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Carrasco, Javier [et al.]. «Metallothionein-I and -III expression in animal models of Alzheimer disease». *Neuroscience*, Vol. 143, Num. 4 (December 2006), p. 911-922 DOI 10.1016/j.neuroscience.2006.08.054, PMID 17027170

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Metallothionein-I&III expression in animal models of Alzheimer disease

Javier Carrasco[1†], Paul Adlard[2,3†], Carl Cotman[3], Albert Quintana[1], Milena Penkowa[4], Feng Xu[5], William E. Van Nostrand[5], Juan Hidalgo[1*]

1 Institute of Neurosciences and Department of Cellular Biology, Physiology and Immunology, Animal Physiology Unit, Faculty of Sciences, Autonomous University of Barcelona, Bellaterra, Barcelona, Spain 08193.

2 Oxidation Disorders Laboratory, The Mental Health Research Institute, Victoria, 3052, Australia

3 Institute for Brain Aging and Dementia, University of California (USA).

4 Section of Neuroprotection, Centre of Inflammation and Metabolism, The Faculty of Health Sciences, University of Copenhagen, Denmark

5 Department of Medicine, HSC T-15/083, Stony Brook University, Stony Brook, NY 11794-8153, USA

†Javier Carrasco and Paul Adlard contributed equally to this paper

* Correspondence with Juan Hidalgo, Telephone: 34935812037. Fax: 34935812390. E-mail: Juan.Hidalgo@uab.es

Running title: metallothionein and AD animal models

Key words: metallothionein-I; metallothionein-III; Alzheimer's Disease; transgenic mice; Tg2576; TgCRND8; TgSwDI

ABSTRACT

Previous studies have described altered expression of metallothioneins (MTs) in neurodegenerative diseases like multiple sclerosis (MS), Down syndrome, and Alzheimer's disease (AD). In order to gain insight into the possible role of MTs in neurodegenerative processes and especially in human diseases, the use of animal models is a valuable tool. Several transgenic mouse models of AD amyloid deposits are currently available. These models express human Aβ precursor protein (AβPP) carrying different mutations that subsequently result in a varied pattern of β-amyloid (Aβ) deposition within the brain. We have evaluated the expression of MT-I and MT-III mRNA by *in situ* hybridization in three different transgenic mice models of AD: Tg2576 (carrying AβPP harbouring the Swedish K670N/M671L mutations), TgCRND8 (Swedish and the Indiana V717F mutations), and Tg-SwDI (Swedish and Dutch/Iowa E693Q/D694N mutations). MT-I mRNA levels were induced in all transgenic lines studied, although the pattern of induction differed between the models. In the Tg2576 mice MT-I was weakly upregulated in cells surrounding Congo red-positive plaques in the cortex and hippocampus. A more potent induction of MT-I was observed in the cortex and hippocampus of the TgCRND8 mice, likely reflecting their higher amyloid plaques content. MT-I upregulation was also more significant in Tg-SwDI mice, especially in the subiculum and hippocampus CA1 area. Immunofluorescence stainings demonstrate that astrocytes and microglia/macrophages surrounding the plaques express MT-I&II. In general, MT-I regulation follow a similar but less potent response than GFAP expression. In contrast to MT-I, MT-III mRNA expression was not significantly altered in any of the models examined suggesting that the various MT isoforms may have different roles in these experimental systems, and perhaps also in human AD.

INTRODUCTION

In the last 10-15 years, metallothionein (MT) research in the brain has shown that this family of proteins play a major role in brain physiology (Hidalgo et al., 2001). There are four closely linked *MT* genes (*MT-1-4)* present in rodents (Palmiter et al., 1992, Quaife et al., 1994). MT-I and MT-II (MT-I&II) are expressed coordinately in most tissues including those of the central nervous system (CNS) (Searle et al., 1984, Yagle and Palmiter, 1985, van Lookeren Campagne et al., 2000), while MT-III and MT-IV show a much more restricted tissue expression (primarily localized in the CNS and stratified squamous epithelia, respectively).

