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Localization of Metallothionein-I and -III Expression in the CNS of Transgenic Mice with Astrocyte-Targeted Expression of Interleukin 6

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The effect of interleukin-6 (IL-6) on metallothionein-I (MT-I) and MT-III expression in the brain has been studied in transgenic mice expressing IL-6 under the regulatory control of the glial fibrillary acidic protein gene promoter (GFAP-IL6 mice), which develop chronic progressive neurodegenerative disease. *In situ* hybridization analysis revealed that GFAP-IL6 (G16-low expressor line, and G36-high expressor line) mice had strongly increased MT-I mRNA levels in the cerebellum (Purkinje and granular layers of the cerebellar cortex and basal nuclei) and, to a lesser degree, in thalamus (only G36 line) and hypothalamus, whereas no significant alterations were observed in other brain areas studied. Microautoradiography and immunocytochemistry studies suggest that the MT-I expression is predominantly localized to astrocytes throughout the cerebrum and especially in Bergman glia in the cerebellum. However, a significant expression was also observed in microglia of the GFAP-IL6 mice. MT-III expression was significantly increased in the Purkinje cell layer and basal nuclei of the cerebellum, which was confirmed by Northern blot analysis of poly(A)$^+$ mRNA and by ELISA of the MT-III protein. In contrast, in the G36 but not G16 mice, transgene expression of IL-6 was associated with significantly decreased MT-III RNA levels in the dentate gyrus and CA3 pyramidal neuron layer of the hippocampus and, in both G36 and G16 mice, in the occipital but not frontal cortex and in ependymal cells. Thus, both the widely expressed MT-I isoform and the CNS specific MT-III isoform are significantly affected in a MT isoform- and CNS area-specific manner in the GFAP-IL6 mice, a chronic model of brain damage. © 1998 Academic Press

Key Words: **IL-6; brain; neurodegeneration; metallothionein; astrocytosis; microgliosis; inflammatory response.**

INTRODUCTION

Metallothioneins (MTs) are cysteine-rich, heavy metal-binding, low-molecular-weight proteins (28, 42). MT-I and MT-II are widely expressed isoforms, whereas MT-III normally exhibits restricted expression to the central nervous system (29, 36, 43). MT-III was initially named growth inhibitory factor after its discovery in human brains because of its potent inhibitory effect in a bioassay with rat neonatal neurons *in vitro* (44). MT-III was suggested to be involved in the pathogenesis of Alzheimer's disease (AD) because of its effect on neuronal survival *in vitro* and its apparent downregulation in AD brains (43, 44), although the latter finding has not been confirmed (10). Subsequently, a number of reports have characterized the response of MT-III in several experimental models of brain damage, but the results have not been consistent. Thus, it has been shown that MT-III protein levels or expression are increased in astrocytes by stab wounds or kainic acid injection (2, 22, 23), whereas after cortical ablation MT-III expression first decreased and latter increased in astrocytes but also in neurons (49). Moreover, brain MT-III levels decreased after ischemia caused by middle cerebral artery occlusion (26), and neuronal MT-III expression decreased in the ipsilateral facial nucleus following facial nerve transection (50). It has been speculated that decreased MT-III expression might be a physiological attempt to facilitate neurite extension in response to injury, whereas increased expression could reflect efforts to suppress oversprouting promoted by neurotrophic factors produced in response to tissue injury (49), but this remains unclear. Nevertheless, the data collectively suggest that this MT isoform may have a role in reparative processes. The widely expressed counterparts, MT-I+II, are also potentially important for brain physiology, since they are upregulated by an enormous number of factors (28, 42), and could serve in the control of Zn and Cu metabolism, in the adaptation to stress, and in cell protection against oxidative stress (11, 12, 17, 18, 20, 21, 32, 34, 38). Interestingly, and contrary to MT-III, MT-I is upregulated during AD (9); furthermore, MT-I is also upregulated in other human neurodegenerative diseases such as Pick's disease and amyotrophic lateral sclerosis (9, 40).

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Contrary to MT-III, the main site of expression of the MT-I+II isoforms is the astrocyte (20, 34, 37, 46, 48, 51).

In order to better understand the physiological functions of these proteins in the brain, it is essential to characterize the factors that control their expression. Previous results indicate that MT-III is controlled differently to MT-I+II, since it is mostly unresponsive to the inducers of MT-I+II (7, 30, 31, 36, 51) and indeed substantial differences exist in their promoter regions (24, 35). Recently (15), we undertook studies in transgenic mice (termed GFAP-IL6) in which IL-6 was expressed under the regulatory control of the glial fibrillary acidic protein (GFAP) gene promoter, which targeted expression of IL-6 to astrocytes (5, 6). These mice are a good model for human inflammatory neurodegenerative diseases since a chronic inflammatory response in the brain is produced by the transgenic expression of IL-6. Thus, in the brain of GPAP-IL6 mice neurodegeneration, astrocytosis, microgliosis, angiogenesis, and upregulation of several inflammatory and other host-response genes including IL-1 α/β, TNFα, GFAP, ICAM-1, the acute-phase protein EB22/5, and complement C3 protein are prominent (3, 5, 6). Moreover, concordant with these structural alterations, GFAP-IL6 mice have impaired hippocampal electrophysiology and develop a progressive learning deficit (16, 41). MT-I+II protein levels measured in tissue homogenates demonstrated that these proteins were increased in the brains of GFAP-IL6 mice, whereas expression of MT-I mRNA (determined by Northern blot) was not consistently increased (15). The spatial distribution of MT gene expression in the brain of the GFAP-IL6 mice has not been determined. Here we present *in situ* hybridization studies which more precisely ascertain the effect of astrocyte produced IL-6 on brain MT-I and MT-III expression. The results demonstrate that IL-6 has a significant role in regulating the expression of the brain MT isoforms which is specific for each isoform and brain area.

