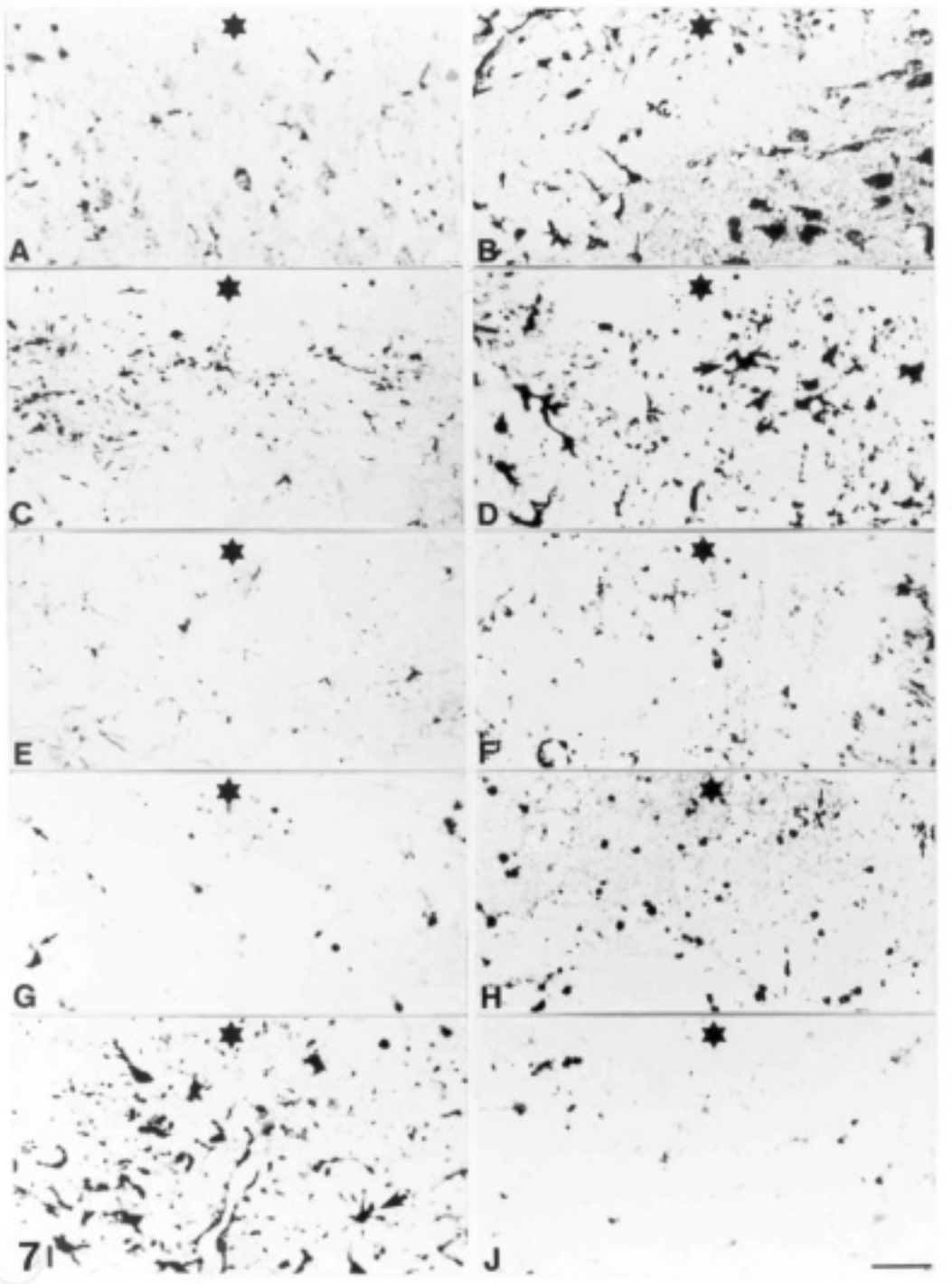
Zinc and copper deficiencies lead to an unbalanced oxidative stress status in the brain

MT-I+II are significant antioxidant proteins (see Discussion) and thus the down-regulation caused by the metal deficient diets could led to increased oxidative stress, which, in turn, could contribute significantly to the increased neuronal death above described. To verify such a possibility, we examined the oxidative stress status of the rats by analyzing a number of variables sensitive to the oxidative stress of the cells, namely MDA, NITT and NF-κB. A dramatic increase of all of them was observed in the border of the lesion, which was further potentiated by zinc and copper deficiencies (Figs. 7 and 8). NITT and MDA were increased in macrophages, astrocytes and neurons, while NF-κB immunoreactivity moved to the nucleus and was increased in macrophages/microglia, astrocytes, vascular endothelium, perivascular cells and neurons.

Besides MT-I+II, other factors could contribute to the observed increased oxidative stress, such as increased prooxidant enzymes and/or decreased antioxidant enzymes. We have evaluated iNOS (prooxidant) and Cu,Zn-SOD (antioxidant) levels and have found that they were significantly increased and decreased, respectively, by the metal deficient diets (Figs. 7 and 8). Therefore, decreased MT-I+II and Cu,Zn-SOD and increased iNOS levels will likely contribute to the increased oxidative stress caused by zinc and copper deficiencies in the TBI paradigm in rats.

MT-I+II deficiency is a major factor contributing to CNS damage during TBI

To ascertain more thoroughly the role of MT-I+II during TBI, we carried out an experiment with normal and MT-I+II KO mice and measured all variables which were used in the rat experiments (Fig. 9). The results obtained were remarkably similar to those observed in rats fed a copper and especially a zinc deficient diet: (a) an increased microgliosis and decreased astrogliosis and an increased neuronal apoptosis rate; and (b) an increased oxidative stress. The only variable which differed significantly from the rat results were the Cu,Zn-SOD levels, which were increased in the MT-I+II KO mice while a decrease was observed in the zinc and copper-deficient rats. This discrepancy is likely related to the fact that zinc and copper are essential components of the Cu,Zn-SOD protein; thus, in metal deficiency conditions, a decrease in the immunoreactivity can be expected (Olanow, 1993). This metal deficiency



Figs 7.
Oxidative stress markers in normal and zinc deficient rats around the lesion (asterix). **A:** MDA levels around the lesion were mildly increased in normally fed rats. **B:** MDA levels around the lesion were increased in zinc deficient rats compared to those of normally fed rats. **C:** NITT levels were increased around the lesion in normally fed rats. **D:** NITT levels were clearly higher around the lesion of zinc deficient rats compared to those of normally fed rats. Primarily astrocytes (arrow) and neurons, but also some macrophages, were expressing the excess NITT. **E:** NF-κB expression translocated from the cytoplasm to the nucleus in many cells around the lesion of normally fed rats. However, some cells still showed NF-κB in the cytoplasm (arrows). **F:** The number of cells showing NF-κB in the nucleus was increased the most in zinc deficient rats. However, some cells showed cytoplasmic NF-κB (arrows). **G:** iNOS expression was slightly increased at the lesion of normally fed rats. **H:** iNOS expression around the lesion of zinc deficient rats was significantly increased in macrophages, reactive astrocytes and neurons. **I:** Cu,Zn-SOD expression was increased around and inside of the lesion of normally fed rats. **J:** Cu,Zn-SOD expression was almost absent around the lesion of zinc deficient rats.
Scale bars: A-J: 44 μm.

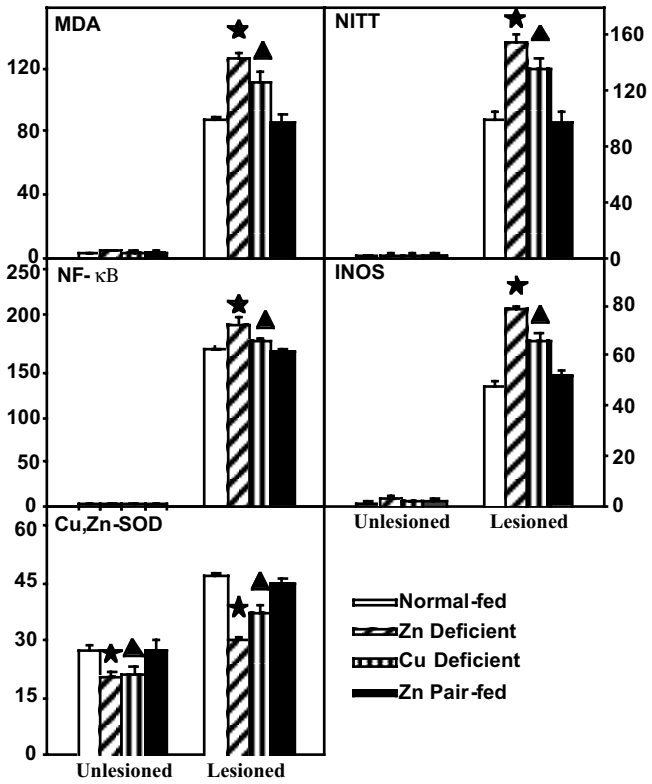


Fig. 8. Immunohistochemical cell countings of oxidative stress markers. Counting was carried out as in Fig. 3. Results are mean ± SE (n=3 independent rats per group). The lesion affected significantly ($p<0.001$) all the variables analyzed. Separate one-way ANOVA followed by post-hoc comparison of the means were the carried out for unlesioned and lesioned rats. * denotes a significant effect ($p<0.05$) of the zinc deficient diet versus the corresponding normally fed rats. °∂denotes a significant effect ($p<0.05$) of the copper deficient versus the corresponding normally fed rats.

is not expected in the MT-I+II KO mice (Penkowa *et al.*, 1999b), and thus the increased Cu,Zn-SOD levels they show could be viewed as an attempt to overcome the increased oxidative stress caused by the MT-I+II deficiency. These results strongly suggest that the MT-I+II deficiency observed in the rat experiment is a major factor contributing to CNS damage during TBI.

DISCUSSION

Zinc and copper are essential for brain physiology. In this report we have examined the effect of feeding zinc and copper deficient diets to rats on the normal brain and on the inflammatory response in the CNS following a traumatic brain injury. In the normal brain, the zinc and copper deficient diets employed decreased zinc and copper total cytosolic levels. However, this decrease did not affect substantially either the morphology or the physiology of the glial cells, although zinc deficiency increased the number of lectin positive cells and decreased that of GFAP positive cells in the cortex. Also, neurons appeared normal as determined by NSE immunoreactivity, and the number of cells engaged in apoptosis was small regardless of the diet used. As could be expected (Gasull *et al.*, 1994; Hidalgo *et al.*, 1994; Ebadi and Wallwork, 1985), the number of brain MT-I+II positive cells tended to decrease in zinc deficient rats and, in contrast to the liver, food restriction had no effect. The potent antioxidant enzyme Cu,Zn-SOD (Olanow, 1993) was decreased by both zinc and copper deficiencies. Since MT-I+II are also significant antioxidant proteins (Sato and Bremner, 1993), the combined Cu,Zn-SOD and MT-I+II deficiencies might render the CNS somewhat compromised against oxidative stress. Indeed, that could contribute to the increased microglia observed in zinc deficient rats. However, MDA and NITT levels were similar in all unlesioned rats, and thus the CNS was not particularly affected by the metal-deficient diets in normal conditions.

More significant effects of the zinc and copper deficiencies were observed during the inflammatory response elicited by a cryolesion of the cortex. Injury to the CNS induces a characteristic inflammatory response orchestrated by resident microglia, invading bone marrow-derived monocytes and astrocytes (Mattson and Scheff, 1994; Stichel and Verner Müller, 1998; Amat *et al.*, 1996; Ridet *et al.*, 1997). In agreement with previous results in rats and mice (Penkowa and Moos, 1995; Penkowa *et al.*, 1999c; Carrasco *et al.*, 1999; Penkowa *et al.*, 1999a), the cryolesion carried out to normally fed rats elicited numerous round or amoeboid macrophages around and inside of the lesion.

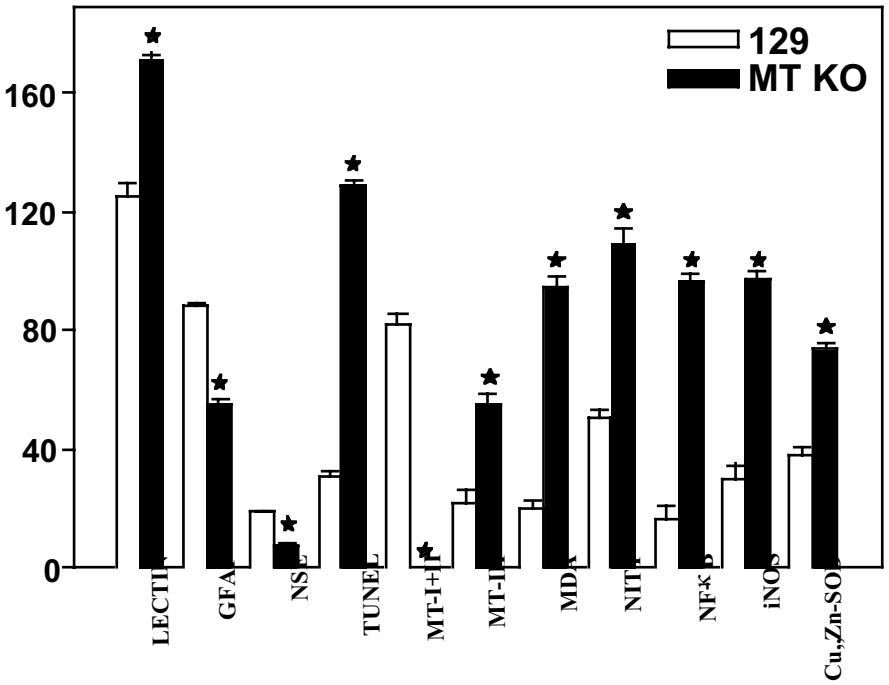


Fig. 9. Immunohistochemical cell countings carried out in 129/SvJ and MT-I+II null mice. Three animals per strain were subjected to a cryolesion and killed two days postlesion. All variables shown were analyzed as described for rats. Results were analyzed with the Student "t" test. * denotes a significant effect ($p<0.05$) of MT-I+II deficiency.

Reactive astrocytosis was apparent 3 days postlesional, and the number of NSE positive neurons was significantly decreased. Moreover, the number of cells engaged in apoptosis was dramatically increased, and the results suggest that neurons, astrocytes and microglia/macrophages all were affected to various degrees. This CNS damage scenario is well known, and the evidence that increased oxidative stress and an excess metal ions due to disruption of the blood-brain barrier and/or release from dying cells are involved in such scenario is compelling (McIntosh *et al.*, 1998; Mattson and Scheff, 1994; Choi and Koh, 1998; Coyle and Puttfarcken, 1993; Cuajungco and Lees, 1997; Bains and Shaw, 1997). In accordance, we found clear evidence that the cryolesion increased the oxidative stress in the damaged areas, with dramatic increases of MDA (reflecting lipid peroxidation) and NITT (reflecting protein tyrosine nitration, which, in turn, reflects increased nitric oxide and/or superoxide) levels, as well as of NF-κB, a major oxidative stress-responsive transcription factor in eukaryotic cells (Schreck *et al.*, 1992)

In this report, we are proposing that the MT family of proteins play a major role for a normal response of the CNS to TBI due to their antioxidant properties (Sato and Bremner, 1993). First, a dramatic upregulation of MT-I+II was observed in the damaged areas caused by the cryolesion, presumably for protective roles. Second, this upregulation was significantly reduced in the rats fed a zinc deficient diet (and tended to it in those fed a copper deficient diet), which showed more apoptotic cells and an impaired glial response to TBI with increased microgliosis and decreased astrogliosis. Moreover, the zinc and copper deficient rats showed increased oxidative stress, with significant increases of the MDA, NITT and NF-κB levels compared to normally fed or the pair-fed rats. Third, this increased oxidative stress could likely be due to the decreased MT-I+II levels, although it must be acknowledged that the increase and decrease of iNOS and Cu,Zn-SOD, respectively, could equally contribute. And fourth, to more directly establish the role of MT-I+II during TBI, we carried out an experiment with MT-I+II null mice. The results indicated that the absence of MT-I+II caused an impaired glial response, increased apoptosis, and potentiated oxidative stress, all of which were remarkably similar to the effects caused by the zinc and copper deficient diets in the rat experiments. These results strongly suggest that most of the effects caused by the metal deficiencies can be causally linked to the MT-I+II down-regulation. The magnitude of the changes observed in the MT-I+II null mice is higher than in the rats fed metal deficient diets, which seems logical since the latter only have a reduction but not a total lack of the MT-I+II proteins. Thus, MT-I+II appear to be of major importance for the CNS coping with TBI. Consistent with such a role, MT-I+II levels have been observed to be increased in several human neurodegenerative disorders as well as in a number of experimental models of brain damage (see Hidalgo *et al.*, 1997 for

review). Interestingly, oxidative stress has been involved in several human neurodegenerative diseases (Bains and Shaw, 1997; Coyle and Puttfarcken, 1993; Hensley *et al.*, 1997; Shohami *et al.*, 1997).

