Metallothionein I + II expression and roles during neuropathology in the CNS

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INTRODUCTION

MT PROTEINS

In historical reports, a protein with high affinity for heavy metals and unusual cysteine abundance was discovered in 1957 in the equine kidney, and the protein was named metallothionein (MT) due to its high content of metals and sulfur (Kägi and Vallee, 1960; Margoshes and Vallee, 1957). Over the years, it was shown that MT constitutes a superfamily of quite unusual proteins, which are subdivided into MT classes and isoforms. The mammalian MTs all belong to class I; and the MT discovered in 1957 comprised the two major isoforms MT-I and MT-II (MT-I+II). MT-I+II are low-molecular-weight (6-7 kDa), nonenzymatic proteins consisting of a single polypeptide chain of 61 amino acids characterized by a high content of sulfur (present as cysteine), zinc (Zn) and copper (Cu). In fact, 20 out of the 61 amino acids are cysteine residues, while aromatic amino acids and histidine are absent in MT-I+II (Ghoshal and Jacob, 2001; Klaassen et al., 1999). The spatial structure resembles a dumbell having two separate metal-thiolate clusters, the N-terminal β domain and the C-terminal α domain (Fischer and Davie, 1998; Kägi and Kojima, 1987).

MT-I+II are regulated and expressed coordinately, and MT-I+II proteins are expressed ubiquitously throughout the animal kingdom in most tissues and cell types including in the CNS (Hidalgo et al., 1997b; Miles et al., 2000; Searle et al., 1984). In fact, MT-I+II are present not only in every animal phyla but also in prokaryotic and eukaryotic bacteria, fungi and plants, as MT-I+II proteins are very old in an evolutionary sense and have changed only little in the course of time (Ghoshal and Jacob, 2001; Kägi and Kojima, 1987; Klaassen, 1999). Besides the ubiquitous MT-I+II, another MT isoform called growth inhibitory factor (GIF) or MT-III has been described in CNS. However, MT-III/GIF regulation, expression and functions differ clearly from those of MT-I+II (Carrasco et al., 2003; Coyle et al., 2002). The fourth, known isoform MT-IV has not been shown in CNS.

MT-I+II EXPRESSION IN THE CNS

MT-I+II expression in the normal CNS

During development and in adults, MT-I+II are expressed in low concentrations in the CNS (Nakajima and Suzuki, 1995; Penkowa et al., 1999b; Young et al., 1991). MT-I+II are seen intracellularly in some dispersed astrocytes, meningeal cells, ependymal cells, and choroid plexus, while microglia, oligodendrocytes, neurons and endothelial cells appear roughly devoid of MT-I+II (Nishimura et al., 1992; Penkowa et al., 2001b).

MT-I+II expression in CNS pathological conditions

Damage to the CNS elicits a characteristic inflammatory response, where resident microglia and blood-derived monocytes are activated and transform into amoeboid or round macrophages (Benveniste et al., 2004). Astrocytes are also prominent and show reactive astrogliosis (Faulkner et al., 2004; Hartlage-Rubsamen et al., 2003), and haematogenous leucocytes such as T and B lymphocytes are recruited (Ransohoff et al., 2003; Trebst et al., 2003). These activated cells generate many proinflammatory mediators such as cytokines, complement proteins, adhesion/costimulatory molecules, enzymes and arachidonic acid metabolites; and ultimately reactive oxygen species (ROS) are generated (Allan and Rothwell, 2003; Floyd et al., 1999; Mhatre et al., 2004; Villoslada and Genain, 2004). In CNS, ROS have detrimental actions including oxidation of DNA, proteins and lipids, which eventually lead to neurodegeneration and cell death (Barnham et al., 2004; Pong, 2003).

Hence, inflammation in CNS may result in delayed (secondary) damage and neuron death. On the other hand, immune responses are important host defense reactions. Accordingly, reactive astrocytes provide antioxidants, energy substrates and trophic factors that promote neuron survival and CNS regeneration (Liberto et al., 2004; Takuma et al., 2004). Also, some cytokines with neurotoxic actions may also mediate significant neuroprotection depending on the intensity and duration of the disorder (Campbell, 2001; Penkowa et al., 2001c, 2003a; Wang et al., 2002). Thus, cerebral inflammation can result in neurotoxicity with progressive neuron death or instead, neuroprotection and CNS tissue repair.