MATERIALS AND METHODS

Production of GFAP-IL6 Transgenic Mice

Construction and characterization of the GFAP-IL6 transgenic mice was described previously (5). Briefly, an expression vector derived from the murine glial fibrillary acidic protein (GFAP) gene was used to target expression of IL-6 to astrocytes. Heterozygous offspring of the low expressor G16 (3 months of age) and the high expressor G36 (3 months of age) lines were studied in parallel with age- and sex-matched nontransgenic littermates.

In Situ Hybridization

The animals were killed and their brains removed, frozen in liquid nitrogen, and stored at −80°C. Serial sagittal sections (20 μm in thickness) were obtained from the frozen brains with a cryostat and mounted on slides coated with poly(L)lysine, which were then maintained at −80°C until the day of analysis.

For MT-I mRNA studies, we used the mouse cDNA kindly provided by Dr. R. D. Palmiter (University of Washington, WA). For MT-III mRNA studies, and 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 region for the terminal 15 amino acids and the 3′ untranslated region until the poly(G) stretch of MT-III mRNA, which was generously provided by Dr. G. K. Andrews (Department of Biochemistry, The University of Kansas Medical Center, Kansas City, KS). Both the MT-I and the MT-III cDNAs were labeled with [α-^{35}S]UTP using a SP6/T7 transcription kit (Boehringer Mannheim, Mannheim, Germany). In brief, the DNA was linearized by digestion with proper restriction endonucleases. Four microliters of the linearized DNA (0.25 μg/μl) was incubated with 2 μl of transcription buffer, 2 μl of 100 mM dithiotreitol, 0.8 μl of RNase inhibitor, 1 μl of 10 mM ATP, CTP, and GTP, 2.4 μl of 0.1 mM cold UTP, 2 μl of [α-^{35}S]UTP (20 μCi/μl) (Amersham), 2.8 μl of RNase free H_2O, and 1 μl of RNA polymerase (T7 RNA polymerase for antisense probes, SP6 RNA polymerase for sense probes) at 37°C for 30 min. After the transcription process, the DNA was digested by adding 1 μl of DNase and incubating for 30 min at 37°C. After DNAse treatment, the RNA was extracted with phenol and phenol:cloroform:isoamyl alcohol (25:24:1). The upper phase (200 μl) was recovered and incubated overnight with 1 μl of tRNA (10 mg/ml), 10 μl of 3 M sodium acetate, and 500 μl of 100% ethanol to precipitate the RNA. After centrifugation the precipitated probe was dissolved in 50 μl of RNAse free H_2O. *In situ* hybridization was performed using procedures described by Yuguchi *et al.* (49) with some modifications: the sections were incubated with 0.1 N HCl instead of proteinase K, and we used RNAse at 10 μg/ml instead of 1 μg/ml to digest the free probe. The concentration of probe used was 1×10^6 dpm/90 μl/slide. Autoradiography was performed exposing the film (hyperfilm-MP, Amersham) to the slides for 6 days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III mRNA levels were semiquantitatively determined in four sections per brain area and animal, by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500 MC system. The MT-I and MT-III mRNA values shown are expressed in arbitrary units (number of pixels × optic density).

Microautoradiography

After macroautoradiography was performed, the slides were coated with Hypercoat LM-1 emulsion (Amersham) following the instructions of the manufacturer. The slides were exposed for 3 weeks at 4°C into a

light-tight box, and then they were developed in D-19 (Kodak). For microscope observation the slides were counterstained with hematoxylin–eosin.

RNA Isolation and Northern Blot Analysis

Brains were removed and the cerebellum immediately snap-frozen in liquid nitrogen. Total and poly(A)$^+$-enriched RNA were isolated and analyzed by Northern blot hybridization as previously described (6, 15). For MT-I and MT-III mRNA analysis the same cDNAs above described were used. For γ-actin mRNA, a cDNA was used, kindly provided by Dr. Albert Boronat (University of Barcelona), through Dr. X. Avilés (Autonomous University of Barcelona). The cDNA for the murine acute-phase response gene EB22/5 (25) was kindly provided by Dr. John Inglis (Medical Research Council Human Genetics Unit, Edinburgh, UK). Autoradiographs were developed by exposing X-ray film (XAR-05, Kodak, Rochester, NY) with a high-plus intensifying screen (Wolf X-Ray Corporation, NY) at −70°C, and the bands were quantitated with Molecular Analyst software (Bio Rad).

Development of an ELISA for MT-III

There are no commercial sources for MT-III antibodies. Therefore, an enzyme-linked immunosorbent assay (ELISA) for rat MT-III was developed, which will be described in full elsewhere (submitted). Rat MT-III is highly homologous to mouse MT-III (29, 36), and presumably the antibodies raised will fully cross-react with the latter. Attempts to purify significant native rat MT-III protein by conventional biochemical techniques were unsuccessful with the amount of tissue available. Thus, recombinant rat MT-III (rMT-III) protein was rather used for raising antibodies. *Escherichia coli* 1B392 Lon Δ1 transformed with the expresion construct pET-29a(+)-rMT-III coding region (generously provided by Dr. P. Kille, School of Molecular and Medical Biosciences, University of Wales, Cardiff) was used to induce and purify rMT-III. Native MT-I and MT-II were isolated from cytosols of rat (male Sprague–Dawley) livers that had been injected intraperitoneally with $ZnS0_4$ (20 mg Zn/kg b.w.).

Antisera for rMT-III were raised following the procedure previously described for rMT-I+II (13). Booster injections were given 9, 14, and 30 weeks after the first rMT-III injection. When significant MT-III antibodies were produced, a competitive double-antibody ELISA assay was developed following a procedure previously described (12). The antibodies did not cross-react with native MT-I+II proteins, and the normal working range of this ELISA was 2–200 ng/well.

