Short title: Metallothioneins protect against IL-6-induced injury

METALLOTHIONEIN-I OVEREXPRESSION DECREASES BRAIN PATHOLOGY IN TRANSGENIC MICE WITH ASTROCYTE-TARGETED EXPRESSION OF INTERLEUKIN 6

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ABSTRACT

Transgenic expression of IL-6 in the CNS under the control of the GFAP gene promoter (GFAP-IL6 mice) causes significant damage and alters the expression of many genes, including a dramatic upregulation of metallothionein-I (MT-I). The findings in this report support the idea that the upregulation of MT-I observed in the GFAP-IL6 mice is an important mechanism for coping with brain damage. Thus, GFAP-IL6 mice that were crossed with TgMTI transgenic mice (GFAP-IL6xTgMTI) and overexpressed MT-I in the brain showed a decreased upregulation of cytokines such as IL-6, and a diminished recruitment and activation of macrophages and T cells throughout the CNS but mainly in the cerebellum. The GFAP-IL6 mice showed clear evidence of increased oxidative stress, which was significantly decreased by MT-I overexpression. Interestingly, MT-I overexpression increased angiogenesis in GFAP-IL6 mice but not in control littermates. Overall, the results strongly suggest that the MT-I+II proteins are valuable factors that protect against cytokine-induced CNS injury.

Key words: Angiogenesis, Cytokines, IL-6, Metallothionein, Oxidative stress

INTRODUCTION

It is now clear that cytokines are essential mediators of cell-cell communication in the CNS, and that astrocytes, microglia and macrophages are major sources of these proteins that profoundly affect glia and neuron physiology (1-4). While the cellular expression of cytokines in the CNS is very low in normal conditions, in a number of neuropathological conditions their expression may be dramatically altered (see (5, 6) for review). It is likely that such alterations could contribute to the clinicopathological features of many neurological disorders, and indeed results obtained in transgenic mice with astrocyte targeted expression of cytokines such as IL-6 (GFAP-IL6 mice) (7-16), IL-3 (15, 17-19), IFN-α (15, 20-22), TNF (23, 24) and IL-12 (25) have demonstrated that an abnormal cytokine production may cause significant neurological disorder. On the other hand, these transgenic mice are useful animal models for the characterization of the mechanisms underlying cytokine-induced pathological conditions in the CNS as well as for identifying potentially important genes for coping with CNS damage. One such family of genes is the metallothionein (MT) family, which we have thoroughly studied in the GFAP-IL6 mice (see below). These transgenic mice show profound effects of IL-6 in the brain, with clear symptoms of neurodegeneration, astrocytosis, microgliosis, angiogenesis and up-regulation of several inflammatory and other host-response genes including IL-1 α/β, TNF, GFAP, ICAM-1, the acute-phase protein EB22/5, and complement C3 protein. Moreover, concordant with these structural alterations, GFAP-IL6 mice have impaired hippocampal electrophysiology and develop a progressive learning deficit.

MTs are cysteine-rich, heavy metal binding, low molecular weight proteins, which in rodents are comprised of four isoforms (MT-I to MT-IV) (26, 27). In the brain only three isoforms are expressed, MT-I, MT-II and MT-III (also known as Growth Inhibitory Factor). MT-I&II are widely expressed and regulated coordinately in most tissues including the CNS (28, 29), while MT-III is mainly expressed in the CNS and shows a very different pattern of regulation compared to that of other MT isoforms (26). MTs bind zinc and copper and presumably function in metal ion regulation and detoxification in peripheral tissues as well as in the CNS, while more recent evidence suggest that MTs could be significant antioxidant proteins (see (30-32) for review). In previous studies we have shown that MT-I mRNA and MT-I&II protein levels are dramatically increased in the brains of the GFAP-IL6 mice (33, 34), which presumably forms part of the physiological repertoire elicited in the brain for coping with tissue injury. To test this

possibility, we recently crossed GFAP-IL6 mice with Mt1&2-null mice and demonstrated that these proteins were important for coping with transgenic IL-6-induced CNS damage (35). In this report we extend that study by crossing GFAP-IL6 mice with transgenic mice overexpressing MT-I (TgMTI) (36) and thus characterizing the effects of MT-I overexpression on the phenotype of GFAP-IL6 mice. The results fully support the hypothesis that MT-I&II are essential proteins for coping with brain injury induced by cytokines such as IL-6.

MATERIALS AND METHODS

Animals

Homozygous TgMTI mice were kindly provided by Dr. Glen K. Andrews, Dept. Biochemistry & Molecular Biology, University of Kansas Medical Center, KS, USA. These mice were derived from heterozygous males that carry 56 copies of a minimally mutated Mt1 (Mt1*) gene on the B6/SJL F_1 background (36), which were outbred to CD-1 females (37). TgMTI mice are routinely genotyped by Southern blot as previously described (38).

Construction and characterization of the GFAP-IL6 transgenic mice has been described previously (7). Briefly, an expression vector derived from the murine glial fibrillary acidic protein (GFAP) gene was used to target expression of murine IL-6 to astrocytes. The GFAP-IL6 mice have a mixed C57B6xSJL genetic background, and are routinely genotyped by PCR as previously described (39).

Production of GFAP-IL6-TgMTI mice

For obtaining GFAP-IL6 mice with genetic MT-I overexpression and proper controls, we crossed homozygous TgMTI male mice with CD1 females. The offspring mice were all heterozygous for the 56 Mt1* genes, and they were crossed with heterozygous GFAP-IL6 mice. The offspring of this second cross were genotyped by PCR (39) and Southern blot (38) for identifying the four possible genotypes: (a) GFAP-IL6+/- TgMTI+/-, (b) GFAP-IL6+/- TgMTI-/-, (c) GFAP-IL6-/- TgMTI+/-, and (d) GFAP-IL6-/- TgMTI-/-. These F2 mice have the same genetic background and thus can be compared directly. This experimental design was followed in two separate experiments. Animals were studied at different ages (2-13 months old); typically 5-10 animals per group were used.

In situ hybridization

The mice were killed and their brains removed, frozen in liquid nitrogen, and stored at -80°C. Later, serial coronal sections (20 μm in thickness) were obtained with a cryostat and mounted on slides coated with poly(L)lysine. The in situ hybridization analysis of the MT-I and GFAP mRNAs was carried out as described previously (34).