Another finding of this study was the specific responses of the rather CNS specific isoform, MT-III. In line with previous brain damage models (Hidalgo *et al.*, 1997), the cryo lesion of the CNS of normal rats produced a mild MT-III upregulation in inflammatory cells surrounding the lesioned area. Since MT-III has an inhibitory effect on neuronal survival in vitro (Uchida *et al.*, 1991; Erickson *et al.*, 1994), it has been speculated that the increase of MT-III levels after brain damage reflects attempts to limit neuronal sprouting (Yuguchi *et al.*, 1995a). However, further studies have shown that MT-III has indeed a neuroprotective role, at least in the CA3 hippocampal area after kainic acid-induced seizures (Erickson *et al.*, 1997). A promoting role of astrocyte migration has also been described in vitro (Carrasco *et al.*, 1999). Somewhat surprisingly, MT-III protein levels were strongly increased in the lesioned zinc deficient rats and moderately increased in copper deficient rats. The comparison of the MT-III changes versus those of MT-I+II suggest a sort of compensatory response of MT-III because of a decrease in the MT-I+II response. This has also been observed in IL-6 deficient mice after TBI (Penkowa *et al.*, 1999c) and in MT-I+II deficient mice after administration of the glial toxin 6-aminonicotinamide (Penkowa *et al.*, 1999b). The mechanisms underlying these effects are unknown, and their putative physiological importance need further experiments.

The present results demonstrate that zinc and copper are important for cellular activation/recruitment and for expression of factors and enzymes synthesized during inflammation in CNS. Not only the glial responses but also the cellular survival are significantly altered by the deficiency of these essential heavy metals. Most of the effects described in this paper could be attributable to the accompanying changes of MT-I+II protein levels, likely because of their antioxidant functions. This and previous studies are identifying an emerging essential role of this family of proteins in the CNS.

ACKNOWLEDGEMENTS

The study was supported by The Novo Nordisk Fonden, Dansk Epilepsi Selskabs Forskningsfond, Dir. Jacob Madsens og Hustrus Fond, Fonden af 17.12.1981, Gerda og Aage Haensch´s Fond, and by Leo Nielsen og Hustrus Legat and by CICYT SAF96-0189, PSPGC PM98-0170 and Fundación “La Caixa” 97/102-00 (JH). J. Carrasco is supported by a fellowship of the CIRIT, FI/96-261

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Trabajo 12

Enhanced seizures and hippocampal neurodegeneration following kainic acid induced seizures in metallothionein-I+II deficient mice

European Journal of Neuroscience, ***12:*** *2311-2322, 2000*

European Journal of Neuroscience

Enhanced seizures and hippocampal neurodegeneration following kainic acid induced seizures in metallothionein-I+II deficient mice.

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Key words: Apoptosis, Caspases, NF-κB, Oxidative stress, Metallothionein-III, Zinc

Abstract

Metallothioneins (MTs) are major zinc binding proteins in the CNS which could be involved in the control of zinc metabolism as well as in protection against oxidative stress. Mice lacking MT-I and MT-II because of targeted gene inactivation were injected with kainic acid (KA), a potent convulsive agent, to examine the neurobiological importance of these MT isoforms. At 35 mg/kg KA, MT-I+II deficient male mice showed a higher number of convulsions and a longer convulsion time than control mice. Three days later, KA-injected mice showed gliosis and neuronal injury in the hippocampus. MT-I+II deficiency decreased both astrogliosis and microgliosis and potentiated neuronal injury and apoptosis as suggested by terminal deoxy-nucleotidyl transferase-mediated in situ end labeling (TUNEL) and detection of single stranded DNA (ssDNA) and by increased interleukin-1β-converting enzyme (ICE) and caspase-3 levels. Histochemically reactive zinc was increased by KA in the hippocampus, to a higher extent in MT-I+II deficient compared to control mice. KA-induced seizures also caused increased oxidative stress, as suggested by the malondialdehyde (MDA) and protein tyrosine nitration (NITT) levels and by the expression of MT-I+II, nuclear factor-κB (NF-κB), and Cu,Zn-superoxide dismutase (Cu,Zn-SOD). MT-I+II deficiency potentiated the oxidative stress caused by KA. Both KA and MT-I+II deficiency affected significantly the expression of MT-III, granulocyte-macrophage colony stimulating factor (GM-CSF) and its receptor (GM-CSFr). The present results indicate MT-I+II as important for neuron survival during KA-induced seizures, and suggest that both impaired zinc regulation and compromised antioxidant activity contribute to the observed neuropathology of the MT-I+II deficient mice.

Introduction

Metallothioneins (MTs) are a family of low molecular-weight, cysteine-rich, heavy metal-binding proteins (Kagi & Schaffer, 1988). There are several isoforms of MTs in mice. MT-I and MT-II are expressed coordinately in all tissues, whereas MT-III and MT-IV are localized mainly in the brain and stratified squamous epithelia, respectively (Palmiter *et al.*, 1992; Quaife *et al.*, 1994).

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The knowledge of the specific roles of the different MT isoforms in the CNS, if any, is still scarce. MT-III was discovered as a factor in brain extract with sequence homology to MT-I and MT-II (Uchida *et al.*, 1991). Previous studies have demonstrated that the localization and regulation of MT-III differs from that of MT-I+II, and that several human neurodegenerative disorders and a number of animal models of brain damage alter significantly the expression of the three CNS MT isoforms (Aschner *et al.*, 1997; Aschner, 1996; Hidalgo *et al.*, 1997).

The development of mice carrying null mutations in the MT-I+II (Masters *et al.*, 1994a) and MT-III (Erickson *et al.*, 1997) genes, as well as mice overexpressing them (Erickson *et al.*, 1997; Erickson *et al.*, 1995), will undoubtedly help in our understanding of MTs functions in the CNS. MT-III deficient mice had decreased concentrations of zinc in several

brain regions, demonstrating a role of this MT isoform in zinc regulation (Erickson *et al.*, 1997). In addition, when these mice were challenged with kainic acid (KA), a well-known excitotoxin (Sperk, 1994; Coyle & Puttfarcken, 1993), they showed increased susceptibility to seizures and exhibited greater neuron injury in the CA3 field of hippocampus (Erickson *et al.*, 1997). Transgenic mice overexpressing MT-III, on the contrary, were more resistant to KA-induced neuronal injury. Thus, these studies demonstrated for the first time a physiological role of MT-III in the CNS, namely, neuronal protection in the CA3 area of the hippocampus.

The mechanisms underlying the protective in vivo role of MT-III have not been established, but they might be related to the proposed role of this protein on zinc regulation (Erickson *et al.*, 1997; Uchida *et al.*, 1991; Erickson *et al.*, 1994; Sewell *et al.*, 1995; Palmiter, 1995; Quaife *et al.*, 1998), since this metal is released during seizures (Frederickson & Moncrieff, 1994). KA-induced seizures may also increase reactive oxygen species (ROS) levels (Bains & Shaw, 1997; Coyle & Puttfarcken, 1993), and thus an antioxidant role of MT-III could also be considered. MT-III is homologous to MT-I+II, and, thus, it is feasible that all three CNS MT isoforms could function similarly despite their very different pattern of expression. The aim of this report was to challenge MT-I+II deficient mice with KA and examine whether or not these MT isoforms appear to share functions with MT-III.

Materials and methods

Animals

Homozygous MT-I+II knockout (MT-KO) mice, generated as previously described (Masters *et al.*, 1994a), were purchased from Jackson labs (Maine, USA). The MT-KO mice were raised on the 129/Sv genetic background; therefore mice from this strain were used as controls.

All the mice were maintained under standard laboratory conditions (12h light/dark circle, temperature 22°C, food and water provided *ad libitum*) for at least 1 week before starting experiments. The handling of the animals were approved by the proper Committees of Animal Research and Ethics of Spain and Denmark.

Experimental procedures

We carried out 5 separate experiments. Male and female mice were treated with kainic acid (KA) (Sigma, MO, USA, code K-0250), dissolved in PBS and injected intraperitoneally (i.p.), and the resulting seizures were observed for 2 hours. Seizure activity, general histopathology and a number of selected physiological variables were evaluated at different timings (see Results). Control mice received PBS.

KA-induced damage is not uniform throughout the brain; the hippocampus is one of the most sensitive areas (Sperk, 1994). Since the CA3 region was most vulnerable to KA in MT-III deficient mice and was protected from KA-induced seizures in MT-III transgenic mice, we focused our studies in this hippocampal area. Cellular counts were carried out in a 1.5 mm^2 area for statistical evaluation of the results.

Immunohistochemistry and Histochemistry

Mice were deeply anesthetized with Brietal (Methohexital 10 mg/ml, Eli Lilly, IN, USA) and perfused intracardially with heparinized saline buffer (5 min), followed by sodium sulfide (1mg/liter in PBS) (5 min) and Zamboni's fixative (15 min). Zamboni´s fixative consists of buffered 4% formaldehyde added 15% picric acid solution (1.2% (saturated) aqueous picric acid). Formaldehyde was prepared shortly before use by alkaline hydrolysis of paraformaldehyde. Brains were post-fixed in Zamboni's fixative at 4ºC for 4 hours, and processed for cryo-sectioning by incubation in 30% sucrose at 4ºC for 2 days. Afterwards, brains were frozen in isopentane (-50°C) for 5 min and kept at –80°C. Ten μm coronal sections were cut on a cryostat, and the sections were immediately collected on glass slides, to be used for general histology, histochemistry, inmunohistochemistry, Neo Timm's staining and TUNEL technique.

For epitope retrieval, sections were pre-incubated in Digest-ALL-3 (Pepsin solution) (Zymed Lab. Inc., CA, USA, code 00-3009) for 5 min followed by incubation in 10% goat serum in TBS (TBS: 0.05 M TRIS, pH 7.4, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet) for 30 min at room temperature. Mouse sections prepared for incubation with monoclonal mouse-derived antibodies, were in addition incubated with Blocking Solutions A+B from HistoMouse-SP Kit to quench endogenous mouse IgG (Zymed Lab. Inc., CA, USA, code 95-9544).

Sections were incubated overnight with one of the following primary antibodies: Polyclonal rabbit anti-cow glial fibrillary acidic protein (GFAP) 1:250 (Dakopatts, DK code Z 334); monoclonal rat anti-bovine GFAP 1:100 (Zymed Laboratories, Inc., CA, USA, code 13-0300); polyclonal rabbit anti-human neuron specific enolase (NSE) 1:1000 or 1:5000 (Dakopatts, DK code A589); monoclonal rat anti-mouse F4/80 1:50 (Sigma-Alldrich, UK,

code MCA 497); mouse anti-human neurofilament (NF) 1:200 (Dakopatts, DK, code M762); polyclonal rabbit anti-rat liver MT-I+II 1:500 (Gasull *et al.*, 1993; Gasull *et al.*, 1994); monoclonal mouse anti-horse MT-I+II 1:50 (Dakopatts, DK, code M 0639); polyclonal rabbit anti-rat MT-III 1:1000 (Carrasco *et al.*, 1999); monoclonal mouse anti-nitrotyrosine (NITT) 1:50 (Alpha Diagnostics, TX, USA, code NITT-11-M); polyclonal rabbit anti-malondialdehyde (MDA) 1:100 (Alpha Diagnostics, TX, USA, code MDA-11-S); monoclonal rat anti-mouse GM-CSF 1:50 (Genzyme Diagnostics, UK code 1723-01); polyclonal rabbit anti-mouse GM-CSFrec α-chain 1:500 (Research Diagnostics Inc., Flanders, NJ, USA); polyclonal rabbit anti-human ferritin 1:500 (Dakopatts, DK code A 0133); monoclonal mouse anti-human Cu/Zn-SOD 1:50 (Sigma, MO, USA, code S2147); monoclonal mouse anti-human single stranded DNA (ssDNA) 1:100 (Alexis Corp., UK, code 804-192-L001); polyclonal rabbit anti-mouse interleukin-1 converting enzyme, ICE 1:100 (Santa Cruz, CA, USA, code sc 1218-R); polyclonal rabbit anti-human caspase-3/CPP32 1:100 (Santa Cruz, CA, USA, code sc 7148); monoclonal mouse anti-human NF-κB 1:100 (Boehringer Mannheim, Germany, code 1697838).

The primary antibodies were detected using biotinylated anti-rabbit IgG 1:400 (Sigma, MO, USA, code B3275), or biotinylated anti-mouse IgG 1:200 (Sigma, MO, USA, code B8774), or biotinylated anti-rat IgG 1:1000 (Amersham, UK, code RPN 1002), followed by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP) (Dakopatts, DK, code K377). These secondary and tertiary steps in the immunoreaction were performed for 30 min at room temperature. Afterwards, sections were incubated with biotinylated tyramide and streptavidin-peroxidase complex (tyramide signal amplification, TSA indirect) (NEN, Life Science Products, MA, USA, code NEL700A). The anti-ssDNA is of IgM subtype and was detected by using biotinylated goat anti-mouse IgM 1:10, (Zymed Lab. Inc., CA, USA, code 62-6840) followed by StreptABComplex/HRP. The immunoreaction was visualized using 0.015% H_2O_2 in DAB/TBS.

Biotinylated tomato lectin from the *Lycopersicon esculentum* (Sigma, MO, USA, code L9389) 1:500, or Texas Red labeled Lectin 1:50 (Sigma-Aldrich, UK, code L-9139) were used as markers for cells of the myelo-monocytic cell lineages, such as microglia/macrophages.

In order to evaluate the extent of non-specific binding in the immunohistochemical experiments the primary antibody step was omitted. Results were considered only if these controls were unstained. For the examination and recording of the stainings, a Zeiss Axioplan2 light microscope was used.

Immunofluorescence and TUNEL

During the course of this study a number of double and triple labelings were carried out for cellular identification by immunofluorescence techniques. Depending on the primary antibody, one of the following secondary antibodies were used: goat anti-rabbit IgG (H+L) linked with FITC (Southern Biotechnology Ass., AL, USA, code 4050-02); fluorescein-conjugated goat anti-rat IgG (H+L) (Calbiochem, Oncogene Res. Products, CA, USA, code 401414); rhodamine-conjugated swine anti-rabbit IgG 1:20 (Dakopatts, DK, code R156); Texas Red conjugated goat anti-mouse IgM (μ chain specific) 1:20 (Jackson Immuno Res. Lab. Inc., PA, USA, code 115-075-020); goat anti-rabbit IgG (H+L) linked with FITC 1:40 (Southern Biotechnology Ass., AL, USA, code 4050-02); goat anti-mouse IgG linked with aminomethylcoumarin (AMCA) 1:20 (Dakopatts, DK, code W0477); goat anti-mouse IgG linked with FITC 1:200 (Zymed Laboratories Inc., CA, USA, code 81-6511); goat anti-rabbit IgG linked with AMCA 1:50 (Dakopatts, DK, code W0478); goat anti-rabbit IgG linked with Texas Red 1:70 (Jackson ImmunoResearch Lab., PA, USA, code 111-075-144); goat anti-rat IgG linked with AMCA 1:50 (Jackson ImmunoResearch Lab., PA, USA, code 112-155-102); donkey anti-goat IgG linked with fluorescein 1:60 (Binding Site, UK, code AF360).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining was accomplished using the Apoptag Plus, *In Situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD, USA, Code S7101-KIT). To detect the type of cells undergoing apoptosis sections were preincubated with one of the following: polyclonal rabbit anti-human NSE (for neurons); polyclonal rabbit anti-cow GFAP (for astrocytes); or monoclonal rat anti-mouse F4/80 (for microglia/macrophages). These sections were afterwards incubated with ApopTag In Situ Apoptosis Detection Kit (TUNEL) linked with fluorescein (Oncor, Gaithersburg, MD, USA, code S7110-KIT) or with rhodamine (Oncor, Gaithersburg, MD, USA, code S7165-KIT) prepared following manufacturer´s recommendations.