Immunocytochemistry and Histochemistry

Simultaneous demonstration of MT-I+II and astroglia or microglia was achieved through the sequential combination of MT-I+II immunostaining and selective markers for these glial cells. MT-I+II were demonstrated by the monoclonal antibody E9 (generous gift of Dr. Pete Kille, School of Molecular and Medical Biosciences, University of Wales, College of Cardiff). Astrocytes were identified immunocytochemically by using an antibody against glial fibrillary acidic protein (GFAP) (4). Microglia were identified by tomato lectin histochemistry, a marker for both normal and reactive microglia (1). These double-labeling techniques have been used successfully in previous studies (46).

Statistical Analysis

Results of control, G16, and G36 mice were normally analyzed with one-way analysis of the variance (ANOVA) since more than two groups were compared, followed by multiple comparisons of the means using the Duncan procedure. Logarithmic transformation of the data was carried out when necessary.

RESULTS

Figure 1 shows representative macroautoradiographies of control and GFAP-IL6 mice for MT-I and MT-III mRNAs. The sense probes produced a very weak signal compared with the antisense probes (data not shown). The pattern of hybridization of the two MT probes were in clear agreement with the published literature (27, 34, 49, 51). Thus, an intense signal of MT-III was observed in control mice in the pyramidal neurons of the hippocampus in the CA1–CA3 regions, as well as in the granule cells of the dentate gyrus. In contrast, MT-I expression was low in all but the CA3 neurons and was prominent in the stratum radiatum, a structure abundantly populated of astrocytes. In the remaining brain areas, the MT-I and MT-III antisense probes produced comparable signals, with clear hybridization throughout the neocortex, thalamus, hypothalamus, brain stem, and cerebellar cortex, especially in the Purkinje cell layer. A prominent signal was also observed in the choroid plexus and ependymal cells.

The transgenic expression of IL-6 produced clear effects on both MT-I and MT-III mRNAs. Thus, MT-I, and to a lesser extent MT-III, mRNA levels were strongly increased in the cerebellum of the two lines of GFAP-IL6 mice (Fig. 1). For statistical purposes, mRNA levels were quantitated in several mice of each group as described under Materials and Methods not only in the cerebellum but also in other brain areas. Figure 2 shows the results for the cerebellum. GFAP-IL6 mice showed significantly ($P < 0.05$) higher MT-I mRNA levels in the Purkinje and granular cell layers and in the basal nuclei. In contrast, MT-III mRNA were increased in the Purkinje cell layer and basal nuclei, but not in the granular cell layer. In addition, the fold induction for MT-III in the Purkinje cell layer was

