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Research Article

Increased demyelination and axonal damage in metallothionein I+II-deficient mice during experimental autoimmune encephalomyelitis

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Abstract. Metallothioneins I+II (MT-I+II) are antioxidant, neuroprotective factors. We previously showed that MT-I+II deficiency during experimental autoimmune encephalomyelitis (EAE) leads to increased disease incidence and clinical symptoms. Moreover, the inflammatory response of macrophages and T cells, oxidative stress, and apoptotic cell death during EAE were increased by MT-I+II deficiency. We now show for the first time that demyelination and axonal damage are significantly increased in MT-I+II deficient mice during EAE. Furthermore, oligodendroglial regeneration, growth cone formation, and tissue repair including expression of trophic factors were significantly reduced in MT-I+II-deficient mice during EAE. Accordingly, MT-I+II have protective and regenerative roles in the brain.

Key words. Metallothionein; EAE/MS; demyelination; neurodegeneration; regeneration; neurotrophic factor.

The animal model for multiple sclerosis (MS) is experimental autoimmune encephalomyelitis (EAE). EAE/MS are inflammatory autoimmune diseases with demyelination and neurodegeneration [1–5]. During EAE/MS, metallothioneins I and II (MT-I+II) are increased [3, 6, 7]. MT-I+II are low-molecular-weight (6–7 kDa) nonenzymatic proteins (60–68 amino acid residues, 25–30% cysteine), and there are increasing data to show a key protective role for MT-I+II during central nervous system (CNS) pathological conditions [8–10].

MT-I+II are significant antiinflammatory and beneficial factors during actively induced EAE in rodents. Thus, MT-I+II inhibit T lymphocytes and macrophages, including their proinflammatory cytokines, during EAE [3, 4, 11]. Furthermore, during EAE, MT-I+II reduce the levels of oxidative stress and neuroglial apoptotic cell death [3, 4, 11]. Next to these roles, MT-I+II ameliorate EAE clinical symptoms and decrease disease mortality and incidence. In addition MT-I+II also have significant tissue-protective functions during other CNS disorders. Accordingly, MT-I+II amend inflammation and inhibit neurodegeneration and cell death as well as enhancing neuronal tissue repair, angiogenesis, and growth factor production [12–16]. They also amend the clinical outcome after epilepsy, cerebral ischemia, and neuroglial degeneration [15–18].

In this study, we examine the roles of MT-I+II in demyelination, oligodendroglial survival/regeneration, and neuronal degeneration such as axonal damage or transec-

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tion during EAE in MT-I+II-deficient mice [MT-I+II knock-out (MTKO) mice]. We demonstrate that EAE-induced demyelination and axonal degeneration and transection are increased significantly in MTKO mice. Furthermore, oligodendroglial regeneration from their precursor cells and tissue repair including growth cone formation and expression of growth factors and neurotrophins are significantly reduced in MTKO mice. As a result, preserving or increasing MT-I and/or MT-II levels could have therapeutic value during MS and other demyelinating disorders.

Materials and methods

Mice

Homozygous MTKO mice were generated as previously described [19]. MTKO mice were raised on the 129/Sv genetic background; therefore, mice from this strain were used as control. Mice were 8–14 weeks old, and were fed with standard chow and had access to water ad libitum. Immunostainings (see below) were carried out in at least eight animals per group, and cell counts of positively stained cells were carried out in three animals per group. Anesthesia was induced by intraperitoneal (i.p.) injection of 37 mg/kg of ketamine (Ketolar; Parke & Davis, Morris Plains, USA) and 5.5 mg/kg of xylazine (Rompun; Bayer, Germany). Animal welfare was observed in compliance with European Community regulations, and all animal experiments adhered to the standards of the National Research Council's Guide for the Care and Use of Laboratory Animals. Furthermore, all experiments performed were in line with 'Ethical Principles and Guidelines for Scientific Experiments on Animals' of the Swiss Academy of Medical Sciences.

EAE immunization

Mice were immunized by a single subcutaneous (s.c.) injection of 0.9% saline containing 100 µg of peptide 40–55 of rat myelin oligodendrocyte glycoprotein (MOG_{40-55}) emulsified in Freund's adjuvant (Sigma, USA) (9:11 v/v) containing 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, USA). Rat MOG_{40-55} was synthesized by Dr. D. Andreu (Departament de Síntesi de Pèptids, Facultat de Química, Universitat de Barcelona, Spain). Mice received 0.05 ml of emulsion s.c. in the four limb flanks. At days 0 and 2 post-immunization, each mouse received 0.2 ml (2 IU/ml) of inactivated *Bordetella pertussis* (Vaxicoq; Pasteur Merieux, France) intravenously.

Clinical evaluation

All mice were weighed and examined daily, from day 7 to the end of the experiment (day 37 post-immunization), for neurological symptoms according to these criteria: 0, no clinical signs; 0.5, partial loss of tail tonus for 2 consecutive days; 1, paralysis of whole tail; 2, mild paraparesis of one or both hind limbs; 3, paraplegia; 4, tetraparesis; 5, tetraplegia; 6, death. All evaluation of symptoms was performed in a blinded manner.

Tissue processing

Animals were sacrificed with CO_2 at day 37 post-immunization and the CNS was resected. Tissues were fixed overnight in 4% paraformaldehyde/PBS. Following fixation, the samples were cryoprotected in 30% sucrose/PBS, frozen in 2-methylbutane cooled in liquid nitrogen and then stored at –80ºC until use.

Brain and spinal cord were cut into 30-µm-thick serial, coronal sections on a cryostat and used for histochemistry and immunohistochemistry. The sections were incubated in 3.0% H_2O_2 in TBS/Nonidet for 15 min at room temperature to quench endogenous peroxidase.

The sections were pre-incubated with pronase E (protease type XIV) (Sigma; code P5147; 0.025 g dissolved in 50 ml TBS for 10 min, pH 7.4, at 37 °C). All sections were incubated in 10% normal goat serum (In Vitro, Denmark; code 04009-1B) or normal donkey serum (The Binding Site, UK; code BP 005.1) in TBS/Nonidet for 30 min at room temperature. Moreover, sections prepared for incubation with monoclonal mouse-derived antibodies were in addition incubated with Blocking Solutions A+B from the HistoMouse-SP Kit to quench endogenous mouse IgG (Zymed, South San Francisco, USA; code 95-9544).