The sections were embedded in 20 _l fluorescent mounting (Dakopatts, DK, code S3023) and kept in darkness at 4°C. Sections were processed in parallel where the primary antibody was omitted to evaluate the extent of non-specific binding. Results were considered only if these controls were unstained. For the

simultaneous examination and recording of the two stains, a Zeiss Axioplan2 light microscope equipped with a tripleband (DAPI/FITC/Texas Red) filter was used.

In situ hybridization of MT-I and MT-III

Serial coronal cryostat sections (20 µm in thickness) were obtained from the frozen brains with a cryostat (Reichert-Jung 2800 Frigocut E, Germany) and mounted on slides coated with poly-L-lysine, which were then maintained at -80°C until the day of the analysis. The sections were obtained between the -1.60mm and -1.80mm bregma points for the analysis of the expression of MT-I and MT-III isoforms in the granular layer of dentate gyrus, CA3 and CA1 pyramidal neurons, lacunosum moleculare, and oriens layer of hippocampus.

MT-I and MT-III were specifically measured by using the cDNAs generously provided by Dr. R.D. Palmiter (University of Washington, Seattle, WA) for MT-I mRNA, and Dr. G.K. Andrews (Dept. Biochemistry, Kansas City, KS, USA), respectively. Preparation of sense and antisense probes and the in situ hybridization procedure were performed as previously described (Carrasco *et al.*, 1998; Hernández *et al.*, 1997). Autoradiography was performed exposing the film (Hyperfilm-MP, Amersham, UK) to the slides for three days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III RNA levels were semiquantitatively determined in three sections of each brain area per animal by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500MC system (Leica, Germany). The MT-I and MT-III RNA values shown are expressed in arbitrary units (number of pixels x optical density).

Microautoradiographies were obtained by exposing the sections to an autoradiographic emulsion (Amersham, UK) and developed according to manufacturer's instruction. Cells were counterstained with thionine.

Neo Timm's staining

Sections were physically (autometallographically) developed for 25 min at room temperature in solution containing silver lactate (Moos, 1993).

Statistical analysis

Results were analyzed with two-way analysis of variance (ANOVA) with strain and KA treatment while main factors. When interaction was significant ($p<0.05$), it was interpreted to be a consequence of a specific effect of the MT-I+II deficiency during the treatment. The Student t-test was used to assay the effect of MT-I+II deficiency in KA induced seizures and the effect of KA administration on MT-I+II expression in the normal mice. When no variance homogeneity was found Mann-Whitney-U test was used.

Results

Seizures

MT-III KO mice have been shown to have increased susceptibility to KA-induced seizures (Erickson *et al.*, 1997). We herewith show that MT-I+II KO (MT-KO) mice behave similarly. In a first experiment, we examined the tolerance of MT-KO animals to the treatment and determined the appropriate dose to cause seizures in the majority of mice; the seizures were observed for 2 hours. From this experiment, a dose of 35 mg/kg KA was selected for the following experiments (data not shown). Control animals were injected with PBS. After the injection the behavior of each mouse was recorded with a video-tape for 100 min for further analysis. The number and duration of limb clonus and tonic-clonic convulsions were quantitated for statistical comparisons. Others seizure-associated behaviors (time of latency, jumping and death) were also measured. We used the total time each animal exhibited limb clonus and/or tonic-clonic convulsions as an index of the total seizure activity.

At the dose of KA used (35 mg/kg), most of the animals showed seizures and some seizure-related behaviors such as catatonic posture, withdrawal and jumping (Figure 1 and Table 1). These behaviors showed considerable variability among animals, which did not seem to be attributable to age or weight (data not shown). While the latency of seizure onset was of about 20 min in both normal and MT-KO male mice, significant differences were found between both groups in the number of convulsions ($p<0.05$) and in the cumulative convulsion time ($p<0.01$). Furthermore, the MT-KO mice showed an increased frequency of seizing ($p<0.01$) and jumping ($p<0.01$), indicating a greater susceptibility of MT-I+II deficient mice to KA-induced seizures.

Metallothioneins

To establish the importance of the MT family in the context of KA-induced seizures, we evaluated the changes in the mRNA and protein levels in both normal (for MT-I+II and MT-III isoforms) and

Table 1. Response of 129/Sv and MT-I+II deficient mice to kainic acid administration

Strain	Weight variation. (% of weight)	% Seizing	% Animals with jumping	Latency time (min)	Mortality (%)
129/Sv	1.59±1.0	90	40	20.9±2.5	0
MT-KO	1.56±0.7	100	86.5*	18.0±1.7	0

Mice were injected intraperitoneally with kainic acid at 35 mg/ kg body weight. Weight variation, latency time, number of convulsions and convulsion time are the mean ± SEM, (129/Sv n=20; MT-I+II deficient n=26). * Significantly greater than 129/Sv mice; $p<0.01$. Frequency data are analyzed with χ^2 test.

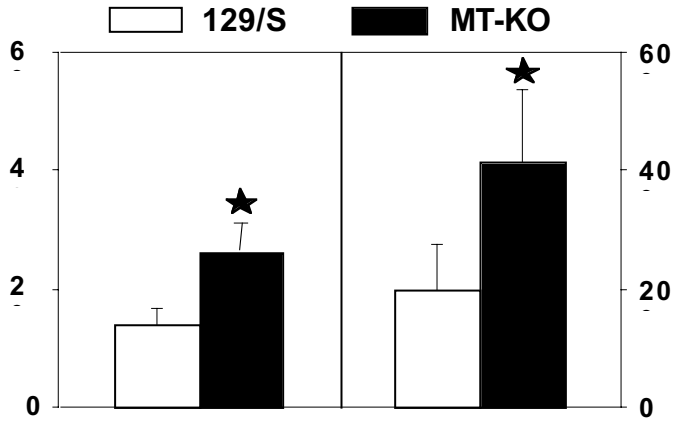


Fig 1. Effect of MT-I+II deficiency on KA-induced seizures. Male mice were injected with 35 mg/kg kainic acid (KA) as described in material and methods and observed for 100 min. The number of convulsions (left) and the total time of convulsions (right) are plotted. Results are mean ± SEM; normal mice (129/Sv), n=20; MT-KO mice, n=26. *p<0.05 vs normal mice.

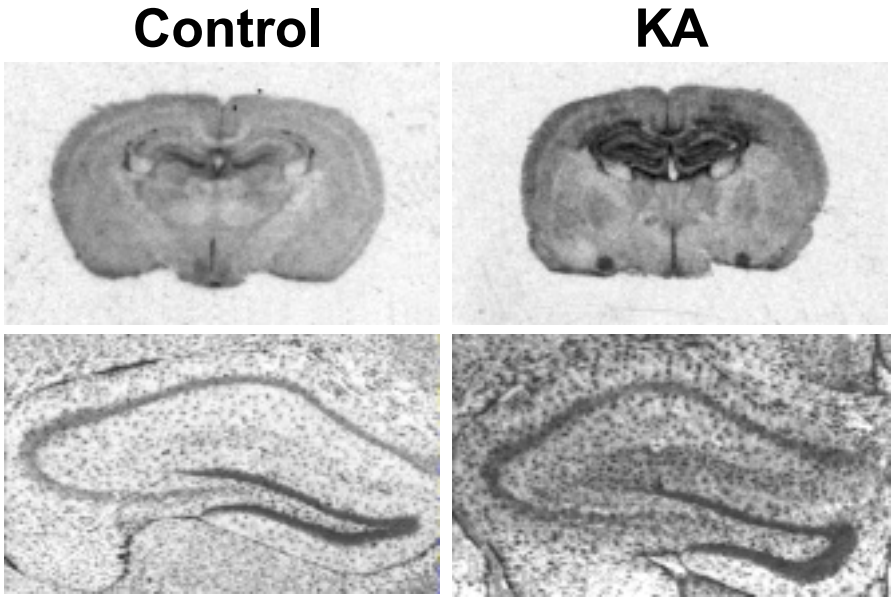


Fig. 2. Effect of KA administration on hippocampal MT-I mRNA levels by in situ hybridization. A representative macroautoradiography showing a coronal section of the brain (top) and a microautoradiography showing specifically the hippocampus (bottom) for the MT-I isoform of control and KA-injected mice. Mice were killed 1 day after the PBS or KA injection. Quantifications were carried out in specific hippocampal areas of several mice for statistical purposes (see Table 2). Scale bar of the microautoradiography: 500 μm.

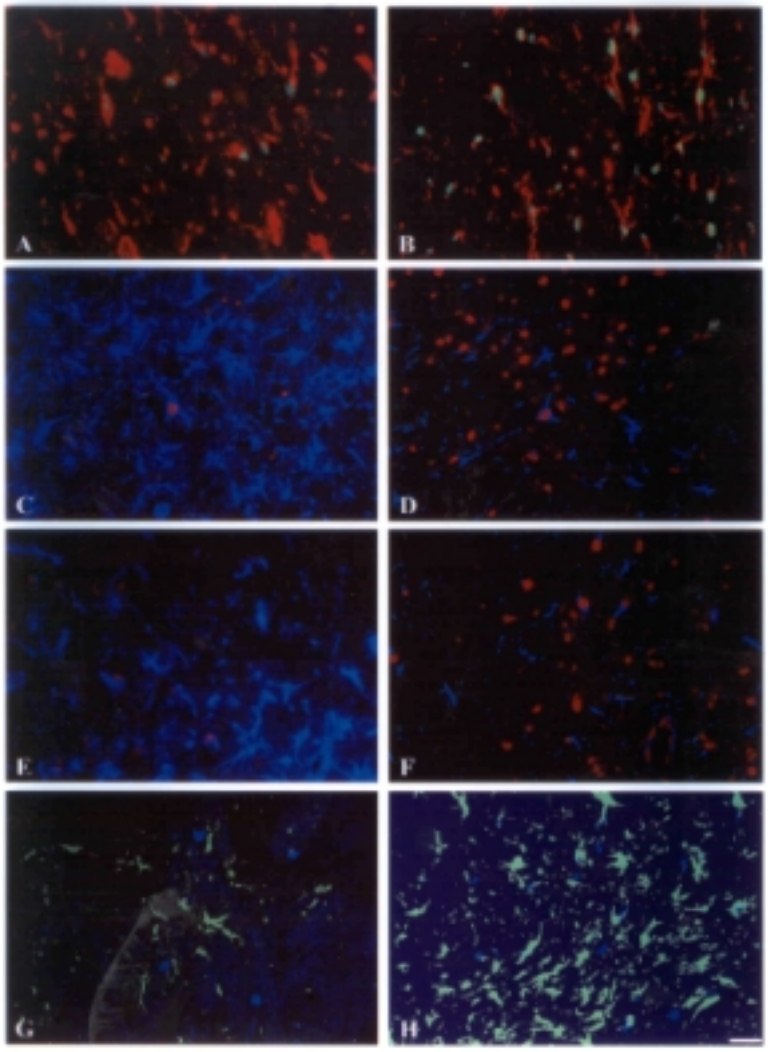
Table 2. Effect of KA administration on hippocampal MT-I and MT-III mRNA levels measured by in situ hybridization.

	129/Sv		MT-KO	
	Control	**KA**	**Control**	**KA**
MT-I mRNA				
Dentate gyrus	762±46	1121±45*	-	-
CA1 pyramidal cell layer	118±27	974±160*	-	-
CA3 pyramidal cell layer	344±27	1154±45*	-	-
Lacunosum moleculare	312±38	1362±47*	-	-
Oriens layer	147±5	862±60*	-	-
MT-III mRNA				
Dentate gyrus	588±102	596±20	523±49	545±35
CA1 pyramidal cell layer	364±81	396±44	339±63	284±49
CA3 pyramidal cell layer	539±91	566±33	494±69	484±20
Lacunosum moleculare	133±10	166±13	103±23	143±21
Oriens layer	130±7	131±13	87±9	91±9

MT-I and MT-III mRNAs were assayed by in situ hybridization (see Figure 2 for representative autoradiographies of the MT-I isoform). Animals were killed 1 day after the PBS or KA injection, and the brains frozen as described in Materials and Methods. RNA levels were semiquantitatively determined in three sections per animal of up to 7 mice per group. Results are mean ± SEM. Control, injected with PBS; KA, injected with 35 mg/kg kainic acid. n=3 in PBS treated mice, and n=7 in KA treated mice. *p<0.001 vs control mice.

Fig.3. Double immunohistoche-mistry for TUNEL and specific cell markers plus MDA and NITT stainings of normal and MT-KO mice following KA-injection. A,B: Mice were killed three days after the PBS or KA injections and the brains fixed as described in Materials and Methods. The figure shows TUNEL staining (green) and NSE staining (red) of KA-injected normal (A) and MT-KO (B) mice. It is obvious that the MT-KO mice show an increased number of apoptotic neurons in the hippocampus CA3 area when compared to that of normal mice. C,D: GFAP (blue) and TUNEL (red) stainings of KA-injected normal (C) and MT-KO (D) mice. E,F: F4/80 (blue) and TUNEL (red) stainings of KA-injected normal (E) and MT-KO (F) mice. G,H: NITT (green) and MDA (blue) stainings of KA-injected normal (G) and MT-KO (H) mice.

Scale bars: A-D, G,H: 30 μm, E,F: 22 μm.



MT-KO (only the MT-III isoform)

mice. MT-I and MT-III mRNA levels were measured by in situ hybridization after KA treatment in two separate experiments. Since MT-I and MT-II are coordinately regulated, we assume that the results described for MT-I are representative of the MT-I+II isoforms (Masters *et al.*, 1994b). MT-I+II and MT-III proteins were evaluated by immunocytochemistry in the same experiment where seizure activity was determined.

In a first experiment, mice were killed by decapitation without tissue fixation 24 hours after KA (n=7 for both control and MT-KO mice) or PBS (n=3) injections. Representative autoradiographies for MT-I mRNA are shown in Fig. 2, and the quantifications carried out in some hippocampal fields of all the animals per group are shown in Table 2. The results show a very prominent upregulation of the MT-I isoform was observed in the hippocampus after the KA-induced seizures. The increase was significant in all the hippocampal fields analyzed, namely the granular layer of dentate gyrus, pyramidal cell layer of CA1 and CA3, lacunosum moleculare and oriens layer ($p<0.001$). The microautoradiography clearly showed that most of the MT-I signal was located in hippocampal layers enriched in glial cells (Fig. 2 bottom). As expected, the MT-III signal was prominent in the CA1-CA3 pyramidal cell layer and dentate gyrus (not shown). In contrast to MT-I, MT-III mRNA levels remained basically unaffected in all areas analyzed after KA treatment (Table 2). Regardless of the KA treatment, a mild tendency to have decreased MT-III mRNA levels in the hippocampus was observed in the MT-KO mice, which was only significant in the oriens layer of hippocampus ($p<0.01$). In a second experiment, MT-I and MT-III mRNAs were evaluated in mice killed up to 21 days after the KA injection (data not shown). MT-I was significantly upregulated for up to 1 week after KA injection, decreasing gradually and returning to normal levels in most hippocampal areas 3 weeks after KA injection. MT-III was basically unaffected 1 day after KA injection in most hippocampal layers, but afterwards this MT isoform increased and 4-7 days after KA administration MT-III mRNA levels were 2-3 fold higher than PBS-injected mice in lacunosum moleculare, stratum radiatum or oriens layer.

As expected, in the brain of PBS-injected normal mice MT-I+II immunoreactivity was observed in ependymal cells, meninges, some grey matter astrocytes and in the glia limitans; in the hippocampus, some grey matter astrocytes expressed MT-I+II and no neuronal cells were immunostained for MT-I+II (not shown). Cell counts of MT-I+II positive cells were carried out in the CA3 subfield for statistical purposes, and are shown in Fig. 6 together with other physiological variables. Three days after KA injection, normal mice increased their MT-I+II expression significantly ($p<0.001$). Both reactive astrocytes and activated microglia showed MT-I+II immunoreactivity (not shown).

In PBS-injected mice, MT-III immunoreactivity was observed in a few hippocampal astrocytes as well as in ependyma and meninges (not shown). Cell counts of MT-III positive cells carried out in the CA3 subfield (Fig. 6) indicated that three days after KA injection, normal and MT-KO mice increased their MT-III expression significantly ($p<0.001$), being the increase higher in the MT-KO mice ($p<0.001$). MT-III was expressed in reactive astrocytes and activated microglia (not shown).