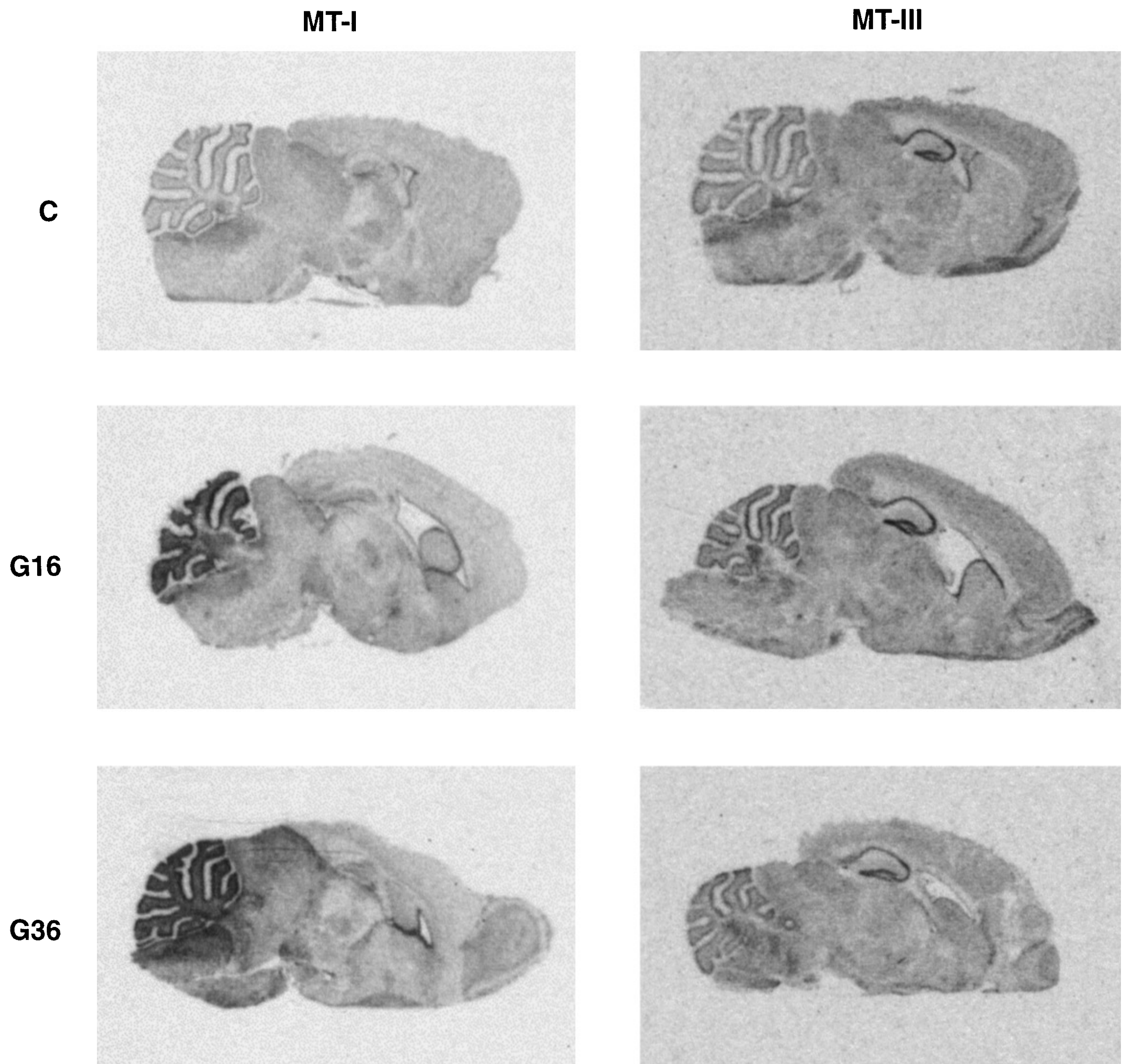


FIG. 1. *In situ* hybridization analysis of brain MT-I and MT-III RNA expression. Mice of the G16 and G36 lines were killed at 3 months of age along appropriate controls and the brains immediately frozen in liquid nitrogen. Sagittal sections were then prepared and *in situ* hybridization performed as described under Materials and Methods. A representative macroautoradiography of each experimental condition is shown. MT-I and MT-III RNA levels of three mice per group were quantitated in specific brain areas, and the results are shown in Figs. 2, 4, and 5.

lower than for MT-I. To confirm these results, a Northern blot analysis of MT-I and MT-III mRNA levels in the cerebellum was carried out (Fig. 3). Total RNA and poly(A)$^+$ mRNA were analyzed in four independent mice per group, the ratios with γ-actin mRNA calculated, and the mean $\pm$ SE values shown in Table 1. In agreement with the *in situ* hybridization results, in poly(A)$^+$ samples both MT-I (up to 3-fold) and MT-III (up to 1.5-fold) mRNA levels were increased by the transgenic expression of IL-6. In total RNA samples the MT-I mRNA increase (2.6-fold) was also clearly observed. Presumably the increase of MT-III was not observed because of the limits of sensitivity of the technique with such samples. An upregulation of the MT-III isoform in the cerebellum of the GFAP-IL6 mice was definitively demonstrated by measuring the MT-III protein, since it increased significantly at the same extent as the messanger. Thus, MT-III protein levels (μg/g) for control, G16, and G36 mice were 1.40 $\pm$ 0.23, 2.07 $\pm$ 0.37, and 2.31 $\pm$ 0.09 (mean $\pm$ SE, $n = 3$).

Figures 4 and 5 show the MT-I and MT-III mRNA quantifications for other brain areas, respectively.

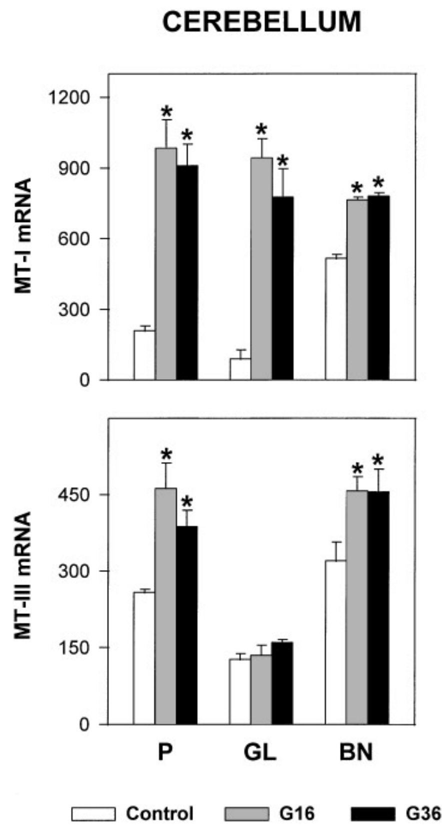


FIG. 2. Effect of transgenic IL-6 expression on cerebellar MT-I (top) and MT-III (bottom) RNA levels. MT RNAs were measured in defined working areas in the Purkinje cell layer (P), granular layer (GL), and basal nuclei (BN). Results are mean ± SE, $n = 3$. *P at least <0.05 vs control mice.

Transgenic expression of IL-6 was associated with increased MT-I mRNA signal in the hypothalamus of the two lines, whereas in the thalamus it was increased only in the G36 mice (significant only when compared with the control and G16 data pooled, Student t test, $P < 0.05$); a trend toward increased expression of MT-I RNA was also observed in the dentate gyrus and in ependymal cells in both mouse lines. In contrast, no alteration in MT-I RNA expression was observed in the brain cortex or striatum of the GFAP-IL6 mice. Transgenic expression of IL-6 affected MT-III expression differently to that of MT-I. Thus, there was a tendency toward decreased MT-III signal in the GFAP-IL6 mice was that observed in all forebrain areas except the striatal tissue. However, this trend reached statistical significance only in dentate gyrus and CA3 field of the hippocampus (only G36 mice), ependymal cells, and occipital cortex (P at least < 0.05). In contrast to the occipital cortex area, which is close to the cerebellum, no change in MT-III expression was observed in frontal cortex of the GFAP-IL6 mice. This is important since the transgenic expression of IL-6 is highest in the

TABLE 1

MT-I, MT-III, and EB22/5.3 mRNA Levels in Control and GFAP-IL6 Mice

	Control	G16	G36
Total RNA			
MT-I	3.41 ± 0.44	6.17 ± 1.21	8.93 ± 1.16*
MT-III	5.91 ± 1.19	4.17 ± 1.14	5.54 ± 1.09
Poly(A)$^+$ RNA			
MT-I	0.73 ± 0.15	1.29 ± 0.41	2.20 ± 0.29*
MT-III	0.78 ± 0.14	0.98 ± 0.13	1.20 ± 0.07*
EB22/5.3	0.31 ± 0.17	3.30 ± 1.40*	8.93 ± 1.46*

Note. Total RNA or poly(A)$^+$ mRNA from cerebellum were isolated and analyzed by Northern blot as shown in Fig. 3. Results are mean ± SE ($n = 3$–4) expressed as ratio vs γ-actin mRNA.
* $P < 0.05$ vs control mice.