There is compelling evidence that MT-I&II are involved in the response of the brain to damage (Hidalgo et al., 2001), and indeed there is a significant upregulation of these proteins in a number of human neurological diseases, including Alzheimer's disease (AD) (Duguid et al., 1989, Uchida, 1993, Adlard et al., 1998, Zambenedetti et al., 1998, Chuah and Getchell, 1999), Pick's disease (Duguid et al., 1989), short-course Creutzfeld-Jakob disease (Kawashima et al., 2000), amyotrophic lateral sclerosis (ALS) (Sillevis Smitt et al., 1992, Sillevis Smitt et al., 1994, Blaauwgeers et al., 1996), and multiple sclerosis (MS) (Lock et al., 2002, Penkowa et al., 2003c). Furthermore, animal studies have demonstrated a neuroprotective role for these proteins following mild focal cerebral ischemia and reperfusion (van Lookeren Campagne et al., 1999, Trendelenburg et al., 2002), kainic acid-induced seizures (Carrasco et al., 2000b), 6-hydroxydopamine (Asanuma et al., 2002), in models of ALS (Nagano et al., 2001, Puttaparthi et al., 2002) and MS (Penkowa et al., 2001, Penkowa et al., 2003b), after traumatic brain injury (Penkowa et al., 1999a, Penkowa et al., 2000, Giralt et al., 2002b) and transgenic IL-6-induced neuropathology (Giralt et al., 2002a, Molinero et al., 2003, Penkowa et al., 2003a). It is likely, therefore, that these proteins are upregulated as a protective mechanism in response to evolving brain damage.

MT-III was discovered in the human brain (Uchida et al., 1991) and originally referred as "Growth Inhibitory Factor" (GIF) due to its activity and ability to inhibit neuronal growth. It was postulated that a decrease of this protein in AD may contribute to the aberrant sprouting characteristic of this disorder (Uchida et al., 1991) and perhaps, therefore, have an underlying involvement in this disease process. There have been numerous studies examining MT-III in the AD brain; however, that MT-III is decreased in AD is not a consistent finding (Uchida et al., 1991, Tsuji et al., 1992, Erickson et al., 1994, Uchida, 1994, Amoureux et al., 1997, Carrasco et al., 1999, Yu et al., 2001). MT-III expression has also been shown to be altered (up- or downregulated) in Down's syndrome (Arai et al., 1997), Creutzfeld-Jakob disease (Kawashima et al., 2000),

pontosubicular necrosis (Isumi et al., 2000), Parkinson's disease, meningitis, and ALS (Uchida, 1994). Similarly, a MT-III modulation (up- or downregulation depending on the model, time, etc.) has been observed in animal models of brain injury (Hidalgo et al., 2001) and been shown to have a neuroprotective role after kainic acid-induced seizures (Erickson et al., 1997), cortical cryolesion (Carrasco et al., 2003) and in models of increased oxidative damage (in the G93A SOD1 mouse that has a mutated superoxide dismutase gene) (Puttaparthi et al., 2002), and peripheral nerve injury (Ceballos et al., 2003). The available evidence strongly suggests that the roles of MT-III will differ substantially from those of MT-I&II.

While the above data support a role for MTs in neurodegenerative diseases such as MS and ALS, to our knowledge there are no studies in mouse AD models that might provide insight into the putative role of MTs in the development of this disease. The aim of this study, therefore, is to evaluate MT-I and MT-III expression in three different AD mouse models, Tg2576 mice (expressing human AβPP harbouring the Swedish K670N/M671L mutations) (Hsiao et al., 1996), TgCRND8 mice (Swedish and Indiana V717F mutations) (Chisthi et al., 2001), and Tg-SwDI mice (Swedish and Dutch/Iowa E693Q/D694N mutations) (Davis et al., 2004). The results show a specific pattern of induction of MT-I in each model, as well as a lack of significant alteration in MT-III expression. Thus, these AD models will be valuable tools for characterizing the role of MTs in AD and for examining their putative therapeutic potential.

MATERIALS AND METHODS

AD amyloid deposits mouse models.