RNase Protection Assay

RNase protection assay for the detection of cytokine and host-gene RNA was performed as described previously (8, 40, 41). The intensity of the bands was measured densitometrically and normalized with the L32 mRNA levels.

Histochemistry and Immunohistochemistry

Animals were studied at 3 and 7 month of age; typically 5-10 animals per group were used, and in some stainings cell counts were carried out in a blinded manner in specific brain areas in four animals per group for statistical purposes.

Tissue processing.

Mice were perfused and sections prepared essentially as previously described (35).

Histochemistry

Sections were incubated overnight at 4º C with biotinylated tomato lectin from Lycopersicon esculentum (Sigma-Aldrich, St. Louis, MO, USA) 1:500, which was used as a marker for cells of the myelo-monocytic cell lineages, such as microglia/macrophages, as well as a marker for vessels. The lectin was developed using streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dako, Glostrup, DK) prepared at manufacturer´s recommended dilutions for 30 min at room temperature. The lectin staining was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature.

Hematoxylin & eosin staining of brain sections was performed according to standard procedures.

Immunohistochemistry

The sections were incubated overnight at 4ºC with one of the following antibodies:

For detection of IL-6 and MT-I+II: rat anti-mouse IL-6 1:50 (Harlan Seralab, Indianapolis, Indiana, USA); and rabbit anti-rat MT-1+2 1:500 (42-44).

For detection of inflammatory cells and cytokines: rabbit anti-bovine GFAP 1:250 (Dako) (as a marker for astrocytes); rat anti-mouse MOMA-2 1:50 (Serotec, Oxford, UK) (marking only peripherally blood-derived monocytes/macrophages); mouse anti-rat

CD3 1:50 (Serotec) (as a marker for all T lymphocytes); mouse anti-human IL-1β 1:50 (Biogenesis, Kingston, NH, USA); and rabbit anti-mouse TNF 1:100 (Biosource, Camarillo, CA, USA).

For detection of oxidative stress and apoptosis: rabbit anti-human iNOS 1:100 (Alexis Biochemicals, San Diego, CA, USA) (marker for oxidative stress); rabbit anti-NITT 1:100 (Alpha Diagnostic Int., San Antonio, TX, USA) (a marker for peroxynitrite-induced nitration of tyrosine residues, which monitors oxidative stress); rabbit anti-MDA 1:100 (Alpha Diagnostic Int.) (a marker for malondialdehyde (MDA) produced as a byproduct of fatty acid peroxidation, which monitors oxidative stress); mouse anti-human Cu/Zn-super oxide dismutase (Cu/Zn-SOD) 1:50 (Sigma-Aldrich); sheep anti-bovine Mn-SOD 1:50 (Biogenesis); sheep anti-human catalase 1:50 (The Binding Site, Birmingham, UK); rabbit anti-human activated caspase-3 1:50 (Cell Signaling Technology Inc., Beverly, MA, USA) (as a marker for apoptotic signaling); and mouse anti-calf ssDNA 1:25 (Alexis Biochemicals) (as a marker for single stranded DNA in apoptotic cells).

For detection of angiogenesis: rabbit anti-human VonWillebrand Factor 1:100 (Neomarkers, Fremont, CA, USA); mouse anti-rat endothelial barrier antigen (EBA) 1:100 (Affinity, Nottingham, UK) (a marker of an endothelial blood-brain barrier antigen); goat anti-human angiopoietin-1/4 1:100 (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) (marking angiogenesis and vascular remodeling); rat anti-mouse perlecan 1:50 (Neomarkers) (marker of angiogenesis and neovascularization); mouse anti-human CD34 1:30 (Neomarkers) (marking proliferating vascular endothelial cells); mouse anti-human vascular endothelial growth factor (VEGF) 1:30 (RD Systems, Minneapolis, MN, USA); rabbit anti-human bFGF 1:100 (Santa Cruz Biotech. Inc.); rabbit anti-human TGF-β1 1:200 (Santa Cruz Biotech. Inc.); and goat anti-human Endoglin/TGFβ-receptorIII 1:100 (Santa Cruz Biotech. Inc.).

The detection of these primary antibodies and the controls for specificity were performed essentially as described previously (35).

Statistical analysis

Results were analyzed with the Student “t” test, the Mann-Whitney “U” test, or with two-way ANOVA.

RESULTS

The frequency of the offspring genotypes is normal

Table 1 shows the data for two separate series of crossings. In general, it can be concluded that neither MT-I overexpression nor GFAP-IL6 expression nor the combination of both had any effect on the development of the offspring, since a normal Mendelian distribution of transgene genotypes was clearly observed, namely 25% for each one of the four groups of the F2 offspring ((a) GFAP-IL6+/- TgMTI+/-, (b) GFAP-IL6+/- TgMTI-/-, (c) GFAP-IL6-/- TgMTI+/-, and (d) GFAP-IL6-/- TgMTI-/-).

Clinical characterization

The GFAP-IL6 mice showed clear symptoms of neurological damage, with hunched posture, tremor, ataxia and hindlimb weakness, as expected (7). The mice were evaluated in some behavioral tests (hole-board, rota-rod, balance bar) but no statistical differences between genotypes were observed for locomotor activity (number of areas crossed in the hole-board) or coordination (rota-rod, balance bar), exploratory activity (head-dips and rearing in the hole-board), and emotionality (number of defecations in the hole-board). As expected (7), old GFAP-IL6 mice tended to weigh less than littermate controls, but no statistically significant effects of MT-I overexpression were observed (data not shown).

MT-I mRNA and MT-I&II protein levels are increased by transgenic IL-6 and MT-I expression

Confirming previous studies (33, 34), the transgenic expression of IL-6 altered MT-I expression in a brain area-specific manner, with the cerebellum the area most affected (Figure 1 and Table 2). MT-I mRNA levels in the cerebellum of TgMTI-/- mice were similarly upregulated by the transgenic IL-6 expression in 2 and 13 month old animals. Also as expected (45), MT-I mRNA levels were significantly higher in the TgMTI+/- mice; we herewith report that MT-I mRNA levels increase with aging significantly (Table 3). MT-I mRNA levels tended to be further increased in the GFAP-IL6+/- TgMTI+/- mice. Interestingly, MT-I mRNA levels of all the TgMTI mice were less upregulated in telencephalic areas than in the remaining brain.