Immunohistochemistry

The sections were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-rat liver MT-I+II 1:500 [20]; rabbit anti-human myelin basic protein (MBP) 1:100 (Dakopatts, Denmark; code A0623); mouse anti-myelin oligodendrocyte-specific protein (MOSP) 1:50 (Neomarkers, USA; code MS-245-P); mouse anti-human CNPase 1:150 (Biogenesis, UK; code 2406-3006) (a marker of mature oligodendrocytes); rabbit anti-human platelet-derived growth factor-α receptor; PDGFα-rec 1:50 (Santa Cruz, USA; code sc-338) (a marker of oligodendrocyte progenitor cells); rabbit anti-rat NG2 (NG2 chondroitin sulfate protein) 1:100 (Chemicon, USA; code AB5320) (a marker of oligodendrocyte progenitor cells); mouse anti-rat PCNA 1:10 (Dakopatts; code M0879) (a marker of cell proliferation); mouse anti-CDC47 1:200 (Neomarkers; code MS-862-P) (a proliferation marker); mouse anti-nonphosphorylated neurofilaments (SMI-32) 1:50 (Sternberger Monoclonals, USA; code SMI-32) (detecting axonal transection and demyelination); goat anti-human amyloid precursor protein (APP) (frame shift mutant 1:50 (Chemicon; code AB5342) (a marker for acute axonal injury/degeneration); mouse anti-rat GAP-43 1:100 (Calbiochem, USA;

code CP09) (a marker for neuronal growth cones); mouse anti-human P-40 1:100 (Serotec, UK; code MCA1712) (a marker for CNS growth cones); rabbit anti-human basic fibroblast growth factor (bFGF) 1:100 (Santa Cruz; code sc-79); rabbit anti-human transforming growth factor-β1 (TGF-β1) 1:200 (Santa Cruz; code sc-146); goat anti-human neurotrophin-3 (NT-3) 1:30 (RD Systems, UK; code AF-267-NA); rabbit anti-human NT-4/5 1:50 (Santa Cruz; code sc-545); goat anti-rat nerve growth factor (NGF) 1:100 (RD Systems; code AF-556-NA).

The primary antibodies were detected using biotinylated mouse anti-rabbit IgG 1:400 (Sigma Aldrich; code B3275), or biotinylated goat anti-mouse IgG 1:200 (Sigma Aldrich; code B8774), or biotinylated goat anti-mouse IgM (µ chain specific) 1:10 (Jackson ImmunoRes., code 115-065-020), or biotinylated donkey anti-sheep/goat IgG 1:20 (Amersham, UK; code RPN 1025) by incubating the sections for 30 min at room temperature followed by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP; Dakopatts; code K377) prepared at the manufacturer's recommended dilutions for 30 min at room temperature. Afterwards, sections were incubated with biotinylated tyramide and streptavidin-peroxidase complex (NEN, Life Science Products, USA; code NEL700A) prepared following the manufacturer's recommendations. The immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature.

Standard control stainings without primary antibody were performed as previously described [3]. A further control for some cytokines and growth factors was to pre-absorb the primary antibodies with their corresponding antigenic proteins. For this purpose we used: bFGF peptide (RD Systems; code 423-F8-025 and 424-FC-025); TGF-β1 peptide (RD Systems; code 240-B-002); NT-3 peptide (RD Systems; code 267-N3-005); NT-4 peptide (Santa Cruz; code sc-545P); NGF peptide (RD Systems; code 556-NG-100). Results were considered only if these controls were negative.

Immunofluorescence

To detect if oligodendrocyte progenitors were proliferating cells, sections were pre-treated as above and then incubated overnight at 4°C with rabbit anti-human PDGFα-rec or rabbit anti-rat NG2 (both as above) and simultaneously with mouse anti-rat PCNA or mouse anti-CDC47 (both as above). The monoclonal antibodies were detected using goat anti-mouse IgG linked with TexasRed (TXRD) (Southern Biotechnology, USA; code 1030-07), while the polyclonal antibodies were detected using goat anti-rabbit linked with aminomethylcoumarin (AMCA) (Jackson Immunoresearch, USA; code 111-155-144).

To detect which cell types express growth factors and neurotrophins during EAE, we performed double and triple immunofluorescence. The sections were pre-treated as above and then incubated overnight at 4°C with goat anti-human GFAP 1:100 (Santa Cruz; code sc-6170) (a marker for astrocytes) and mouse anti-rat ED1 1:500 (Serotec; code MCA 341R) (as a marker for macrophages/microglia) and simultaneously with rabbit anti-human bFGF (as above) or rabbit anti-human NT-4/5 (as above). Afterwards, these antibodies were detected using donkey anti-goat IgG linked with AMCA (Jackson Immunoresearch; code 705-155-147) and donkey anti-mouse IgG linked with TXRD (Jackson Immunoresearch; code 715-075-151) and donkey anti-rabbit IgG linked with fluorescein (FITC) (Jackson Immunoresearch; code 711-095-152). Other sections were incubated simultaneously with goat anti-human GFAP and mouse anti-rat ED1 (as above) and rabbit anti-human TGF-β1 (as above). These antibodies were detected using donkey anti-goat IgG linked with AMCA (as above) and donkey anti-mouse IgG linked with FITC (Jackson Immunoresearch; code 715-095-151) and donkey anti-rabbit IgG linked with TXRD (Jackson Immunoresearch; code 711-075-152).

Other sections were incubated simultaneously with rabbit anti-cow GFAP 1:250 (Dakopatts; code Z334) and mouse anti-rat ED1 (as above) and goat anti-rat NGF (as above) or goat anti-human NT-3 (as above). These antibodies were detected using donkey anti-rabbit IgG linked with AMCA (Jackson Immunoresearch; code 711-155-152) and donkey anti-mouse IgG linked with TXRD (as above) and donkey anti-goat IgG linked with FITC (Jackson Immunoresearch; code 705-095-147).

Other sections were incubated simultaneously with mouse anti-human NF IgG ready-to-use (Biogenex, USA; code AM073-10M) and mouse anti-MOSP IgM 1:50 (Neomarkers; code MS-245-P) and rabbit anti-human bFGF (as above); or rabbit anti-human NT-4/5 (as above). These antibodies were detected using goat anti-mouse IgG linked with TXRD (Jackson Immunoresearch; code 115-075-100) and goat anti-mouse IgM linked with FITC (Jackson Immunoresearch; code 115-095-020) and goat anti-rabbit linked with AMCA (as above). Other sections were incubated simultaneously with mouse anti-human NF IgG ready-to-use (as above) and mouse anti-MOSP IgM (as above) and goat anti-rat NGF (as above) or goat anti-human NT-3 (as above). These antibodies were detected using donkey anti-goat IgG linked with AMCA (as above) and donkey anti-mouse IgG linked with TXRD (as above) and donkey anti-mouse IgM linked with FITC (Jackson Immunoresearch; code 715-095-140).

