urally. By gross examination of the brains, a focal hemorrhagic injury was seen in the right fronto-parietal cortex. In toluidine blue–stained frontal sections, the freeze lesion was seen as a necrotic area with no neuronal cells. The lesioned area became infiltrated by reactive astrocytes and microglia.

Following brain injury, both MT-III and MT-I+II were upregulated in the ipsilateral cortex. Thus, in representative cell counts carried out in 1 mm^2 of the area adjacent to the lesion where all MT isoforms were upregulated, MT-III–positive cells were $63.5 \pm 2.84/mm^2$ (10.5 ± 1.32 in unlesioned cortex), and MT-I+II positive cells were $87.5 \pm 4.48/mm^2$ (3.0 ± 0.91 in unlesioned cortex).

MT-III expression was seen in reactive astrocytes lying in a circle around the lesioned area, and MT-III immunoreactivity was lost from the meninges above the lesion (Fig. 6C). The MT-III expressing astrocytes showed hypertrophy with swollen cytoplasm and short cell processes. Between the necrotic area and the MT-III–positive cells, a zone without MT-III immunoreaction product was seen (Fig. 6C). MT-III expression was always intracellular, in the perinuclear cytoplasm and cell processes (Fig. 6E,G). MT-III was also expressed in round microglia/macrophages, and the immunoreaction product in these cells was also observed in both nucleus and cytoplasm (Fig. 6H).

In the ipsilateral hemisphere of the freeze lesioned rats, a strong MT-I+II immunoreactivity was seen in reactive astrocytes and macrophages in the lesioned area. The anatomical distribution of MT-I+II–positive cells significantly differed from that of MT-III–positive cells, since MT-I+II–positive cells were prominent in the immediate vicinity of the necrotic area as well as in deeper cortical layers (Fig. 6D). In contrast, MT-III was upregulated only in the latter. The most prominent upregulation of MT-I+II was seen in astrocytic cells adjacent to the necrotic tissue of the lesion, whereas microglia/macrophages upregulated them to a lower extent (Fig. 6D,F,I,J). Interestingly, somewhat the opposite trend happened for MT-III.

In order to investigate the type of cells expressing MT-III and MT-I+II, we carried out triple immunofluorescence stainings for MT-I+II, MT-III, GFAP, and lectin (Fig. 7). They clearly demonstrate that all MT isoforms are expressed in both astrocytes and microglia/macrophages, in both unlesioned and lesioned mice.

Effect of rMT-III on Astrocyte Migration in Vitro

The results of Fig. 8 clearly demonstrate that rMT-III shows bioactivity in this bioassay. Furthermore, when rMT-III and native MT-I+II were compared in several cell preparations (Fig. 9), a clear dose-response effect of all MT isoforms was observed, and rMT-III promoted astrocyte/cell processes migration to a higher degree than MT-I+II ($p < 0.001$).

We attempted to set up this bioassay for rat neurons, without success, since even serum failed to promote neuron/cell processes migration into the scratched area (data not shown).

MT-III Protein and MT-III mRNA Levels in AD Brains

In order to get a useful technique for the mesurement of tissue native MT-III levels, we developed a competitive double-antibody ELISA similar to that previously described for MT-I+II (Gasull et al., 1994). A typical result is shown in Fig. 10, which again shows a reaction of this polyclonal antibody with rMT-III but not with native rat MT-I+II. We have estimated the concentration of MT-III in the normal adult rat brain and found to be between 300 and 600 ng/g tissue. However, this concentration has been calculated by using rMT-III as the competitor. To find out the actual absolute native MT-III concentration, it should be used the native protein and not the recombinant one, since they might behave differently in the ELISA. The technique seems to be reliable at least for relative MT-III levels, since we have observed higher MT-III protein levels in the cerebellum of GFAP-IL6 mice (Campbell et al., 1993), in agreement with the *in situ* hybridization results for MT-III mRNA (Carrasco et al., 1999).

As stated above, severe discrepancies exist in the literature regarding the putative effect of AD on MT-III expression. Given the small number of brains analyzed, we have measured MT-III mRNA and MT-III protein levels of 8 AD brains and 8 proper control brains by Northern blot and the ELISA above described, respectively (Fig. 11). Somewhat surprisingly the MT-III mRNA levels of AD brains doubled those of control brains; furthermore, MT-III protein showed clearly the same tendency.