MT-I+II protein levels were also analyzed by immunohistochemistry (Figure 2). In GFAP-IL6-/- TgMTI-/- mice, MT-I+II expression was seen in some astrocytes

dispersed throughout the CNS as well as in meningeal cells, ependymal cells, and choroid plexus, while microglia, oligodendrocytes and neurons appeared devoid of MT-I+II expression. Confirming the above results, MT-I+II immunoreactivity was significantly increased by the transgenic IL-6 expression throughout the brain but especially in the cerebellum. Also as expected, MT-I+II immunoreactivity was dramatically increased in the GFAPIL6-/- TgMT+/- mice. The highest levels of MT-I+II immunoreactivity were seen in the GFAP-IL6+/- TgMTI+/- mice. Bergmann glia, astrocytes and microglia/macrophages were the main cellular sources of MT-I+II in the cerebellum of GFAPIL6+/- TgMTI-/- and GFAP-IL6-/- TgMTI+/- mice, but the latter showed some staining also in endothelial cells and in a few neurons.

In other brain areas such as the forebrain and the brain stem of the GFAP-IL6+/- TgMTI-/- and GFAP-IL6-/- TgMTI+/- mice, astrocytes and microglia/macrophages were the main cells that expressed MT-I+II, but again the latter mice showed some MT-I+II staining in some endothelial cells and a few neurons (not shown).

MT-I overexpression decreases GFAP-IL6-induced cytokine expression

Figure 2 shows IL-6 immunostaining results for the cerebellum of 7 month-old mice of the four genotypes studied. In GFAP-IL6-/- TgMTI-/- and GFAP-IL6-/- TgMTI+/- mice IL-6 immunoreactivity was only seen in the meninges, while intraparenchymal cells of the CNS were negative. In the GFAP-IL6+/- TgMTI-/- mice IL-6 immunoreactivity increased throughout the CNS but especially in the cerebellum. IL-6 immunoreactivity was mainly seen in Bergmann glia, round or amoeboid microglia/macrophages, and in some astrocytes and a few T cells of the cerebellum white and grey matter. In the forebrain and brain stem, IL-6 immunoreactivity was only detected in some scattered astrocytes (not shown). IL-6 immunoreactivity was significantly lower in GFAP-IL6+/- TgMTI+/- than in GFAP-IL6+/- TgMTI-/- mice, suggesting that MT-I overexpression decreased IL-6 expression. This effect was also seen in the forebrain and brain stem (not shown).

To confirm the immunohistochemistry data, IL-6 mRNA levels were measured in the cerebellum of 7 month-old animals by a specific ribonuclease protection assay that measures a number of cytokines simultaneously (Figure 3). Densitometric quantitation and normalization of the cytokine mRNA with the L32 mRNA levels demonstrated that MT-I overexpression decreased GFAP-IL6-induced IL-6 expression

significantly (Table 3), in accordance with the immunohistochemistry results. IL-1α, IL-1β and TNF expression was clearly increased in the GFAP-IL6 mice, although to a lower extent than that of IL-6 (Table 3). MT-I overexpression tended to decrease such responses, although statistical significance was not achieved for any of these three cytokines. Overall the same results were observed for IL-1β and TNF protein levels as detected by immunohistochemistry (not shown).

MT-I overexpression decreases microglia/macrophage and T lymphocyte recruitment and activation.

Figure 4 shows the results for lectin staining, a marker of microglia/macrophages and blood vessels. In all the CNS areas of GFAP-IL6-/- TgMTI-/- mice, lectin staining was seen in vessels and in a very few ramified microglial cells, while round macrophages were rare. Lectin staining in the GFAPIL6+/- TgMTI-/- mice showed that round macrophages and activated microglia heavily infiltrated the cerebellum mainly in the white matter. In the brain stem, only some activated microglia were seen around vessels in the GFAPIL6+/- TgMTI-/- mice, while the cerebral cortex of these mice only showed a few microglia/macrophage cells (not shown). MT-I overexpression was without effect on lectin staining in GFAP-IL6-/- mice, but it dramatically decreased the recruitment and activation of microglia/macrophages induced by IL-6 throughout the CNS judging by number of cells and morphology. The data obtained using lectin histochemistry was confirmed when using the macrophage marker MOMA-2.

In accordance with the lectin staining, Mac1 mRNA levels were higher in GFAP-IL6 mice, and MT-I overexpression tended to decrease this (Figure 5 and Table 3).

MT-I overexpresssion decreases T lymphocyte recruitment and activation

T cells were detected by examining immunoreactivity for CD3. In the GFAP-IL6-/- TgMTI-/- mice, T lymphocytes were rarely detected throughout the CNS. In contrast, the GFAPIL6+/- TgMTI-/- mice showed recruitment of CD3+ cells, mainly to the cerebellum, which displayed many T lymphocytes around the vessels in white matter. In brain stem and forebrain of these mice, the number of recruited T cells was low. The GFAPIL6-/- TgMT+/- mice showed immunoreactivity for CD3 that was comparable to that of GFAPIL6-/- TgMT-/- mice. The GFAPIL6+/- TgMT+/- mice

showed clearly reduced numbers of T cells relative to the GFAPIL6+/- TgMT-/- mice, which indicated that MT reduced T cell recruitment by IL-6 (data not shown).

MT-I overexpression alters astrocyte/Bergmann glia reactivity

GFAP immunostaining of the GFAP-IL6-/- TgMTI-/- mice showed a faint staining throughout the CNS (Figure 6). In GFAP-IL6+/- TgMTI-/- mice GFAP staining increased progressively with age (not shown), and at 7 month of age reactive astrogliosis was obvious, especially in the Purkinje and molecular layers and in white matter of the cerebellum (Figure 6) and to a lesser extent in brain stem and cortex (not shown).

Somewhat surprisingly, GFAP immunoreactivity was increased in 7 month-old GFAP-IL6-/- TgMTI+/- mice throughout the CNS (Figure 6) compared with 3 month-old animals (not shown). In the cerebellum of 7 month-old GFAP-IL6-/- TgMTI+/- mice most of the GFAP staining was seen in the Purkinje and molecular layers. This was in sharp contrast to GFAP-IL6+/- TgMTI-/- mice, which in addition to these two layers showed a significant reactive astrocytosis in the white matter. Overall, the transgenic IL-6 expression seemed to increase GFAP staining of the cerebellum to a greater extent than the MT-I overexpression, at least at the ages studied. In contrast, in the remaining brain areas the GFAP-IL6-/- TgMTI+/- mice showed significantly higher GFAP staining than GFAP-IL6+/- TgMTI-/- mice (not shown). GFAP-IL6+/- TgMTI+/- mice tended to have a greater GFAP immunoreactivity than either GFAP-IL6+/- TgMTI-/- or GFAP-IL6-/- TgMTI+/- mice, especially in the cerebellum (Figure 6) and brain stem (not shown).