Tg2576 male mice (Hsiao et al., 1996) were purchased from Taconic Europe A/S, and crossed with C57BL/6 females; female offspring 14-18 months old were used in this study (n=9 APP+/-, n=12 APP-/-). TgCRND8 male and female mice (Chisthi et al., 2001), courtesy of David Westaway (University of Toronto), were maintained on a B6C3F1/J genetic background and used at 18months of age along age- and gender-matched controls (n=3 in the four groups). Tg-SwDI male mice (Davis et al., 2004) at 5-6, 12 and 18 months of age were used along with littermate controls (4 mouse per group).

All animal procedures were approved by the appropriate ethical committees of the institutions involved in this study. All mice to be used for in situ hybridization were killed by decapitation, and the brains were immediately removed and one hemisphere frozen on dry ice or liquid nitrogen and used later for in situ hybridization analyses; the other hemisphere was also frozen, in some cases being microdissected into several brain areas (frontal cortex, hippocampus and remaining brain excluding cerebellum). All tissues were stored at –80ºC.

In situ hybridization.

Serial sagittal sections (20 μm in thickness for the Tg2576, 14 μm for the TgCRND8 and the Tg-SwDI) were cut on a cryostat and mounted on poly-L-lysine coated slides. All sections were then maintained at -80°C until the day of analysis. The *in situ* hybridization analysis of the MT-I and MT-III isoforms and of GFAP was carried out as previously described (Carrasco et al., 1998b, Molinero et al., 2003). As the MT-I and MT-II isoforms are coordinately regulated in both the periphery (Yagle and Palmiter, 1985) and the brain (Masters et al., 1994, van Lookeren Campagne et al., 2000), we assumed that the results for MT-I are representative of those of MT-II. Autoradiography was performed by exposing the slides to the film (hyperfilm-MP, Amersham) for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I, MT-III and GFAP mRNA levels were semiquantitatively determined in several 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 mRNA values shown are expressed in

arbitrary units (number of pixels x optic density). Quantitations were carried out as described for the autoradiographic films.

Microautoradiography.

After macroautoradiography was performed, the slides were coated with Hypercoat LM-1 emulsion (Amersham) following the instructions of the manufacturer. The slides were the exposed for 3 weeks at 4°C and subsequently they were developed in D-19 (Kodak). For microscope observation the slides were counterstained with hematoxylin-eosin.

Plaques.

Amyloid plaques were demonstrated by the Congo Red method using the Sigma kit HT60 following the recommendations of the manufacturer. In some occasions Congo Red staining was performed after the *in situ* hybridization was carried out, followed by microautoradiography to simultaneously demonstrate MT-I, MT-III or GFAP expression surrounding the amyloid plaques.

MT-I+II protein and Aβ assays.

MT-I+II levels were measured by radioimmunoassay as described previously (Gasull et al., 1993) using a polyclonal antibody which cross-reacts with rat and mouse MT-I and MT-II. To this end, TgCRND8 and WT samples were manually homogenized in 1 ml of 50 mM Tris-HCl (pH 8.2), and spun (16000g, 4ºC) for 45 minutes and the supernatant used for the RIA. The pellet fraction from the hippocampal tissue was resuspended in 70% formic acid (150 µl per sample), sonicated (0.5 sec pulses for 10 sec), spun (16000g, 4ºC) for 90 minutes and then the supernatant used for an Aβ ELISA, as previously described (Adlard et al., 2005). TgSwDI and WT samples (an aliquot of powdered whole hemispheres) were reconstituted in water, sonicated and a clear supernatant obtained by centrifugation for measuring MT-I+II by RIA. With other aliquots, Aβ40 and Aβ42 levels, soluble and insoluble, were measured by ELISA as described ((Johnson-Wood et al., 1997, DeMattos et al., 2002, Davis et al., 2004)).

Immunofluorescence.

