ELSEVIER

Brain Research 767 (1997) 345–355

BRAIN RESEARCH

Research report

Induction of metallothionein in astrocytes and microglia in the spinal cord from the myelin-deficient jimpy mouse

José Miguel Vela [a,*], Juan Hidalgo [b], Berta González [a], Bernardo Castellano [a]

[a] *Department of Cell Biology and Physiology, Unit of Histology, Faculty of Medicine, Torre M-5, Autonomous University of Barcelona, Bellaterra, 08193 Barcelona, Spain*

[b] *Department of Cell Biology and Physiology, Unit of Animal Physiology, Faculty of Sciences, Autonomous University of Barcelona, Bellaterra, 08193 Barcelona, Spain*

Accepted 6 May 1997

Abstract

Jimpy is a shortened life-span murine mutant whose genetic disorder results in severe pathological alterations in the CNS, including hypomyelination, oligodendrocyte death and strong astroglial and microglial reaction. The knowledge of metallothionein (MT) regulation in the CNS and especially of MT presence in specific glial cell types under pathological conditions is scarce. In the present study, immunocytochemical detection of MT-I + II has been performed in spinal cord sections from 10–12- and 20–22-day-old jimpy and normal animals. The identification of MT-positive glial cells was achieved through double labeling combining MT immunocytochemistry and selective markers for oligodendrocytes, astrocytes and microglia. MT was found in glial cells and was present in the spinal cord of jimpy and normal mice at both ages, but there were remarkable differences in MT expression and in the nature of MT-positive glial cells depending on the type of mouse. The number of MT-positive cells was higher in jimpy than in normal spinal cords. This was apparent in all spinal cord areas, although it was more pronounced in white than in the gray matter and at 20–22 days than at 10–12 days. The mean number of MT-positive glia in the jimpy white matter was 1.9-fold (10–12 days) and 2.4-fold (20–22 days) higher than in the normal one. Astrocytes were the only parenchymal glial cells that were positively identified as MT-producing cells in normal animals. Interestingly, MT in the jimpy spinal cord was localized not only in astrocytes but also in microglial cells. The occurrence of MT induction in relation to reactive astrocytes and microglia, and its role in neuropathological conditions is discussed. © 1997 Elsevier Science B.V.

Keywords: Neuroglia; Hypomyelination; Immunohistochemistry; Lectin histochemistry; Double staining

1. Introduction

Jimpy is an X-linked recessive disorder in which central nervous system (CNS) pathology in affected male mice results in a 95% reduction in the amount of CNS myelin [65]. Jimpy animals begin to exhibit body tremors at 8–10 days of age and die before day 30. The genetic defect consists in a point mutation in the gene coding for a major CNS myelin component, the proteolipid protein (PLP) [17]. Besides the myelin deficit, histological studies revealed severe alterations in relation to glial cells. Alterations in oligodendroglial cells include protracted proliferation [57], cell cycle irregularities [30,64] and a drastic reduction in the number of well-differentiated oligodendrocytes as a consequence of their premature death [33]. Astrocytes in jimpy exhibit a pronounced gliosis with the occurrence of numerous hypertrophied astrocytes [6,56] showing an increased glial fibrillary acidic protein (GFAP) content and mRNA expression [38]. In addition, jimpy astrocytes are unable to increase oxygen uptake in response to K^+-induced stimulation [27], show an increased intracellular Ph [32] and their cell cycle is lengthened [31]. In regard to the microglial cell population, an intense microglial reaction has been described in the jimpy spinal cord [61]. In contrast to the typical ramified appearance and regularly-spaced distribution of 'resting' microglia observed in different normal CNS areas [10,34,62], the jimpy microglia show reactive morphological features and their number is largely increased [61]. In the spinal cord, the mean microglial densities in the mutant white matter are about threefold (10–12 days) and fivefold (20–22 days) higher than in normal animals. These increased numbers of microglia are in accordance with the reactive proliferation of these cells [64]. A specific physical relation

* Corresponding author. Fax: +34 (3) 581-2392.

between microglial cells and apoptotic oligodendrocytes has also been described in the jimpy spinal cord [63]. These reactive glial alterations strongly suggest a disturbance in the development and relations of glial cells resulting from an intrinsic CNS damage.

Metallothioneins (MTs) constitute a class of metal-binding proteins whose expression is known to markedly increase following injury and inflammation in several tissues [24,50]. In fact, MT is now considered to play a significant role in cellular defense mechanisms and is thought to play a protective role against oxidative damage in tissue injury and inflammation [50]. Cytokines which are mediators of various kinds of inflammatory and immune responses, such as interleukin-1 (IL-1) and -6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), are effective inducers of hepatic MT [18,51]. Interestingly, glucocorticoids, LPS and the cytokines IL-1 and IL-6 induce astrocytic MT in vitro [14,18,28,51,52]. In vivo, MT expression in astrocytes is also increased in injured CNS [46,55], but only limited attention has been paid to its distribution, regulation and function in CNS pathology. In addition, there are no available studies focused on the unambiguous identification of MT-positive cells and it remains to be determined if glial cells other than astrocytes might express MT under pathological conditions.

The aim of the present study was to analyze the production of MT and to determine the nature of MT-positive cells in spinal cords from the hypomyelinated jimpy mouse. MT expression in spinal cord sections from jimpy and normal male littermates of 10–12 and 20–22 days old was assessed by immunocytochemistry using a monoclonal antibody specific for the MT-I and MT-II isoforms. The identification of MT-positive cells was achieved through double labeling combining MT immunocytochemistry with selective markers for oligodendrocytes, astrocytes and microglia.

2. Materials and methods

2.1. Experimental animals

C57BL/6J mice used in this study were males raised here in the UAB animal center by mating jimpy carrier females ($jp/+$) obtained from the Institute Pasteur with normal males ($+/Y$). Jimpy hemizygous males (jp/Y) were distinguished from normal control males ($+/Y$) by their characteristic motor tremors and drastic myelin reduction. Five jimpy mice of 10–12 and five of 20–22 days old were used in this study. The same number of normal male littermates of each age were used as controls.

2.2. Preparation of sections

Animals were anesthetized with sodium pentobarbital (100 mg/kg body weight) and sacrificed by intracardiac perfusion with a 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.2, for 5 min. The cervical segment of the spinal cord was removed and placed in the same fixative for 4 h at 4°C. Samples were then rinsed in phosphate buffer for 4 h, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Spinal cords were coronally sliced with a microtome to obtain 10-μm thick sections that were mounted on glass slides pretreated with 1:10 poly-L-lysine solution (Sigma). Finally, sections were dewaxed, hydrated and immunocytochemically processed.