GFAP mRNA levels were also measured by RPA and in situ hybridization (Figure 5 and Table 4). As expected, GFAP-IL6 mice showed a dramatic increase of GFAP mRNA levels in the cerebellum. In contrast to GFAP immunoreactivity, MT-I overexpression did not affect GFAP mRNA levels. The reasons for such discrepancies between GFAP mRNA and protein are unknown and deserve further attention.

MT-I overexpression decreases oxidative stress

In non-transgenic littermate animals, MDA (not shown) and NITT immunoreactivity was barely detectable in the CNS (Figure 7). In contrast, in GFAP-IL6+/- TgMTI-/- mice, MDA and NITT staining was clearly increased in the CNS, particularly in the cerebellum. These markers of oxidative stress were also significantly

decreased in GFAP-IL6+/- TgMTI+/- mice at all ages studied. Both oxidative stress markers were detected mainly in neuronal cells but also in some astrocytes and microglia/macrophages.

MT-I overexpression increases angiogenesis in GFAP-IL6 mice but not in control littermates

H&E stainings of the cerebellum of the different genotypes studied (not shown) revealed the expected granular layer disorganization and increased angiogenesis caused by the transgenic expression of IL-6 (7, 13). Figure 8 shows angiopoietin staining as a marker of angiogenesis, which showed an obvious increased angiogenesis in the GFAP-IL6+/- mice, in accordance with the H&E stainings. While not having any observable effect in GFAP-IL6-/- animals, MT-I overexpression significantly increased angiogenesis in GFAP-IL6+/- mice. These results were further supported by immunostaining for VEGF, VonWillebrand Factor, EBA (endothelial barrier antigen), perlecan, CD34, bFGF, TGF-β1, and endoglin/TGFβ-receptorIII (not shown), all supporting a proangiogenic effect of MT-I&II during IL-6 induced CNS injury.

DISCUSSION

In a recent study (35), we analyzed the phenotype of GFAP-IL6 mice made deficient for MT-I&II. Here we have extended that study by generating GFAP-IL6 mice with MT-I overexpression, to verify if an excess of MT-I can have the opposite effects to those caused by MT-I+II deficiency. Overall, the results demonstrated that such upregulation is important in terms of tissue protection, presumably by controlling the inflammatory response and oxidative stress in the brain.

In line with previous observations (7), the GFAP-IL6 mice showed clear symptoms of neurological damage, with hunched posture, tremor, ataxia and hindlimb weakness. The previously reported histopathological features were also clearly observed, namely neurodegeneration, microgliosis, astrocytosis, oxidative stress, angiogenesis and up-regulation of several inflammatory and other host-response genes including IL-1 α/β, TNF, GFAP, ICAM-1 and the acute-phase protein EB22/5, being the cerebellum the most affected area (7-16). We showed in the present study that GFAP-IL6 mice with MT-I overexpression have a significantly decreased inflammatory response in the brain, especially in the cerebellum. Thus, the recruitment and activation of macrophages and T cells, the production of inflammatory cytokines such as IL-6, IL-1α,β and TNF, as well as oxidative stress, were all lower in GFAP-IL6 mice with MT-I overexpression compared with littermate controls. Reactive astrocytosis, in contrast, showed the opposite response. Thus, GFAP-IL6 mice with MT-I overexpression showed a reversed pattern of responses for all the variables analyzed compared with those with MT-I&II deficiency (35). Overall, the results strongly support the idea that MT-I+II are important proteins for coping with cytokine-induced CNS damage. Similar conclusions have been obtained from studies with animals subjected to cryolesion (39, 44-46), experimental autoimmune encephalomyelitis (EAE) (47-49), convulsions (50) or to mild focal cerebral ischemia (51). MT-I&II deficiency has also been shown to increase degenerative pathology in a mouse model of familial amyotrophic lateral sclerosis (52).

Altogether, these results suggest that MT-I&II are involved in the control of the inflammatory response in the CNS, although the mechanisms underlying this control are uncertain. A number of studies suggest that these proteins act at different levels of the immune system. First, binding sites for MT-I&II have been described in T cells, B cells and macrophages (53, 54), and it is essential to realize that despite the absence of the

typical signal peptide sequences in the Mt genes, extracellular MT-I&II presence and response to factors such as stress and inflammation is well-documented (55-58). Second, exogenous MTs are able to induce lymphocyte proliferation (54, 59, 60), although this cannot be considered a general effect, since the same group has reported that MTs decrease lymphocyte proliferation mediated by macrophages activity (53). Third, MTs potentiate the capacity of macrophages to produce increased levels of oxygen radicals and to kill engulfed yeast cells (53); in agreement with this study, decreased levels of oxygen radicals in response to LPS were observed in MT-depleted THP-1 cells (a human monocyte-derived cell line) (61). Fourth, MTs have been found to inhibit significantly humoral responses to specific antigens (62) as well as cytotoxic T lymphocyte activity (60). Finally, it is noteworthy the fact that results obtained with MT immunoneutralization by using the UC1MT monoclonal antibody (62, 63) as well those obtained in Mt1&2-null mice (64) support a role of MT in the control of the humoral immune response, but conflicting results are obvious regarding the role of MTs on the lymphoproliferative response (64, 65). Other recent studies add more controversy to the interaction between MTs and the immune system. Thus, Kimura et al. reported that Mt1&2-null mice were more sensitive to LPS/GalN-induced lethality, suggesting a protective role of MTs (66). This has been confirmed (67), but yet these authors report that Mt1&2-null mice are protected against TNF-induced shock while MT-I overexpressing mice are sensitized, therefore suggesting a deleterious role for MTs. It seems thus clear that MTs could affect cell proliferation, differentiation and function separately, and that depending on the specific insult activating the immune system quite different physiological outcomes might occur.

Regardless of the mechanisms involved, it is clear that MT-I&II have the potential to dramatically decrease the inflammatory response in the CNS, as demonstrated in the present study. The decreased macrophage and T cell infiltration of the CNS of animals with astrocyte-targeted expression of IL-6 caused by MT-I overexpression, together with the downregulation of several proinflammatory cytokines and the increased astrogliosis would undoubtedly contribute to the control of oxidative stress since these cells are key factors controlling free radical production, antioxidant mechanisms and apoptosis pathways (3, 68-71). It is also likely that MT-I&II per se are important as antioxidant and antiapoptotic factors (29, 72-80).