Tg2576 and WT mice were deeply anesthetized with 10 mg/100 g body weight of Brietal (Methohexital 10 mg/ml, Eli Lilly) and were transcardially perfused with 0.9% saline with 0.3% heparin (15000 IU/liter) for 3-5 min followed by perfusion with 4% paraformaldehyde, pH 7.4. Afterwards, all the brains were fixed by immersion in 4% paraformaldehyde for 4 h, pH 7.4 at room temperature. Brains were dehydrated according to standard procedures, embedded in paraffin, and cut in serial, coronal 5 µm thick sections. Sections were rehydrated and were incubated in 1.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40) (Sigma-Aldrich, USA, code N-6507) for 15 min at room temperature to quench endogenous peroxidase. Afterwards, sections were incubated with 10% goat serum (In Vitro, DK, code 04009-1B) or donkey serum (The Binding Site, UK, code BP 005.1) in TBS/Nonidet for 30 min at room temperature in order to block nonspecific binding. Since we used monoclonal mouse-derived antibodies for MT-I&II, sections were in addition incubated with Blocking Solutions A+B from HistoMouse-SP Kit (Zymed Lab. Inc., USA, code 95-9544) to quench endogenous mouse IgG.

For triple IHCs for GFAP & MT-I+II & Amyloid beta 42 ($A\beta_{42}$), we used these primary antiboides: Goat anti-GFAP, diluted 1:100 (Santa Cruz Biotech. Inc., CA, USA, code: sc-6170); Mouse anti-MT-I+II diluted 1:50 (Dako, Glostrup, DK, code M0639); and Rabbit anti-$A\beta_{42}$ diluted 1:100 (Calbiochem, USA, code 171609). These primary antibodies were detected by the following secondary antibodies (1:50): Donkey-anti-mouse IgG linked with AMCA (Jackson ImmunoRes. Lab. Inc., code 715-155-150); Donkey-anti-goat IgG linked with FITC (Jackson ImmunoRes. Lab. Inc., code 705-095-147); and Donkey-anti-rabbit IgG linked with TXRD (Jackson ImmunoRes. Lab. Inc., code 711-075-152).

For triple IHCs for lectin & MT-I+II & Amyloid beta 42 ($A\beta_{42}$), we used TXRD-linked tomato lectin from *Lycopersicon esculentum* 1:50 (Sigma-Aldrich, USA, code L9139); Mouse anti-MT-I+II diluted 1:50 (Dako, Glostrup, DK, code M0639); and Rabbit anti-$A\beta_{42}$ diluted 1:100 (Calbiochem, USA, code 171609). As secondary antibodies we used (1:50) Goat-anti-mouse IgG linked with FITC (Jackson ImmunoRes. Lab. Inc., code 115-095166); and Goat-anti-rabbit IgG linked with AMCA (Jackson ImmunoRes. Lab. Inc., code 111-155-144

Statistical analyses.

When only two groups were to be compared, the Student “t” test was used. When age and genotypes were to be compared, two-way ANOVA was employed.

RESULTS

Figure 1 shows representative autoradiographies for MT-I, MT-III and GFAP *in situ* hybridizations obtained in the Tg2576 AD model. Figure 2 shows representative microautoradiographies. It is quite clear that, as expected, GFAP was prominently upregulated in areas surrounding amyloid plaques, which were abundantly present in the cortex and hippocampus. Quantifications carried out in the autoradiographic films in both areas demonstrated highly significant ($p<0.01$) increases in GFAP mRNA levels (Table 1). Such increases were also evident in the microautoradiographies (Figures 2 and 3). This is consistent with the well-established astrocytosis that occurs as a function of amyloid deposition in these mice at an advanced age (Hsiao et al., 1996).

In contrast to GFAP, MT-I did not appear to be induced in the Tg2576 mice since quantifications of the autoradiographic films failed to show any difference between WT and Tg2576 mice (Figure 1, Table 1). However, a more thorough analysis revealed that MT-I mRNA levels were in fact increased in cells surrounding Congo Red-positive plaques (Figure 2). This upregulation was smaller than that for GFAP, and combined with the highest basal expression in the normal brain, the plaque-specific MT-I induction could only be detected in the microautoradiographies: quantitation of MT-I mRNA levels in them demonstrated an approximately 2-fold induction (a 7-fold induction for GFAP) in the plaque-containing areas compared to the non-plaque areas (Figure 3). The cell surrounding the plaques expressing MT-I+II were examined by immunofluorescence. As expected, both astrocytes and microglia/macrophages appeared to express predominantly MT-I+II (Figure 4a,b).