In general, the immunocytochemical data for MT-I+II confirmed the in situ hybridization analysis for MT-I mRNA carried out. This was not the case for the MT-III isoform, since although MT-III mRNA levels did increase several days after the KA injection, the increase was similar in control and MT-KO mice. To rule out methodological problems with both the *in situ* and the immunocytochemistry for MT-III, we have analyzed the brains of MT-III null mice, and as expected, the MT-III RNA signal disappears (Penkowa *et al.*, 1999c) as does the MT-III immunoreactivity (Carrasco *et al.*, 1999).

Apoptosis: TUNEL, ssDNA, ICE and Caspase-3

The number of cells undergoing apoptosis, as determined by using in situ labeling TUNEL technique as well as by immunohistochemical detection of single stranded DNA (ssDNAir), is shown in Table 3. ssDNA stainings were carried out because they are more sensitive and specific than TUNEL (Frankfurt *et al.*, 1996). Only a few TUNEL positive or ssDNA positive cells were detected in the hippocampus of PBS-injected mice. Following the KA injection, the number of apoptotic cells increased throughout the hippocampus of normal mice ($p<0.001$), and, to a greater extent, of MT-KO mice ($p<0.05$). To further characterize the apoptotic process, we also analyzed the ICE and caspase-3 immunostainings, which were in accordance with the TUNEL and ssDNA levels (Table 3).

To establish the identity of the cells undergoing apoptosis in the CA3 hippocampal area, we carried out double fluorescence stainings with TUNEL and anti-NSE (for neurons), anti-GFAP (for astrocytes) or anti-F4/80 (for microglia/macrophages) (Fig. 3A-F). Counts carried out in five mice per group of the number of cells positive for TUNEL and NSE,

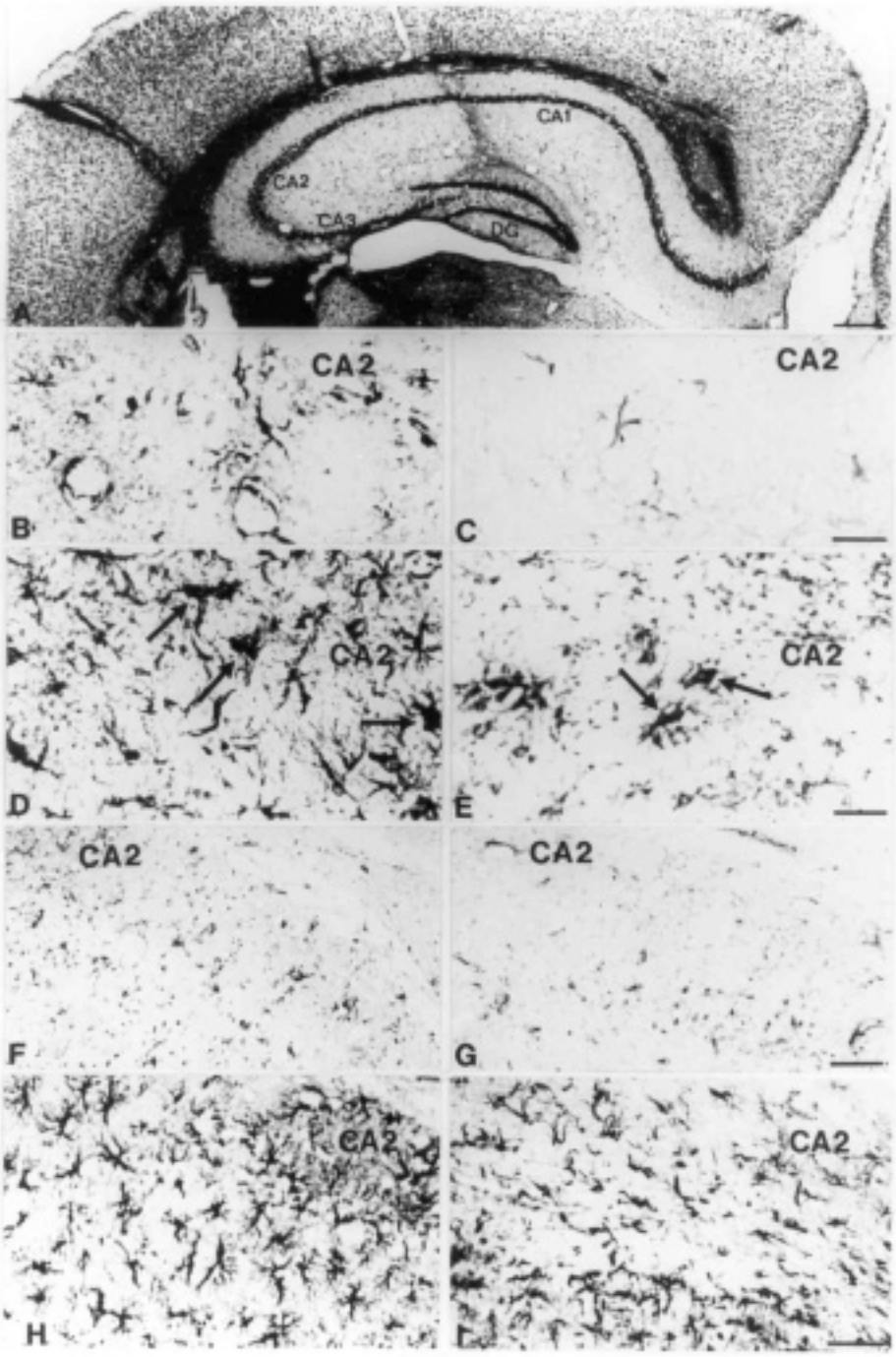


Fig. 4. GFAP immunohistochemistry and lectin histochemistry of uninjected and KA injected mice. Mice were killed three days after the PBS or KA injections and the brains fixed as described in Materials and Methods. A. Toluidine blue staining of brain, showing hippocampal areas CA1-CA3 and dentate gyrus (DG) of a normal mouse. B: GFAP immunohistochemistry, showing astrocytes of the hippocampal CA2 region in PBS-injected normal mice. C: GFAP immunohistochemistry, showing astrocytes of the hippocampal CA2 region in PBS-injected MT-KO mice. D: The CA2 region displays numerous reactive astrocytes with swollen cell bodies (arrows) following KA injection in normal mice. E: The CA2 region of KA injected MT-KO mice displays fewer reactive astrocytes (arrows) than those of normal mice. F: Lectin histochemistry showing resting microglia of the hippocampal CA2 region in PBS-injected normal mice. G: Lectin histochemistry, showing resting microglia of the hippocampal CA2 region in PBS-injected MT-KO mice. H: Following KA injection, the number of microglial cells is significantly increased in normal mice. Activated microglial cells have maintained their ramified morphology even though they are hypertrophic. I: Following KA injection, the number of microglial cells is also significantly increased in MT-KO mice hippocampus. However, the reactive microglial cells are rod-like with less ramification compared to that of normal mice.

Scale bars: A: 290 μm; B-E: 29 μm; F-I: 57 μm

GFAP or F4/F80 are shown in Table 3. It is clear that KA- induced seizures tended to increase apoptosis in the three types of cells but that the ones significantly affected were neurons.

Reactive astrocytes

Astrocytes are essential for coping with CNS damage, and thus we evaluated astrocyte reactivity by mean of GFAP stainings. A general view of the hippocampus by toluidine blue staining is shown for comparison (Fig. 4A). In PBS-injected mice, the number of GFAP positive astrocytes was decreased in MT-KO mice compared to that of normal mice (Figs. 4 and 6). Morphologically, the astrocytes of MT-KO mice appeared stellate as seen in normal mice (Fig. 4B,C). Following KA injection in normal mice, GFAP immunoreactive astrocytes were observed throughout the hippocampal subfields and the number of GFAP positive cells was significantly ($p<0.001$) increased compared to that seen in PBS-injected mice (Fig. 6). The reactive astrocytes were hypertrophic with swollen cell bodies and thick processes (Fig. 4D). In KA-injected MT-KO mice, reactive astrogliosis was reduced ($p<0.001$) compared to that of normal mice (Fig. 6). Some astrocytes displayed hypertrophy with swollen cell bodies and retracted thick processes; however, most astrocytes maintained the morphology of PBS-injected mice as determined from GFAP immunoreactivity (Fig. 4E).

Microglia

Microglia/macrophages are also essential for coping with CNS damage, and thus lectin histochemistry was carried out to analyze their reactivity toward KA-induced damage in the hippocampus. In all PBS-injected mice, microglial cells were similar in morphologic appearance as verified from lectin histochemistry showing ramified quiescent microglia (Fig. 4F,G). In normal mice, the KA injection induced a significant activation of microglial cells in all subfields of hippocampus and the number of lectin positive cells increased significantly with the highest increase in CA1-CA3 ($p<0.001$) (Fig. 6). The activated microglia showed coarsening and hypertrophy of the cytoplasm. However, microglial cells remained ramified and bushy in morphology, while no round monocytoid macrophages were present at 3 days post-injection (Fig. 4H). In MT-KO mice, the KA injection was also followed by microgliosis; however, the number of lectin positive cells was decreased compared to that of normal mice ($p<0.05$) (Fig. 6). Also the morphologic appearance of microglia was affected by MT-I+II deficiency, in that the microglia appeared rod-like or amoeboid without the characteristic bushy arborisation seen in microglia of normal mice (Fig. 4H,I).

Histochemically reactive zinc

MTs are major zinc binding proteins and their absence could impair the normal control of this essential heavy metal in the hippocampus, one of the richest areas regarding zinc levels. Thus, histochemically reactive (free) zinc levels were evaluated by the Timm's staining. In PBS-injected mice, no difference in reactive zinc was observed between normal and MT-KO mice (Figs. 5A,B). The intensity of the Timm staining in PBS-injected mice was kept low by developing a short time in order to detect KA-induced increases. Thus, in KA-injected normal mice a dramatic increase in histochemically reactive zinc was clearly seen in hippocampus as measured three days after KA injection (Fig. 5C). In KA-injected MT-KO mice, a further increase was observed (Fig. 5D).

Oxidative stress response: MDA, NITT, NF-κB and Cu/Zn-SOD expression

MTs are major antioxidant proteins, and therefore the MT-KO mice could be subjected to an increased oxidative stress during KA-induced seizures. To establish such a possibility, we analyzed two general indices of increased oxidative stress, MDA (reflecting lipid peroxidation) and NITT (reflecting protein tyrosine nitration, which, in turn, reflects peroxynitrite formation by increased nitric oxide and superoxide levels). KA injection caused a dramatic increase of the number of cells positive for MDA and NITT (Fig. 6). NITT levels were increased in the MT-KO mice compared to normal mice (Figs. 3G,H and 6).

We also evaluated the major oxidative stress-responsive transcription factor NF-κB and the antioxidant enzyme Cu,Zn-SOD (Fig. 6). Both were increased by KA-induced seizures ($p<0.001$) in the CA1-CA3 fields, and to a higher extent in MT-KO mice ($p<0.05$). After KA administration, NF-κB immunoreactivity moved to the nucleus (not shown), and as verified from triple immunofluorescence histochemistry, the cells expressing NF-kB were ssDNA positive neurons. In uninjected mice Cu/Zn-SOD was observed in a few glial cells of the hippocampus, and following KA-injection, Cu/Zn-SOD expression was observed in reactive glia and in

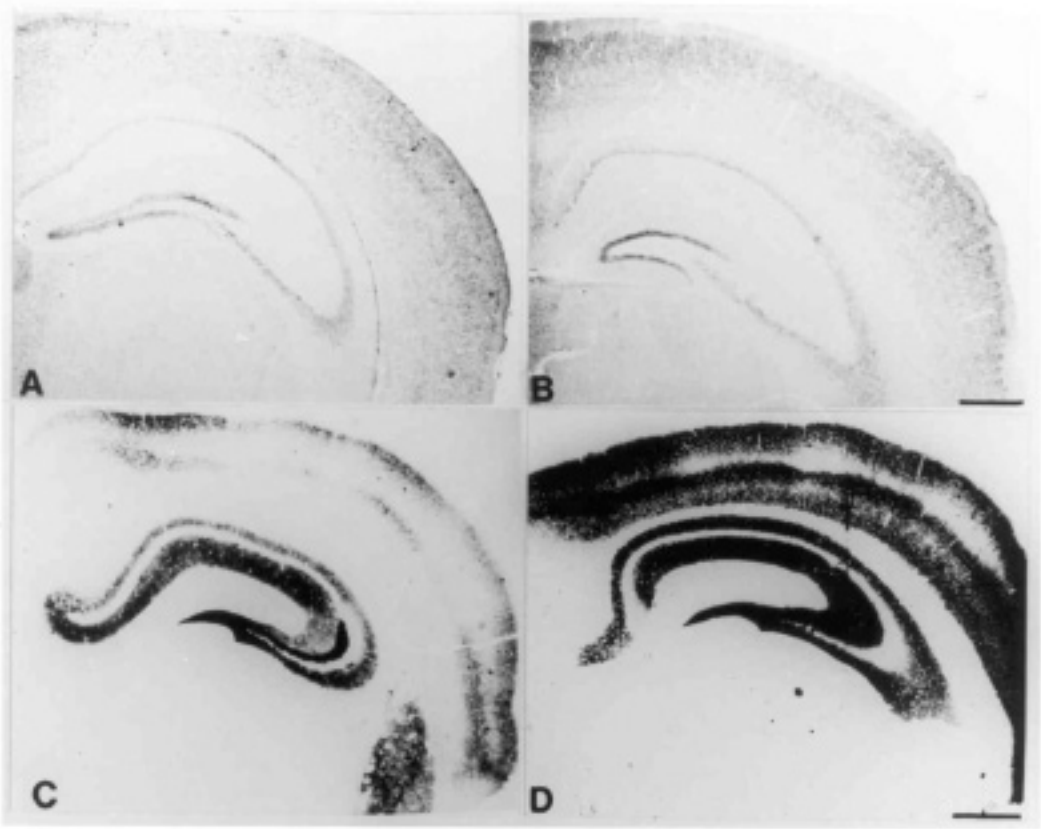


Fig. 5. Timm´s staining of normal and MT-KO mice. Mice were killed three days after the PBS or KA injections and the brains fixed as described in Materials and Methods. A: In PBS-injected normal mice, zinc staining is seen primarily in hippocampus and mildly in the cortex. B: In PBS-injected MT-KO mice, zinc staining is seen primarily in hippocampus and mildly in the cortex. C: Following KA-injection, zinc levels of normal mice are clearly increased in hippocampus and cortex. D: Following KA-injection, zinc levels of MT-KO mice are further increased, especially in the cortex.
Scale bars: A-D: 400 μm

surviving neuronal cells of the CA1-CA3 fields and in dentate gyrus (not shown).

GM-CSF and GM-CSFr

The previous results indicated a compromised glial response to KA-induced seizures in the MT-KO mice, so we suspected that the production of growth factors important for glial activation could also be affected. In this report we have evaluated the effect of KA and MT-I+II deficiency on GM-CSF immunoreactivity and on that of its receptor, GM-CSFr. In both cases, MT-I+II deficiency tended to decrease their levels in PBS-injected mice (Fig. 6). KA treatment raised the number of GM-CSF positive cells ($p<0.01$), but again MT-I+II deficiency significantly ($p<0.05$) blunted these responses (Fig 6). The main cell type expressing GM-CSF was reactive astrocytes, but some microglial cells were also positively stained; GM-CSFr expression was seen primarily in activated microglia, and otherwise in a few astrocytes (not shown).

Discussion

The effect of KA on MT-I+II regulation has been analyzed only in a few reports, but they suggest that this glutamate analog upregulates MT-I+II expression (Montpied *et al.*, 1998; Zheng *et al.*, 1995; Dalton *et al.*, 1995). We herewith show that MT-I+II are upregulated in the CNS by the systemic administration of KA, and that the main area where this occurs is the hippocampus, with tendency in some cortex areas such as the pyriform cortex, and in some amygdaline nuclei. This pattern of expression is associated with the KA related seizure syndrome (Ben-Ari, 1985; Sperk, 1994), and strongly suggests that MT-I+II are important proteins for coping with KA-induced seizures. The results show that MT-I+II deficient mice are susceptible to KA-induced seizures. Thus, the percentage of animals jumping, the number of convulsions, and the convulsion time

Table 3. Effect of MT-I+II deficiency on KA-induced apoptosis in the CA3 subfield of the hippocampus (positive cells/1.5mm^2).