It is also noteworthy that the present results support an important role of MT-I+II in the control of angiogenesis in the injured CNS. We previously demonstrated a decreased

angiogenesis in cryolesioned Mt1&2 null mice (39) and the reverse phenomenon in MT-I overexpressing mice (45). Such a proangiogenic role of MT-I+II is not observed in unlesioned animals, which suggests that these proteins interact specifically with tissue injury-elicited factors. In a very different animal model of CNS injury, the GFAP-IL6 mice, we have now demonstrated that MT-I overexpression also seems to promote angiogenesis. These mice appeared to have a higher number of vessels with a higher percentage of which are enlarged in comparison to those of GFP-IL6+/- TgMTI-/-. Although a more quantitative approach to precisely determine the extent of the increased vessel density is desirable in future studies, we did also note here that the angiogenic factor angiopoietin-1/4 (as well as VEGF, VonWillebrand Factor, EBA, perlecan, CD34, bFGF, TGF-β1, and endoglin/TGFβ-receptorIII) was also consistently increased. These findings are replicated in both models of CNS injury, namely the cryolesion and the astrocyte targeted IL-6 expression, and thus it is likely that they might be underlying the MT-I+II-related angiogenic effects (see (39) for further discussion and references). Importantly, GFAP-IL6 mice with MT-I&II deficiency show decreased angiogenesis (35), in accordance with the above results

In summary, the present results provide a rationale for the control of the Mt1&2 genes by IL-6 in the CNS, and demonstrate that these proteins are likely important factors for coping with cytokine-induced CNS injury.

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REFERENCES

1. Hopkins S, Rothwell N. Cytokines and the nervous system. I: Expression and recognition. Trends Neurosci 1995;18**:**83-88.
2. Rothwell NJ, Hopkins SJ. Cytokines and the nervous system II: actions and mechanisms of action. Trends Neurol Sci 1995;18**:**130-136.
3. Stichel C, Verner Müller H. Experimental strategies to promote axonal regeneration after traumatic central nervous system injury. Progr Neurobiol 1998;56**:**119-148.
4. McIntosh T, Juhler M, Wieloch T. Novel pharmacologic strategies in the treatment of experimental traumatic brain injury. J Neurotrauma 1998;15**:**731-769.
5. Campbell IL. Transgenic mice and cytokine actions in the brain: bridging the gap between structural and functional neuropathology. Brain Res Rev 1998;26**:**327-336.
6. Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. Nature Rev Neurosci 2001;2**:**734-744.
7. Campbell IL, Abraham CR, Masliah E et al. Neurologic disease in transgenic mice by cerebral overexpression of interleukin 6. Proc Natl Acad Sci USA 1993;90**:**10061-10065.
8. Chiang C-S, Stalder A, Samimi A, Campbell IL. Reactive gliosis as a consequence of interleukin 6 expression in the brain: studies in transgenic mice. Dev Neurosci 1994;16**:**212-221.
9. Heyser CJ, Masliah E, Samimi A, Campbell IL, Gold LH. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. Proc Natl Acad Sci USA 1997;94**:**1500-1505.
10. Powell HC, Campbell IL. Ultrastructural abnormalities of glia and blood-brain barrier in a transgenic mouse overexpressing interleukin-6. Brain Pathol 1994;4**:**278-279.
11. Steffensen SC, Campbell IL, Henriksen SJ. Site-specific hippocampal pathophysiology due to cerebral overexpression of interleukin-6 in transgenic mice. Brain Res 1994;652**:**149-153.
12. Castelnau PA, Garrett RS, Palinski W, Witztum JL, Campbell IL, Powell HC. Abnormal iron deposition associated with lipid peroxidation in transgenic mice

expressing interleukin-6 in the brain. J Neuropathol Exp Neurol 1998;57:268-282.

13. Brett FM, Mizisin AP, Powell HC, Campbell IL. Evolution of neuropathological abnormalities associated with blood-brain barrier breakdown in transgenic mice expressing interleukin-6 in astrocytes. J Neuropathol Exp Neurol 1995;54:766-775.
14. Barnum SR, Jones JL, Müller-Ladner U, Samimi A, Campbell IL. Chronic complement C3 gene expression in the CNS of transgenic mice with astrocyte-targeted interleukin-6 expression. Glia 1996;18:107-117.
15. Asensio CC, Lassmann S, Pagenstecher A, Steffensen SC, Henriksen SJ, Campbell IL. C10 is a novel chemokine expressed in experimental inflammatory demyelinating disorders that promotes inflammatory recruitment of macrophages to the central nervous system. Am J Pathol 1999;154:1181-1191.
16. Nelson TE, Campbell IL, Gruol DL. Altered physiology of Purkinje neurons in cerebellar slices from transgenic mice with chronic central nervous system expression of interleukin-6. Neuroscience 1999;89:127-136.
17. Chiang C-S, Powell HC, Gold LS, Samimi A, Campbell IL. Macropahe/microglial-mediated primary demyelination and motor disease induced by the central nervous system production of interleukin-3 in transgenic mice. J Clin Invest 1996;97:1512-1524.
18. Powell HC, Garrett RS, Brett FM et al. Response of glia, mast cells and the blood brain barrier, in transgenic mice expressing interleukin-3 in astrocytes, an experimental model for CNS demyelination. Brain Pathol 1999;9:219-235.
19. Powell HC, Garrett RS, Muehlenbachs A, Brett FM, Campbell IL. Crystalloid inclusions in brain macrophages and hemopoietic tissue in GFAP-IL3 mice resemble inclusions identified in multiple sclerosis. Ultrastruct Pathol 1999;23:285-297.
20. Akwa Y, Hassett DE, Eloranta M-L et al. Transgenic expression of IFN-α in the central nervous system of mice protects against lethal neurotropic viral infection but induce inflammation and neurodegeneration. J Immunol 1998;161:5016-5026.
21. Campbell IL, Krucker T, Steffensen S et al. Structural and functional neuropathology in transgenic mice with CNS expression of IFN-α. Brain Res 1999;835:46-61.