MT-III showed the expected different pattern of expression compared to MT-I, with a prominent hippocampal signal being the most distinctive feature. MT-III expression remained essentially unaltered in the Tg2576 mice (Figs. 1, 2, Table 1); nevertheless, a tendency to show increased MT-III mRNA levels was seen in the hippocampal CA1 area, and the opposite in the cortex. In quantitations carried out in the microautoradiographies, MT-III mRNA levels showed also the same tendency to be decreased in the cortex (245378±28802 in WT, 160846±31250 in Tg2576, mean±SE, $p=0.07$). This was not an effect specifically related to plaques (in contrast to MT-I), since quantitations performed in plaque areas demonstrated similar MT-III expression to that of control areas (Figure 3).

In the TgCRND8 mouse model MT-I upregulation was clearly more prominent and widespread, being detected by both microautoradiography (Figure 5) and autoradiography (not shown). Quantitations carried out in the autoradiographic films show a significant increase of MT-I mRNA levels in the cortex and hippocampus (Figure 6). This pattern of response was also observed for GFAP expression (Figures 5,6). This in principle is consistent with the more aggressive phenotype of the double mutant AD mouse model, with a significantly higher plaque load (Figure 4c,d). Somewhat surprisingly, MT-I&II protein levels tended to be increased in the TgCRND8 mice but this was not statistically significant (not shown). Nevertheless, there was a clear correlation between Aβ and MT-I&II levels (Figure 7). Despite this clear alteration in MT-I expression, MT-III remained unaffected (Figures 5,6).

We have also examined MT-I&III as well as GFAP expression in the triple mutant Tg-SwDI at different ages, first in a preliminary experiment with one mouse per group (not shown), and afterwards with 3 mice per group (Figures 8,9). In contrast to the Tg2576 and TgCRND8 models that produce wild-type Aβ peptides, the Tg-SwDI produce vasculotropic Dutch/Iowa mutant forms of Aβ. The data clearly suggests MT-I is upregulated at all ages in the cortex and hippocampal formation, and in particular in the subiculum, which exhibits high levels of fibrillar microvascular amyloid, showing a prominent signal compared to the other two models. There was also a clear tendency in the thalamus, where high deposits of fibrillar microvascular amyloid are also observed, but variability among mice precluded statistical significance. MT-I+II protein levels measured in whole hemispheres also tended to be higher at all ages, although this was significant only at 18 months age (Table II). As also noticed in the other two models, MT-I upregulation was similar to but less potent than GFAP expression (Figures 8,9). Analyses of the Aβ levels (insoluble and soluble) clearly show that GFAP expression is more closely related to plaque load than MT-I (Table II). Once again, MT-III remained basically unaltered (Figure 8).

DISCUSSION.

Alzheimer disease, the major human neurodegenerative disease, is a progressive neurodegenerative disease of the CNS. AD is characterized by senile plaques, neurofibrillary tangles and neuronal loss. MT-I&II have consistently been shown to be upregulated in AD brains; considering what is known in several animal models of brain injury (Hidalgo et al., 2001), one could envision this response as a neuroprotective mechanism, but to test this in AD patients will be a formidable task. MT-III, on the other hand, has been shown to be decreased in AD brains in some reports but not in others (see Introduction), which creates uncertainties on its putative physiological role. Thus, there is a need for analyzing MT-I&III expression in animal models of AD to give some insight in this regard. We herewith report for the first time the analysis of MT-I&III expression in three different mouse AD models.

With the discovery that β-amyloid is the major constituent of plaques, a very durable hypothesis has developed, the so-called amyloid cascade/neuroinflammation hypothesis. According to it, deposits of Aβ are responsible for (1) causing tau phosphorylation and neurofibrillary tangle formation, and (2) activating microglia, which would release proinflammatory cytokines and other inflammatory mediators, as well as oxidative species, that bring about altogether neurodegenerative changes and eventually neuronal death and dementia (Streit, 2004). The identification of Aβ as a key factor has led to the generation of transgenic mice that overexpress human amyloid precursor protein (APP) with specific mutations normally found in patients with familial AD (Quon et al., 1991 , Games et al., 1995 , Hsiao et al., 1996, Sturchler-Pierrat and Sommer, 1999). The Tg2576 mouse model is one of the best characterized strains of APP transgenic mice. They develop normally until approximately 12 months of age, when they begin to form β-amyloid deposits in cortical and hippocampal areas that are accompanied by clear signs of inflammation, behavioural deficits and learning disabilities (Hsiao et al., 1996). In this study we have used 14-18 months old mice, and thus with a very mature amyloid phenotype. The TgCRND8 mouse model is characterised by a very rapid onset of Aß plaque pathology and initially deposits Aß1-42 at two-fold higher levels than Aß1-40, matching the course of Aß deposition in AD. As such, this represents a good model for AD. It has previously been reported that the deposition of plaques in the TgCRND8 mouse model is associated with an increase in