	CONTROL		KA	
Single stainings	**129/Sv**	**MT-KO**	**129/Sv**	**MT-KO**
TUNEL	2.0±0.3	2.2±0.4	6.8±0.7*	9.4±0.7*,–
ssDNA	1.6±0.4	2.0±0.4	9.0±0.5*	12.4±0.8*,–
ICE	1.8±0.4	2.6±0.5	8.4±0.6*	13.3±0.9*,–
CASPASE-3	1.6±0.4	2.2±0.6	8.2±0.8*	13.8±0.7*,–
Double stainings				
TUNEL +/ GFAP +			1.2±0.37	2.6±0.51
TUNEL +/ F4/80 +			0.6±0.24	2.2±0.73
TUNEL +/ NSE +			5.8±0.73	8.4±0.51–

Cellular counts shown are mean ± SEM (n=5). Mice were killed three days after the PBS or KA injections and the brains fixed as described in Materials and Methods. For the single stainings, results were evaluated by two-way ANOVA with strain and treatment as main factors. The effect of the KA-induced seizures (*) and the MT-I+II deficiency (–) was significant (p at least <0.05) in all cases. For the double immunofluorescence stainings (see also Fig. 3), the results were evaluated with the Student "t" test. The number of cells positive for both TUNEL and NSE were significantly higher in the MT-KO mice (–, p<0.05).

was about twice in the MT-KO compared to control mice. In contrast, the latency time was not affected by MT-I+II deficiency. Overall, these results are remarkably similar to those observed in MT-III deficient mice (Erickson *et al.*, 1997). Surprisingly, males and females clearly differed in their responses to KA injection; females showed 3-4 times more convulsions (number and time) than males, only some of the females died after the KA administration, and MT-I+II deficiency did not alter their susceptibility to KA even at lower KA doses (data not shown). While the greater susceptibility of females to KA could be expected (Wetmore & Nance, 1991), the mechanisms underlying this gender difference regarding the effect of MT-I+II deficiency remain to be established.

To characterize putative mechanisms related to the increased susceptibility of the MT-KO male mice, we have carried out a detailed histopathological analysis of the CA3 hippocampal subfield, the most vulnerable region to the KA-induced seizures. It is important to realize that this is the area where a significant neuroprotective role of MT-III was found (Erickson *et al.*, 1997). Three days after the KA administration to normal mice, severe damage was caused to the neurons of the hippocampus, and particularly those of the CA3 field, as verified by toluidine blue or hematoxylin-eosin staining (not shown). At this time, many of the neurons were undergoing apoptosis, as shown by TUNEL, ssDNA, ICE and caspase-3 levels. A significant glial response to neuronal damage was also obvious, since both reactive astrocytes and activated microglia were present. When these responses were evaluated in the MT-KO mice, significant differences were observed: the number of neurons undergoing apoptosis was increased, while the number of activated microglia and especially of reactive astrocytes was significantly reduced.

Pathologic release of excitatory amino acids, such as the neurotransmitters glutamate and aspartate, leads to an increased neuronal influx of cations (sodium, calcium) followed by high frequency neuronal firing. In seizure-prone hippocampus, synaptic transmission co-releases zinc from presynaptic vesicles, which appears to be an endogenous modulator of excitatory neurotransmission since it decreases NMDA receptor mediated excitation and enhances AMPA/Kainate receptor mediated excitation (Lees *et al.*, 1990; Koh & Choi, 1988; Yin & Weiss, 1995; Weiss *et al.*, 1993). Excess Zn is toxic to neurons, and following seizures degenerating neurons are positively stained for zinc (Frederickson *et al.*, 1989; Koh & Choi, 1994; Koh *et al.*, 1996). MT-I+II are major zinc binding proteins, and thus their deficiency could have a significant impact in the control of zinc metabolism in the CNS. Indeed, we have observed a clear tendency of total zinc levels to be decreased in the CNS of MT-KO mice (Penkowa *et al.*, 1999b). During KA-induced seizures, it could be predicted that the absence of MT-I+II could lead to increased free zinc levels in the hippocampus, especially following glutamatergic neuronal firing. Indeed, the pool of chelatable zinc identified by the Timm staining (Moos, 1993) was clearly increased. Therefore, increased free zinc levels in the MT-KO mice could contribute to the observed neurodegeneration after KA-induced seizures (Nave & Connor, 1993; Frederickson *et al.*, 1989). Interestingly, zinc is implicated in the pathophysiology of several neurologic disorders (Cuajungco & Lees, 1997).

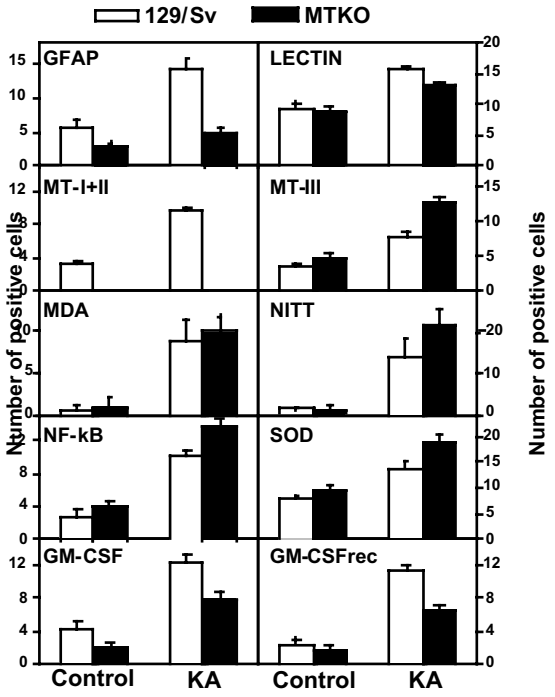


Fig 6. Immunohistochemical cell counts in the CA3 subfield of the hippocampus (positive cells/1.5mm^2). Mice were injected with either PBS (control) or with kainic acid (KA) and killed 3 days later. Cellular counts shown are mean ± SEM (n=5-10). Results were evaluated by two-way ANOVA with strain and treatment as main factors. The effect of the KA-induced seizures and the MT-I+II deficiency was significant (p at least <0.05) in all cases.

Another possible explanation for the increased neuronal apoptosis of the MT-KO mice is an increased oxidative stress. Activation of glutamate receptors may increase free radical production, which may then lead to further receptor activation, a self-perpetuating cycle that contributes significantly to neuronal death (reviewed in Bains & Shaw, 1997). Interestingly, oxidative stress has been involved in several human neurodegenerative diseases (Bains & Shaw, 1997; Coyle & Puttfarcken, 1993; Hensley *et al.*, 1997; Shohami *et al.*, 1997). MT-I+II are considered significant antioxidant proteins (Sato & Bremner, 1993; Lazo *et al.*, 1995), and, again, it could be predicted that MT-I+II deficiency could have a significant impact on the antioxidant capacity of the CNS. The increase in NF-κB, a major oxidative stress-responsive transcription factor in eukaryotic cells (Schreck *et al.*, 1992), clearly suggests an increased oxidative stress in MT-KO mice challenged with KA. The results for Cu,Zn-SOD fully support that conclusion, since the upregulation of this antioxidant enzyme could be viewed as a compensatory response. The NITT analysis clearly demonstrates that the absence of MT-I+II leads to increased oxidative stress. Protein tyrosine nitration is an index of increased peroxynitrite formation by nitric oxide (NO) and superoxide. MT-I+II have been shown to interact significantly with NO (Kennedy *et al.*, 1993; Misra *et al.*, 1996; Kroncke *et al.*, 1994). Furthermore, MT-I+II appear to protect against the cytotoxic and DNA-damaging effects of NO in NIH 3T3 cells (Schwarz *et al.*, 1995). Although these proteins are unlikely important quenching factors of the superoxide radical, their deficiency might increase superoxide levels through increased free Cu^{2+} levels (Sato & Bremner, 1993). These results provide a rationale for the increased protein tyrosine nitration observed in the MT-KO mice, although other mechanisms cannot be ruled out with the present data.

Therefore, both impaired zinc metabolism and increased oxidative stress could contribute to the neuronal death observed in the MT-KO mice injected with KA. This has also been suggested for a different model of CNS damage, the cryolesion (Penkowa *et al.*, 1999a). It is noteworthy that the neurons were the most susceptible cells to the damage caused by the cryolesion. By immunocytochemistry, these MT isoforms are not readily detected in such cells. In cultured cells, MT-I+II have been shown to be expressed to a much lower extent in neurons than in other brain cells (Aschner *et al.*, 1997; Aschner, 1996; Hidalgo *et al.*, 1997). Overall, the decreased MT-I+II protein levels in neurons could contribute significantly to their increased damage following brain injury compared to other brain cells.

The astrocytic and microglial responses to KA-induced neuronal injury were impaired in the MT-KO despite increased neuronal damage as revealed by increased apoptosis. This could also contribute to increased neuronal death because astrocytosis and microgliosis are believed to be essential for neuronal protection during CNS damage (Gebicke-Haerter *et al.*, 1996; Ridet *et al.*, 1997). The exact mechanisms responsible for the impairment of glial function in the MT-KO mice remain to be established. It could be argued that the same factors that affect neuronal survival discussed above, namely, excess zinc levels and oxidative stress, could also affect glial normal function after KA-induced seizures. It is also possible that intrinsic mechanisms of the astrocytes and/or microglia of the MT-KO are altered, e.g. GFAP and GM-CSF levels are reduced in MT-KO mice prior to KA treatment. GM-CSF, mainly expressed in reactive astrocytes, is a potent microglia/macrophage mitogen (Giulian & Ingemann, 1988). Thus, the decreased

astrocytic production of GM-CSF, together with the decreased microglial expression of GM-CSFr, could explain, at least in part, the observed down-regulated microgliosis in the MT-KO. Further analysis of other cytokines/growth factors in this and other models of excitotoxicity and CNS damage is warranted.

MT-III was described as a neuronal 'growth inhibitory factor' because it inhibited survival of rat cortical neurons in culture in the presence of brain extracts, (Uchida *et al.*, 1991; Erickson *et al.*, 1994; Sewell *et al.*, 1995; Palmiter, 1995). Conversely, MT-III is neuroprotective factor in vitro in the absence of brain extracts and in vivo (Erickson *et al.*, 1994; Sewell *et al.*, 1995; Erickson *et al.*, 1997). We have also observed a growth promoting effect of MT-III for cultured rat astrocytes (Carrasco *et al.*, 1999), and thus the physiological role of this protein does not necessarily relate only to neurons. In this report, we show that MT-III mRNA and protein levels are increased by KA-induced seizures in normal mice, and that the protein but not the mRNA levels are further increased in MT-KO mice. The reasons for the inability of the *in situ* method for detecting the expected increase of MT-III RNA levels in the MT-KO are unknown. Changes in protein stability might be a factor to consider. Although very differently regulated, the available evidence suggest that MT-III is very similar structurally and regarding its metal-binding properties to MT-I+II (Sewell *et al.*, 1995; Pountney *et al.*, 1994), which suggests that the three isoforms could share some functions. Thus, one could speculate that the absence of MT-I+II causes a sort of compensatory response of MT-III. Studies similar to those described here in MT-III KO mice will surely help in elucidating this problem.

Acknowledgements

The authors acknowledge Dr. R.D. Palmiter for critically reading the manuscript and for the MT-I probe. Thanks are given to Pernille S. Thomsen and Jordi Canto for excellent technical assistance, and to Keld Stub and Birgit Risto for superb photographic assistance. The help of the Laboratori d'Anàlisi Bioquimica del Departament de Bioquimica i Biologia Molecular is acknowledged. The study was supported by PSPGC PM98-0170, Comissionat per a Universitats I Recerca 1999SGR 00330 and Fundación "La Caixa" 97/102-00 (JH), and by The Novo Nordisk Fonden, Dansk Epilepsi Selskabs Forskningsfond, Dir. Jacob Madsens og Hustrus Fond, Fonden af 17.12.1981, Gerda og Aage Haensch´s Fond, and Københavns Universitets Lægevidenskabelige Fakultetsfond (MP). JC is a fellow of CIRIT FI 96/2613.

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Discusión

DISCUSION

1. Regulación de las MTs durante el estrés y la inflamación

El principal objetivo de la presente tesis ha sido el estudio de la regulación de las MTs durante situaciones fisiológicas. El hígado es el órgano más estudiado desde el punto de vista de la regulación de estas proteínas. No obstante en la última década el estudio de las MTs cerebrales ha ganado en interés a raíz del descubrimiento de la MT-III, debido a su posible implicación en la patología de enfermedades neurodegenerativas (Uchida *et al*., 1991). Por tanto he centrado el interés experimental en las MTs cerebrales y hepáticas, lo que ha permitido hacer una valoración comparativa de la regulación de las MTs en ambos órganos. Son muchas las situaciones en las que las MTs ven alterada su expresión aunque desde un punto de vista fisiológico el estrés y la inflamación son las más relevantes.

El estrés psicológico (por inmovilización) ha sido durante años el principal modelo de estudio en nuestro laboratorio. El estrés por inmovilización induce la síntesis de MT-I+II tanto a nivel hepático (Hidalgo *et al*., 1986a; Hidalgo *et al*., 1986b; Hidalgo *et al*., 1988a) como cerebral (Hidalgo *et al*., 1990), no obstante los factores que median esta inducción no están completamente establecidos. Debido a que la exposición a un estímulo estresante activa el eje HPA, generalmente se ha considerado que los glucocorticoides mediaban la mayoría de las respuestas al estrés, incluida la inducción de las MT-I y -II. Los glucocorticoides son liberados masivamente a la circulación durante el estrés y en los genes de MT-I y –II del ratón se han detectado elementos de respuesta a estas hormonas (Kelly *et al*., 1997). De hecho se ha demostrado que los glucocorticoides participan en el control de las MTs hepáticas durante el estrés. En respuesta a un estímulo psicológico estresante se produce activación del receptor de los glucocorticoides y unión a los elementos de respuesta para estas hormonas presentes en el promotor de las MT-I y -II (Ghoshal *et al*., 1998). En nuestro laboratorio hemos observado que la ADX, y sobre todo la administración del antagonista del receptor de los glucocorticoides RU 486, disminuye la respuesta de las MTs hepáticas al estrés en ratones. No obstante, el efecto es parcial e incluso en el caso de la ADX no afecta a los niveles de mRNA para estas proteínas. Además tampoco parece ser que los glucocorticoides sean los principales reguladores de las MTs cerebrales durante el estrés, ya que la ADX, aunque provoca una tendencia a la disminución de la inducción de la MT-I, no afecta significativamente a sus niveles en el ratón (Belloso *et al*., 1996), aunque sí en rata (Hidalgo *et al*., 1997).

Por otra parte se conoce que las MTs se inducen durante la inflamación tanto en el hígado (Sobocinski and Canterbury, 1982) como en el cerebro (Itano *et al*., 1991). Se piensa que las citoquinas serian los principales mediadores de esta respuesta aunque no se ha podido demostrar ni precisar cuales de ellas. En este sentido se ha observado que los ratones C3H/HeJ, que debido a la mutación *lps* no producen citoquinas en respuesta la endotoxina, presentan menor inducción de las MTs hepáticas durante la inflamación provocada por la administración de LPS (De *et al*., 1990).

En apoyo de un papel regulador, sobre las MTs, para las citoquinas, se sabe que algunas de ellas, como la IL-1, IL-6 y IFN-γ inducen la síntesis de MT-I y -II en cultivo de hepatocitos (Karin *et al*., 1985; Schroeder and Cousins, 1990; Farber, 1992; Vanguri, 1995). Además cuando son administradas *in vivo* provocan un incremento de la síntesis de estas proteínas tanto a nivel hepático (De *et al*., 1990) como cerebral (Hernández and Hidalgo, 1998). Por tanto es probable que las citoquinas controlen la expresión de las MTs en situación fisiológica.

Frecuentemente las citoquinas son capaces de provocar cascadas de estas moléculas que conducen a la amplificación y diversificación de la respuesta. La IL-6 es una de las citoquinas más destacadas y puede provocar cascadas del tipo mencionado. Además se considera que es uno de los principales reguladores de la respuesta de fase aguda durante la inflamación y es liberada a la circulación durante el estrés por inmovilización (Lemay *et al*., 1990). Por tanto, debido a la importancia de esta citoquina y a la disponibilidad de animales KO para la IL-6 (IL6-KO) (Kopf *et al*., 1994) y transgénicos con expresión dirigida a astrocitos de IL-6, (Campbell *et al*., 1993) nos planteamos estudiar su posible implicación en la regulación de las MTs cerebrales y hepáticas durante el estrés y la inflamación.