2.3. Immunocytochemical reaction for MT

Sections were treated for 10 min with 2% H_2O_2 in 100% methanol to block endogenous peroxidase, rinsed 3×5 min in 0.05 M Trizma base saline (TBS) and sequentially incubated for: (1) 30 min at room temperature (RT) in TBS containing 10% fetal calf serum and 0.1% bovine serum albumin (BSA) (blocking buffer; BB); (2) overnight at 4°C with the primary monoclonal mouse anti-MT antibody E9 (generous gift from Dr. Pete Kille) diluted 1:8000 in BB; (3) 60 min at RT with anti-mouse IgG biotinylated antibody (Amersham) in a 1:200 dilution in BB; and (4) 60 min at RT with a 1:600 dilution of avidin–peroxidase complex (Sigma) in BB. Between successive incubation steps, the sections were rinsed 3×5 min in TBS with 0.1% Triton X-100. The peroxidase reaction was visualized by transferring the sections for 3 min to 100 ml of 0.05 M Trizma base (TB), pH 7.4, containing 50 mg 3,3′-diaminobenzidine (DAB; Sigma), 4 ml of 1% ammonium nickel sulfate, 5 ml of 1% cobalt chloride and 66 μl H_2O_2. Finally, sections were rinsed, dehydrated in graded ethanol, cleared in xylene, and coverslipped in DPX. As a control for the immunocytochemical staining, the primary antibody was omitted.

2.4. Double-labeling technique

Simultaneous demonstration of MT and astroglia, oligodendroglia or microglia was achieved through the sequential combination of MT immunostaining and selective markers for these glial cells. Astrocytes were demonstrated by immunocytochemical detection of glial fibrillary acidic protein (GFAP) [7] and oligodendroglial cells by immunodetection of myelin basic protein (MBP) [58]. The microglial cell population was demonstrated by tomato lectin histochemistry, a marker for both normal [2] and reactive microglia [1,63].

In brief, sections were rinsed 3×5 min in TBS, treated for 10 min with 2% H_2O_2 in 100% methanol to block peroxidase and rinsed again 3×5 min in TBS including 0.1% Triton X-100. Sections were placed in BB for 30 min to reduce unspecific staining, and incubated overnight at 4°C either with the rabbit anti-GFAP antibody (Dakopatts) diluted 1:200 in BB, with the rabbit anti-MBP antibody (Dakopatts) diluted 1:500 in BB or with the biotinylated tomato lectin (*Lycopersicon esculentum*; Sigma) diluted to

15 μg/ml in TBS. After rinsing 3 × 5 min in TBS with 0.1% Triton X-100, the sections reacted with primary anti-GFAP and anti-MBP antibodies were incubated for 60 min at RT with a secondary anti-rabbit IgG biotinylated antibody (Amersham) in a 1:200 dilution in BB. Finally, all sections were rinsed 3 × 5 min in TBS with 0.1% Triton X-100 and incubated for 60 min at RT with avidin–peroxidase (Sigma) in a 1:600 dilution in BB. The peroxidase reaction was visualized using DAB as chromogen but without metal intensification. As a control for the double staining, either the primary antibodies or the lectin were omitted.

2.5. Quantification and identification of MT-positive cells

The quantitative study was carried out by counting MT-positive glial cells in randomly selected spinal cords hemisections. Five hemisections per animal, amounting a total of 25 jimpy and 25 normal spinal cord hemisections

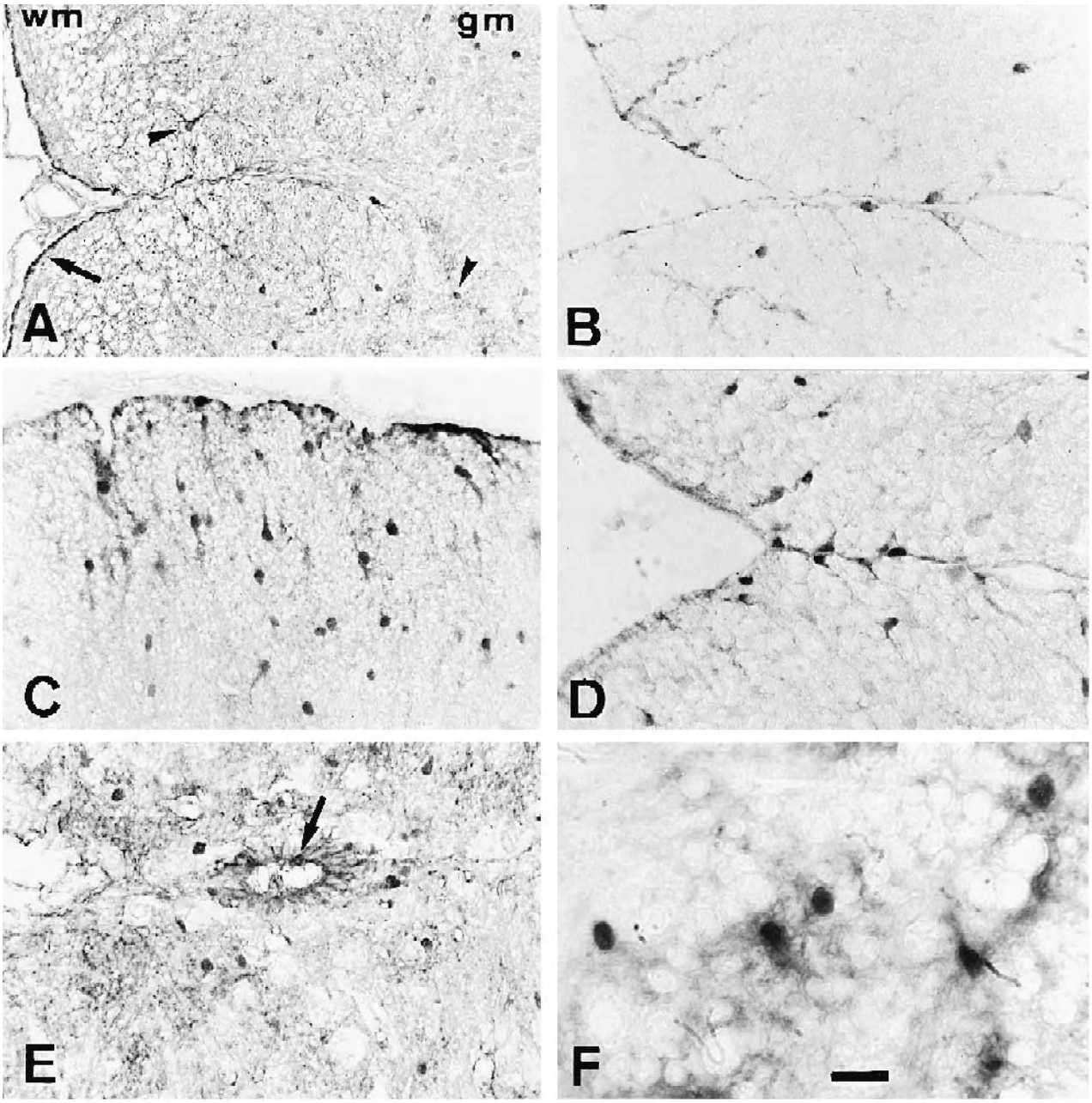


Fig. 1. Photomicrographs showing MT immunoreactivity in spinal cord sections from normal (A, B) and jimpy (C–F) mice. A, B: MT-positive glial cells (arrowheads) in 20–22-day-old normal animals (A) were more abundant in the gray matter (gm) than in the white matter (wm). At 10–12 days, MT-positive glial cells were clearly recognized in the white matter (B). Note the presence of MT immunoreactivity in relation to pia mater (arrow in A). C–F: in jimpy, numerous MT-positive cells were seen in the white (C, D, F) and gray matter (E) at both ages. Note that ependymal cells of the central canal showed MT immunostaining (arrow in E) and that MT immunostaining in parenchymal glial cells had a predominant nuclear location. Bars: A = 50 μm; B–E = 25 μm; F = 10 μm.