inflammatory processes (Dudal et al., 2004), with CD11b-positive microglia appearing in the cortex and hippocampus as early as 9-10 weeks of age. The numbers of these cells increased progressively with age and showed a clustering around both diffuse and compact plaques. Also, there was a significant upregulation and specific colocalization of activated astrocytes with plaques. In the present study we utilized 18 month old animals, thus with a widespread Aß plaque deposition and inflammation throughout the cortical and hippocampal areas of the brain. Finally, we used up to 18 month old TgSwDI mice, thus again with a very mature phenotype characteristic of this model. These mice also display robust symptoms of inflammation including increased numbers of activated astrocytes and microglia and elevated levels of pro-inflammatory cytokines (Miao et al., 2005). However, in contrast to the other two Tg mouse models the neuroinflammatory response in Tg-SwDI mice is largely restricted to the vicinity of the fibrillar cerebral microvascular amyloid deposits, which are most abundant in the subiculum and thalamus.

MT-I was upregulated in the three AD models used in this study. Induction was greater and wider in the TgCRND8 and TgSwDI mouse models than in the Tg2576 model, which is consistent with the severity of the phenotypes of each of these mouse models. It is noteworthy that MT-I upregulation seems to be closely related to plaques, and indeed this was clearly demonstrated in the Tg2576 model, where cells intimately related to Congo red-positive plaques showed an increase of the MT-I signal. This was not seen in areas away from plaques, ruling out an unspecific induction caused by stress factors such as glucocorticoids (Gasull et al., 1994, Hidalgo et al., 1997). Similarly, robust induction of MT-I signal was observed in the subiculum of Tg-SwDI mice, a region rich in microvascular amyloid deposition. A significant upregulation of MT-I around amyloid deposits is consistent with the inflammatory scenario normally found in them. It has been shown in many instances that reactive astrocytes and microglia upregulate MT-I&II synthesis (Hidalgo et al., 2001), and we have shown this is also the case for the Tg2576 mice (preliminary results also suggest that for the other two mouse AD models). Also, inflammatory cytokines such as IL-6 and TNF are major inducers of these MT isoforms (Carrasco et al., 1998a, Carrasco et al., 1998b, Penkowa et al., 1999b, Carrasco et al., 2000a), and, furthermore, oxidative stress is normally coupled to MT-I&II synthesis (Andrews, 2000). Moreover, heavy metals such as zinc and copper are known to accumulate in plaques (Huang et al., 2000), and these are primary inducers of the MT-I&II genes through the MTF-1 transcription factor (Andrews, 2000).

MT-III, in contrast to MT-I, remained essentially unaltered in the three AD mouse models, with cells surrounding plaques showing the same MT-III signal than those away from plaques, suggesting that the above scenario known to affect MT-I expression (gliosis, oxidative stress, inflammation, metals) is not important for MT-III regulation. Nevertheless, it is important to mention that MT-III mRNA levels as a whole (i.e. not specifically related to plaques) tended to be decreased in the Tg2576 mice; a similar (and significant) result has been found independently for MT-III protein (Martin et al., 2006). The reasons for these overall MT-III decreases are unknown, but it is remarkably similar to results found in some of the AD studies (Uchida et al., 1991, Yu et al., 2001). If the mechanisms underlying these are similar, the present results do not support a plaque-specific effect but a rather unspecific, stress-like phenomenon. Nevertheless, as already indicated, MT-III regulation is in many instances of a biphasic nature (Hidalgo et al., 2001); in a chronic situation like this that may be a significant confounding factor for understanding how this protein is regulated. But yet this is the physiological model for understanding AD, where acute, transient responses are of dubious importance. A number of studies suggest that MT isoforms may be regulated in opposite directions to the same stimulus (e.g. (Zheng et al., 1995, Kramer et al., 1996)). This differential regulation of the MT isoforms might be due to the presence of specific MT-I&II inducing factors or to the concomitant upregulation of a factor that opposes the regulation of MT-III. Another possibility, however, is based on previous studies that have demonstrated that an LPS-challenge to a young brain will elicit an upregulation of MT-III whereas the same treatment in the aged brain has no effect (Miyazaki et al., 2002). The lack of change in MT-III in the current study, therefore, may represent a change in sensitivity to particular stimuli (such as Aß accumulation).