The secondary antibodies were used simultaneously and were diluted 1:30 and incubated for 30 min at room temperature.

The sections were embedded in 20 µl fluorescent mounting (Dakopatts; code S3023) and kept in darkness at 4°C.

Standard control stainings were performed as previously described [3, 14].

Cell counts

For statistical purposes, cellular counts of positively stained cells were performed in a blinded fashion in matched 0.5-mm^2 areas of each mouse. Hence, we counted from the anterior part of the brain stem (pons), the anterior part of the cervical spinal cord, and from the anterior part of the cerebellum right behind the fourth ventricle. Positively stained cells were defined as cells with staining of cell soma or of both cell soma and processes.

Cell counts were carried out in three mice per group of non-EAE and EAE-sensitized normal and MTKO mice.

Statistical analysis

Cell counts were carried out in independent animals in representative experiments as described above. As appropriate, results were evaluated by two-way analysis of variance (ANOVA), with treatment and timing as main factors, or with one-way ANOVA.

Results

We observed increased EAE incidence and increased clinical EAE symptoms in the MTKO mice when compared to normal mice [11]. The inflammatory responses of macrophages and T lymphocytes including levels of proinflammatory cytokines were also significantly increased in MTKO mice relative to normal mice during EAE, as were the levels of oxidative stress and apoptotic cell death [11].

The following is a detailed description of the new histopathological analysis carried out in this experimental paradigm.

Myelin and oligodendrocytes

Myelination was evaluated using immunohistochemistry for MBP (fig. 1) and MOSP (not shown). During EAE in normal mice, demyelinated areas were observed in both brain stem, spinal cord, and cerebellum white matter, as determined by loss of MBP staining (fig. 1 A, C). However, the EAE-induced demyelination was significantly more pronounced in the MTKO mice (fig. 1 B, D), which showed an increased number of demyelinated areas in both brain stem, spinal cord, and cerebellum white matter.

Oligodendrocytes were identified by CNPase immunohistochemistry, which during EAE showed a significant decrease in the number of oligodendrocytes of MTKO mice relative to normal mice (fig. 1 E, F, table 1). However, in all the mice, the number of CNPase+ oligodendrocytes decreased simultaneously with demyelination during EAE, but in MTKO mice this reduction was much more pronounced than in normal mice (table 1).

Oligodendroglial progenitor cells were detected using PDGFα-rec and NG2 immunoreactivity. In the MTKO mice during EAE, the numbers of PDGFα-rec+ and NG2+ progenitors were always significantly reduced relative to normal mice (fig. 2, table 1). Thus, throughout the CNS during EAE, only the normal mice showed numerous PDGFα-rec+ and NG2+ progenitor cells in both white and gray matter. In contrast, the MTKO mice showed almost no increases in the number of oligodendroglial progenitor cells relative to healthy control mice without EAE (fig. 2, table 1).

In all mice with EAE, the number of PCNA+ and CDC-47+ proliferating cells was at all time points significantly increased relative to healthy mice without EAE (table 1). The proliferating cells were mainly the oligodendroglial progenitor cells as verified using double immunofluorescence (fig. 3 A, B). According to the above, the number of PCNA+ and CDC-47+ proliferating cells was at all time points significantly increased in normal mice with EAE relative to MTKO mice (table 1).

Table 1. Effect of EAE and MT-I+II deficiency.

Cells positive for	Control	MTKO	Control + EAE	MTKO + EAE
NG2	12.0 ± 2.6	12.0 ± 1.7	27.0 ± 4.0	12.3 ± 2.33
CNPase	59.3 ± 5.2	55.3 ± 3.5	33.3 ± 3.2	12.0 ± 3.2
APP	1.33 ± 0.9	3.0 ± 1.0	38.3 ± 2.7	124.3 ± 7.6
SMI	2.0 ± 0.58	2.0 ± 1.0	25.0 ± 4.1	84.0 ± 3.2
CDC	5.33 ± 1.4	4.0 ± 1.5	44.7 ± 8.4	14.3 ± 3.8
PCNA	5.3 ± 0.9	4.0 ± 1.0	43.7 ± 5.2	13.0 ± 3.1
bFGF	6.33 ± 0.9	4.3 ± 1.2	86.0 ± 5.1	22.7 ± 5.2
TGFβ	10.3 ± 2.0	8.3 ± 0.7	109.3 ± 8.1	27.3 ± 3.5
NT-3	3.3 ± 1.5	1.7 ± 0.7	67.0 ± 6.3	20.3 ± 2.3
NGF	2.7 ± 1.2	1.3 ± 0.7	61.7 ± 6.4	18.7 ± 2.3

Cell counts were carried out in three sections (0.5-mm^2 areas) from three different CNS areas: brain stem, spinal cord, and cerebellum. A mean value was calculated for each animal, and cell counts were carried out in three mice in each group. Results (mean ± SE) were evaluated with two-way ANOVA with strain and EAE as main factors. Both EAE and MT-I+II deficiency had a significant effect ($p<0.001$) in all cases.