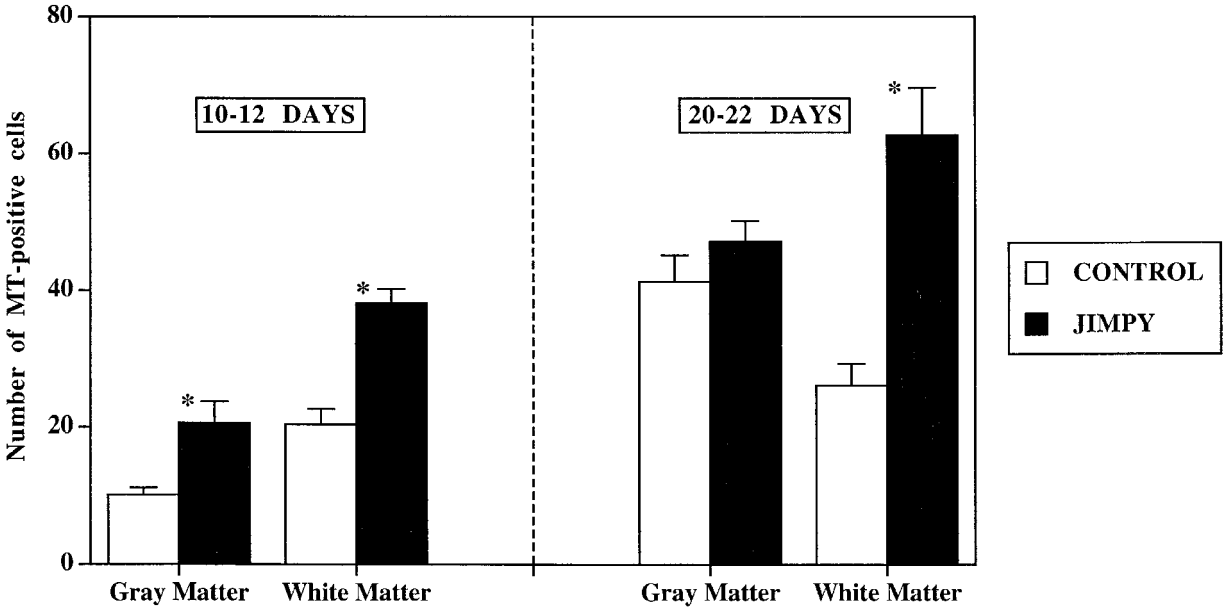


Fig. 2. Histogram showing the mean number of MT-positive cells estimated in the gray and white matter spinal cord from 10–12- and 20–22-day-old normal and jimpy mice. Values were obtained by counting MT-positive cells in spinal cord hemisections. Note that, at both ages, the number of MT-positive cells was higher in jimpy than in normal spinal cords. Asterisks at the upper left corner of jimpy columns indicate significant differences ($P < 0.05$) as compared to values in normal mice (analysis of variance and Scheffé test). Error bars indicate standard error.

per age, were analyzed under the microscope by using a 25× objective. Analysis of variance (ANOVA) and Scheffé test was applied to determine statistical differences in the numbers of glial cells showing MT immunostaining. The effect of the following variables: type of mouse (normal or jimpy), age (10–12 or 20–22 days), and location (gray or white matter), was considered.

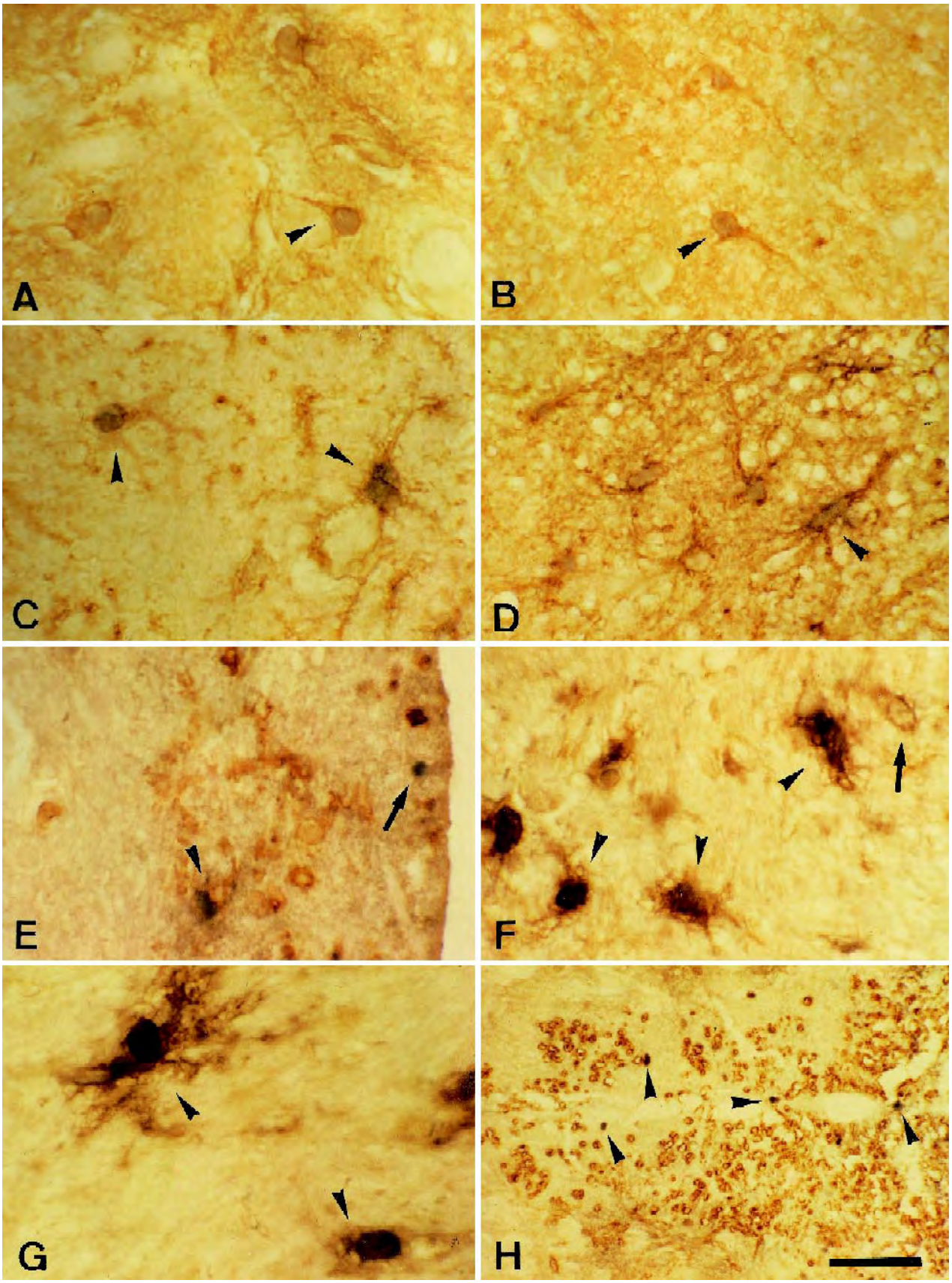
In order to estimate the percentage of MT-positive cells simultaneously expressing either the oligodendrocytic, the astrocytic or the microglial marker, two sections per animal, from a total of 10 jimpy and 10 normal spinal cord sections per age, processed for the three different double-labeling techniques, were analyzed under the microscope by using a 40× objective, or even a 100× objective when necessary.