1.1 Efecto de la IL-6 sobre las MTs hepáticas y cerebrales

Los animales GFAP-IL6 poseen el gen de la IL-6 bajo control transcripcional del promotor de la GFAP. Como consecuencia se produce expresión de la IL-6 dirigida a astrocitos. La exposición prolongada a niveles elevados de esta citoquina provoca una inflamación crónica y progresiva del parénquima cerebral. Estos animales muestran evidencias de neurodegeneración, astrocitosis y microgliosis progresiva. Paralelamente presentan inducción de genes inflamatorios, entre ellos los de varias citoquinas y moléculas de adhesión, así como de proteínas de fase aguda (Campbell *et al*., 1993; Chiang *et al*., 1994).

Resultados previos realizados en nuestro laboratorio obtenidos mediante radioinmunoanálisis mostraban que los niveles proteicos de MT-I+II están inducidos en estos animales (Hernández *et al*., 1997b). En el presente trabajo hemos extendido estos estudios a nivel de mRNA mediante hibridación *in situ* para intentar diseccionar mejor el efecto de la IL-6 sobre la expresión de las MTs. Debido a que la MT-I y –II de ratón se regulan de manera coordinada (Yagle and Palmiter, 1985) hemos considerado los niveles del mRNA de la MT-I como representativos de la expresión de las MTs ubicuas. Nuestros resultados con estos animales demuestran que la expresión de mRNA de MT-I está incrementada significativamente en cerebelo y tallo encefálico. Los niveles de mRNA de MT-I reflejan los observados para las proteínas sugiriendo que la regulación en este modelo experimental es básicamente a nivel transcripcional.

La expresión de la MT-I en estos animales sigue un patrón espacial bien definido que correlaciona con las áreas de máxima expresión de la IL-6 y del gen de fase aguda cerebral EB22/5 (Campbell *et al*., 1993). Esto sugiere que la inducción de la MT-I no formaría parte de una respuesta inespecífica de estrés sino que la IL-6 regularía de manera directa la expresión

de esta proteína. Por otro lado la colocalización con EB22/5 nos permite suponer que la MT-I podría comportarse en el cerebro, de manera análoga a lo considerado en el hígado, como una proteína de fase aguda.

Por el contrario no hemos observado efectos significativos sobre la expresión de la MT-III, exceptuando una ligera inducción en el cerebelo. Por tanto la MT-III parece estar regulada de manera distinta a las isoformas ubicuas. Estos resultados están en consonancia con estudios realizados por otros grupos en los que ha podido observarse que la MT-III no responde a inductores clásicos de las MT-I y –II como los glucocorticoides o los metales (Palmiter *et al*., 1992; Zheng *et al*., 1995a).

Aunque todos los resultados obtenidos parecen indicar que la IL-6 sería el factor implicado en la inducción de las MTs en los animales GFAP-IL6, es arriesgado concluirlo categóricamente. En los animales GFAP-IL6, como consecuencia de la sobreexpresión de IL-6 se produce inducción de la síntesis de otras citoquinas proinflamatorias como la IL-1, el TNF-α e incluso la IL-6 endógena (Chiang *et al*., 1994). A su vez estas citoquinas pueden iniciar toda una serie de respuestas que contribuyen a orquestar la inflamación que observamos en estos animales. Además, como consecuencia de la inflamación se produce lesión del tejido cerebral (Campbell, 1998) que seguramente comporta un incremento de la liberación de radicales libres y metales pesados (Zn, Cu). Tanto las citoquinas (DiSilvestro and Cousins, 1984a; De *et al*., 1990; Sato *et al*., 1992), como el estrés oxidativo (Sato and Bremner, 1993) y los metales pesados (Kägi, 1993) son capaces de inducir la síntesis de las MTs. Por tanto las alteraciones producidas en los animales GFAP-IL6 podrían ser las últimas responsables de la inducción de la MT-I y no la IL-6 *per se.*

Para intentar diseccionar con más precisión los efectos atribuibles a la IL-6 sobre la expresión de las MTs hemos estudiado la regulación de estas proteínas durante el estrés por inmovilización y la inflamación aguda provocada por la administración de LPS y turpentina en los animales IL-6-KO. El hígado es el órgano más estudiado desde el punto de vista de la regulación de las MTs y diversas proteínas sintetizadas en el hígado (proteínas de fase aguda), entre las cuales se piensa que podría incluirse a las MTs, sufren notables alteraciones durante la inflamación. Por otro lado, *in vitro,* la IL-6 posee efectos dispares sobre las MTs en cultivos de hepatocitos o de células procedentes del SNC. Por tanto nos ha parecido interesante comparar el efecto de la IL-6 sobre las MTs cerebrales y hepáticas.

Como era esperable, la MT-I incrementó su expresión en el cerebro tras su exposición a estrés por inmovilización y la inflamación provocada por la administración de LPS o turpentina. Por el contrario, hemos observado que los tratamientos experimentales utilizados no inducen la síntesis de la MT-III, exceptuando un ligero incremento en la capa de Purkinje del cerebelo. Durante el estrés por inmovilización y la inflamación provocada por turpentina, la deficiencia de IL-6 no provocó ningún efecto sobre la inducción de la MTs cerebrales. Por el contrario, los animales IL-6-KO presentaron una menor inducción de la MT-I cerebral por el LPS, aproximadamente el 50%. Respecto a la MT-III no observamos efectos de la deficiencia de IL-6 en ninguno de los tratamientos experimentales utilizados. Estos resultados sugieren

que la IL-6 intervendría en el control de la MT-I durante la inflamación aunque dependería del agente utilizado para provocarla. Por el contrario la MT-I estaría regulada de distinta manera durante el estrés.

Las discrepancias observadas durante la inflamación causada por LPS o turpentina quizás podrían tener una explicación en el conjunto de citoquinas proinflamatorias que incrementan su síntesis en respuesta a ambos tratamientos. En este sentido, se ha observado que la administración de LPS pero no la de turpentina provoca regulación al alza de IL-6, IL-1 y TNF-α en monocitos circulantes, macrófagos peritoneales y en el hígado (Scotte *et al*., 1996). Es posible que la turpentina no provoque la expresión de la IL-6 en el cerebro y por tanto la deficiencia de esta citoquina no posea ningún efecto sobre la expresión de las MTs cerebrales. En cualquier caso la inducción de la MT-I cerebral por la administración de turpentina es notoria, aunque podría ser debida no a la inflamación propiamente dicha sino a una respuesta de estrés asociada al tratamiento. En este caso, a la luz de los resultados expuestos acerca del estrés por inmovilización, la falta de efecto de la IL-6 sobre las MTs no sería sorprendente.

La carencia de efecto de la IL-6 sobre la inducción de la MT-I durante el estrés y el efecto parcial provocado tras la administración de LPS, indican que otros factores adicionales están implicados en el control de esta proteína. El Zn, que es un potente regulador de las MT-I y –II, sufre cambios locales de concentración durante el estrés y la inflamación, por tanto podría ser un factor a tener en cuenta. En este sentido hemos observado que animales sometidos a dietas deficientes en este metal presentan una menor inducción de la MT-I durante el estrés. Por el contrario este efecto no lo hemos observado durante la inflamación provocada por LPS. De nuevo no pudimos notar ningún efecto sobre la expresión de la MT-III. De todas maneras no puede explicarse todo el incremento de los niveles de MT-I como consecuencia de variaciones en las concentraciones locales de Zn. Más adelante se comenta el efecto que otra citoquina, el TNF-α puede tener sobre las MTs cerebrales.

A nivel hepático el estrés por inmovilización y la inflamación provocaron un drástico incremento de la expresión de la MT-I. En este órgano, la deficiencia de IL-6 causó un descenso significativo de la inducción de los niveles de mRNA de MT-I por el estrés aunque el efecto se manifestó sólo en las 4 primeras horas después de la exposición al estímulo estresante, sugiriendo una acción tiempo dependiente. Por otro lado, la respuesta de la MT-I a la inflamación está prácticamente bloqueada en los animales IL-6-KO lo que sugiere que esta citoquina es el principal agente regulador de las MTs hepáticas durante esta situación.

Estos resultados demuestran que la IL-6 controla la inducción de las MTs hepáticas tanto durante el estrés como en la respuesta inflamatoria. No obstante, la importancia relativa de esta citoquina difiere en cada caso. Mientras que durante la inflamación parece ser el principal factor regulador, en el caso del estrés por inmovilización deben de intervenir otros factores adicionales puesto que los ratones IL-6-KO, a las 8 horas de experimento, alcanzan los mismos niveles de mRNA de MT-I que los animales controles. Según los resultados expuestos previamente, los glucocorticoides podrían colaborar con la IL-6 en la regulación de las MTs hepáticas durante el estrés. De hecho, se conoce que estas hormonas participan

conjuntamente con la IL-6 en la regulación de determinadas proteínas hepáticas durante la respuesta de fase aguda (Baumann and Gauldie, 1994).

El amiloide sérico A (SAA) es una de las principales proteínas de fase aguda (Jensen and Whitehead, 1998). Se considera que la IL-6 juega un papel fundamental en el control de la respuesta de fase aguda en general y sobre la expresión de las proteínas de fase aguda en particular (Heinrich *et al*., 1990). Para situar la respuesta de las MTs en el contexto de la respuesta de fase de aguda hemos estudiado simultáneamente la expresión del SAA en el hígado y el efecto que la IL-6 ejerce sobre él.

Tanto el estrés como la inflamación incrementan considerablemente la expresión del SAA. Además esta proteína presenta una dinámica de expresión muy similar a la observada para la MT-I. Si tenemos en cuenta esto y que la MT-I parece regulada por la IL-6 podríamos considerar a las MTs hepáticas como proteínas de fase aguda. De manera análoga a lo observado para la MT-I la deficiencia de IL-6 bloquea casi completamente la respuesta del SAA a la inflamación, confirmando resultados previamente publicados por otros grupos (Kopf *et al*., 1994). No obstante, una notable diferencia emerge en el caso del estrés por inmovilización. La deficiencia de IL-6 provoca la ausencia de inducción de SAA en todos los tiempos estudiados en contraposición a lo observado para la MT-I que sólo estaba afectada en las primeras horas de experimento. Estos resultados confirman que la IL-6 es un factor regulador de notoria importancia no sólo durante la inflamación sino también durante el estrés. Por otra parte, es evidente que no todas las proteínas de fase aguda están reguladas de igual forma (Baumann and Gauldie, 1994), hecho que se pone de manifiesto en las diferencias observadas entre MT-I y SAA.

La IL-6, como otras citoquinas similares, actúa a través de receptores de membrana que tras su unión al ligando activan proteínas quinasas del tipo Jak. La activación de las quinasas Jak puede provocar la fosforilación y consiguiente dimerización y translocación nuclear de los factores de transcripción de la familia STAT que de este modo pueden regular la expresión génica (Heinrich *et al*., 1998). Esta vía de regulación génica ha sido demostrada para varias proteínas de fase aguda (Zhang *et al*., 1996; Heinrich *et al*., 1998; Kim and Baumann, 1999). Uno de los primeros miembros de esta familia de factores de transcripción que se descubrieron fue el STAT 3 (Zhong *et al*., 1994) que presenta una distribución ubicua (Heinrich *et al*., 1998). El STAT3 media algunos de los efectos de la IL-6 tanto a nivel hepático (Gregory *et al*., 1998; Kim and Baumann, 1999) como cerebral (Wu and Bradshaw, 1996; Ihara *et al*., 1997). Por tanto esta proteína podría mediar la regulación de la MT-I en respuesta a la IL-6 tanto en el hígado como en el cerebro. En este sentido, en un trabajo realizado conjuntamente con el grupo de investigación del Dr. Andrews (Univ. Of Kansas City Medical Center) se ha observado que en la zona proximal del promotor de la MT-I existen varias secuencias consenso de respuesta a la IL-6. Como consecuencia de la administración de LPS, en el hígado, se produce translocación de este factor de transcripción al núcleo celular. Paralelamente se ha demostrado que en estas condiciones el STAT3 se une al promotor de la MT-I hepática pudiendo así regular su inducción (Lee *et al*., 1999). En el caso del estrés por inmovilización

hemos observado que el STAT3 incrementa su presencia en el núcleo de células hepáticas, aunque desafortunadamente no hemos podido demostrar que interaccione con el promotor de la MT-I.

En conjunto, de estos resultados podemos concluir que la IL-6 es un importante regulador de las MTs hepáticas durante el estrés y la inflamación; y en este último caso también de las MT-I+II cerebrales. Los efectos de esta citoquina podrían estar mediados por el STAT3.

1.2 Efecto del TNF-α sobre las MTs cerebrales durante el estrés y la inflamación

Aunque la IL-6 parece ser importante en la regulación de las MTs cerebrales durante la inflamación, su efecto cuantitativo es muy diferente respecto al observado en el hígado, ya que mientras que en este último órgano prácticamente se ha eliminado la respuesta, en el cerebro sólo se ha reducido a la mitad. Por tanto, otros factores deben intervenir en la inducción de las MT-I y –II durante la respuesta inflamatoria. Además, ninguno de los factores testados hasta el momento (a excepción de la IL-6) ha resultado ser relevante en el control de las MTs cerebrales durante el estrés.

Las citoquinas son mediadores intercelulares que en muchos casos poseen funciones redundantes entre sí. En concreto, se conoce que la IL-6 y el TNF-α presentan algunas funciones solapadas (Bluethmann *et al*., 1994). El TNF-α se expresa en el parénquima cerebral en respuesta a la administración de LPS (Dunn, 1992; Laye *et al*., 1994) y su administración exógena induce las MTs (De *et al*., 1990; Sato *et al*., 1992). Por otra parte, se produce liberación de esta citoquina a la circulación durante el estrés (Yamasu *et al*., 1992). Por tanto, esta citoquina podría estar implicada en el control de las MTs cerebrales durante la inflamación y el estrés. En la presente tesis se ha evaluado esta posibilidad mediante el uso de animales que expresan de manera constitutiva en el SNC el TNF-α (GFAP-TNFα) y en animales KO para el receptor tipo 1 de esta citoquina (TNFR1-KO).

Los animales GFAP-TNFα poseen expresión dirigida a astrocitos de manera análoga a los GFAP-IL6. Como se utilizó la misma tecnología para generarlos las zonas de expresión del transgen son muy similares a las del caso anterior. No obstante, el fenotipo que presentan estos animales es claramente diferente. A pesar de que el transgen se expresa a lo largo de toda la vida del individuo, las manifestaciones clínicas aparecen en la etapa adulta y sólo en determinado porcentaje de animales. Cuando estas aparecen se caracterizan por meningoencefalomielitis, neurodegeneración y desmielinización con parálisis (Stalder *et al*., 1998). En virtud de la presencia o no de ataxia podemos clasificar los animales en asintomáticos y sintomáticos.

El estudio de la expresión de las MTs ha demostrado que sólo los animales sintomáticos presentan una elevada expresión de MT-I+II, tanto a nivel de mRNA como de proteína, en comparación con los controles. Por tanto, puesto que el TNF-α se expresa de manera continua durante toda la vida del individuo, debemos concluir que al menos en este modelo, la inducción de las MT-I+II es consecuencia de la lesión provocada en el tejido más

que por la expresión de la citoquina *per se*. Contribuyendo a la misma conclusión, la inducción de la MT-I+II no se correlaciona con los lugares de expresión del TNF-α sino con las áreas lesionadas que, en este caso, es fundamentalmente el cerebelo (Stalder *et al*., 1998).

Respecto a la MT-III, prácticamente no se ve afectada su expresión en este modelo experimental. De manera similar a lo observado durante el estrés, el tratamiento con LPS y en los animales GFAP-IL6, se produce cierta inducción en el cerebelo.

Aunque los resultados obtenidos con los animales GFAP-TNFα sugieren que el efecto de esta citoquina sería indirecto, datos generados en los animales TNFR1-KO demuestran lo contrario. La deficiencia funcional de TNF-α no provocó ningún efecto sobre la inducción de la MT-I cerebral por el estrés de inmovilización. Por el contrario disminuyó a un 50% la inducción por LPS en todas las áreas estudiadas. Estos resultados, junto con los comentados en el apartado dedicado a la IL-6, indican que durante el estrés y la inflamación se desencadenan respuestas específicas en las que estarían implicados mediadores diferentes. Así, las citoquinas, en concreto la IL-6 y el TNF-α parecen tener un papel muy importante en la regulación de la MT-I cerebral durante la inflamación provocada por LPS, pero por el contrario no participarían en la respuesta al estrés.