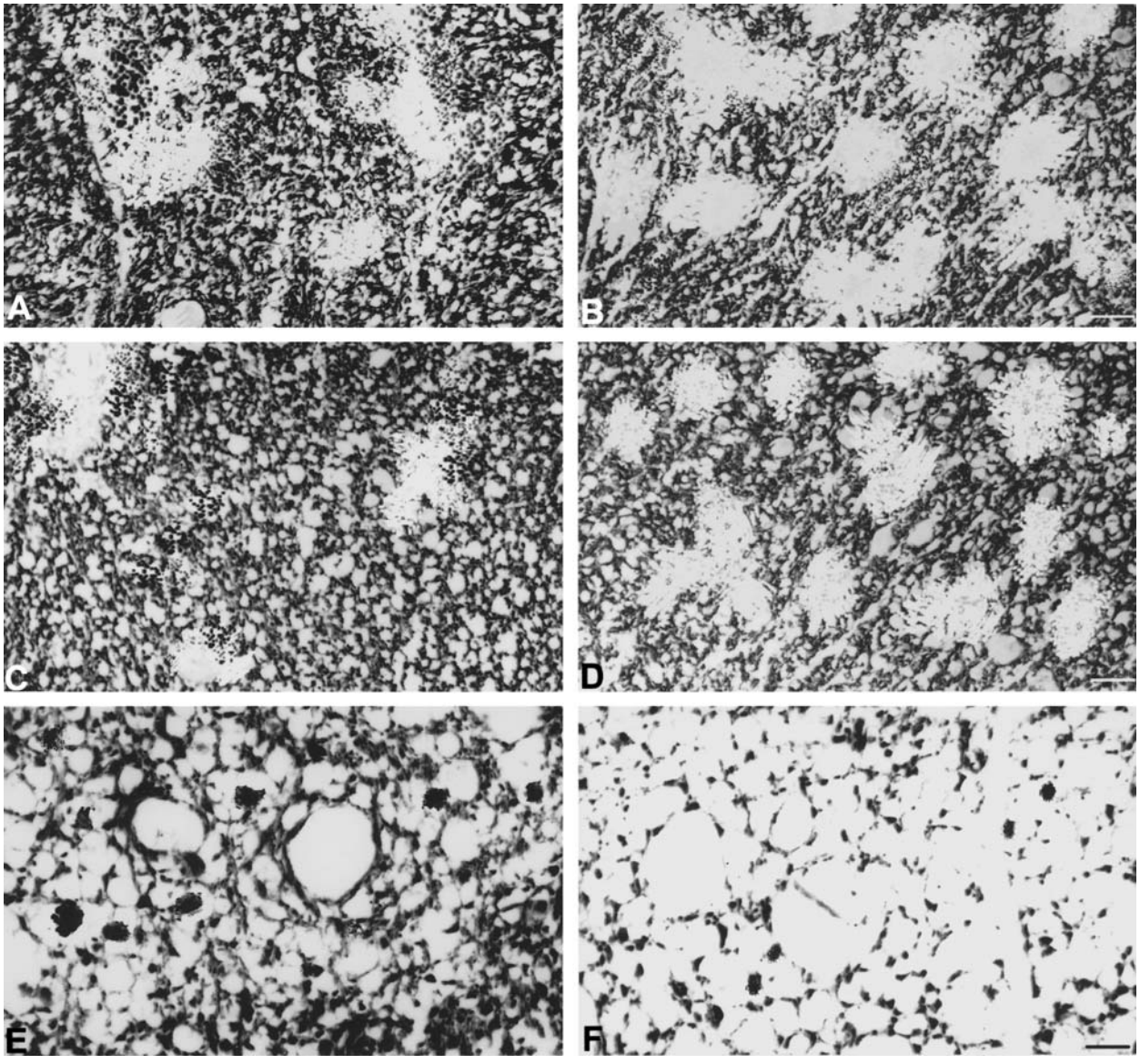

Figure 1. MBP immunoreactivity in brain stem (*A, B*) and spinal cord (*C, D*), and CNPase stainings (*E, F*) of normal and MTKO mice during EAE. (*A*) In the normal EAE immunized mice, demyelination is seen in brain stem where the MBP staining is lost. (*B*) The MTKO mice with EAE showed increased brain stem demyelination. (*C*) In the spinal cord, normal mice also showed demyelination. (*D*) MTKO mice displayed significantly increased demyelination of the spinal cord, whiched showed many areas without MBP staining. (*E*) CNPase immunostainings of oligodendrocytes in the brain stem of normal mice. (*F*) CNPase+ oligodendrocytes were reduced during EAE in MTKO mice relative to the normal mice Scale bars:, A–F: 46 μm.

Neurodegeneration and axonal injury

Neurodegeneration was examined using immunostaining for SMI-32 (nonphosphorylated neurofilaments), which marks axonal transection and damage, and acute axonal injury/degeneration was detected using APP immunoreactivity. In healthy control mice without EAE, the levels of both SMI-32 and APP immunostaining were low or absent (table 1), while during EAE, several axons were positive for both. However, the EAE-induced increase in SMI-32+ and APP+ cells was significantly higher in the MTKO mice when compared to normal mice (fig. 4, table 1). In the case of SMI-32 immunoreactivity of MTKO mice with EAE, many axons showed axonal swellings and terminal spheroids as well as discontinuous immunostaining (fig. 4), indicating that axonal transection and demyelination had occurred. In comparison, normal mice showed much lower SMI-32 and APP immunoreactivity during EAE (fig. 4, table 1). According to these results MT-I+II may significantly protect the CNS cells from neurodegeneration.