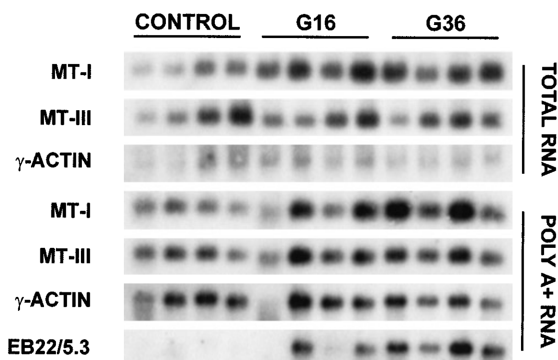


FIG. 3. Northern blot analysis of MT-I, MT-III, γ-actin, and the acute phase gene EB22/5.3 of samples of total RNA and poly(A)$^+$ mRNA isolated from the cerebellum of control and GFAP-IL 6 (G16, low expressor; G36, high expressor) mice, from four independent animals per group. The bands were quantitated and the data normalized with the γ-actin mRNA (see Table 1). Poly(A)$^+$ sample of the animal 1 of the G16 mice was not used because of degradation.

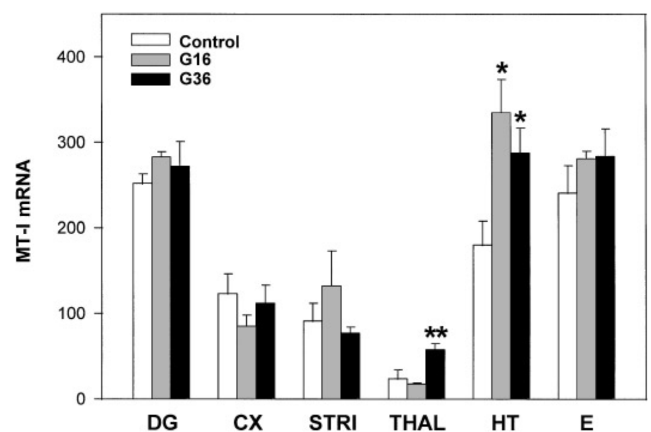


FIG. 4. Effect of transgenic IL-6 expression on MT-I RNA levels of the dentate gyrus (DG), cortex (CX), striatum (STRI), thalamus (THAL), hypothalamus (HT), and ependymal cells (E). Results are mean ± SE, $n = 3$. *P at least <0.05 vs control mice. **$P < 0.05$ vs the other two groups pooled: in the thalamus, the one-way ANOVA was not significant and thus no post hoc comparisons of the means could be done; since the low-expressor G16 line showed comparable MT-I mRNA levels to those control mice, they were pooled and compared with the G36 MT-I mRNA levels with the Student t test.

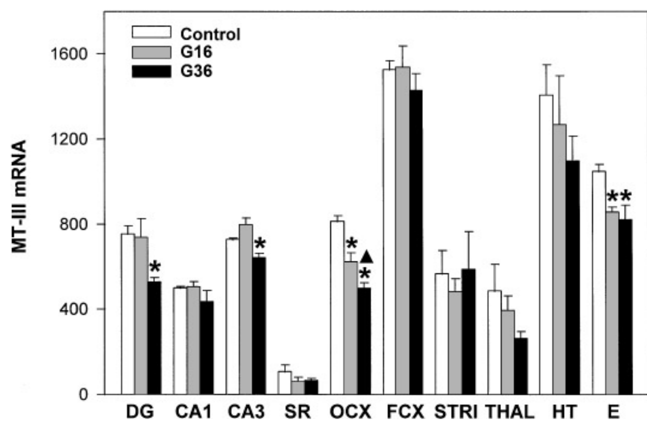


FIG. 5. Effect of transgenic IL-6 expression on MT-III RNA levels of the dentate gyrus (DG), CA1 and CA3 pyramidal layer, and stratum radiatum (SR) areas of the hippocampus, occipital cortex (OCX), frontal cortex (FCX), striatum (STRI), thalamus (THAL), hypothalamus (HT), and ependymal cells (E). Results are mean ± SE, $n = 3$. *P at least <0.05 vs control mice. ▲ $P < 0.05$ vs the G16 line.

cerebellum and progressively decreases in the frontal direction. Interestingly, the decrease of the MT-III signal was higher in the high expressor line G36 than in the low expressor line, G16.

Figures 6 and 7 show microautoradiographies for MT-I and MT-III mRNAs in selected brain areas, which confirmed and expanded the previous results. Thus, in the cerebellum the MT-I mRNA signal was strongly increased in the Purkinje cell layer in GFAP-IL6 mice (Figs. 6a and 6b). The signal was not in the Purkinje neurons but surrounding them, likely in the Bergman glia. This is supported by the distinct MT-I signal observed in the molecular layer (Figs. 6c and 6d) and by the immunocytochemical results (Fig. 6k). The MT-III mRNA signal of the Purkinje cell layer was also significantly increased in the GFAP-IL6 mice (Figs. 6e and 6f), but to a lesser extent than that of MT-I. Significant astrogliosis and microgliosis were observed in the GFAP-IL6 mice, as expected (not shown). Double-labeling studies demonstrated MT-I+II in astrocytes (Fig. 6i, arrowheads, nuclear localization), but also in microglia (Fig. 6j, arrowheads). Some astrocytes and microglia were devoid of MT-I+II (Figs. 6i and 6j, arrows).