2.6. Cross-reactivity of the monoclonal antibody E9 for MT isoforms

In brain studies it is essential to distinguish between the classic MT isoforms, MT-I and MT-II, and the more recently discovered brain-specific MT isoform, MT-III. The monoclonal antibody E9 has been extensively used by several investigators and is now commercially available from Dakopatts (M 0639), but the specificity of this antibody regarding its cross-reaction with MT-III is unknown. In this study, we have developed an enzyme-linked immunosorbent assay (ELISA) in order to characterize the cross-reactivity of the monoclonal mouse anti-MT antibody E9 with the different MT isoforms.

Polystyrene plates were coated overnight with increasing amounts (up to 100 ng/well) of purified rat liver MT-I and MT-II [22] or recombinant rat MT-III (plasmid generously provided by Dr. Pete Kille) in 100 mM carbonate buffer, pH 9.6, at room temperature and in a water-saturated atmosphere. Plates were rinsed three times for 5 min with 0.05% Tween-20 in phosphate-buffered saline (T-PBS), and the remaining active groups were blocked by incubation for 2 h with 200 μl/well of T-PBS containing 1% BSA. The plates were rinsed as above and incubated for 2 h at 37°C in a water-saturated atmosphere with 100 μl/well of a 1:10 000 dilution in T-PBS + BSA of the E9 antibody. After rinsing, the plates were incubated for 2 h with 200 μl/well of anti-mouse IgG biotinylated antibody

Fig. 3. Simultaneous visualization of MT-positive cells and astroglia (A–D), microglia (E–G) and oligodendroglia (H) in the normal (A–B) and jimpy (C–H) spinal cord. MT immunostaining was blackish and predominantly located in the nucleus, whereas staining of glial cells was brownish and cytoplasmatic. Oligodendroglial and astroglial cells were demonstrated by immunocytochemical detection of MBP and GFAP, respectively, and microglial cells by tomato lectin histochemistry. A, B: astrocytes showing double MT/GFAP immunostaining (arrowheads) were present both in the gray (A) and white matter (B) of normal animals. C, D: in jimpy, astrocytes showed increased GFAP immunoreactivity and MT-positive astrocytes were clearly recognized both in the gray (C) and white matter (D). E, G: microglial cells showing MT immunoreactivity (arrowheads) were also found in jimpy. Note both the presence of MT-positive cells lacking tomato lectin reactivity (arrow in E) and the presence of microglial cells lacking MT immunostaining (arrow in F). H: MT-positive cells (arrowheads) located in the jimpy white matter were MBP-negative. Bars: A–D, F, G = 20 μm; E = 50 μm; H = 80 μm.


A
B
C
D
E
F
G
H

(Amersham) diluted 1:200 in T-PBS + BSA, rinsed again, and further incubated for 1 h with 200 μl/well of a 1:600 dilution in T-PBS + BSA of avidin–peroxidase complex (Sigma). The plates were then rinsed, the peroxidase substrate solution containing 40 mM 4-dimethylaminobenzoic acid (4-DMAB), 0.8 mM 3-methyl-2-benzothiazolinone hydrochloride (MBTH), and 3 mM H_2O_2 diluted in 100 mM phosphate buffer, pH 7, was added, and finally the absorbance was read at 600 nm.

3. Results

3.1. Distribution and number of MT-positive cells

Microscopical examination of spinal cord sections immunocytochemically processed for MT using the E9 monoclonal antibody revealed that MT-positive cells were present in the gray and white matter of both the 10–12- and the 20–22-day-old normal and jimpy animals (Fig. 1). MT was found mainly and abundantly in parenchymal cells, identified as glial cells on the basis of their location in the parenchyma, their nuclear size and their morphological features. MT immunostaining had a predominant nuclear location, although the perinuclear cytoplasm and processes of some cells also showed MT immunostaining.

The quantitative analysis indicated that the distribution and number of MT-positive glial cells in the mutant differed from those of the normal animal at both ages (Fig. 2). In normal animals, MT-positive cells were found in the gray and white matter at both ages, but at 20–22 days the number of MT-positive cells was significantly higher, particularly in gray matter. In jimpy, MT-positive cells were numerous throughout the spinal cord and the mean number of MT-positive cells was higher at 20–22 days than at 10–12 days, and it was 1.9- (10–12 days) to 1.3-fold (20–22 days) higher in white than in gray matter.

When comparing jimpy and normal animals, the mean number of MT-positive cells in the spinal cord was significantly higher in the mutants, this difference being more pronounced in white than in the gray matter and at 20–22 days than at 10–12 days. In white matter, the number of MT-positive cells at 10–12 days was about 1.9-fold higher in jimpy than in normal animals, whereas it was 2.4-fold higher at 20–22 days. In gray matter, the number of MT-positive cells at 10–12 days was 2-fold higher in jimpy than in normal cords, but the difference was not statistically significant at 20–22 days. Differences not only in the numbers of MT-positive cells but also in the intensity of MT immunostaining were found when jimpy and control animals were compared. Although quantitative image analysis has not been performed, an increased intensity of immunostaining in jimpy MT-positive glial cells was clearly noticed in the white matter (Fig. 1).

In addition to parenchymal glial cells, MT immunoreactivity was found in ependymal cells of the central canal, meningeal cells of the pia mater and, occasionally, in vascular endothelial cells and neurons. Positive MT immunostaining of the ependyma was located in the nucleus and cytoplasm, and was very often concentrated at the apical cytoplasmic portion. Both nuclear and cytoplasmic immunostaining was found in pial cells. Immunostaining of endothelial cells was predominantly located in the cytoplasm. Neurons showing a weak cytoplasmic MT immunostaining were rarely recognized. We were not able to establish differences in the number and distribution of MT-positive blood vessels, neurons, pial cells and ependymal cells when comparing sections from jimpy and normal animals.

3.2. Identification of MT-positive cells

In parenchymal glial cells, the MT immunostaining was predominantly found in the nucleus, whereas the cytoplasm and processes were frequently devoid of staining or stained weakly and/or incompletely. Thus, the unambiguous and exhaustive identification of MT-positive cells on the basis of their morphological features is unfeasible in sections exclusively reacted for MT. The double staining procedure combining MT immunostaining with specific glial cell markers enabled us to simultaneously visualize MT-positive cells (black staining) and the different glial cell types (brownish staining). The percentages of MT-positive cells expressing the different glial cell markers are shown in Table 1, and photomicrographs showing double-labeled glial cells are presented in Fig. 3.