22. Carr DJ, Veress LA, Noisakran S, Campbell IL. Astrocyte-targeted expression of IFN-alpha1 protects mice from acute ocular herpes simplex virus type 1 infection. J Immunol 1998;161**:**4859-4865.
23. Stalder AK, Carson MJ, Pagenstecher A et al. Late-onset chronic inflammatory encephalopathy in immune-competent and severe combined immune-deficient (SCID) mice with astrocyte-targeted expression of tumor necrosis factor. Am J Pathol 1998;153**:**767-783.
24. DeLeo JA, Rutkowski MD, Stalder AK, Campbell IL. Transgenic expression of TNF by astrocytes increases mechanical allodynia in a mouse neuropathy model. Neuroreport 2000;11**:**599-602.
25. Pagenstecher A, Lassmann S, Carson MJ, Kincaid CL, Stalder AK, Campbell IL. Astrocyte-targeted expression of IL-12 induces active cellular immune responses in the central nervous system and modulates experimental allergic encephalomyelitis. J Immunol 2000;164**:**4481-4492.
26. Palmiter RD, Findley SD, Whitmore TE, Durnam DM. MT-III, a brain-specific member of the metallothionein gene family. Proc Natl Acad Sci U S A 1992;89**:**6333-6337.
27. Quaife CJ, Findley SD, Erickson JC et al. Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. Biochemistry 1994;33**:**7250-9.
28. Yagle MK, Palmiter RD. Coordinate regulation of mouse metallothionein I and II genes by heavy metals and glucocorticoids. Mol Cell Biol 1985;5**:**291-4.
29. van Lookeren Campagne M, Thiobodeaux H, van Bruggen N, Cairns B, Lowe DG. Increased binding activity at an antioxidant-responsive element in the metallothionein-1 promoter and rapid induction of metallothionein-1 and -2 in response to cerebral ischemia and reperfusion. J Neurosci 2000;20**:**5200-5207.
30. Aschner M, Cherian MG, Klaassen CD, Palmiter RD, Erickson JC, Bush AI. Metallothioneins in brain--the role in physiology and pathology. Toxicol Appl Pharmacol 1997;142**:**229-42.
31. Hidalgo J, Castellano B, Campbell IL. Regulation of brain metallothioneins. Current Topics Neurochem 1997;1**:**1-26.
32. Hidalgo J, Aschner M, Zatta P, Vasák M. Roles of the metallothionein family of proteins in the central nervous system. Brain Res Bull 2001;55**:**133-145.

33. Hernández J, Molinero A, Campbell IL, Hidalgo J. Transgenic expression of interleukin 6 in the central nervous system regulates brain metallothionein-I and -III expression in mice. Brain Res Mol Brain Res 1997;48**:**125-31.
34. Carrasco J, Hernández J, González B, Campbell IL, Hidalgo J. Localization of metallothionein-I and -III expression in the CNS of transgenic mice with astrocyte-targeted expression of interleukin 6. Exp Neurol 1998;153**:**184-194.
35. Giralt M, Penkowa M, Hernández J et al. Metallothionein-1+2 deficiency increases brain pathology in transgenic mice with astrocyte-targeted expression of interleukin 6. Neurobiol Dis 2002;9**:**319-338.
36. Palmiter RD, Sandgren EP, Koeller DM, Brinster RL. Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. Mol Cell Biol 1993;13**:**5266-75.
37. Dalton T, Fu K, Enders GC, Palmiter RD, Andrews GK. Analysis of the effects of overexpression of metallothionein-I in transgenic mice on the reproductive toxicology of cadmium. Environ Health Perspect 1996;104**:**68-76.
38. Masters BA, Kelly EJ, Quaife CJ, Brinster RL, Palmiter RD. Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. Proc Natl Acad Sci U S A 1994;91**:**584-8.
39. Penkowa M, Carrasco J, Giralt M et al. Altered central nervous system cytokine-growth factor expression profiles and angiogenesis in metallothionein-I+II deficient mice. J Cerebral Blood Flow Metab 2000;20**:**1174-1189.
40. Hobbs MV, Weigle WO, Noonan DJ et al. Patterns of cytokine gene expression by CD4+ T cells from young and old mice. J Immunol 1993;150**:**3602-3608.
41. Campbell IL, Hobbs MV, Kemper P, Oldstone MBA. Cerebral expression of multiple cytokine genes in mice with lymphocytic choriomeningitis. J Immunol 1994;152**:**716-723.
42. Gasull T, Rebollo DV, Romero B, Hidalgo J. Development of a competitive double antibody radioimmunoassay for rat metallothionein. J Immunoassay 1993;14**:**209-25.
43. Gasull T, Giralt M, Hernández J, Martínez P, Bremner I, Hidalgo J. Regulation of metallothionein concentrations in rat brain: effect of glucocorticoids, zinc, copper, and endotoxin. Am J Physiol 1994;266**:**E760-7.

44. Penkowa M, Carrasco J, Giralt M, Moos T, Hidalgo J. CNS wound healing is severely depressed in metallothionein I- and II-deficient mice. J Neurosci 1999;19:2535-45.
45. Giralt M, Penkowa M, Lago N, Molinero A, Hidalgo J. Metallothionein-1+2 protect the CNS after a focal brain injury. Exp Neurol 2002;173:114-128.
46. Penkowa M, Giralt M, Thomsen P, Carrasco J, Hidalgo J. Zinc or copper deficiency-induced impaired inflammatory response to brain trauma may be caused by the concomitant metallothionein changes. J Neurotrauma 2001;18:447-463.
47. Penkowa M, Hidalgo J. Metallothionein I+II expression and their role in experimental autoimmune encephalomyelitis. Glia 2000;32:247-263.
48. Penkowa M, Hidalgo J. Metallothionein treatment reduces proinflammatory cytokines IL-6 and TNF-a and apoptotic cell death during experimental autoimmune encephalomyelitis. Exp Neurol 2001;170:1-14.
49. Penkowa M, Espejo C, Martínez-Cáceres EM, Poulsen CB, Montalban X, Hidalgo J. Altered inflammatory response and increased neurodegeneration in metallothionein I+II deficient mice during experimental autoimmune encephalomyelitis. J Neuroimmunol 2001;119:248-260.
50. Carrasco J, Penkowa M, Hadberg H, Molinero A, Hidalgo J. Enhanced seizures and hippocampal neurodegeneration following kainic acid induced seizures in metallothionein-I+II deficient mice. Eur J Neurosci 2000;12:2311-2322.
51. van Lookeren Campagne M, Thibodeaux H, van Bruggen N et al. Evidence for a protective role of metallothionein-1 in focal cerebral ischemia. Proc Natl Acad Sci USA 1999;96:12870-5.
52. Nagano S, Satoh M, Sumi H et al. Reduction of metallothioneins promotes the disease expression of familial amyotrophic lateral sclerosis mice in a dose-dependent manner. Eur J Neurosci 2001;13:1363-1370.
53. Youn J, Borghesi LA, Olson EA, Lynes MA. Immunomodulatory activities of extracellular metallothionein. II. Effects on macrophage functions. J Toxicol Environ Health 1995;45:397-413.
54. Borghesi LA, Youn J, Olson EA, Lynes MA. Interactions of metallothionein with murine lymphocytes: plasma membrane binding and proliferation. Toxicology 1996;108:129-40.