DISCUSSION

The main aim of this study was to obtain polyclonal antibodies against the MT-III isoform in order to obtain an essential tool for analyzing the putative physiological differences between the widely expressed and coordinately regulated MT-I+II and the CNS specific isoform MT-III. The recombinant protein was purified using well-established procedures, and exhibited properties in accordance with the metallothionein family and with published reports for MT-III (Pountney et al., 1994; Faller and Vasák, 1997). Furthermore, the antibody raised fulfills exigent criteria of specificity such as absence of immunoreactivity of brains from MT-III–null mice.

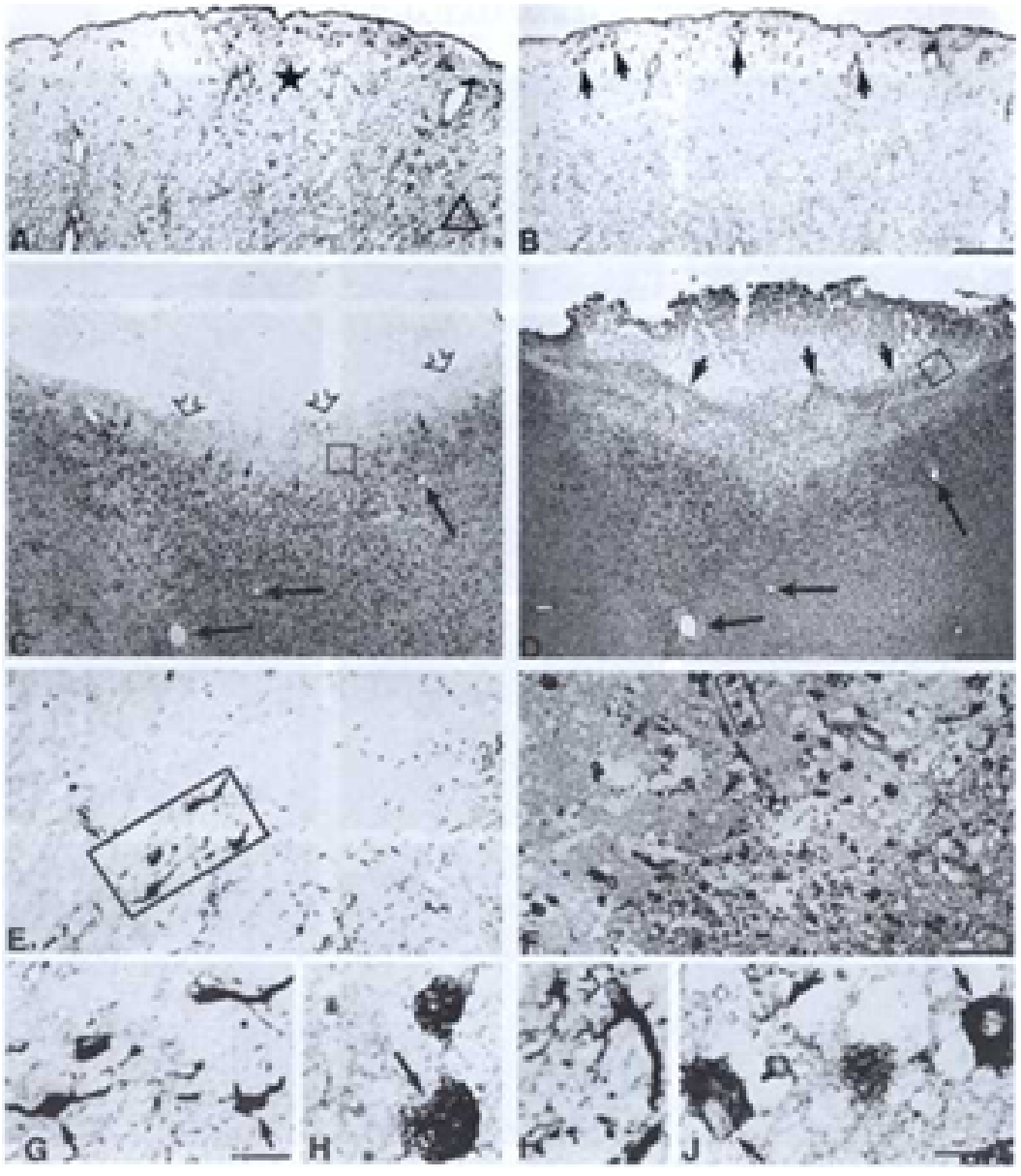


FIG. 6. MT-III and MT-I+II immunostainings of normal unlesioned and lesioned rats. (A) MT-III immunostainings of normal unlesioned rat brain, showing MT-III+ cells distributed widely in both the superficial cortex (*asterisk*) and in deeper areas (*triangle*). (B) MT–I+II immunostainings of normal unlesioned rat brain, showing MT-I+II cells located primarily superficial in the cortex (*arrows*). (C) MT-III expression following the freeze lesion, showing an increase in cells expressing MT-III. The area adjacent to the lesioned necrotic area are devoid of MT-III (*open arrows*), while deeper in the parenchyma numerous cells are MT-III positive. Small arrows are depicting the transitional zone between the area absent of MT-III and the area containing MT-III+ cells. The section is a neighboring section to that of D, with big arrows depicting vessels for similarity. (D) MT-I+II expression is increased following the freeze lesion. The immunoreactivity of MT-I+II is observed both adjacent and distant to the lesion. In contrast to the expression pattern of MT-III, MT-I+II immunoreactivity is observed in the borderline of the lesion (*small arrows*). Large arrows are depicting vessels of the neighboring sections seen in C and D. (E) Higher magnification of the framed area in C, showing both the zone devoid of MT-III immunoreactivity and some of the MT-III expressing cells. (F) Higher magnification of the framed area in D, showing widely distributed MT-I+II expressing cells. (G) Higher magnification of the framed area in E. The immunoreaction staining product of MT-III is seen in both nucleus, perinuclear cytoplasm and cell processes (*arrows*). (H) Also round microglia/macrophages are expressing MT-III, and the immunoreaction product in these cells is also observed in both nucleus and cytoplasm. (I) Also astrocytic cells are expressing MT-I+II. The MT-I+II immunoreaction staining product is mostly seen in the cytoplasm and less pronounced in the nuclei (*arrow*). (J) Higher magnification of the framed area in F, showing round microglia/macrophages expressing MT-I+II. The MT-I+II immunoreactivity is primarily observed in the cytoplasm (*arrows*). Bar = 114 μm (A,B), 455 μm (C,D), 40 μm (E,F), 20 μm (G,I), 8 μm (H,J).