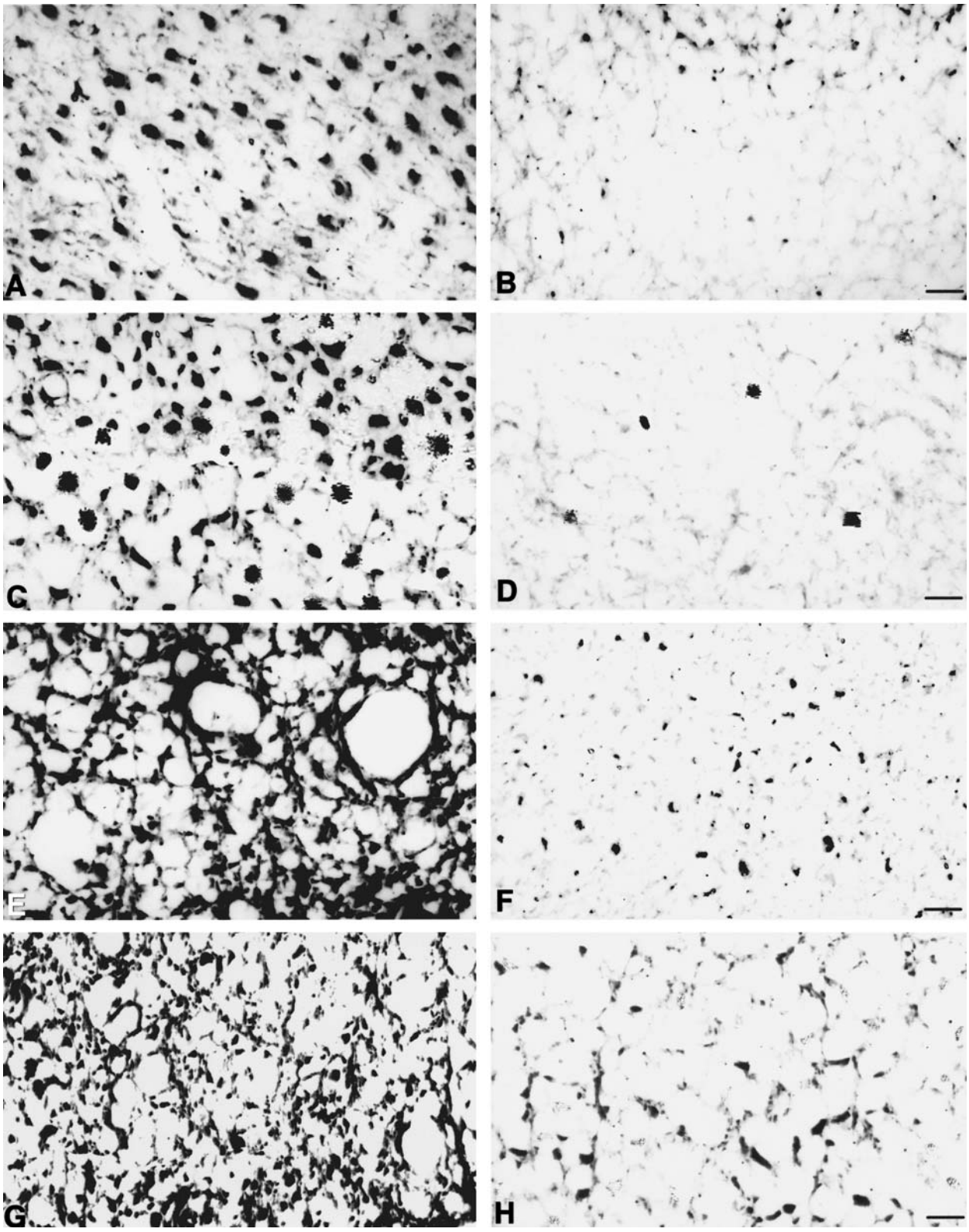

Figure 2. PDGFα-rec and NG2 immunoreactivity in brain stem and spinal cord of normal and MTKO mice during EAE. (*A*) PDGFα-rec+ oligodendrocyte precursor cells in the brain stem of EAE immunized normal mice. (*B*) The number of PDGFα-rec+ oligodendrocyte progenitors was significantly decreased in the brain stem of MTKO mice with EAE. (*C*) In the spinal cord of normal mice with EAE, several PDGFα-rec+ cells were observed. (*D*) MTKO mice with EAE showed a significantly reduced number of PDGFα-rec+ progenitors in the spinal cord. (*E*) NG2 immunostaining in brain stem of normal mice with EAE. (*F*) In MTKO mice, the number of NG2+ progenitors cells of the brain stem was significantly decreased relative to normal mice. (*G*) NG2 immunoreactivity in spinal cord of normal mice during EAE. (*H*) The number of NG2+ oligodendrocyte progenitors was clearly reduced in MTKO mice with EAE. Scale bars: A–D, 19 µm. E–H, 18 µm.