La IL-6 y el TNF-α respecto al control de la MT-I en el contexto de la inflamación, tendrían una importancia relativa similar ya que la deficiencia funcional de cada una de ellas posee un efecto parecido. De hecho, animales KO para la IL-6 y el receptor tipo 1 del TNF-α simultáneamente, no presentan inducción cerebral de MT-I en respuesta al LPS. Estos resultados sugieren que no es necesario el concurso de ninguna otra molécula para regular la MT-I en estas condiciones. Además, probablemente el mecanismo de acción de cada citoquina es totalmente independiente del de la otra puesto que el efecto observado es aditivo.

Por otra parte, los animales TNFR1-KO y los que presentan deficiencia funcional combinada de IL-6 y TNF-α muestran expresión de MT-III similar a la observada en sus controles. Estos resultados demuestran, junto con los expuestos en los párrafos anteriores, que la MT-III posee mecanismos de regulación claramente diferenciados al resto de isoformas. Por tanto, es probable que la MT-III posea funciones específicas que la diferencien del resto de isoformas. Más adelante se comenta el posible papel funcional de la MT-III y las MTs en general.

2. MTs y lesiones cerebrales

La inducción de las MTs durante la inflamación y su control por parte de la IL-6 y el TNF-α nos llevan a pensar que quizás las MTs, al menos las MT-I y –II, podrían jugar un papel relevante en situaciones de inflamación, quizás como proteínas de fase aguda. No obstante, la/s función/es concretas que pueden realizar no se conocen con claridad.

En apoyo a un posible papel de las MTs durante la inflamación, las MT-I+II presentan niveles elevados en el SNC de pacientes afectados de enfermedades neurodegenerativas que poseen un componente inflamatorio importante. Entre ellas, las MT-I+II se inducen en la

enfermedad de Alzheimer (Duguid *et al*., 1989), la esclerosis múltiple (Sillevis Smitt *et al*., 1992a; Sillevis Smitt *et al*., 1994) y la enfermedad de Pick (Duguid *et al*., 1989). Además se sabe que tanto las MT-I y –II como la MT-III sufren cambios en su expresión en respuesta a varios modelos experimentales de lesión cerebral, entre ellas lesiones traumáticas (Hozumi *et al*., 1995; Penkowa and Moos, 1995), excitotóxicas por administración i.p de ácido kaínico (KA) (Anezaki *et al*., 1995; Dalton *et al*., 1995; Zheng *et al*., 1995a) o intracerebral de NMDA (Acarin *et al*., 1999a; Acarin *et al*., 1999b), y la isquemia (Neal *et al*., 1996; Yuguchi *et al*., 1997). No obstante no se ha realizado un estudio comparativo de la expresión de las diferentes isoformas, y no se conocen los posibles mediadores que controlan su síntesis ni las funciones que desempeñan las MTs en situación de lesión del SNC.

En la presente tesis hemos estudiado la expresión y el papel funcional de las MT-I+II en dos modelos de lesión cerebral: criolesión y lesión excitotóxica por administración i.p de KA, en animales KO para estas proteínas. En ambos casos, y de manera análoga a lo descrito en otros modelos de lesión cerebral, se provoca una respuesta inflamatoria en la que las citoquinas, y en concreto la IL-6, poseen un papel coordinador esencial (Minami *et al*., 1991; Woodroofe *et al*., 1991; Taupin *et al*., 1993; de Bock *et al*., 1996; Merrill and Benveniste, 1996; Klusman and Schwab, 1997).

2.1 Regulación de las MTs durante lesiones cerebrales

La criolesión y la administración i.p de KA provocan un incremento de la expresión de MT-I. Estos resultados están en consonancia con los obtenidos con otros modelos de lesión, en los que la administración de una gliotoxina (6-aminonicotinamida, 6-AN) (Penkowa *et al*., 1997) o de NMDA intracerebralmente (Acarin *et al*., 1999b), provocan una regulación al alza de las MT-I+II. No se observa una respuesta general de las MT-I+II sino que la inducción de estas proteínas es claramente dependiente de la lesión ya que sólo se produce en las zonas en las que hay daño tisular. En concreto, la criolesión causa inducción del mRNA de MT-I sobretodo alrededor de la zona necrótica provocada por la lesión, mientras que tras la administración de KA se produce incremento de los niveles de mRNA de MT-I, principalmente en el hipocampo que es la zona más sensible a la acción de este aminoácido excitatorio (Sperk, 1994). Estos resultados están en concordancia con los comentados para los animales GFAP-TNFα que presentaban inducción de la MT-I exclusivamente en aquellas zonas lesionadas como consecuencia de la inflamación crónica. De igual manera la administración de 6-AN provoca inducción de MT-I+II alrededor de las áreas lesionadas por esta gliotoxina, mientras que por el contrario no se observan efectos aparentes en el resto del encéfalo (Penkowa *et al*., 1997) y la administración i.c.v de otro análogo del glutamato, el NMDA, causa máxima inducción de las MT-I y -II en las regiones corticales que han recibido la excitotoxina (Acarin *et al*., 1999b). En conjunto, estos resultados sugieren que la inducción de las MTs como consecuencia de las lesiones cerebrales no es una respuesta general al estrés sino que depende del daño tisular infligido y que por tanto podría estar relacionada con procesos de protección y/o regeneración.

Hasta el momento, las únicas situaciones en las que se ha observado un cambio consistente en la expresión de la MT-III son las lesiones cerebrales (Hozumi *et al*., 1998). Por tanto, *a priori* es razonable pensar que tanto la criolesión como la administración de KA posean un efecto sobre los niveles de mRNA de MT-III. De hecho, tanto lesiones traumáticas (Hozumi *et al*., 1995; Yuguchi *et al*., 1995a) como excitotóxicas (Anezaki *et al*., 1995; Acarin *et al*., 1999a) han demostrado afectar a la síntesis de esta isoforma a nivel de mRNA y proteína. No obstante, la inyección i.p de KA no afectó a la expresión de mRNA de MT-III durante la duración del experimento (24 horas). Por el contrario, en los experimentos de criolesión hemos observado que el mRNA de MT-III varía su expresión en respuesta a la lesión, aunque sólo en la zona inmediatamente adyacente a la zona lesionada. Este modelo de lesión posee un efecto bifásico tiempo-dependiente sobre la MT-III. Tras una criolesión, el mRNA de MT-III disminuye transitoriamente su expresión, y posteriormente (a partir del tercer día) incrementan sus niveles. Este tipo de cinética de la expresión de la MT-III en respuesta a lesiones se ha observado en varios modelos experimentales de lesión (Hozumi *et al*., 1998; Acarin *et al*., 1999a).

Una notable diferencia en la respuesta de la MT-I y la MT-III a la criolesión, además de la dinámica, estriba en la zona de inducción de cada una de las isoformas. La inducción del mRNA de MT-III se limita a la zona inmediatamente adyacente a la lesión, formada por una barrera de células gliales, habitualmente denominada como cicatriz glial, que limita el parénquima cerebral de la zona necrótica (Acarin *et al*., 1999a). Por el contrario el área de inducción de la MT-I es considerablemente más amplia. Este hecho implica que la MT-III incrementa su expresión en células gliales, mientras que en condiciones basales esta isoforma se encuentra principalmente en neuronas (Masters *et al*., 1994b). Estos resultados, en cierta medida, están en consonancia con los obtenidos durante el estrés y la inflamación, en los que observábamos inducción de MT-III en la glia de Bergman del cerebelo, y con estudios realizados en otros laboratorios en condición de lesión cerebral (Uchida, 1994; Hozumi *et al*., 1998).

El mRNA de MT-III incrementa su expresión en respuesta a lesiones excitotóxicas corticales (Acarin *et al*., 1999a), aunque como hemos visto no se induce tras la administración de KA. Estos resultados podrían considerarse como contradictorios. No obstante debemos tener en cuenta que ambos modelos no son totalmente equiparables ya que no se han utilizado los mismos agonistas del glutamato, la vía de administración es distinta y porque el efecto provocado por cada uno de ellos es diferente. Mientras que la administración de NMDA causa muerte celular masiva y localizada en la corteza, la inyección i.p de KA provoca muerte celular en el hipocampo pero de una manera menos acusada y dispersa. Como consecuencia de la zona necrótica que se provoca en el modelo de administración i.c.v se genera una cicatriz glial, por el contrario no hemos observado ninguna estructura de este tipo en los animales tratados con KA. Finalmente, teniendo en cuenta la dependencia del tiempo postlesión que posee la expresión de la MT-III, no podemos comparar directamente un estudio realizado de forma

subaguda (24h tras la administración de KA) respecto a otro más prolongado en el tiempo (del orden de semanas), como fue el caso de la administración de NMDA.

Como he comentado, las lesiones excitotóxicas y de criolesión provocan una respuesta inflamatoria. Las principales moléculas mediadoras de esta respuesta, las citoquinas, son capaces de inducir la síntesis de las MT-I y -II tanto *in vivo* como *in vitro*. En particular la IL-6 parece ser importante en el control de la síntesis de estas proteínas sobretodo durante la inflamación. Por tanto es razonable pensar que la IL-6 podría estar implicada en el control de la respuesta de las MT-I y -II en condiciones de lesión del SNC como las estudiadas en esta tesis. Nuestros resultados confirman esta hipótesis en el caso de la criolesión en el que observamos una drástica disminución de los niveles de mRNA para MT-I en la zona circundante a la lesión en los animales IL-6-KO. Por el contrario la deficiencia de IL-6 no afectó, a corto plazo (24 horas), a los niveles de mRNA para MT-III.

Estos resultados sugieren que de igual manera que ocurría en el caso del estrés y la inflamación, la MT-III está regulada de manera distinta a las isoformas ubicuas. Además, las diferentes dinámicas y magnitudes de inducción sugieren que cada una posee funciones diferentes durante las lesiones cerebrales. El estudio de la regulación de la MT-III en ausencia de las isoformas ubicuas nos puede ayudar a profundizar en este punto. Si las MT-I+II y la MT-III tuvieran las mismas funciones sería esperable que en animales deficientes en las isoformas I y –II, la MT-III compensase mediante su inducción la carencia de las anteriores. Para comprobar cual es el comportamiento de la MT-III en las condiciones comentadas, hemos realizado experimentos de criolesión y administración de KA a ratones KO para la MT-I+II. En ninguno de los modelos observamos ningún tipo de mecanismo compensatorio entre las diferentes isoformas de MTs, de tal manera que la respuesta de la MT-III en los animales MT-KO es esencialmente idéntica a lo observado en los ratones controles.

En conclusión, tanto las isoformas ubicuas como la MT-III se inducen en respuesta a lesiones cerebrales. Este efecto podría estar mediado, al menos en el caso de la MT-I, por la IL-6. Nuestros resultados, junto con los obtenidos por otros grupos, sugieren que las MTs pueden jugar un papel funcional importante en situaciones de lesión del SNC. La MT-III presenta una dinámica de expresión y mecanismos de regulación diferentes al resto de isoformas. Por tanto, es posible que las MT-I y –II y la MT-III se diferencien también en cuanto a sus funciones durante una situación de lesión.

2.2. Funciones de las MTs en situaciones de lesión del SNC

2.2.1 Metalotioneínas –I y –II

La mejor manera de estudiar la contribución específica de las MT-I+II durante procesos inflamatorios del SNC, es el análisis de la respuesta a estas situaciones en animales deficientes para estas proteínas. En la presente tesis para este efecto hemos utilizado dos aproximaciones experimentales diferentes. En una de ellas hemos utilizado animales genéticamente deficientes para las MT-I+II (MT-KO) a los que hemos administrado KA. La

segunda aproximación ha consistido en la obtención de ratas deficientes en Zn, a las que se les ha practicado la criolesión. Estos animales Zn-deficientes, tal como se esperaba presentan niveles inferiores de MT-I+II en el cerebro (Ebadi and Wallwork, 1985; Gasull *et al*., 1994b). Los resultados de estos experimentos, obtenidos en colaboración con M. Penkowa de la Universidad de Copenhague, realizados en animales con deficiencia total o parcial de las MT-I+II nos han permitido obtener conclusiones acerca de la función/es de estas proteínas en el cerebro.

La criolesión, tal y como se esperaba, no provocó ningún cambio evidente en el comportamiento de los animales en los que se les practicó (Penkowa and Moos, 1995). Por el contrario la administración de KA provocó convulsiones generalizadas en prácticamente la totalidad de los animales estudiados. Las propiedades proconvulsionantes del KA son bien conocidas (Ben Ari, 1985). Cuando se administra i.p, sólo un pequeño porcentaje (<1%) del KA atraviesa la barrera hematoencefálica. No obstante, las concentraciones alcanzadas en el SNC son suficientes para iniciar una actividad epileptiforme en el cortex entorrinal que posteriormente se extiende hasta otras áreas del encéfalo (Sperk, 1994). Este estado se caracteriza por una sobreestimulación de las sinapsis excitadoras, y particularmente de las glutamatérgicas.

En los experimentos realizados en nuestro laboratorio, hemos observado que los ratones machos deficientes en MT-I+II presentan mayor sensibilidad, en términos de número de convulsiones y tiempo de duración de estas convulsiones, que los animales controles. No obstante en ambas cepas de animales la eficacia con la que el KA alcanza el SNC es similar, tal como se deduce de los tiempos de latencia observados antes del inicio de las convulsiones. Es interesante notar que estos resultados concuerdan con los obtenidos en animales KO para la MT-III (Erickson *et al*., 1997).

Una posible explicación estriba en la capacidad de las MTs para unir metales pesados, en concreto el Zn. Durante las convulsiones se libera glutamato al espacio intercelular (Benveniste *et al*., 1984). El Zn colocalizado en las vesículas de glutamato (Assaf and Chung, 1984) podría liberarse junto con el neurotransmisor tras la administración de KA (Frederickson *et al*., 1989). El Zn es un factor a tener en cuenta durante las convulsiones puesto que modula la actividad de los receptores de glutamato, disminuye la velocidad de recaptación desde el espacio sináptico de este neurotransmisor y es proconvulsionante *per se* (Ben Ari, 1986; Rassendren *et al*., 1990; Hollmann *et al*., 1993; Cuajungco and Lees, 1997b). Debido a las propiedades de las MT-I y -II, es posible que su deficiencia altere la homeostasis de este metal. De hecho, tras la estimulación con KA, se observa una mayor cantidad de Zn quelable en los animales MT-KO respecto a sus controles. Por tanto, las alteraciones en la concentración de Zn libre en el parénquima cerebral podrían constituir la base de la diferente susceptibilidad al KA observada entre animales MT-KO y controles.

Tanto la criolesión como la administración de KA provocan muerte celular. Aunque en ambos casos los mecanismos por los cuales ocurren presentan puntos en común, también existen notables diferencias entre ellos. El KA administrado i.p provoca muerte neuronal

retardada (Coyle, 1983). Las células más afectadas por este aminoácido son las neuronas que presentan inervación glutamatérgica (Ben Ari, 1985). Son varias las vías por las cuales se cree que el KA puede provocar muerte celular. En primer lugar, cierta cantidad del KA administrado puede alcanzar el SNC y actuar directamente sobre las neuronas que posean su receptor. No obstante, la mayor parte del daño provocado en el SNC es secundario, dependiente de la presencia de convulsiones. Debido a que durante las convulsiones se libera masivamente glutamato (Benveniste *et al*., 1984) y que en cantidades elevadas este neurotransmisor es muy tóxico (Lucas and Newhouse, 1957; Choi *et al*., 1989; Choi, 1992), se produce muerte celular selectiva de las neuronas que reciben sinapsis glutamatérgicas (Sperk, 1994). Este tipo de muerte celular es referido generalmente como excitotoxicidad (Choi, 1992; Dugan and Choi, 1994). Adicionalmente, el Zn coliberado con el glutamato podría estar implicado en la muerte celular selectiva (Frederickson *et al*., 1989; Koh *et al*., 1996). Estos dos mecanismos explican la muerte celular retardada y selectiva. No obstante, la administración i.p de KA, no sólo afecta a las neuronas que reciben sinapsis glutamatérgicas sino también a células gliales. Esta afectación inespecífica no puede ser explicada mediante los mecanismos anteriores y probablemente sea la consecuencia de fenómenos asociados al tratamiento, como la formación de edema, disrupción del flujo sanguíneo normal, isquemia i/o anoxia, y también como consecuencia de la inflamación provocada (Sperk, 1994).