55. Sato M, Mehra RK, Bremner I. Measurement of plasma metallothionein-I in the assessment of the zinc status of zinc-deficient and stressed rats. J Nutr 1984;114:1683-9.
56. Sato M, Bremner I. Biliary excretion of metallothionein and a possible degradation product in rats injected with copper and zinc. Biochem J 1984;223:475-9.
57. Hidalgo J, Giralt M, Garvey JS, Armario A. Physiological role of glucocorticoids on rat serum and liver metallothionein in basal and stress conditions. Am J Physiol 1988;254:E71-8.
58. De Lisle RC, Sarras MP, Jr., Hidalgo J, Andrews GK. Metallothionein is a component of exocrine pancreas secretion: implications for zinc homeostasis. Am J Physiol 1996;271:C1103-10.
59. Lynes MA, Garvey JS, Lawrence DA. Extracellular metallothionein effects on lymphocyte activities. Mol Immunol 1990;27:211-9.
60. Youn J, Lynes MA. Metallothionein-induced suppression of cytotoxic T lymphocyte function: an important immunoregulatory control. Toxicol Sci 1999;52:199-208.
61. Leibbrandt ME, Khokha R, Koropatnick J. Antisense down-regulation of metallothionein in a human monocytic cell line alters adherence, invasion, and the respiratory burst. Cell Growth Differ 1994;5:17-25.
62. Lynes MA, Borghesi LA, Youn J, Olson EA. Immunomodulatory activities of extracellular metallothionein. I. Metallothionein effects on antibody production. Toxicology 1993;85:161-77.
63. Canpolat E, Lynes MA. In vivo manipulation of endogenous metallothionein with a monoclonal antibody enhances a T-dependent humoral immune response. Toxicol Sci 2001;62:61-70.
64. Crowthers KC, Kline V, Giardina C, Lynes MA. Augmented humoral immune function in metallothionein-null mice. Toxicol Appl Pharmacol 2000;166:161-172.
65. Mita M, Imura N, Kumazawa Y, Himeno S. Suppressed proliferative response of spleen T cells from metallothionein null mice. Microbiol Immunol 2002;46:101-107.

66. Kimura T, Itoh N, Takehara M et al. Sensitivity of metallothionein-null mice to LPS/D-galactosamine-induced lethality. Biochem Biophys Res Commun 2001;280**:**358-362.
67. Waelput W, Broekaert D, Vandekerckhove J, Brouckaert P, Tavernier J, C. L. A mediator role for metallothionein in tumor necrosis factor-induced lethal shock. J Exp Med 2001;194**:**1617-1624.
68. Hartung HP, Jung S, Stoll G et al. Inflammatory mediators in demyelinating disorders of the CNS and PNS. J Neuroimmunol 1992;40**:**197-210.
69. Mattson M, Scheff S. Endogenous neuroprotection factors and traumatic brain injury: mechanisms of action and implications for therapy. J Neurotrauma 1994;11**:**3-33.
70. Eddleston M, Mucke L. Molecular profile of reactive astrocytes-Implications for their role in neurological disease. Neuroscience 1993;1**:**15-36.
71. Ridet JL, Malhotra A, Gage F. Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 1997;20**:**570-577.
72. Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophys Acta 1985;827**:**36-44.
73. Hidalgo J, Campmany L, Borras M, Garvey JS, Armario A. Metallothionein response to stress in rats: role in free radical scavenging. Am J Physiol 1988;255**:**E518-24.
74. Sato M, Bremner I. Oxygen free radicals and metallothionein. Free Radical Biology Medicine 1993;14**:**325-337.
75. Lazo JS, Kondo Y, Dellapiazza D, Michalska AE, Choo KH, Pitt BR. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J Biol Chem 1995;270**:**5506-10.
76. Kondo Y, Rusnak J, Hoyt D, Settineri C, Pitt B, Lazo J. Enhanced apoptosis in metallothionein null cells. Mol Pharmacol 1997;52**:**195-201.
77. Sakurai A, Hara S, Okano N, Kondo Y, Inoue J, Imura N. Regulatory role of metallothionein in NF-kappaB activation. FEBS Lett 1999;455**:**55-8.
78. Pearce LL, Gandley RE, Han W et al. Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. Proc Natl Acad Sci 2000;97**:**477-482.

79. Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem Pharmacol 2000;59**:**95-104.

80. Wang GW, Klein JB, Kang YJ. Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiomyocytes. J Pharmacol Exp Ther 2001;298**:**461-468.

Table 1. Gender frequencies and genotypes of the F2 offspring

MALES (Exp. 1 / 2: n= 111 / 38)				FEMALES (Exp. 1 / 2: n= 110 / 33)			
GFAP-IL6+/- (n= 64 / 20)		GFAP-IL6-/- (n= 47 / 18)		GFAP-IL6+/- (n= 53 / 14)		GFAP-IL6-/- (n= 57 / 19)	
TgMTI+/-	TgMTI-/-	TgMTI+/-	TgMTI-/-	TgMTI+/-	TgMTI-/-	TgMTI+/-	TgMTI-/-
27 / 11	37 / 9	22 / 6	25 / 12	27 / 6	26 / 8	33 / 11	24 / 8

Two separate crossings were carried out as described in Materials and Methods (Exp. 1 / 2). Mendelian distribution of the GFAP-IL6 and TgMTI transgenes are normal.