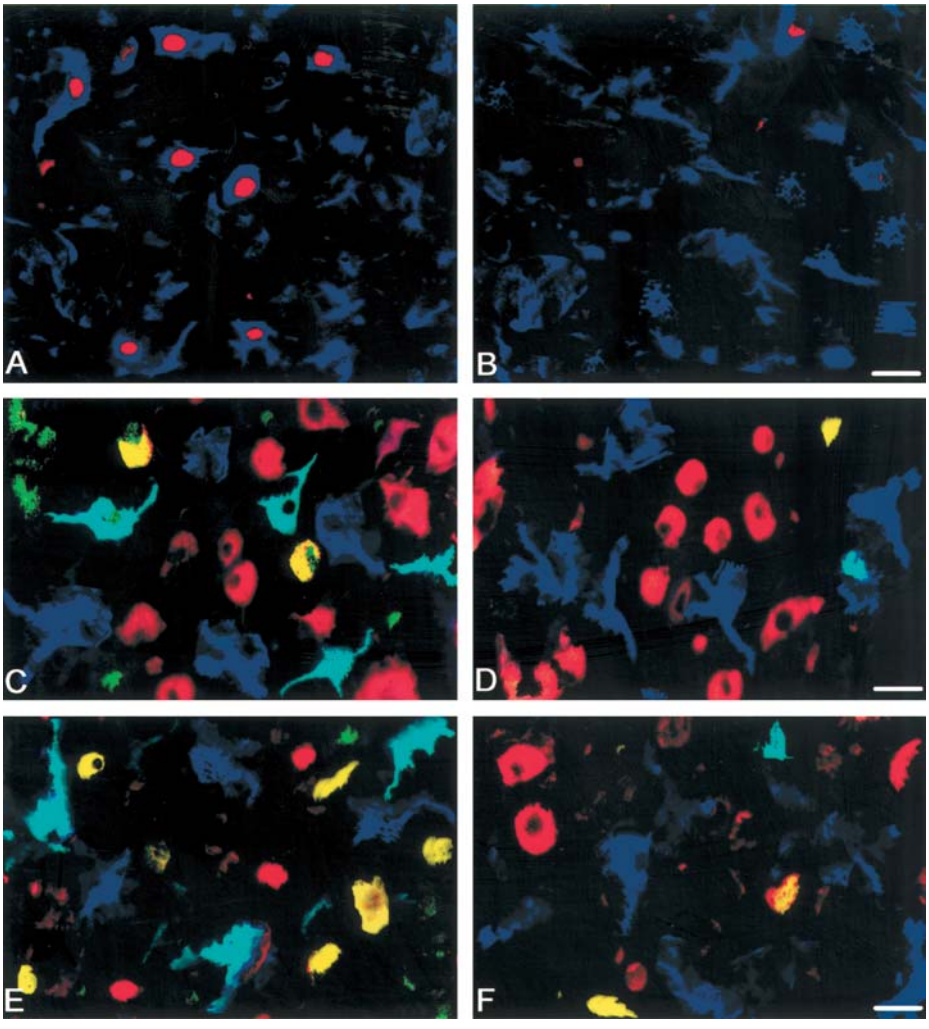

Figure 3. Double and triple immunofluorescence in the brain stem of EAE immunized mice. (*A, B*) Double staining for NG2 (blue) and PCNA (red). (*A*) Oligodendrocyte precursor cells in the brain stem of EAE immunized normal mice were proliferating. (*B*) Oligodendrocyte precursors were often not proliferating in MTKO mice with EAE. (*C, D*) Triple immunofluorescence of GFAP (blue), ED1 (red), and bFGF (green). (*C*) In normal mice with EAE, bFGF was increased in both macrophages (yellow) and astrocytes (turquoise). bFGF was also seen in a few other cells, mostly neurons and oligodendroglial cells. (*D*) In EAE immunized MTKO mice, bFGF was only seen in a very few macrophages (yellow) and astrocytes (turquoise). (*E, F*) Triple immunofluorescence of GFAP (blue), ED1 (red), and NT-4/5 (green). (*E*) In normal mice, NT-4/5 increased in both macrophages (yellow) and astrocytes (turquoise). Moreover, NT-4/5 also appeared in a few neurons and oligodendroglial cells. (*F*) In MTKO mice with EAE, NT-4/5 could only be seen in a few macrophages (yellow) and very few astrocytes (turquoise). Scale bars: *A,B*, 11 µm; *C–F*, 25 µm.

Neuronal growth and repair

By using immunoreactivity for P-40 and GAP-43, growth cones and neurite extension in the CNS could be detected. In healthy mice without EAE, both P-40 and GAP-43 expression throughout the CNS were very low or absent (not shown). However, during EAE, P-40- and GAP-43-expressing growth cones and neurites were increased in both brain stem, spinal cord, and cerebellum (fig. 5). Interestingly, MT-I+II deficiency significantly decreased the number of growth cones and neurites expressing P-40 and GAP-43 in both gray and white matter throughout the CNS during EAE (fig. 5).

Growth factor expression

Growth factors and neurotrophins are important for tissue repair and remyelination [21–23], and accordingly we examined the levels of bFGF, TGF-β1, NTs, and NGF expression during EAE. In healthy mice without EAE, expression of bFGF, TGF-β1, NTs, and NGF was low and seen in some dispersed glial cells throughout the CNS, but following EAE, their expression was increased in all the mice (table 1). However, the number of cells increasing these growth factors and neurotrophins was significantly reduced in MTKO mice relative to normal mice (table 1). bFGF, TGF-β1, NTs, and NGF expression could

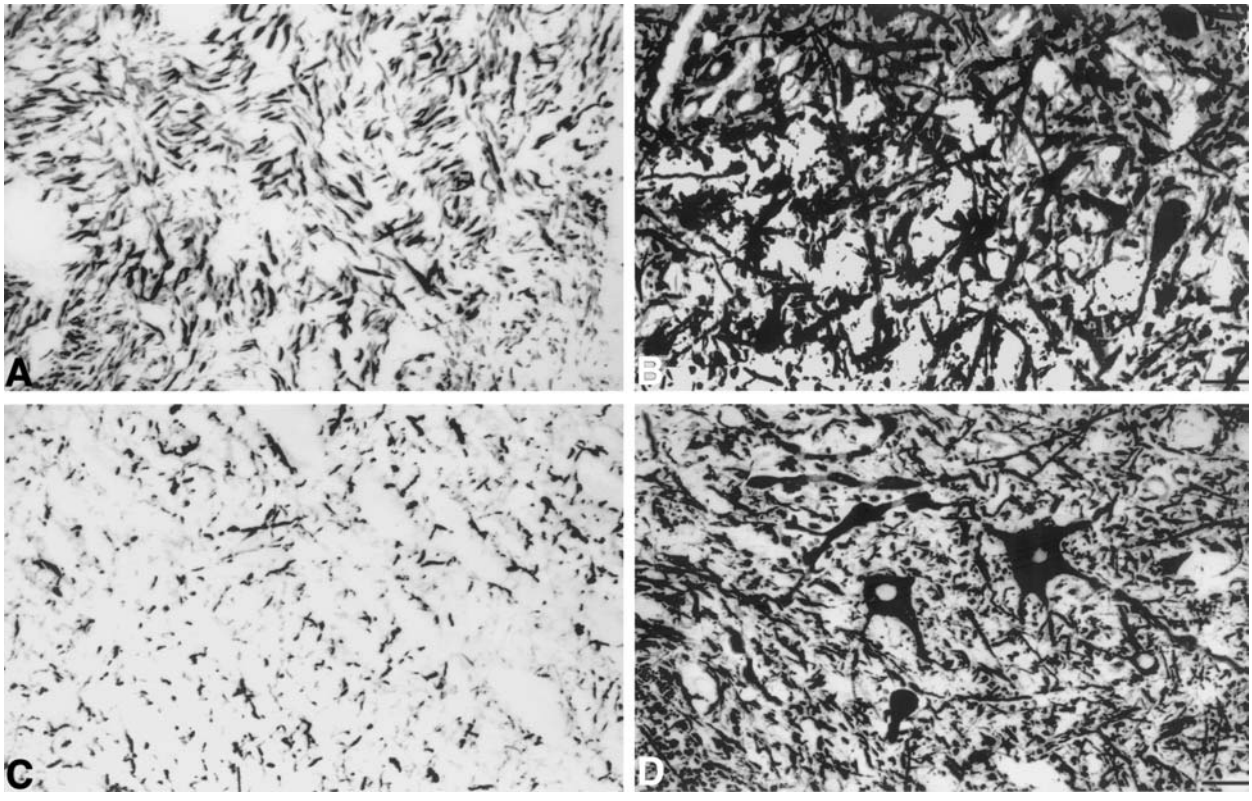

Figure 4. SMI-32 staining of axonal pathology in brain stem and spinal cord of normal and MTKO mice during EAE. (*A*) SMI-32 staining in axons of the brain stem of normal mice. (*B*) In MTKO mice with EAE, significantly increased SMI-32 staining is seen in the brain stem. As shown, SMI-32-positive axons and ovoids are abundant, and some of the degenerating axons show constrictions, dilatations, or large swellings. (*C*) SMI-32+ axons in the spinal cord of normal mice with EAE. (*D*) MTKO mice showed significantly increased SMI-32 staining. Hence, axons are seen with discontinuous SMI-32 staining, which is characteristic of axonal demyelination and degeneration. Axons with constrictions, dilatations, or large swellings can also be seen. Scale bars, 30 µm.

be detected in both reactive astrocytes, macrophages/microglia, and in some neurons and oligodendroglial cells, as verified using double or triple immunofluorescence (fig. 3C–F).