In normal mice, at both ages, there was a consistent correspondence between the location of MT immunoreactivity and the localization of GFAP-positive astrocytes in white matter. Most GFAP-positive astrocytes showed MT immunostaining and nearly 100% of MT-positive cells were GFAP-positive in the white matter. The exhaustive identification of MT-positive cells through double MT/GFAP immunostaining was not possible in gray matter, since GFAP immunoreactivity was mainly located in white matter and most astrocytes in the gray matter of the normal spinal cord showed weak or no detectable GFAP immunostaining. Microglial cells present in the gray and white matter were negative for MT in the normal spinal cord at both ages. The identification of MT-positive cells after double MT/MBP immunostaining could not be accomplished in the normal spinal cord due to the strong MBP immunoreactivity of the densely packed myelinated fibers.

In jimpy, most glial cells showing MT immunoreactivity were astrocytes, although a significant number of MT-positive cells were microglial cells (Table 1). In the white matter, the percentage of MT-positive cells that were GFAP-positive ranged from 86% (10–12 days) to 82% (20–22 days). GFAP-positive astrocytes lacking MT-immunoreactivity in the mutant white matter were very scarce. In the gray matter, at both ages, about 70% of MT-positive

Table 1
Classification of MT-positive glial cells in the normal and jimpy spinal cord

	Normal				Jimpy			
	10–12 days		20–22 days		10–12 days		20–22 days	
	gray matter	white matter	gray matter	white matter	gray matter	white matter	gray matter	white matter
Astroglia (GFAP-positive)	32.2 ± 7.8	93.4 ± 3.8	41.2 ± 6.8	96.2 ± 2.7	68.5 ± 4.5	86.2 ± 3.1	70.6 ± 5.7	82.3 ± 3.8
Microglia (tomato lectin-positive)	0	0	0	0	4.3 ± 1.7	11.8 ± 2.8	5.2 ± 2.1	18.6 ± 3.7
Oligodendroglia (MBP-positive)	–	–	–	–	0	0	0	0
Unclassifiable cells	67.8	6.6	58.8	3.8	27.2	2.0	24.2	0

Percentages of MT-positive glial cells simultaneously expressing different glial cell markers. Values are mean percentages ± S.E.M.
The exhaustive identification of MT-positive cells in normal mice could not be carried out due either to the strong MBP immunoreactivity of the densely packed myelinated fibers or to the weak and/or incomplete astrocytic GFAP immunostaining. In jimpy, the presence of scattered MBP-positive fibers and the upregulation in the expression of GFAP enabled us to classify most MT-positive cells. Note that nearly 100% of MT-positive glial cells were unambiguously classified in the white matter.

cells in the mutant spinal cord showed GFAP immunoreactivity, and the great majority of GFAP-positive astrocytes showed MT immunostaining. It must be noted that hypertrophied jimpy astrocytes showed higher GFAP levels than astrocytes in normal animals, thus enabling us to visualize abundant astrocytes when sections immunoreacted for GFAP were analyzed. Microglial cells represented almost 20% of the white matter MT-positive cell population at 20–22 days, and 12% at 10–12 days. At both ages, about 5% of MT-positive cells in gray matter were microglia. Microglial cells devoid of MT immunoreactivity were frequently seen. The presence of scattered myelinated fibers in jimpy allowed us to simultaneously recognize individual MBP-positive oligodendroglial cells and MT immunoreaction, but no MBP-positive oligodendroglial cells showing MT immunostaining were observed. Although nearly 100% of MT-positive cells in the jimpy white matter were positively identified as astrocytes or microglia, we can not discard on the basis of the present study that some immature MBP-negative oligodendrocyte might express MT.

3.3. Specificity of the monoclonal antibody E9 for MT isoforms

The cross-reactivity of the E9 monoclonal antibody with the different brain MT isoforms was assessed by ELISA. The results indicated that the antibody E9 reacts with MT-I and MT-II, but no cross-reactivity was observed with MT-III.

4. Discussion

In the present study, immunocytochemical detection of MT was assessed by using a monoclonal antibody specific for the MT-I and MT-II isoforms. In rodents, the multiple MT isoforms have been subdivided into four structurally distinct subgroups (MT-I, MT-II, MT-III and MT-IV). The MT-I and MT-II proteins are widely distributed in mammalian tissues, including brain, and their expression is inducible by metals and glucocorticoids [24]; MT-III, also called growth inhibitory factor (GIF), is expressed mostly in the brain and does not appear to be regulated by metals or glucocorticoids [45,60]; and MT-IV is exclusively expressed in stratified squamous epithelia [48]. In this article, we use the term 'metallothionein (MT)' to refer to the classic MT-I and MT-II isoforms.

MT was present both in the spinal cord of 10–12- and 20–22-day-old jimpy and normal mice, but there were remarkable differences in the numbers of MT-positive glial cells depending on the type of mouse (normal or jimpy), the age of the animal (10–12 or 20–22 days), and the location (gray or white matter). Differences not only in numbers but also in the nature of MT-positive glial cells were found when jimpy and normal animals were compared.

4.1. Distribution of MT in normal animals

In the normal spinal cord, the number of MT-positive cells was higher at 20–22 days than at 10–12 days, this difference being mainly due to an increase with age in the number of MT immunopositive cells present in the gray matter. This is in agreement with previous observations reporting that MT is expressed only in minute amounts in the embryonic and neonatal rodent brain and that its expression progressively increases during the first weeks of the postnatal development [43,59]. In addition to age-related differences, MT is not uniformly distributed through the normal CNS and regional differences in the MT protein and MT mRNA content [14,20,21,25,28], as well as in the distribution of MT-positive cells [8,28], have been described. The regional differences are difficult to analyze since the distribution of MT depends on the area and even, within each area, on the specific layer examined. Nevertheless, MT immunoreactivity is preferentially located in gray matter areas and the number of MT-positive cells is usually higher in gray than in white matter [8]. Our quantitative study in the mouse spinal cord is in accordance with these observations.

Astrocytes showing double MT/GFAP immunostaining were regularly seen through the normal spinal cord and there was a consistent correspondence between the location of MT and GFAP, particularly in white matter. In gray matter, most GFAP-positive astrocytes showed MT immunoreactivity, but it must be noted that a considerable percentage of MT-positive glial cells were devoid of apparent GFAP immunostaining. This may be explained by the fact that GFAP immunoreactivity is mainly located in white matter astrocytes and astrocytes in the gray matter of the normal spinal cord often show insufficient GFAP immunoreactivity to be unequivocally detected by immunohistochemical techniques [19]. Parenchymal glial cells showing MT immunoreactivity showed negative staining for the tomato lectin microglial marker. The identification of MT-positive glial cells after double MT/MBP immunostaining could not be accomplished due to the strong MBP immunoreactivity of myelinated fibers. Thus, rigorously, we cannot exclude the possibility that some MT-positive glial cells were oligodendrocytes, but our observations in the normal mouse spinal cord, taken together with the observations of other authors in different normal brain areas and species [42,43,66], strongly suggest that astrocytes are the only parenchymal glial cell that constitutively express MT in normal adult CNS.