Given the results in the three transgenic AD models it is apparent that MT-III is not consistently altered, showing only a modest alteration in the Tg2576 model. The examination of earlier timepoints, when the Aß burden is not so overwhelming, might yield different results, although the preliminary examination of the Tg-SwDI mice did not reveal a change in MT-III expression in the young (5-6 months) or old (18 months) mice that were examined. These data do, however, support the hypothesis that there is an interaction between MT-I&II and Aß deposition, although further studies are required to fully delineate the mechanisms involved.

ACKNOWLEDGEMENTS.

Support by the Ministerio de Ciencia y Tecnología and Feder (SAF2002-01268) and Ministerio de Educación y Ciencia and Feder SAF2005-00671 (JH) and NIH grant NS36645 (WEVN) is fully acknowledged. The support of The Lundbeck Foundation, IMK Almene Fond, Vera og Carl Michaelsens Legat, Kathrine og Vigo Skovgaards Fond, Scleroseforeningen, Karen A Tolstrup, Hørslev-fonden, Toyota Fonden, Dir. Leo Nielsens Legat, The Danish Medical Association Research Fund, The Wacherhausens Legat, Grosserer Johan Quentin og Hustrus Legat, Dagmar Marshalls Fond, Eva og Henry Frænkels Mindefond, Warwara Larsens Fond, Fru Lily Benthine Lunds Fond, Ragnhild Ibsens Legat for Medicinsk Forskning, Fonden til Lægevidenskabens Fremme, Haensch´s Fond, Dir. Ejnar Jonasson, kaldet Johnsen, og Hustrus Fond is also fully appreciated (MP). We thank Lilly Research Laboratories for generously providing the antibody reagents for performing the Aβ40 and Aβ42 ELISA measurements.

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FIGURE LEGENDS

FIGURE 1.

Representative autoradiographies for MT-I, MT-III and GFAP *in situ* hybridizations in wildtype (control) and Tg2576 mice. GFAP expression was dramatically upregulated in amyloid plaques-containing areas (see also Figures 2 and 3), namely cortex and hippocampus. In contrast, MT-I and MT-III showed no noticeable changes by this approach (see also Table I).

FIGURE 2.

Representative microautoradiographies for MT-I, MT-III and GFAP *in situ* hybridizations, as well as Congo Red stainings (a marker of amyloid plaques) in wildtype (control) and Tg2576 mice. As expected, GFAP was prominently upregulated in the vicinity of amyloid plaques. The MT-I signal was also increased in cells surrounding the plaques in comparison to areas away from them, but such upregulation was not as dramatic as for GFAP mRNA (see also Figure 3), which likely explains why this was not detected in autoradiographic films. In contrast, the MT-III signal in the vicinity of the plaques was not altered in the Tg2576 mice; yet, an overall tendency to be decreased in comparison to control mice was noticed regardless of the presence of plaques.

FIGURE 3.

mRNA quantitations in the microautoradiographies were carried out in plaque-containing areas (Plaque area) versus plaque-devoid areas (Control area) of the cortex. Considering an average size of the plaques of approximately 0.0025 mm^2, quantitation was done in areas 10 times higher (0.025 mm^2); a mean value was calculated for each animal from 3 areas quantitated. These measurements showed significant ($p<0.05$) but weak increases of the MT-I signal and prominent increases of the GFAP signal ($p<0.001$), while MT-III was unaffected by the plaques. Results are shown in arbitrary units, mean ± SE, n=9.