Table 2. Metallothionein-I mRNA levels in the cerebellum of the F2 offspring

	Age	
Genotype	2 month	13 month
GFAP-IL6+/- TgMTI+/-	4852 ± 564***▲ (n=5)	6541 ± 170▲ (n=5)
GFAP-IL6+/- TgMTI-/-	3055 ± 420*** (n=4)	3199 ± 383*** (n=7)
GFAP-IL6-/- TgMTI+/-	2976 ± 1055▲ (n=2)	5590 ± 830▲ (n=5)
GFAP-IL6-/- TgMTI-/-	657 ± 113 (n=5)	910 ± 26 (n=5)

In situ hybridization analysis for MT-I mRNA was carried out as described in Materials and Methods. All samples were processed in parallel and exposed to the same autoradiographic film. A representative section of each genotype is shown in Figure 1. Results are arbitrary units, mean ± SE. ***$p<0.001$ versus respective GFAP-IL6-/- mice. ▲$p<0.002$ vs respective TgMTI-/- mice.

Table 3. Analyses of cytokine and host-gene expression in the cerebellum by RNase protection assay in 7 month-old mice (see Figures 3 and 5).

	GFAPIL6-/- TgMTI-/-	GFAPIL6-/- TgMTI+/-	GFAPIL6+/- TgMTI-/-	GFAPIL6+/- TgMTI+/-
IL-6	2.68±0.91	0.73±0.22▲	69.8±10.8***	34.0±13.6***▲
IL-1α	ND	ND	2.28±0.38	1.68±0.36
IL-1β	ND	ND	9.61±1.98	5.64±1.89
TNF	ND	ND	2.49±0.33	1.80±0.48
GFAP	9.26±1.27	9.1±2.04	172.3±15.2***	140.4±22.5***
Mac1	ND	ND	2.22±0.43	1.92±0.38
ICAM	ND	ND	0.41±0.08	0.46±0.15
EB22	ND	ND	25.2±5.39	21.3±8.85

Results (arbitrary units; mean ± SE; n=4-7) were evaluated with two-way ANOVA with GFAP-IL6 transgene and MT-I overexpression as main factors. When only two groups were compared, the Student "t" test was employed. ND, not detectable. ***$p<0.001$ versus respective GFAP-IL6-/- mice. ▲$p<0.002$ vs TgMTI-/- mice.

FIGURE LEGENDS

Figure 1. Representative in situ hybridization analysis for MT-I mRNA of the four genotypes studied at 13 months.
TgMT-I mice showed dramatically increased MT-I mRNA levels throughout the CNS, although telencephalic areas showed a smaller increase. The transgenic expression of IL-6 upregulated MT-I expression in the cerebellum, while the combination of both transgenes tended to further increase MT-I mRNA levels (see Table 2 for quantitative measurements).

Figure 2. IL-6 and MT-I+II immunoreactivity in the cerebellum from 7 month old mice.
Transgenic GFAP-IL6 mice showed significantly increased levels of IL-6 primarily in Bergmann glia, astrocytes and microglia/macrophages. MT-I overexpression on its own did not alter IL-6 immunostaining, but dramatically reduced that caused by transgenic IL-6 expression. Both IL-6 and MT-I transgenic expression on their own strongly increased MT-I+II expression in white (large, black star) and grey (small, white star) matter, while in the GFAP-IL6+/- TgMTI+/- mice the expression of MT-I+II tended to be further increased.
Scale bars: 42 μm.

Figure 3. Analysis of cytokine gene expression.
mRNA levels of a number of cytokines in the cerebellum of 7 month old mice were measured by RNase protection assay. The sample labeled with a star belongs to the GFAP-IL6+/- TgMTI+/- group. As expected, some cytokines were upregulated in those animals with transgenic expression of IL-6, namely TNF, IL-1α, IL-6 and IL-1β. MT-I overexpression decreased this upregulation as shown by the densitometric analysis of the bands and normalization with the L32 ribosomal mRNA (see Table 3).

Figure 4. Lectin immunoreactivity in the cerebellum from 7 month-old mice.
In GFAP-IL6-/- TgMTI-/- mice, lectin staining was mainly seen in vessels but also in a very few ramified microglial cells in cerebellum, both in the white (small star) and grey matter (large star). Transgenic IL-6 expression caused a significant activation of

microglia/macrophages, especially in white matter. This response was dramatically blunted by MT-I overexpression.
Scale bars: 44 μm.

Figure 5. Analyses of host-response gene expression.
Cerebellum mRNA levels of several host-response genes in 7 month old mice were analyzed by RNase protection assay. The sample labeled with a star belongs to the GFAP-IL6+/- TgMTI+/- group. As expected, ICAM-I, Mac1, EB22/5 and GFAP expression was significantly increased in those animals with transgenic expression of IL-6. MT-I overexpression did not significantly alter any of the genes analyzed, although tended to decrease Mac1 and GFAP upregulation (see Table 3). A representative in situ hybridization analysis of GFAP mRNA in the four genotypes studied at 13 months of age is shown in the insert. In accordance with the RPA, the transgenic expression of IL-6 upregulated GFAP expression, which occurred mainly in the cerebellum. MT-I overexpression did not alter significantly GFAP mRNA levels, in agreement with the RPA results but in sharp contrast with the GFAP immunoreactivity results. The GFAP-IL6+/- TgMTI+/- mice had a similar GFAP expression to that of the GFAP-IL6+/- TgMTI-/- mice.

Figure 6. GFAP immunoreactivity in cerebellum from 7 month-old mice.
Significant reactive astrogliosis was observed in the white and grey matter of the cerebellum of the GFAP-IL6+/- TgMTI-/- mice. In some of the GFAP-IL6-/- TgMTI+/- mice, reactive astrogliosis could be observed throughout the CNS at this age; this was not seen in 3 month-old mice. In contrast to the mice with transgenic expression of IL-6, the GFAP immunostaining of the cerebellum was mainly observed in the molecular layer. In the GFAP-IL6+/- TgMTI+/- mice, the cerebellum showed a slightly increased GFAP staining relative to both the GFAP-IL6+/- TgMTI-/- and GFAP-IL6-/- TgMTI+/- mice.
Scale bars: 29 μm.

Figure 7. NITT immunostaining in the cerebellum as marker of oxidative stress.
The transgenic expression of IL-6 dramatically increased NITT stainings in the cerebellum, which indicates significant oxidative stress. This was significantly decreased by MT-I overexpression.
Scale bars: 48 μm

Figure 8. Angiopoietin immunostaining as a marker of angiogenesis.
In normal mice the immunoreactivity for angiopoietin and VEGF was barely detectable, but it was strongly increased by the transgenic expression of IL-6. The transgenic expression of MT-I was without effect on its own, but tended to increase IL-6-elicited angiopoietin staining.
Scale bars: 50 μm.