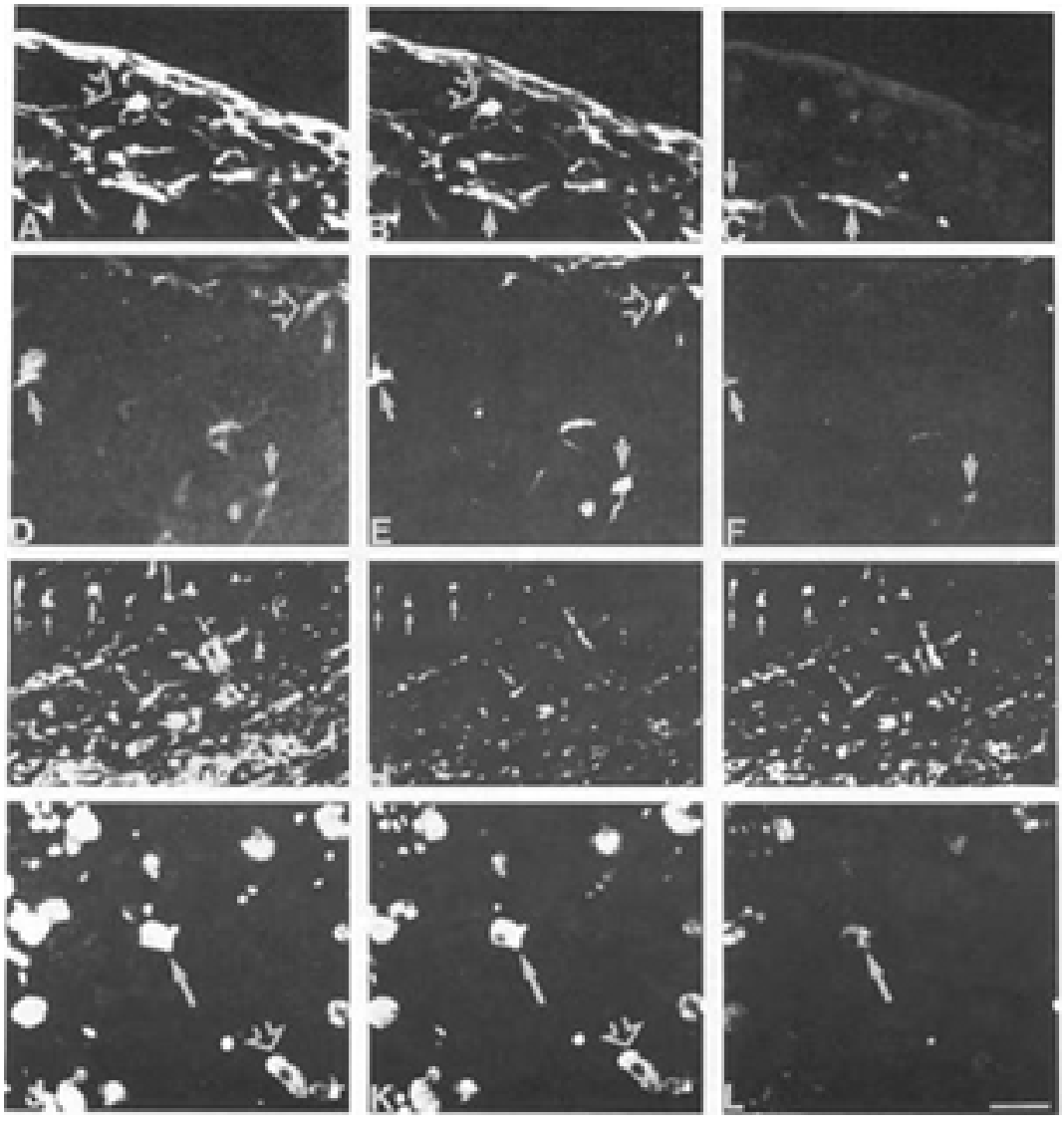


FIG. 7. Triple immunofluorescence histochemistry of unlesioned and lesioned rats. Arrows are depicting cells positive for either GFAP or lectin, and MT-III and MT-I+II, respectively. (A) GFAP immunostaining in unlesioned rat brain, showing astrocytes of the glia limitans and cortex. (B) MT-III immuno-staining in unlesioned rat brain, showing MT-III in many of the GFAP+ cells (*arrows*). (C) MT-I+II immunostaining in unlesioned rat, showing MT-I+II in a few GFAP+ cells. Also some of the cells expressing both GFAP and MT-III are MT-I+II+ (*arrows*). (D) Lectin immunostaining in unlesioned rat, showing ramified microglia of the cortex. (E) MT-III immunostaining in unlesioned rat, showing MT-III in lectin+ cells (*arrows*). (F) MT-I+II immunostaining in unlesioned rat, showing a faint MT-I+II expression in cells also positive for lectin and MT-III (*arrows*). (G) GFAP immunostaining in lesioned rat, showing astrocytes surrounding the lesion (arrows are depicting astrocytes positive for MT-I+II and MT-III). (H) MT-III immunostaining in lesioned rat, is observed in GFAP+ reactive astrocytes (*arrows*). (I) MT-I+II immunostaining in lesioned rat, showing MT-I+II in cells also positive for GFAP and MT-III (*arrows*). (J) Lectin immunostaining in lesioned rat, showing round microglia/macrophages (arrows are depicting microglia/macrophages positive for MT-I+II and MT-III). (K) MT-III immunostaining is primarily observed in lectin+ cells (*arrows*) following the lesion. (L) MT-I+II immunostaining in microglia/macrophages also positive for MT-III (*arrows*). Bar = 20 μm (A–L).

MT-III was discovered by Uchida et al. (1991) while studying factors underlying the AD pathogenesis. In this pioneering study, MT-III was found to be down-regulated in AD brains, and together with other evidence this suggested that MT-III might be important in the development of AD. A number of studies have been published showing that MT-III expression is very sensitive to brain damage (Anezaki et al., 1995; Hozumi et al., 1995, 1996;