MT-containing neurons were very rare in the spinal cord. In accordance with the present study, most studies on MT expression in CNS describe the absence or scarcity of MT-immunopositive neurons [8,42,43,66]. On the other hand, by using polyclonal antibodies, Hidalgo et al. [28] found MT immunoreactivity in neurons of different rat cerebral and cerebellar areas. MT-I levels, measured by radioimmunoassay, were, however, 10-times lower in cul-

tured neurons than in cultured astrocytes. The expression of low MT levels by neurons is also supported by the observations of Kiningham et al. [29], who reported MT immunoreactivity in some motoneurons of both rat and human spinal cords by using the same monoclonal antibody used by us in this study, but including a trypsin treatment previous to the application of antibodies.

4.2. Induction of MT in jimpy

The number of glial cells showing MT immunostaining was higher in the jimpy spinal cord than in normal cords, and MT-positive glial cells in jimpy stained more intensely than positive cells in normal animals. This suggests that there is an induction of MT expression in jimpy and that MT induction implies not only an increase in the number of glial cells synthesizing MT but also an increase in the production of MT by glial cells. Interestingly, MT in the jimpy spinal cord was localized not only in astrocytes but also in microglial cells. The production of MT by microglia is a differential feature in jimpy, but the amount of microglial cells showing MT immunoreactivity is insufficient to explain the increase in number of MT-positive glial cells observed in the mutant. Thus, the de novo MT synthesis is apparently induced in both microglia and astrocytes. Moreover, MT induction was more pronounced in white than in gray matter, and it is known that the degree of microglial [61] and astroglial reactivity [6,56] is also higher in the jimpy white than in the gray matter, thus suggesting a close correspondence between the degree of MT induction and the degree of glial reactivity.

Microglia belong to the monocyte/macrophage system and are the specialized macrophages in the CNS [47]. There is no previous observation in the literature describing MT in microglia, but some studies have described MT induction in monocytes and macrophages in other tissues. Human monocytes are induced to express MT after exposure to zinc, cadmium and the glucocorticoid dexamethasone [40]. In the same way, treatment with cadmium induces MT expression in alveolar macrophages [26], Kupffer cells [39] and mesangial cells [13]. Thus, the ability to express MT when conveniently stimulated seems to be an intrinsic feature of monocytes/macrophages. Interestingly, bacterial lipopolysaccharide (LPS) induces MT expression in both primary human monocytes and a monocyte-derived cell line, and this MT induction is concurrent with increased production of H_2O_2 and enhanced adherence and invasiveness, suggesting that MT expression is associated with the reactive activation of these cells [36,37].

The induction of MT in reactive astrocytes has been previously described in brain injury [46] and in neurodegenerative disorders such as amyotrophic lateral sclerosis [55]. Factors known to increase MT production by astrocytes include metals [28,49,52] and, interestingly, factors involved in the regulation of inflammatory and immunological responses, such as glucocorticoids, LPS and the cytokines IL-1 and IL-6 [14,18,28,51,52]. Following experimental invasive injury, induction of MT is accompanied by massive extravasation of plasma metals in the damaged area [46]. Metals arising from the blood may potentially induce MT expression in astrocytes and microglia in the case of CNS invasive lesions, but this is unlikely in jimpy mice since its tissue damage results from an intrinsic genetic lesion, without the occurrence of traumatic injuries. On the contrary, molecules generated locally in the jimpy parenchyma seem to be the most likely signals for this local induction of MT in certain, but not all, astrocytes and microglial cells. Among known MT inducers, cytokines seem to be the best candidates for the induction of MT in injured CNS. In fact, the induction of MT synthesis by some interleukins has been demonstrated in the brain [18,51], and it is known that glial cells possess receptors for these cytokines [44] and that, under pathological conditions, these cytokines are released into the CNS parenchyma [5]. The intense reaction of astrocytes [56] and microglia [61] observed in jimpy may have special relevance since these cells, when they become reactive, are the main source of cytokines in the damaged brain [5,23]. Accordingly, the occurrence of anomalous glial–glial interactions involving the secretion of defective or abnormal soluble factors has been proposed in jimpy [3].

4.3. Functional significance of MT

It is generally accepted that the principal role of MT in normal tissues lies in the homeostasis of essential trace metals [24]. MT has the capability to bind group II metal ions, particularly zinc and copper. These metals are essential for normal brain functions, but it is important to keep the levels of free metals within physiological limits. MT is a good candidate to chelate excess metals and release them when needed. The presence of MT in endothelial cells, ependymal cells, pial cells and especially in astrocytes, a widely distributed and regularly-spaced cell population which is in contact with the meningeal covering and blood vessels, give support to this hypothesis. There is, however, an accumulating body of evidence which indicate that MT plays a protective role against oxidative damage in pathological conditions [50]. In fact, the role of MT as antioxidant could not be completely separated from its role in the storage/detoxification of metals, since some metal ions are capable of generating reactive oxygen species by the redox Fenton reaction [50]. Chemicals that produce oxidative stress are effective MT inducers [4] and it is known that MT confers resistance to cells from the cytotoxic effects of oxidants by acting as a free radical scavenger [50,53,54]. Accordingly, embryonic cells from transgenic mice deficient in MT-I and -II genes were more sensitive to the cytotoxic effects of oxidants than normal embryonic cells [35]. The subcellular location of MT may be important for its antioxidant function. Most of the MT was

concentrated in the nucleus and there is evidence for DNA damage by reactive oxygen species [50]. MT may protect DNA by sequestering metals and preventing their participation in redox reactions generating reactive oxygen species [9] or by directly interacting with oxygen free radicals and precluding the strand scission of DNA [15]. Cytoplasmic MT also protects cells from reactive oxygen species and other oxidants that induce cytotoxic peroxidation [54]. A role for MT in reducing nitric oxide-induced cytoplasmic and nuclear toxicity has also been described [53].

Reactive microglia generate reactive oxygen species, i.e. superoxide anion, hydrogen peroxide and hydroxyl radical [16], and nitric oxide [12], the production of these free radicals being regulated by cytokines [11]. There is no direct evidence for the production of free radicals by jimpy reactive microglial cells. Nevertheless, jimpy microglia actively remove oligodendrocyte and myelin debris through phagocytosis [61,63] and it is known that phagocytes, and microglia in particular, increase the consumption of oxygen and release reactive oxygen species and nitric oxide during phagocytosis [16,41]. The microglial production of free radicals mediating oxidative damage is essential, in our opinion, for understanding the role of MT induction in injured CNS. Further studies on MT are needed for the accurate identification of glial-inducing factors and a better understanding of its function during pathological conditions.

Acknowledgements

The authors are grateful to Dr. Pete Kille (School of Molecular and Medical Biosciences, University of Wales, College of Cardiff) for his generous gift of the anti-MT antibody E9 and the plasmid for rat MT-III. We thank Miguel Angel Martil and Anna Garrit for their excellent technical assistance. This work was partially supported by DGICYT Grants PB95-0662 and PB94-0667.

References

[1] L. Acarín, B. González, B. Castellano, A.J. Castro, Microglial response to *N*-methyl-D-aspartate-mediated excitotoxicity in the immature rat brain, J. Comp. Neurol. 367 (1996) 361–374.