Discussion

The present study demonstrates that genetic MT-I+II deficiency has detrimental effects during actively induced EAE in MTKO mice. During EAE, MTKO mice showed increased clinical symptoms, incidence, and histopathological signs [11]. In the present paper, we examined if MT-I+II have significant roles in EAE-induced demyelination, neurodegeneration, and axonal injury, and if MT-I+II influence neuroglial regeneration and expression of growth factors and neurotrophins.

We showed that MTKO mice display significantly increased demyelination during EAE as verified using immunoreactivity for MBP, MOSP, and CNPase; oligodendroglial regeneration and the number of PDGFα-rec+ and NG2+ oligodendrocyte progenitors were also significantly reduced in MTKO mice. Additionally, MT-I+II prevented EAE-induced neurodegeneration and axonal injury and/or transection as shown using APP and SMI-32 stainings. Both APP and SMI-32 immunostainings are sensitive and early signs of axonal pathology [2, 24]. Accordingly, throughout the CNS, immunostainings for APP and SMI-32 were drastically increased in MTKO mice relative to normal mice. MT-I+II may inhibit axonal injury and transection because they are extraordinarily efficient antioxidants and scavengers of free radicals (reactive oxygen species, ROS) [25–27]. ROS destroy brain cells and tissue [28, 29] and ROS-induced tissue damage and oxidative stress are implicated in EAE/MS pathophysiology [30–33]. Thus, scavengers and inhibitors of ROS can reduce EAE onset and clinical symptoms and the histopathological signs of EAE [33–35]. Accordingly, MT-I+II expression, which is increased significantly during EAE [11] likely protects the brain tissue from ROS-induced injury and oxidative stress. Supporting this, many studies have shown how MT-I+II radically inhibit CNS oxidative stress and ROS-induced tissue damage [8]. In fact, MT-II treatment during EAE in rats could significantly inhibit oxidative stress and the formation of ROS [3]. Additionally, we have previously shown

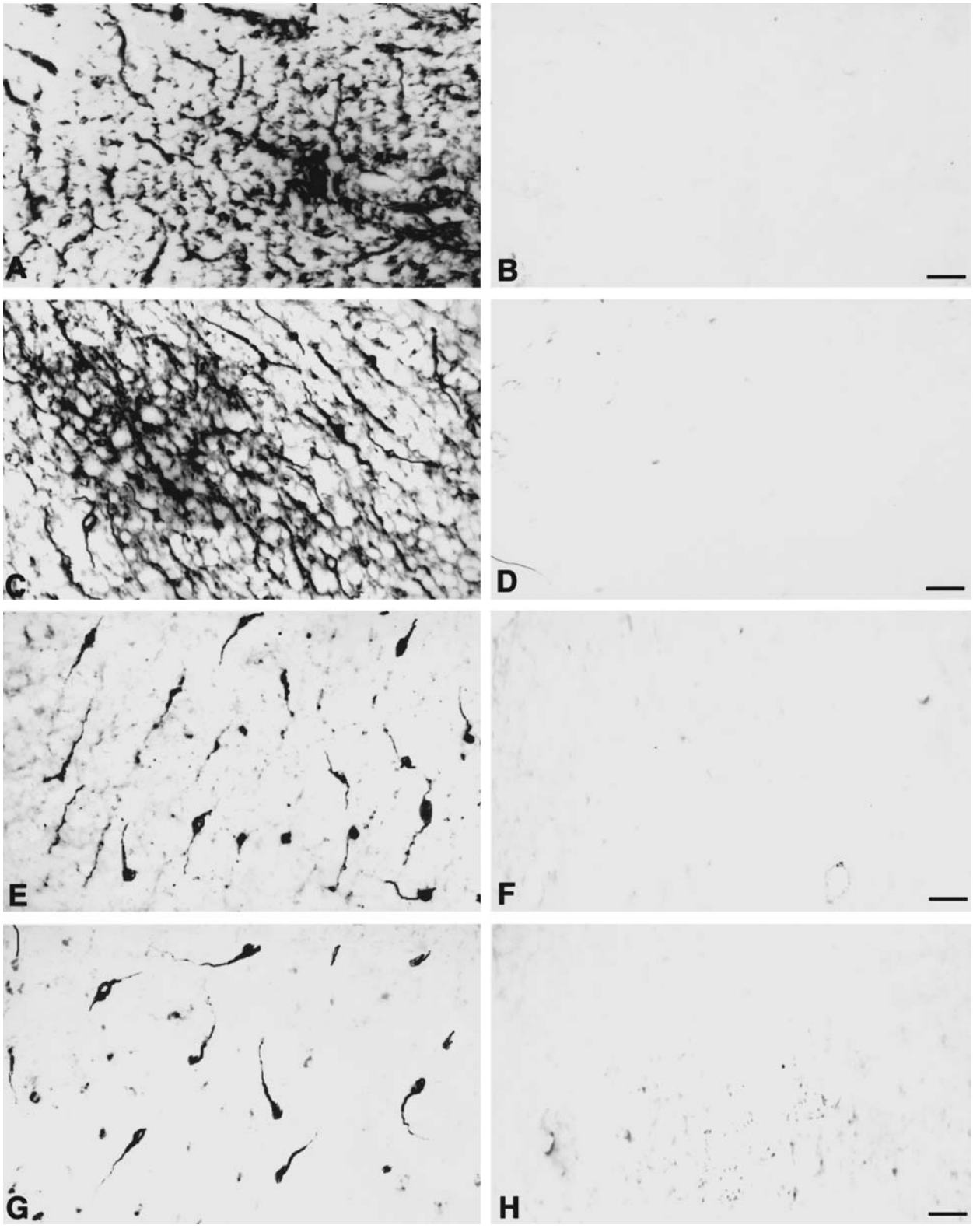

Figure 5. Immunoreactivity of GAP-43 and P-40 in the brain stem and spinal cord of EAE immunized mice. (*A*) GAP-43 staining in brain stem of normal mice, which show neuronal growth cones and neurites. (*B*) In the MTKO mice with EAE, GAP-43 immunoreactivity decreased significantly in the brain stem. (*C*) In the spinal cord, many GAP-43+ neurons and neurites were seen in normal mice. (*D*) MT-I+II deficiency significantly reduced the amount of GAP-43-expressing neuronal growth cones and neurites. (*E*) In brain stem, P-40 immunoreactivity was seen in growth cones of normal mice. (*F*) In MTKO mice, P-40+ growth cones were clearly decreased in the brain stem. (*G*) P-40 staining in spinal cord of normal mice. (*H*) P-40+ growth cones were significantly reduced in spinal cord of MTKO mice. Scale bars, 33 µm.

that MT-I+II deficiency during EAE was followed by significantly increased oxidative stress associated with increased tissue damage and cell death [11]. This supports the idea that MT-I+II protect actions against oxidative stress. Thus, the antioxidant actions of MT-I+II could explain why normal mice show reduced demyelination, neurodegeneration and axonal injury and/or transection during EAE relative to MTKO mice.