FIGURE 4.

Triple immunostainings for identifying the cellular source of MT-I&II proteins surrounding the plaques. In panel (A), immunostaining is demonstrated for GFAP (green), MT-I+II (blue) and Ab42 (red); in panel (B), it is shown staining for lectin (red), MT-I+II (green) and Ab42 (blue). MT is clearly expressed in astrocytes and microglia/macrophages, although significant variability is seen among cells and plaques. C,D: Demonstration of amyloid plaques by Congo-red staining in Tg2576 (C) and TgCRND8 (D) mice.
Scale bars: A,B: 55 μm; C,D: 1 mm.

FIGURE 5.

Representative microautoradiographies for MT-I and MT-III *in situ* hybridizations in wildtype (WT) and TgCRND8 mice. MT-I but not MT-III was clearly upregulated in cortex and hippocampus (see also Figure 6).

FIGURE 6.

Quantitations carried out in the autoradiographic films for MT-I, MT-III and GFAP *in situ* hybridizations in wildtype and TgCRND8 mice. Measures were done in cortex and the hippocampal areas dentate gyrus (DG), laconosum moleculare (LM) and CA1 pyramidal layer. Results (arbitrary units) are mean ± SE, n=6 (gender were grouped since no differences were seen between males and females). * $p<0.05$ vs WT mice.

FIGURE 7.

Correlation analysis for MT-I&II protein levels with Aβ1-42 levels in TgCRND8 mice showed a correlation of 0.826 with a p value of 0.04 (r-squared value of 0.682).

FIGURE 8.

Representative autoradiographies for MT-I, GFAP and MT-III *in situ* hybridizations in wildtype (control) and Tg-SwDI mice at 18 months of age. MT-I was somewhat

upregulated throughout the cortex and hippocampal formation, and a prominent induction was observed in the subiculum and thalamus, resembling GFAP expression. MT-III, in contrast, was not affected (see also Figure 9).

FIGURE 9.

Quantifications of the autoradiographic films of WT and TgSwDI mice (see Figure 8). Measures were done in the specified brain areas at 5, 12 and 18 months of age. Results (arbitrary units) are mean ± SE, n=3. * p at least <0.05 vs WT mice.

Table I. Quantitations carried out in the autoradiographic films for MT-I, MT-III and GFAP in situ hybridizations in WT and Tg2576 mice.

	Wild type				Tg2576			
	Cortex	**GD**	**LM**	**CA1**	**Cortex**	**GD**	**LM**	**CA1**
MT-I	6038±383	1396±171	1048±107	550±60	5527±393	1607±192	1039±149	425±59
MT-III	4468±342	1214±40	966±72	1242±171	3982±290	1240±74	894±87	1742±374
GFAP	1416±237		188±27		11237±3672*		568±97*	

Measures were done in same size areas for all mice in cortex and the hippocampal areas dentate gyrus (DG), laconosum moleculare (LM) and CA1 pyramidal layer for MT-I and MT-III (arbitrary units). For GFAP, measures were done in bigger areas in cortex and hippocampus (values shown in the column of LM). Measurements were done in three sections per animal. Results are mean ± SE, n=9-12 mice per group. *$p<0.01$ vs WT mice.

Table II. MT-I+II protein and Aβ levels in WT and Tg-SwDI mice.

	Wild type			Tg-SwDI		
	5	**12**	**18**	**5**	**12**	**18**
MT-I+II (µg/g)	5.97±0.08	5.92±0.21	5.89±0.17	7.22±1.41	6.79±0.24	7.51±0.14*
Aβ40 soluble (pg/mg)				997±210	1728±105	6744±573
Aβ40 insoluble (pg/mg)				4855±1055	5106±528	24616±2208
Aβ42 soluble (pg/mg)				260±27	310±22	972±60
Aβ42 insoluble (pg/mg)				1106±221	1232±133	4825±558

MT-I+II protein was measured by RIA, and Aβ peptides by ELISA, in 5, 12 and 18 months old mice. Results are mean ± SE, n=3 mice per group. *p<0.01 vs WT mice.