[2] L. Acarín, J.M. Vela, B. González, B. Castellano, Demonstration of poly-*N*-acetyl lactosamine residues in ameboid and ramified microglial cells in rat brain by tomato lectin binding, J. Histochem. Cytochem. 42 (1994) 1033–1041.

[3] W.P. Bartlett, P.E. Knapp, R.P. Skoff, Glial conditioned medium enables jimpy oligodendrocytes to express properties of normal oligodendrocytes: production of myelin antigens and membranes, Glia 1 (1988) 253–259.

[4] J.W. Bauman, J. Liu, Y.P. Liu, C.D. Klaassen, Increase in metallothionein produced by chemicals that induce oxidative stress, Toxicol. Appl. Pharmacol. 110 (1991) 347–354.

[5] E.N. Benveniste, Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action, Am. J. Physiol. 263 (1992) C1–C16.

[6] T.T. Best, R.P. Skoff, W.P. Bartlett, Astroglial plasticity in hemizygous and heterozygous jimpy mice, Int. J. Dev. Neurosci. 6 (1988) 39–57.

[7] A. Bignami, L.F. Eng, D. Dahl, T. Uyeda, Localization of glial fibrillary acidic protein in astrocytes by immunofluorescence, Brain Res. 43 (1972) 429–435.

[8] H.G. Blaauwgeers, P.A. Sillevis Smitt, J.M. De Jong, D. Troost, Distribution of metallothionein in the human central nervous system, Glia 8 (1993) 62–70.

[9] L. Cai, J. Koropatnick, M.G. Cherian, Metallothionein protects DNA from copper-induced but not iron-induced cleavage in vitro, Chem.-Biol. Interact. 96 (1995) 143–155.

[10] B. Castellano, B. González, I. Dalmau, J.M. Vela, Identification and distribution of microglial cells in the cerebral cortex of the lizard: a histochemical study, J. Comp. Neurol. 311 (1991) 434–444.

[11] C.C. Chao, S. Hu, P.K. Peterson, Modulation of human microglial cell superoxide production by cytokines, J. Leukoc. Biol. 58 (1995) 65–70.

[12] C.C. Chao, S. Hu, T.W. Molitor, E.G. Shaskan, P.K. Peterson, Activated microglia mediate neuronal cell injury via a nitric oxide mechanism, J. Immunol. 149 (1992) 2736–2741.

[13] T.A. Chin, D.M. Templeton, Effects of $CdCl_2$ and Cd-metallothionein on cultured mesangial cells, Toxicol. Appl. Pharmacol. 116 (1992) 133–141.

[14] S. Choudhuri, K.K. Kramer, N.E. Berman, T.P. Dalton, G.K. Andrews, C.D. Klaassen, Constitutive expression of metallothionein genes in mouse brain, Toxicol. Appl. Pharmacol. 131 (1995) 144–154.

[15] L.S. Chubatsu, R. Meneghini, Metallothionein protects DNA from oxidative damage, Biochem. J. 291 (1993) 193–198.

[16] C.A. Colton, D.L. Gilbert, Microglia, an in vivo source of reactive oxygen species in the brain, in: F.J. Seil (Ed.), Advances in Neurology. Neural Injury and Regeneration, vol. 59, Raven, New York, 1993, pp. 321–326.

[17] A. Dautigny, M.-G. Mattei, D. Morello, P.M. Alliel, D. Pham-Dinh, L. Amar, D. Arnaud, D. Simon, J.-F. Mattei, J.-L. Guenet, P. Jollès, P. Avner, The structural gene coding for myelin-associated proteolipid protein is mutated in jimpy mice, Nature 321 (1986) 867–869.

[18] S.K. De, M.T. McMaster, G.K. Andrews, Endotoxin induction of murine metallothionein gene expression, J. Biol. Chem. 265 (1990) 15267–15274.

[19] L.F. Eng, Y.L. Lee, Intermediate filaments in astrocytes, in: H. Kettenmann, B.R. Ransom (Eds.), Neuroglia, Oxford University Press, New York, 1995, pp. 650–667.

[20] T. Gasull, M. Giralt, A. Garcia, J. Hidalgo, Regulation of metallothionein-I + II levels in specific brain areas and liver in the rat: role of catecholamines, Glia 12 (1994) 135–143.

[21] T. Gasull, M. Giralt, J. Hernandez, P. Martinez, I. Bremner, J. Hidalgo, Regulation of metallothionein concentrations in rat brain: effect of glucocorticoids, zinc, copper, and endotoxin, Am. J. Physiol. 266 (1994) E760–E767.

[22] T. Gasull, D.V. Rebollo, B. Romero, J. Hidalgo, Development of a competitive double antibody radioimmunoassay for rat metallothionein, J. Immunoassay 14 (1993) 209–225.

[23] D. Giulian, The consequences of inflammation after injury to the central nervous system, in: S.K. Salzman, A.I. Faden (Eds.), The Neurobiology of Central Nervous System Trauma, Oxford University Press, New York, 1994, pp. 155–164.

[24] D.H. Hamer, Metallothionein, Annu. Rev. Biochem. 55 (1986) 913–951.

[25] R. Hao, D.R. Cerutis, H.S. Blaxall, J.F. Rodriguez-Sierra, R.F. Pfeiffer, M. Ebadi, Distribution of zinc metallothionein I mRNA in rat brain using in situ hybridization, Neurochem. Res. 19 (1994) 761–767.

[26] B.A. Hart, Q. Gong, J.D. Eneman, C.C. Durieux-Lu, In vivo expression of metallothionein in rat alveolar macrophages and type II