Moreover, MT-I+II also have some specific antiapoptotic functions [8, 10]. Thus, during EAE, apoptotic cell death of neurons and oligodendrocytes was significantly increased in MTKO mice [4]. Furthermore, treatment with MT-II during EAE could significantly reduce apoptosis of neurons and oligodendrocytes [3]. Likewise, in the brains of MS patients, MT-I+II-expressing cells never suffered from apoptotic cell death (own unpublished observations). In agreement with this, a positive correlation was observed between MT-I+II expression and cancer cell proliferation, while MT-I+II were not correlated with apoptosis [36]. In different carcinomas, an inverse relationship between MT-I+II and apoptosis exists [37, 38]. The actual molecular mechanisms by which MT-I+II inhibit apoptosis are not completely clarified, but a number of options are possible. As mentioned above, MT-I+II are particularly efficient antioxidants, which in itself could inhibit apoptosis [28, 39, 40]. Second, MT-I+II are major factors controlling Zn and Cu metabolism [25, 26, 41–43], and thereby control Zn and Cu concentrations, which could induce neuronal toxicity and death [44–46]. Third, MT-I+II inhibit effectively neuroinflammatory responses and levels of proinflammatory cytokines [3, 4, 11, 12, 47], which otherwise increase proteases, eicosanoids, complement factors, vasoactive amines, adhesion/costimulatory molecules, and ROS, which can affect the apoptosis pathway [48–50]. As MT-I+II inhibit CNS immune responses during various brain disorders and neuropathological conditions [10], they likely employ a common antiinflammatory mechanism in the nervous system. Another possibility is that MT-I+II affect cell proliferation and apoptosis by altering molecular pathways that determine cell survival and/or death. Thus, MT-I+II can induce a p53-null state, whereby cell survival and growth are powerfully promoted [51]. Hence, the levels of p53 were increased in MTKO mice relative to normal mice [52], and antisense down-regulation of MT-I+II was followed by increased levels of p53 [53]. This response of p53 was reversed completely in MT-II-overexpressing cells [53]. Future studies should delineate more precisely the role of MTs by crossing MTKO mice (or MT-I-overexpressing mice) with mice genetically modified for other proteins of the apoptotic pathway.

Furthermore, we examined CNS growth cones and neurite extension using P-40 and GAP-43 immunoreactivity. Interestingly, the MTKO mice displayed significantly fewer growth cones and sprouting cells than normal mice during EAE. This is in agreement with previous studies showing that brain tissue regrowth/repair and angiogenesis are significantly impaired in MT-I+II-deficient mice [13, 14], while neuroregeneration and brain wound healing are drastically improved in MT-I-overexpressing and MT-II-treated mice following brain trauma [12]. Additionally, astrocytes affect regrowth of injured axons in the CNS, and astrocyte-induced changes in the extracellular matrix can induce neurite outgrowth [54–56]. Of note is that MT-I+II are considerable stimulators of astrogliosis [3, 12, 13, 17], and that could likely contribute to the reduced neuronal regrowth and growth cone formation observed in MTKO mice.

Since brain tissue repair and remyelination are affected by antiinflammatory growth factors and neurotrophins [21–26, 57], we also examined the levels of bFGF, TGF-β1, NTs, and NGF during EAE. Interestingly, MTKO mice showed significantly reduced levels of bFGF, TGF-β1, NT-3, NT-4/5, and NGF during EAE relative to normal mice. This is in agreement with other studies showing that growth factors and neurotrophins are significantly stimulated by MT-I+II during pathology [12, 14, 47]. This could likely contribute to the previously observed MT-I+II-induced amelioration of EAE symptoms, incidence, and mortality [3, 11]. bFGF can revert the clinical and histopathological signs of EAE [58], while TGF-β affects resistance to and suppression of EAE [59]. NGF improves the EAE clinical course and delays the onset of disease [60], while neurotrophins are considered important during neuroprotection and neuroregeneration including remyelination [21, 57, 61]. Therefore, stimulation of these growth factors and neurotrophins is likely an important molecular mechanism by which MT-I+II reduce EAE.

Additionally, MT-I+II likely have protective roles during EAE by inhibiting proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, which are significantly involved in EAE induction and pathogenesis [62–65]. In fact, we have shown previously that MT-II treatment significantly reduces TNF-α and IL-6 during EAE [4], and MTKO mice show significantly increased levels of EAE-induced proinflammatory cytokines [11].

Compatible with the present study, MT-I+II were recently shown to be useful as medical treatment for experimential rheumatoid arthritis [47], an inflammatory autoimmune disease like EAE/MS. Thus, MT-I+II successfully inhibit experimential rheumatoid arthritis by inducing the antiinflammatory cytokine TGF-β as well as reducing proinflammatory molecules like TNF-α and cyclooxygenase-2 [47]. Furthermore, increased MT-I+II production or transgenic MT-II overexpression in murine pancreatic islets prevents streptozotocin-induced diabetes [66, 67]. Thus, transgenic MT-II overexpression could radically diminish pancreatic islet disruption, DNA damage, cell

death, and depletion of NAD+ as well as clinical disease and hyperglycemia [66]. In addition, MT-I+II have anti-inflammatory and regenerative roles during wound healing in the skin [68]. Hence, MT-I+II are seen in wound margins especially in areas with high mitotic activity, reflecting MT-I+II promotion of cell proliferation and tissue regeneration [68].

In conclusion, our results as well as data in the literature strongly and consistently indicate that MT-I and/or MT-II therapy during the course of inflammatory autoimmune diseases can radically inhibit overall disease progression, and could very likely become a therapeutic approach for the future.

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