Por el contrario, la criolesión provoca muerte celular de una manera indiscriminada y de forma aguda, principalmente por necrosis. No obstante, después del periodo inicial se produce muerte celular por otros mecanismos, como apoptosis, que pueden prolongarse durante varios días (Penkowa *et al*., 1999a). En este caso, la rotura de la barrera hematoencefálica, la liberación de radicales libres (Coyle and Puttfarcken, 1993; Olanow, 1993) y Zn (Choi *et al*., 1988; Koh and Choi, 1994; Choi, 1996; Koh *et al*., 1996) al medio, y la inflamación (Feuerstein *et al*., 1994; Arvin *et al*., 1996; Merrill and Benveniste, 1996) que acompaña a la lesión pueden ser responsables de la muerte celular causada una vez finalizado el estímulo nocivo.

Generalmente se considera que las MT-I y -II son proteínas que participan en la homeostasis de metales pesados (Kägi, 1993; Masters *et al*., 1994a) y como antioxidantes (Sato and Bremner, 1993). En función de lo expuesto en el párrafo anterior las MT-I y -II podrían jugar un papel importante en el contexto de una lesión. De hecho, tanto los animales KO para la MT-I+II como los deficientes en Zn presentan mayor muerte celular como consecuencia de la criolesión o el tratamiento con KA. Este fenómeno también se ha observado en otro de los modelos utilizados en nuestro laboratorio, la lesión provocada por la gliotoxina 6-AN (Penkowa *et al*., 1999b). Dos de los marcadores de daño oxidativo más utilizados experimentalmente son la peroxidación lipídica y la nitrosilación de las tirosinas de las proteínas. Los animales MT-KO y los deficientes en Zn presentan más cantidad de ambos marcadores en respuesta a la criolesión y el KA. Por otra parte, en ambos modelos experimentales, los animales deficientes total o parcialmente en MT-I+II presentan mayor cantidad de Zn libre. Estos resultados sugieren que las MT-I+II son importantes agentes protectores frente a una lesión, posiblemente debido a sus propiedades antioxidantes y de

unión a metales pesados. Esto podría explicar las diferencias observadas en cuanto a muerte celular no selectiva entre animales controles y MT-KO o sometidos a dietas deficientes en Zn.

Respecto a la excitotoxicidad, las MTs podrían influenciar de una manera similar. El Zn puede alterar la toxicidad de los agonistas del glutamato (Koh and Choi, 1988) y se ha observado que este metal podría jugar un papel muy importante en la muerte celular selectiva en determinados modelos experimentales como las convulsiones y la isquemia (Frederickson, 1989; Koh *et al*., 1996). Por otra parte, tras la estimulación por glutamato se inducen en la célula múltiples vías que conducen a la producción de radicales libres (Coyle and Puttfarcken, 1993) que podrían contribuir significativamente a los mecanismos de la excitotoxicidad (Dugan and Choi, 1994). Adicionalmente el exceso de radicales libres y Zn provoca liberación de glutamato e inhibición de su recaptación lo que puede conducir a un ciclo autoperpetuable de muerte celular (Olanow, 1993). Por tanto en los animales MT-KO, debido al exceso de radicales libres y Zn que se puede acumular respecto a los controles, los efectos nocivos de la excitotoxicidad podrían ser más notorios.

Los dos modelos de lesión utilizados van acompañados de inflamación. La inflamación en el SNC posee rasgos característicos ya que participan células provenientes de la médula ósea pero sobretodo está orquestada por células residentes como los astrocitos y la microglia (Mattson and Scheff, 1994; Ridet *et al*., 1997). En este contexto, los astrocitos y la microglia cambian su morfología y actividad convirtiéndose en células productoras de citoquinas, factores quimiotácticos y de crecimiento, que contribuirán a modular la respuesta inflamatoria. Generalmente se considera que esta respuesta es necesaria para minimizar el daño provocado y organizar la regeneración del tejido (Gebicke-Haerter *et al*., 1996; Ridet *et al*., 1997).

Como era esperable tras la administración de KA y la criolesión se provocó astrocitosis y microgliosis. No obstante esta respuesta presentó notables diferencias entre los animales controles y los deficientes en MT-I+II. En estos últimos la respuesta astrocitaria y microglial inicial (3 días) estaba significativamente disminuida respecto a sus controles tras la administración de KA. La deficiencia de MT-I+II provocó efectos similares sobre la astrocitosis tras la criolesión, aunque incrementó el número de células microgliales. Las diferencias observadas entre animales controles y MT-KO tras la administración de 6-AN son muy similares a los descritos para el KA (Penkowa *et al*., 1999b). En general podríamos decir, que la respuesta inflamatoria, en los animales deficientes en MT-I+II es anómala, destacando el descenso de la activación de astrocitos. El déficit en el reclutamiento de astrocitos podría explicarse debido al descenso observado en los animales MT-KO en la inmunoreactividad del GM-CSF, un potente factor de crecimiento para este tipo de células, tras el tratamiento con KA y 6-AN. En cualquier caso, la deficiente respuesta glial que observamos en estos animales podría contribuir al mayor daño tisular observado durante la lesión.

Estos resultados sugieren que las MT-I+II son unas proteínas importantes para la fisiología de las células gliales. No obstante su posible importancia parece estar limitada a periodos de activación de este tipo de células, como ocurre durante la inflamación, ya que en situación basal tanto el número como aspecto morfológico de astrocitos y microglia no se ven

afectados. El mecanismo concreto por el cual las MT-I+II influyen sobre la actividad de astrocitos y microglia durante la inflamación no se conoce. Los monocitos son células funcionalmente relacionadas, en periodos de inflamación, con astrocitos y microglia. Con estas últimas también les une una relación ontogénica puesto que se considera que las células microgliales provienen de monocitos infiltrados en el SNC durante el desarrollo. En una línea celular derivada de monocitos humanos se ha observado que la manipulación experimental a la baja de los niveles de MT-I+II provoca un descenso de su actividad (Leibbrandt *et al*., 1994; Leibbrandt and Koropatnick, 1994; Koropatnick and Zalups, 1997). Más interesante aún es el déficit de actividad detectado en monocitos de ratones MT-KO (Koropatnick, comunicación personal). Además, las MT-I+II pueden afectar a la actividad de otras células del sistema inmunitario como linfocitos y macrófagos (Lynes *et al*., 1990; Lynes *et al*., 1993; Youn *et al*., 1995; Borghesi and Lynes, 1996; Lynes *et al*., 1999).

Paralelamente a los resultados presentados en esta tesis, hemos observado que tras la criolesión, en los animales MT-KO, la respuesta inflamatoria no se resuelve de manera normal y se prolonga en el tiempo (Penkowa *et al*., 1999a), presumiblemente debido a una actividad incorrecta de los monocitos/microglia. Aunque se considera que la inflamación es una respuesta beneficiosa, también es cierto que si se prolonga esta situación se puede agudizar el daño tisular (Campbell, 1998). En este sentido, los animales MT-KO poseen mayor daño tisular y no regeneran el tejido durante el periodo de tiempo considerado normal para los animales controles (Penkowa *et al*., 1999a). Con estos resultados, es razonable pensar que las MT-I+II poseen un papel inmunomodulador en situación de lesión cerebral. Aunque no se conoce el mecanismo por el cual podrían actuar, en otros contextos también se han atribuido funciones similares a las MT-I+II (Lynes *et al*., 1999).

A la luz de los resultados presentados en esta tesis, y otros realizados por otros miembros de nuestro grupo investigador, podríamos concluir que las MT-I+II juegan un papel protector durante situaciones de lesión cerebral. Estos efectos serian debidos a sus propiedades antioxidantes y de unión a metales pesados aunque su posible actividad inmunomoduladora no puede ser despreciada.

2.2.2 Metalotioneína –III

La MT-III se descubrió en cerebros humanos como una proteína que se encontraba disminuida en pacientes de Alzheimer (Uchida *et al*., 1991). Además, se observó que la MT-III, pero no el resto de isoformas, poseía actividad inhibitoria del crecimiento de neuronas en cultivo en presencia de extractos cerebrales. Por estas razones se propuso que podría participar en la patogénesis de la enfermedad de Alzheimer (Uchida *et al*., 1991). La actividad inhibitoria a sido demostrada por otros laboratorios (Erickson *et al*., 1994; Sewell *et al*., 1995) aunque el descenso de sus niveles en enfermos de AD no ha podido ser confirmado (Erickson *et al*., 1994; Amoureux *et al*., 1997).

El número de muestras procedentes de enfermos de AD que se han analizado en los estudios previos no es suficiente como para poder extraer conclusiones acerca de la

implicación de la MT-III en esta enfermedad. Por tanto consideramos interesante analizar muestras adicionales en nuestro laboratorio. En nuestro caso hemos observado un incremento de la expresión de MT-III. Debido a la disparidad de resultados obtenidos entre laboratorios debemos concluir que esta proteína no sería un factor principal a tener en cuenta en la AD.

Respecto a la función de la MT-III, se ha propuesto que podría poseer actividad moduladora del crecimiento y desarrollo de células nerviosas tras una lesión (Hozumi *et al*., 1998). Estos cambios habitualmente se interpretan en función de la posible actividad neurotrófica de esta proteína (Uchida *et al*., 1991; Hozumi *et al*., 1998). Basándose en la actividad inhibitoria del crecimiento neuronal en cultivo de la MT-III (Uchida *et al*., 1991), se ha especulado que el descenso inicial favorecería el desarrollo de neuritas para restablecer en la medida de lo posible las conexiones intercelulares. Por el contrario su posterior inducción respondería a la necesidad de evitar un crecimiento excesivo.

Por otra parte, recientemente se ha observado que posee actividad neurotrófica sobre las neuronas piramidales de la capa CA3 del hipocampo tras la administración de KA (Erickson *et al*., 1997). No obstante no se sabe si este efecto lo ejerce directamente sobre las neuronas o lo realiza por mediación de los astrocitos, que en circunstancias de lesión cerebral protegen el tejido de daños adicionales (Gebicke-Haerter *et al*., 1996; Ridet *et al*., 1997). De hecho inmunocitoquímicamente, en casi todos los estudios realizados, se ha detectado la MT-III principalmente en células gliales (Uchida, 1994; Yamada *et al*., 1996; Hozumi *et al*., 1998). Además, tanto nuestros resultados como los publicados por otros grupos apuntan que las células en las que se producen cambios de expresión de la MT-III en respuesta a lesión cerebral son astrocitos. En este sentido es destacable la inducción de esta proteína en la cicatriz glial formada tras la criolesión (datos presentados en esta tesis) y excitotoxicidad provocada por NMDA (Acarin *et al*., 1999a).

A pesar de que la MT-III se ha detectado en astrocitos, no se ha evaluado la posible acción trófica que pudiera tener sobre este tipo de células. En la presente tesis hemos considerado oportuno estudiar el efecto de la MT-III sobre astrocitos en cultivo, utilizando un bioensayo para la migración astrocitaria tras una lesión mecánica, similar al descrito por Faber-Elman y colaboradores (Faber-Elman *et al*., 1995). La MT-III ha demostrado poseer un potente efecto activador de la migración astrocitaria *in vitro* superior al observado para las MT-I+II. Desafortunadamente este bioensayo no ha podido ser aplicado a cultivos neuronales. No sabemos si la acción de la MT-III se manifestará también *in vivo*, aunque es tentador pensar que la inducción de la expresión de esta proteína en la cicatriz glial tras una lesión obedezca a su posible intervención en la migración astrocitaria.

De todas maneras, potencialmente la MT-III podría actuar como antioxidante e intervenir en la homeostasis de metales pesados (Aschner, 1996). Por tanto, a pesar de las reiteradas diferencias observadas respecto al resto de isoformas, no podemos descartar que también intervenga en la protección del tejido frente a una lesión mediante mecanismos similares a los supuestos para las isoformas ubicuas. En este sentido se ha observado que ratones KO para la MT-III son más sensibles que sus controles, en términos de muerte

neuronal en el área CA3 del hipocampo, tras la administración i.p de KA (Erickson *et al*., 1997). Actualmente estamos realizando en nuestro laboratorio experimentos con animales KO para la MT-III que posiblemente contribuyan a esclarecer la/s función/es de esta proteína.

Conclusiones

CONCLUSIONES

1. Las MT-I+II se inducen por una variedad de tratamientos de distinta naturaleza que en general provocan estrés y respuestas inflamatorias, como la inmovilización, la administración de agentes inflamatorios (LPS y turpentina), citoquinas proinflamatorias (IL-6 y TNF-α) y lesiones del SNC (criolesión y administración i.p de KA).

2. La MT-III está regulada de manera distinta al resto de isoformas. La MT-III no responde, como el caso de lesiones por administración de KA, o presenta cambios mucho más atenuados que las MT-I+II a los estímulos que inducen notablemente estas últimas. Cuando se producen cambios en la expresión de MT-III pueden darse en la misma dirección que los observados para las MT-I+II, como durante el estrés, la inflamación causada por LPS, o la expresión continuada de TNF-α, o en sentido contrario, como ocurre en algunas áreas cerebrales debido la expresión sostenida de IL-6. Un caso particular es la criolesión donde se observa una respuesta bifásica, disminuyendo los niveles de MT-III a corto plazo (1 día postlesión) para posteriormente inducirse su expresión (a partir de 3 días postlesión).

3. A nivel de mRNA la MT-I y la MT-III presentan un patrón de expresión en el SNC claramente diferenciado. De acuerdo con la literatura, el análisis mediante hibridación *in situ* revela que la MT-I se expresa fundamentalmente en células gliales mientras que la MT-III es más abundante en neuronas. El estudio inmunocitoquímico, usando el suero policlonal obtenido frente a la MT-III, revela diferencias sustanciales respecto a lo observado a nivel de mRNA. La inmunoreactividad para MT-III se localiza mayoritariamente en células gliales aunque también se ha detectado en neuronas. El suero policlonal anti-MT-III reconoce la MT-III recombinante de rata pero no a las MT-I+II, por lo que las diferencias entre mensajero y niveles proteicos no puede atribuirse a problemas de reacción cruzada con el resto de isoformas.

4. La IL-6 es un factor muy importante en el control de las MT-I+II hepáticas y cerebrales durante la respuesta inflamatoria como lo demuestran los datos obtenidos en los animales GFAP-IL6 y IL6-KO. La expresión sostenida de IL-6 en el SNC de los animales GFAP-IL6 incrementa significativamente los niveles de mRNA para MT-I+II. Por el contrario la deficiencia de IL-6 provoca una disminución significativa en la inducción de la MT-I por agentes inflamatorios y durante lesiones del SNC. El STAT-3 podría mediar los efectos de la IL-6 sobre la MT-I durante la inflamación, al menos en el hígado.

5. Durante el estrés, la IL-6 afecta a la inducción de la MT-I en el hígado pero no en el cerebro. El efecto de la IL-6 sobre la MT-I hepática es transitorio, afectando solo durante las primeras 4-5 horas de estrés. De manera análoga a la inflamación, el STAT-3 podría

intervenir en esta respuesta. Otros factores, distintos de los glucocorticoides, intervendrían en el control de la MT-I en el contexto del estrés.

6. El TNF-α interviene en la inducción de las MT-I+II cerebrales durante la inflamación provocada por LPS. Su efecto sería aditivo al de la IL-6 puesto que la deficiencia combinada de ambas citoquinas provoca efectos superiores a los observados para la deficiencia individual de cada una de ellas, de tal manera que en los animales KO para ambas citoquinas no se produce respuesta de la MT-I al LPS.

7. Los animales deficientes en MT-I+II presentan mayor susceptibilidad a lesiones del SNC (criolesión y administración i.p de KA) que los animales controles, que se manifiesta en una menor astrocitosis, mayor microgliosis, desajustes en las concentraciones locales de Zn libre, incremento del estrés oxidativo y muerte celular por apoptosis. Estos datos sugieren que las MT-I+II cerebrales podrían actuar como proteínas protectoras frente a lesiones cerebrales gracias a sus propiedades antioxidantes, de unión a metales pesados y quizás modulando la respuesta inflamatoria.

8. Los animales MT-KO no presentan alteraciones en la expresión del mRNA de MT-III en situación basal ni durante lesiones del SNC (criolesión y administración de KA), respecto a los animales controles (con niveles normales de MT-I+II), indicando que no existen mecanismos compensatorios entre las diferentes isoformas. Por el contrario la deficiencia de MT-I+II provoca un incremento de los niveles proteicos de MT-III en respuesta a lesiones del SNC (criolesión y administración de KA), observándose de nuevo discrepancias entre los datos de inmunocitoquímica y de hibridación *in situ*.

9. La MT-III y en menor medida la MT-I, en cultivo, promueven la migración astrocitaria sugiriendo que estas proteínas poseen efectos tróficos sobre astrocitos.

10. La MT-III no parece ser un factor de relevancia en el desarrollo de la enfermedad de Alzheimer.

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