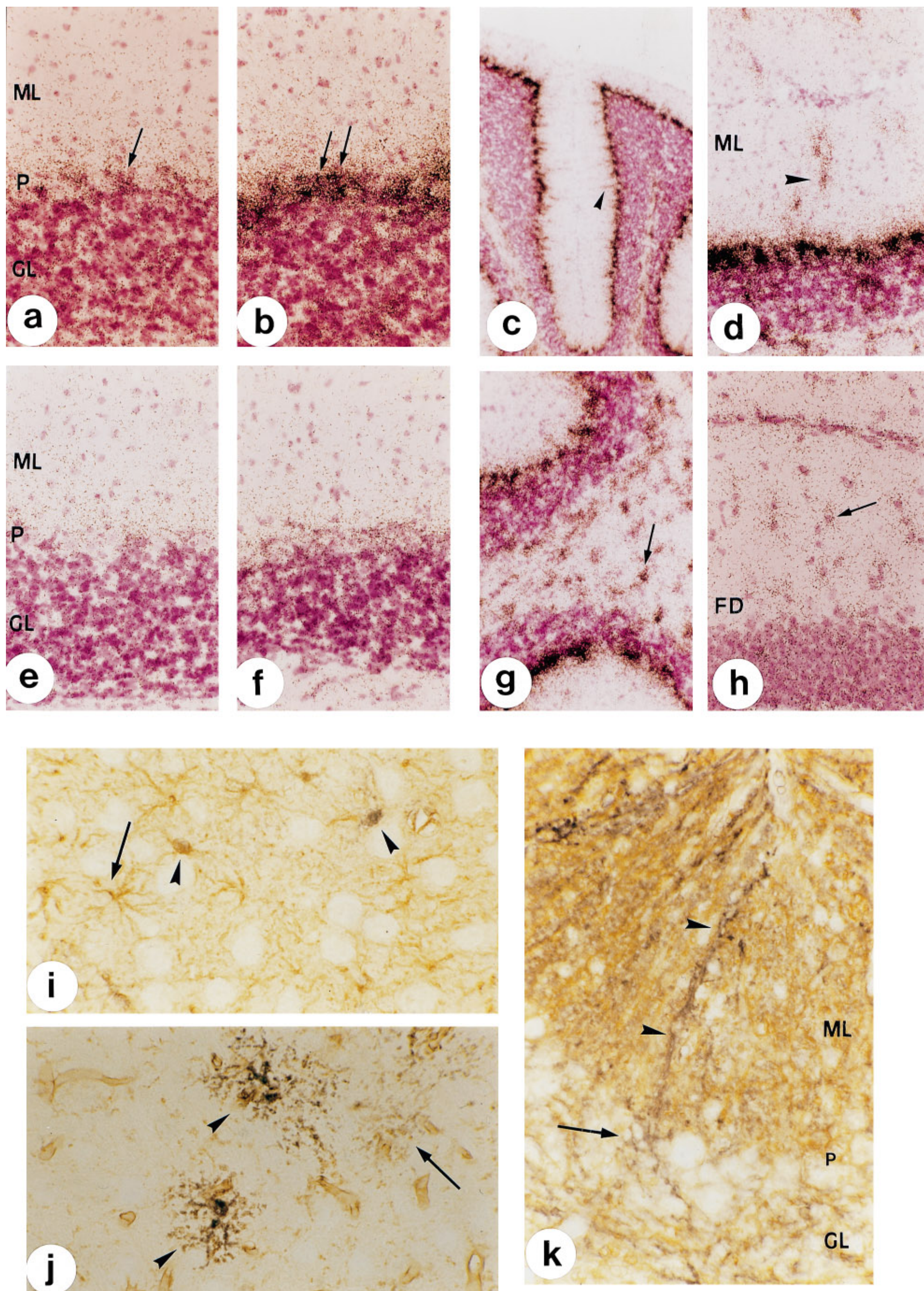
As expected, a clear MT-I and MT-III mRNA signal was observed in ependymal cells (Figs. 7a–7f). MT-III but not MT-I mRNA was decreased in GFAP-IL6 mice. Also, in the hippocampus the MT-III signal was prominently located in the pyramidal neuron layer. In the CA3 field, a significant reduction of the MT-III signal was observed in the G36 mice (Figs. 7g–7i).

DISCUSSION

In this study the spatial regulation of brain MT gene expression was examined by means of *in situ* hybridization studies in transgenic mice in which chronic expression of IL-6 was targeted to astrocytes under the regulatory control of the GFAP promoter. These mice suffer a chronic inflammatory process in the brain and show clear evidence of neurodegeneration and other cellular and molecular processes that make them a valuable model for the study of several human neuropathologies. Furthermore, they are a unique tool for examining the role of the MT family in brain physiology and pathobiology.

In a previous study (15), MT-I+II protein levels were measured by radioimmunoassay in homogenates, and they were found to be significantly increased by transgenic IL-6 expression in specific brain areas, namely, cerebellum (highest induction), medulla plus pons, hypothalamus and remaining brain (lowest induction), but not in the hippocampus. This correlated well with the IL-6-associated inflammatory response observed in these brain areas (5, 6), and agrees with the well-known IL-6 effect on MT-I+II synthesis in other tissues (8, 39). In contrast to the MT-I+II protein changes, Northern blot analysis of isolated poly(A)$^+$ mRNA was inconclusive and indicated that MT-I mRNA levels, once normalized with γ-actin mRNA levels, were only marginally increased in the GFAP-IL6 mice, even in the cerebellum (15). This was puzzling and in principle suggestive of posttranscriptional control of MT gene expression in this chronic situation, which has also been demonstrated in other reports (33, 45). The present *in situ* hybridization results, however, demonstrate that MT-I RNA changes clearly reflect the MT-I+II protein levels. Since the previous Northern blot studies were carried out with poly(A)$^+$ mRNA (15), in this report we examined total RNA and poly(A)$^+$ mRNA isolated from the same animals to rule out an effect of the poly(A)$^+$ tail. The results were alike in both cases and on this occasion in agreement with those of the *in situ* experiments and the MT-I + II protein levels. The discrepancy between the present findings and the previously reported Northern results is likely the consequence of using γ-actin mRNA content to normalize against since it was somewhat elevated in the GFAP-IL6 mice. Why the expression of this gene was increased in the former GFAP-IL6 mice analyzed and not in the present mice remains unclear.