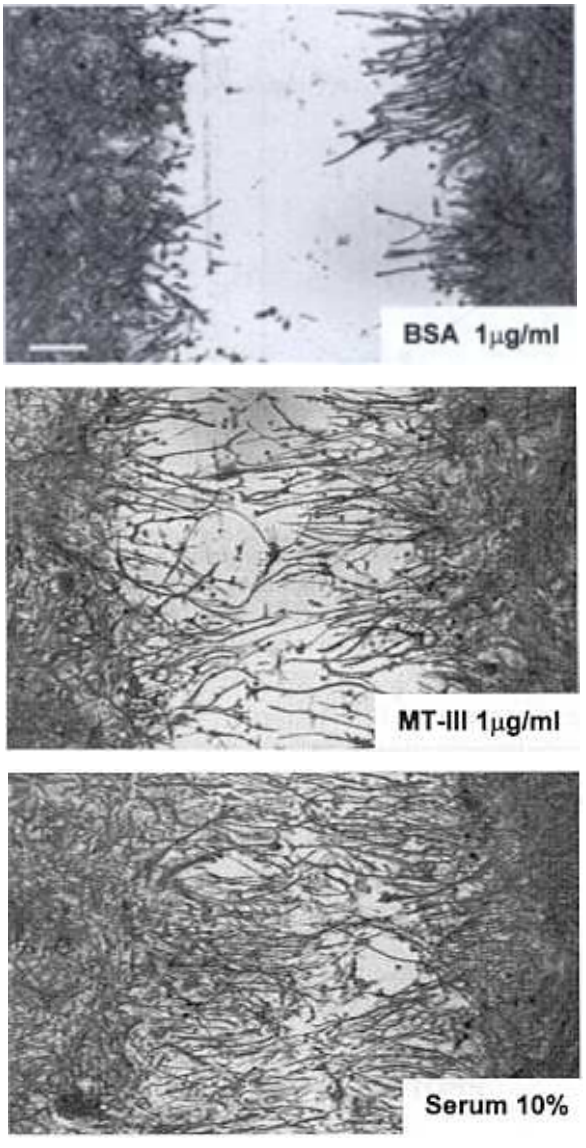


FIG. 8. Effect of MT-III on astrocyte migration *in vitro*. A monolayer was prepared as described in Materials and Methods. The monolayer was scratched with a sterile pipette tip, and 2 ml of fresh culture media (DMEM) was added supplemented with either 10% fetal calf serum, BSA, or MT-III at the indicated concentrations and maintained 4 days in 10% CO_2 at 37°C, and then stained with a GFAP antibody. Similar results were obtained in three separate cell preparations. Dose-response curves were generated in additional cultures (Fig. 9).

Inuzuka et al., 1996; Yamada et al., 1996; Yuguchi et al., 1995a,b), supporting a role of this protein during traumatic brain injury. However, rather surprisingly the MT-I+II isoforms have been neglected in such studies, which is noteworthy since these isoforms are strongly induced in the brain by a number of factors including inflammation and have been suggested to be significant antioxidant proteins *in vivo* (for further discussion, see Hidalgo et al., 1997). Whether or not MT-III will have different functions to those of MT-I+II is still unknown, but the direct comparison of the responses of these MT isoforms to brain damage could help in our understanding of their physiological roles. Therefore, we have carried out comparative studies with the freeze lesioned rat brain, an animal model of brain damage that we have characterized in detail previously regarding MT-I+II responses (Penkowa and Moos, 1995). The results suggest that substantial differences exist in the response of the brain MT isoforms to brain injury. Thus, although both astrocytes and microglia express MT-I+II and MT-III in both unlesioned and lesioned rats, not all cells express all MT isoforms in a given area, and, most important, the anatomical distribution of MT-I+II and MT-III positive cells was significantly different in the ipsilateral hemisphere of lesioned rats. Thus, the strongest MT-I+II immunoreactivity was seen in reactive astrocytes and macrophages in the cells adjacent to the necrotic tissue of the lesion, while cells distant from the lesion were showing less immunoreactivity. In contrast, MT-III levels reamined unaltered in the vicinity of the border of the lesion and increased in deeper cortical layers. This is the first time that the response to brain damage of the widely expressed MT isoforms, MT-I+II, is compared with that of the brain specific isoform, MT-III. The results clearly suggest that the physiological functions of these MT iso-