epithelial cells following repeated cadmium aerosol exposures, Toxicol. Appl. Pharmacol. 133 (1995) 82–90.
[27] L. Hertz, G. Chaban, E. Hertz, Abnormal metabolic response to excess potassium in astrocytes from the jimpy mouse, a convulsing neurological mutant, Brain Res. 181 (1980) 482–487.
[28] J. Hidalgo, A. Garcia, A.M. Oliva, M. Giralt, T. Gasull, B. Gonzalez, H. Milnerowicz, A. Wood, I. Bremner, Effect of zinc, copper and glucocorticoids on metallothionein levels of cultured neurons and astrocytes from rat brain, Chem.-Biol. Interact. 93 (1994) 197–219.
[29] K. Kiningham, X. Bi, E.J. Kasarskis, Neuronal localization of metallothioneins in rat and human spinal cord, Neurochem. Int. 27 (1995) 105–109.
[30] P.E. Knapp, R.P. Skoff, A defect in the cell cycle of neuroglia in the myelin deficient jimpy mouse, Dev. Brain Res. 35 (1987) 301–306.
[31] P.E. Knapp, R.P. Skoff, Jimpy mutation affects astrocytes: lengthening of the cell cycle in vitro, Dev. Neurosci. 15 (1993) 31–36.
[32] P.E. Knapp, C.S. Booth, R.P. Skoff, The pH of Jimpy glia is increased: intracellular measurements using fluorescent laser cytometry, Int. J. Dev. Neurosci. 11 (1993) 215–226.
[33] P.E. Knapp, R.P. Skoff, D.W. Redstone, Oligodendroglial cell death in jimpy mice: an explanation for the myelin deficit, J. Neurosci. 6 (1986) 2813–2822.
[34] L.J. Lawson, V.H. Perry, P. Dri, S. Gordon, Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain, Neuroscience 39 (1990) 151–170.
[35] J.S. Lazo, Y. Kondo, D. Dellapiazza, A.E. Michalska, K.H. Choo, B.R. Pitt, Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes, J. Biol. Chem. 270 (1995) 5506–5510.
[36] M.E. Leibbrandt, J. Koropatnick, Activation of human monocytes with lipopolysaccharide induces metallothionein expression and is diminished by zinc, Toxicol. Appl. Pharmacol. 124 (1994) 72–81.
[37] M.E. Leibbrandt, R. Khokha, J. Koropatnick, Antisense down-regulation of metallothionein in a human monocytic cell line alters adherence, invasion, and the respiratory burst, Cell. Growth Differ. 5 (1994) 17–25.
[38] X. Li, W.P. Bartlett, Developmental expression of glial fibrillary acidic protein and glutamine synthetase mRNAs in normal and jimpy mice, Mol. Brain Res. 9 (1991) 313–317.
[39] J.M. McKim Jr., J. Liu, Y.P. Liu, C.D. Klaassen, Distribution of cadmium chloride and cadmium-metallothionein to liver parenchymal, Kupffer, and endothelial cells: their relative ability to express metallothionein, Toxicol. Appl. Pharmacol. 112 (1992) 324–330.
[40] O.J. Mesna, I.L. Steffensen, H. Hjertholm, R.A. Andersen, Accumulation of metallothionein and its multiple forms by zinc, cadmium and dexamethasone in human peripheral T and B lymphocytes and monocytes, Chem.-Biol. Interact. 94 (1995) 225–242.
[41] K. Mosley, M.L. Cuzner, Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents, Neurochem. Res. 21 (1996) 481–487.
[42] K. Nakajima, K. Suzuki, Immunochemical detection of metallothionein in brain, Neurochem. Int. 27 (1995) 73–87.
[43] N. Nishimura, H. Nishimura, A. Ghaffar, C. Tohyama, Localization of metallothionein in the brain of rat and mouse, J. Histochem. Cytochem. 40 (1992) 309–315.
[44] G.C. Otero, J.E. Merrill, Cytokine receptors on glial cells, Glia 11 (1994) 117–128.
[45] R.D. Palmiter, S.D. Findley, T.E. Whitmore, D.M. Durnam, MT-III, a brain-specific member of the metallothionein gene family, Proc. Natl. Acad. Sci. USA 89 (1992) 6333–6337.
[46] M. Penkowa, T. Moos, Disruption of the blood-brain interface in neonatal rat neocortex induces a transient expression of metallothionein in reactive astrocytes, Glia 13 (1995) 217–227.
[47] V.H. Perry, S. Gordon, Macrophages and microglia in the nervous system, Trends Neurosci. 11 (1988) 273–277.
[48] C.J. Quaife, S.D. Findley, J.C. Erickson, G.J. Froelick, E.J. Kelly, B.P. Zambrowicz, R.D. Palmiter, Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia, Biochemistry 33 (1994) 7250–7259.
[49] L. Rising, D. Vitarella, H.K. Kimelberg, M. Aschner, Cadmium chloride ($CdCl_2$)-induced metallothionein (MT) expression in neonatal rat primary astrocyte cultures, Brain Res. 678 (1995) 91–98.
[50] M. Sato, I. Bremner, Oxygen free radicals and metallothionein, Free Radicals Biol. Med. 14 (1993) 325–337.
[51] M. Sato, M. Sasaki, H. Hojo, Differential induction of metallothionein synthesis by interleukin-6 and tumor necrosis factor-α in rat tissues, Int. J. Immunopharmacol. 16 (1994) 187–195.
[52] J. Sawada, Y. Kikuchi, M. Shibutani, K. Mitsumori, K. Inoue, T. Kasahara, Induction of metallothionein in astrocytes by cytokines and heavy metals, Biol. Signals 3 (1994) 157–168.
[53] M.A. Schwarz, J.S. Lazo, J.C. Yalowich, W.P. Allen, M. Whitmore, H.A. Bergonia, E. Tzeng, T.R. Billiar, P.D. Robbins, J.R. Lancaster Jr., B.R. Pitt, Metallothionein protects against the cytotoxic and DNA-damaging effects of nitric oxide, Proc. Natl. Acad. Sci. USA 92 (1995) 4452–4456.
[54] M.A. Schwarz, J.S. Lazo, J.C. Yalowich, I. Reynolds, V.E. Kagan, V. Tyurin, Y.M. Kim, S.C. Watkins, B.R. Pitt, Cytoplasmic metallothionein overexpression protects NIH 3T3 cells from tert-butyl hydroperoxide toxicity, J. Biol. Chem. 269 (1994) 15238–15243.
[55] P.A. Sillevis Smitt, H.G. Blaauwgeers, D. Troost, J.M. de Jong, Metallothionein immunoreactivity is increased in the spinal cord of patients with amyotrophic lateral sclerosis, Neurosci. Lett. 144 (1992) 107–110.
[56] R.P. Skoff, Myelin deficit in the jimpy mouse may be due to cellular abnormalities in astroglia, Nature 264 (1976) 560–562.
[57] R.P. Skoff, Increased proliferation of oligodendrocytes in the hypomyelinated mouse mutant-jimpy, Brain Res. 248 (1982) 19–31.
[58] N.H. Sternberger, Y. Itoyama, M.W. Kies, H. deF. Webster, Immunocytochemical method to identify basic protein in myelin-forming oligodendrocytes of newborn rat CNS, J. Neurocytol. 7 (1978) 251–263.
[59] K. Suzuki, K. Nakajima, N. Otaki, M. Kimura, Metallothionein in developing human brain, Biol. Signals 3 (1994) 188–192.
[60] Y. Uchida, K. Takio, K. Titani, Y. Ihara, M. Tomonaga, The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein, Neuron 7 (1991) 337–347.
[61] J.M. Vela, I. Dalmau, L. Acarín, B. González, B. Castellano, Microglial cell reaction in the gray and white matter of the jimpy spinal cord. An enzyme histochemical study at the light and electron microscope level, Brain Res. 694 (1995) 287–298.
[62] J.M. Vela, I. Dalmau, B. González, B. Castellano, Morphology and distribution of microglial cells in the young and adult mouse cerebellum, J. Comp. Neurol. 361 (1995) 602–616.
[63] J.M. Vela, I. Dalmau, B. González, B. Castellano, The microglial reaction in spinal cords of jimpy mice is related to apoptotic oligodendrocytes, Brain Res. 712 (1996) 134–142.
[64] J.M. Vela, I. Dalmau, B. González, B. Castellano, Abnormal expression of PCNA in the spinal cord of the hypomyelinated jimpy mutant mice, Brain Res. 747 (1997) 130–139.
[65] K. Yanagisawa, R.H. Quarles, Jimpy mice: quantitation of myelin-associated glycoprotein and other proteins, J. Neurochem. 47 (1986) 322–325.
[66] J.K. Young, J.S. Garvey, P.C. Huang, Glial immunoreactivity for metallothionein in the rat brain, Glia 4 (1991) 602–610.