Thus, *in situ* hybridization analysis indicated that MT-I RNA levels were strongly increased in the cerebellum of both GFAP-IL6 mice, and detailed analyses indicated that the MT-I signal increased in the Purkinje cell layer, the granular layer, white matter, and the basal nuclei, but not in the molecular layer. MT-I RNA levels also increased in other brain areas, such as the thalamus and hypothalamus, but the induction was smaller than in the cerebellum. These MT-I RNA changes are fully in agreement with the protein levels (15) and clearly suggest that the transcription rate of the MT-I gene is increased in these transgenic mice. An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important point to consider is that MT-I induction in the GFAP-IL6 mice could rather be due to the inflammatory reaction elicited in the brain and not to the cytokine per se, especially considering that this is a chronic situation. However, we have recently shown in the rat that the administration of IL-6 into the third ventricle increases MT-I+II protein levels in several areas including the cerebellum (14). Although these results suggest that that IL-6 induces these MT isoforms directly, they do not rule out that other factors in addition to IL-6 could also contribute to MT-I induction in the GFAP-IL6 mice. Since the pattern of the MT-I induction throughout the brain of the GFAP-IL6 mice correlates with IL-6 transgenic expression and is similar to that of the acute-phase response genes EB22/5 and complement C3 (3, 5, 6), and given the coordinated nature of the regulation of the MT-I and MT-II genes (47), the results suggest that the MT-I+II isoforms could be considered acute-phase genes in the brain.

Regarding MT-III, previous reports have demonstrated a substantial differential control of this isoform compared to the widely expressed MT-I+II isoforms (see Introduction). The results with GFAP-IL6 mice also demonstrate that the cerebral regulation of MT-III differs to that of the other isoforms, since a general tendency of MT-III to be decreased throughout the cerebrum was evident, while MT-I, if anything, showed the opposite tendency. In contrast, in the cerebellum the MT-III gene expression, similar to MT-I, was upregulated by the transgenic IL-6 expression. This was confirmed by Northern blot analysis and by measuring the MT-III protein. Again (see above), the discrepancies with the previous Northern results (15) are likely due to concomitant changes in the γ-actin mRNA observed in the former GFAP-IL6 mice which were not observed in the present report. Expression of the different MTs differed in the granular layer, where only MT-I was induced in the GFAP-IL6 mice. Thus, the regulation of MT-I and MT-III genes may differ, but the nature of the difference depends on the brain area. MT-III has been proposed to be involved in reparative processes in the brain by a number of reports dealing with brain damage (see Introduction), but the results are conflicting since, depending on the nature of the insult used to inflict damage to the brain, MT-III expression is either up- or downregulated, and, furthermore, it changes over time. The GFAP-IL6 mice is a chronic model of brain damage, and probably is more indicative of the physiological needs of the brain for each MT isoform. In the cerebellum, where the damage inflicted by the chronic inflammation is the greatest, both MT-I and MT-III genes are clearly upregulated, suggesting that in these conditions increased levels of the two proteins are needed to cope with the tissue damage. In contrast, opposing trends between MT isoforms are observed in the cerebrum, indicating therefore that the functions served by the two proteins must differ, at least in these brain areas.

As stated above, several studies suggest that the brain astrocytes are the main sites of MT-I+II production, and neurons that of MT-III. The microautoradiography and immunocytochemical results are consistent with these reports. Moreover, our findings also suggest that the chronic expression of IL-6 did not modify that general pattern of MT-I and MT-III expression. Thus, although a strong upregulation of the MT-I gene was evident in the cerebellum and other brain areas, the MT-I signal seemed to be always associated with cells of a glial nature. However, the double-labeling techniques employed revealed that in the GFAP-IL6 mice the MT-I+II immunostaining was significantly associated not only with astrocytes but also with microglia. It is well-known that microglia of control animals are devoid of MT-I+II immunostaining (19), and this has also been the case in this report. However, when we examined MT-I+II immunoreactivity of the spinal cord of the myelin-deficient jimpy mouse, which exhibit a pronounced astrogliosis and microgliosis where a number of cytokines and free radicals are likely to be involved, it was clear that a significant number of microglial cells were MT-I+II positive (46). Since the GFAP-IL6 mice also show pronounced astrogliosis and microgliosis, it may be concluded that in such situations (gliosis) MT-I+II synthesis is upregulated in both astrocytes and microglia. Given the antioxidant properties of these proteins (38), MT-I+II induction may be viewed as a protective mechanism within the CNS (see (19) for further discussion).