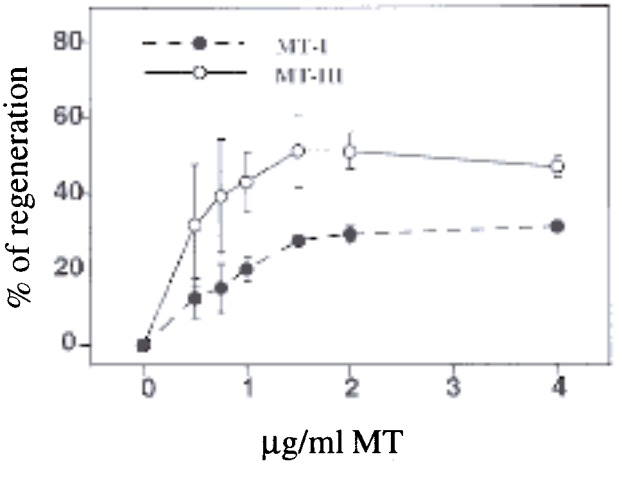


FIG. 9. Comparison of MT-III and MT-I+II on astrocyte migration *in vitro*. Semiquantitative measurements of the cell migration into the scratch (Fig. 8) were done with a Leica Q 500 MC system in several separate cell preparations as described in Materials and Methods. Regeneration caused by fetal calf serum was considered 100%, and that caused by BSA, 0%. Results are mean ± SE ($n = 2$ in the 0.75 and 4 μg/ml protein concentrations; $n = 3$ in the remaining protein concentrations). Two-way ANOVA indicated a significant effect of the MT isoform additions and that MT-III promoted a higher astrocyte regeneration than MT-I+II ($p < 0.001$).

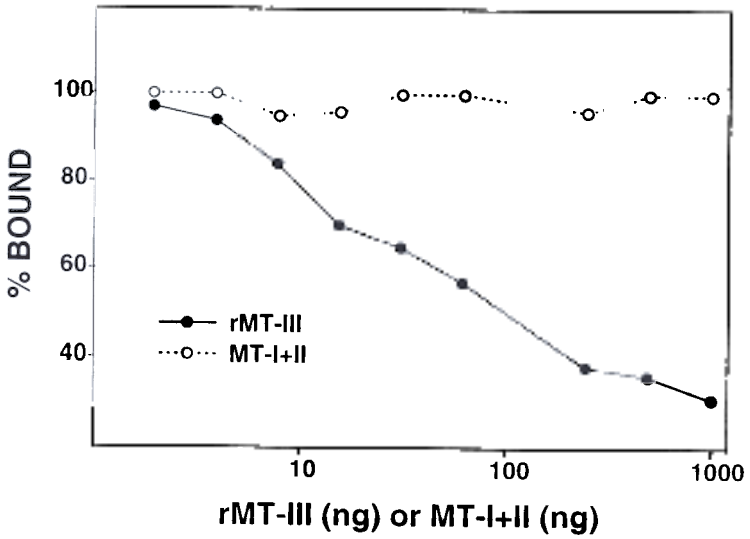
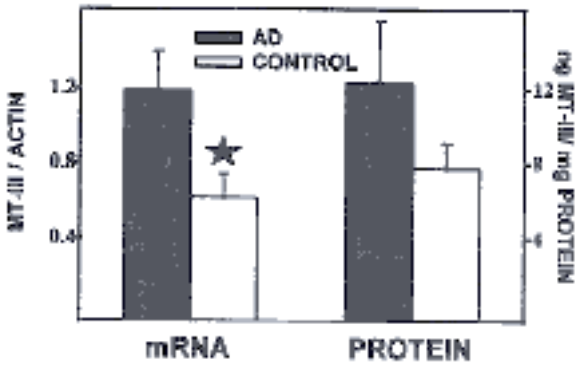


FIG. 10. Competitive assay. Recombinant rat MT-III coated plates were incubated with rMT-III or native MT-I+II standard curves and 4-month serum (1:400). The exact procedure is described in Material and Methods. Results are expressed in % bound [(sample OD_{540mm}/total bound OD_{540mm}) × 100] versus competitor quantity (log scale). When rMT-III was the competitor, percentage of binding decreased linearly. No significant effect was observed if native MT-I+II was added as competitor.



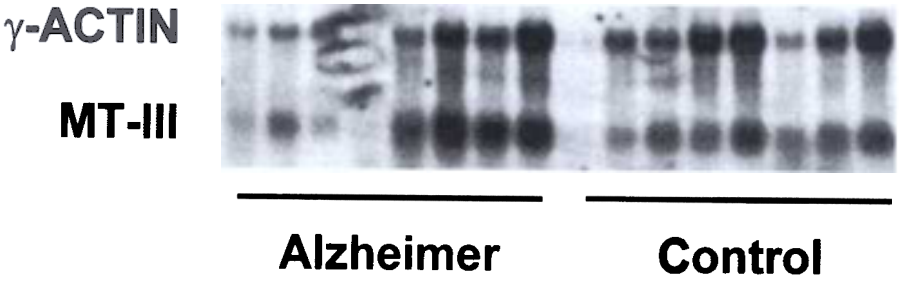


FIG. 11. MT-III mRNA and protein levels of Alzheimer's disease and control brains. Northern blot analysis of MT-III mRNA (bottom) were carried out as described in Materials and Methods. The blot was hybridized with both MT-III and γ-actin probes, and after the autoradiography the bands were quantitated and the ratio MT-III/γ-actin mRNA calculated (top). The results indicated that the AD brains had higher MT-III mRNA levels than control brains. The ELISA also indicated that the MT-III protein levels tended to be higher in AD brains.

forms in the brain should be different, given their different responses to the freeze lesion model.

Previous studies have suggested that MT-III could have an inhibitory neurotrophic role (Erickson et al., 1994; Swewll et al., 1995; Uchida et al., 1991; Yuguchi et al., 1995a). However, such a role is observed in a bioassay *in vitro*. Whether or not it will be relevant *in vivo* is unknown. Recent results with mice overexpressing MT-III and with MT-III null mice actually suggest that MT-III could rather have a stimulatory neurotrophic role, at least in mice convulsing after kainic acid administration and in the hippocampus (Erickson et al., 1997). Whether this was a direct effect of MT-III on neurons, or an indirect effect through actions on the glial cells, was not established. As demonstrated in this and other reports, MT-III is expressed thoroughly in glial cells, especially after a traumatic brain injury, and thus a significant role of this protein on this cell population could be predicted. While searching for a suitable bioassay for testing the bioactivity of the rMT-III obtained in this study, a simple but elegant bioassay for vibronectin and TNF-α was published, which measures astrocyte migration into a scratched area in a monolayer (Faber-Elman et al., 1995). The results demonstrate that MT-III has a singificant effect on astrocyte migration, indicating that this protein may have a role on glial rather than on neuronal cells. Such a role could have to do with the above mentioned results for neuronal survival in the hippocampus in transgenic and MT-III null mice (Erickson et al., 1997). Interestingly, the MT-III isoform promoted more astrocyte migration than MT-I+II, again suggesting differences between the MT isoforms. Nevertheless, to establish whether the results obtained in this bioassay with astrocytes are relevant in vivo needs further studies.

Finally, we measured MT-III protein levels in AD brains. In a pioneering study (Uchida et al., 1991), MT-III was found to be down-regulated in AD brains, and together with other evidence this suggested that MT-III might be important in the development of AD. However, in another study neither the MT-III mRNA (measured by quantitative solution hybridization) nor the MT-III protein (measured indirectly by cadmium binding) were found to be decreased (Erickson et al., 1994), casting doubts about the putative importance of MT-III in AD. Since a few brains were used in each study, it seemed worthwhile to examine MT-III expression in more AD brains. Thus, in the present report we measured MT-III mRNA levels of 8 AD brains and 8 proper control brains by Northern blot. MT-III mRNA levels of AD brains doubled those of control brains, and, actually, these results are rather consistent with those obtained by Northern blot by Erickson et al. (1994). Furthermore, MT-III protein measured by the ELISA developed in this report showed clearly the same tendency. Taken together, the results indicate that it is unlikely that MT-III is a major etiologic factor of AD since the protein levels are not consistently decreased in the AD brains evaluated.

The present study reports the development of a polyclonal antibody raised against recombinant rat MT-III that is useful for measuring native MT-III protein by ELISA and for immunocytochemistry. This is the first time that MT-III expression is compared directly to their widely expressed counterparts, MT-I+II, in a animal model of brain damage, the freeze lesion of one brain hemisphere. The results demonstrate that in the vicinity of the lesion MT-I+II are significantly upregulated, while MT-III is not, indicating that these MT isoforms should have significant different physiological functions in the brain. The results indeed indicate that MT-III promotes astrocyte migration in vitro to a higher extent than MT-I+II. Finally, the results do not support a down-regulation of MT-III during AD.

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Trabajo 3

Localization of metallothionein-I and –III expression in the CNS of transgenic mice astrocyte-targeted expression of interleukin-6

Experimental Neurology ***153****: 184-194, 1998*

Trabajo 4

Metallothioneins are upregulated in symptomatic mice with astrocyte-targeted expression of tumor necrosis factor-α

Experimental Neurology ***163****: 46-54, 2000*