In summary, the present results demonstrate that the widely expressed MT isoform MT-I is strongly

FIG. 6. Microautoradiographic detection of MT-I and MT-III RNA expression in the hindbrain. MT-I RNA (a, b, c, d, g, h). MT-III RNA (e, f). In the cerebellum, the MT-I RNA signal was strongly increased in the Purkinje cell layer in GFAP-IL6 mice (a, b), presumably in astrocytes (arrows) which were surrounding the Purkinje neurons. A distinct MT-I signal was observed in the G36 line (c, d) suggestive of Bergman glia (arrowhead). The transgenic expression of IL-6 also increased the MT-III RNA signal of the Purkinje cell layer (e, f). A prominent MT-I signal was observed in astrocytes of cerebellar white matter (g) or in the fascia dentata (h). Double-labeling techniques for demonstrating MT-I+II in astrocytes (GFAP immunocytochemistry, i, k) and in microglia (tomato lectin histochemistry, j) are also shown. MT-I+II immunostaining is blackish and predominantly located in the nucleus, whereas staining of glial cells is brownish and cytoplasmatic. In both astrocytes and microglia, MT-I+II are present in some cells (arrowheads) but other are devoid of them (arrows) (i, j). In the cerebellum (k) the MT-I+II presence in the Bergman glia is confirmed and both the cell body (arrow) and the process (arrowhead) through the molecular layer are clearly observed. (ML, molecular layer; P, Purkinje cell layer; GL, granular layer; FD, fascia dentata. Original magnifications: a, b, e, f, h, ×160; c, ×16; d, g, ×64; i–k, ×400.

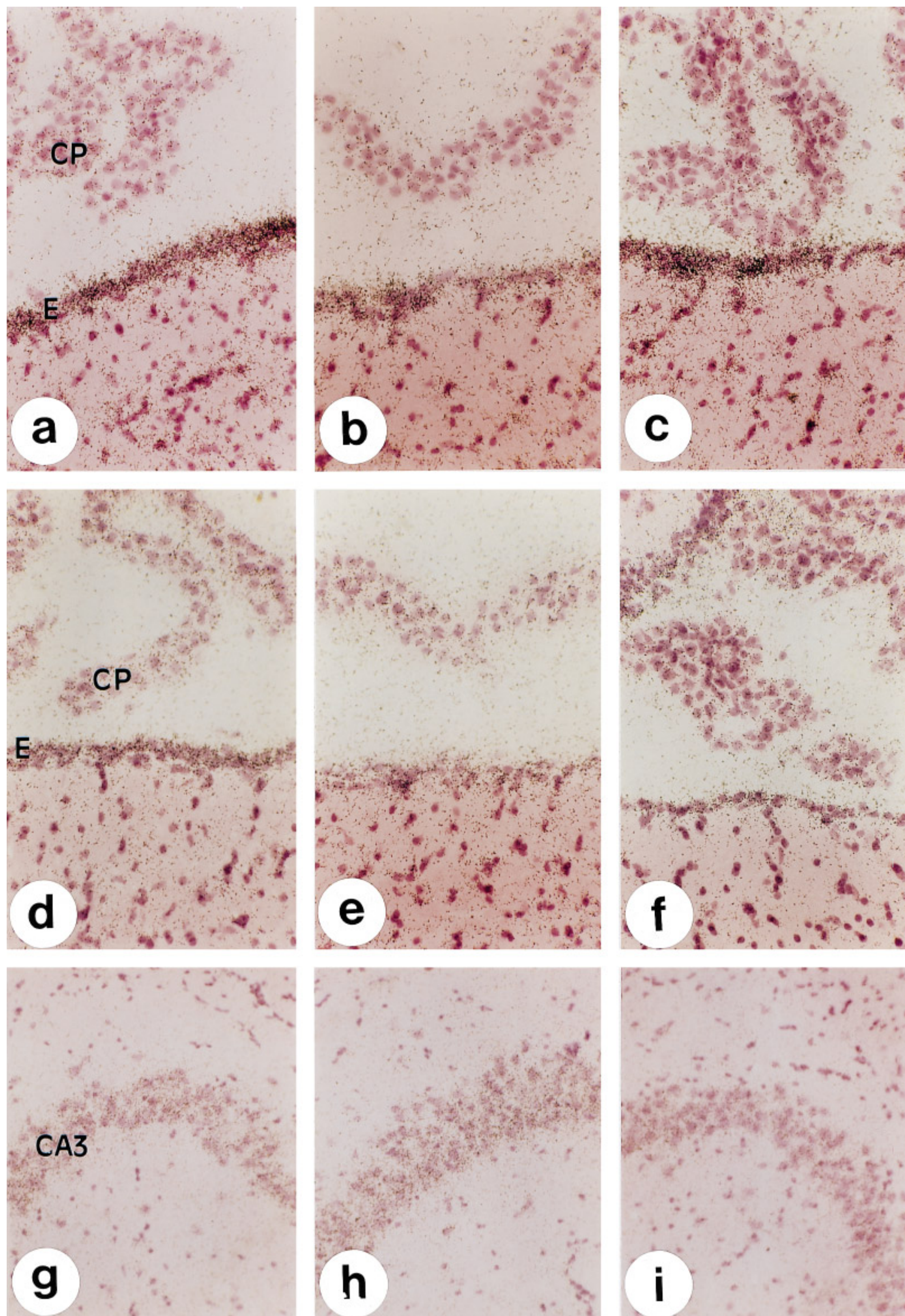

FIG. 7. Microautoradiographic detection of MT-I and MT-III RNA expression in the forebrain. Expression of MT-I (a–c) and MT-III (d–f) RNA in ependymal cells. The transgenic expression of IL-6 significantly decreased the MT-III signal. In the hippocampus (g–i), the MT-III signal was prominently located in the pyramidal neuron layer, and in the CA3 field a significant reduction of the MT-III signal was observed in the GFAP-IL6 G36 mice. CP, choroid plexus; E, ependymal cells. Original magnifications: a–f, ×160; g–i, ×64.

induced in the brain of transgenic mice which have the cytokine IL-6 under the control of the GFAP promoter. The induction correlates with the inflammatory response caused by IL-6 and suggests that this MT isoform could be considered an acute-phase protein in the brain as appears to be the case in the liver. The transgenic expression of IL-6 also affected dramatically the expression of the MT-III gene, which was upregulated in the cerebellum and downregulated in many parts of the cerebrum. The results suggest that all MT isoforms are involved in the physiological response of the brain to damage, but that they should accomplish different functions.

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