By the early 1990's, MT-I+II expression during various CNS disorders had not been examined. However, it soon became clear that basically any pathological condition could increase MT-I+II mRNA and proteins in CNS. Some of the first in vivo studies describing the MT-I+II induction during brain injury are presented in this thesis. Subsequent studies have validated that MT-I+II mRNA and proteins are rapidly increased following brain disorders like traumatic injury (Penkowa et al., 1999a, 2001b) neurotoxicity (Penkowa and Hidalgo, 2000a; Penkowa et al., 1997, 2002); epilepsy (Carrasco et al., 2000b; Penkowa et al., 2001c), brain ischemia (Trendelenburg et al., 2002; Van Lookeren Campagne et al., 1999); EAE (an animal model for MS) (Espejo et al., 2001a, 2002; Penkowa and Hidalgo, 2000b); and inflammatory encephalopathies (Carrasco et al., 1998b, 2000a; Giralt et al., 2001); and in a model of familial ALS (Gong and Elliott, 2000). In fact, MT-I+II are induced even by immobilization for 6-18 hours (Hidalgo et al., 1997a) or subjection to total darkness for 2-8 weeks (Beltramini et al., 2004). Also in humans, MT-I+II increase in astroglia during degenerative diseases such as AD, Pick's disease and ALS (Adlard et al., 1998; Nakajima and Suzuki, 1995; Sillevis Smitt et al., 1992); and during MS, Binswanger's encephalopathy and ischaemia (Neal et al., 1996; Penkowa et al., 2003c; Zambenedetti et al., 2002).

MT-I+II regulation in the CNS

MT-I+II are regulated in a coordinate manner in mice (Searle et al., 1984) and can be induced by almost any inflammatory or pathological stimulus (Ghoshal and Jacob, 2001; Klaassen et al., 1999; Miles et al., 2000). The inducibility of MT-I+II occurs mainly at transcription levels; where several cis-acting DNA elements in the promoter region are binding sites for trans-acting transcription factors; although post-transcriptional events may also affect MT-I+II biosynthesis (Haq et al., 2003; Hernandez et al., 1997).

MT-I+II are induced by essential and heavy metals (Zn, Cu, Cd, Hg) (Aschner, 1997; Penkowa et al., 2001b), glucocorticoids and catecholamines (Beattie et al., 2000; Hidalgo et al., 1997a,b), ROS and oxidative stress (Andrews, 2000; Sato and Bremner, 1993) and lipopolysaccharide (LPS) (Hidalgo et al., 2001; Leibbrandt and Koro-

patnick, 1994). Proinflammatory cytokines, for example IL-3, IL-6, TNF-α, M-CSF, IFN-α and IFN-γ have also been suggested to regulate MT-I+II, as demonstrated by using genetically cytokine-modified mice (Carrasco et al., 1998a,b,2000a; Espejo et al., 2001b; Giralt et al., 2001; Penkowa et al., 2002).

THE FUNCTIONAL SIGNIFICANCE OF MT-I+II

Even four decades after the discovery of MT-I+II in 1957, the precise physiological functions of these proteins remained elusive. However, genetically modified mice with MT-I+II deficiency (MT-KO mice) or MT-I overexpression (TgMT mice) have provided valuable tools for the analysis of MT-I+II functions in vivo during various disorders. Some of the first studies of such mice showing that MT-I+II induce significant neuroprotection and CNS repair after brain injury are presented in this thesis.

By the time we began to study in vivo brain pathology in MT-KO and TgMT mice, in vitro studies had indicated that MT-I+II have different roles in response to a variety of stresses.

Hence, MT-I+II bind and release essential metal ions such as Zn and Cu and also heavy metals such as Cd, Pb and Hg (Aschner and Walker, 2002; Coyle et al., 2002; Vasak and Hasler, 2000), which in excess amounts are highly toxic in CNS (Frederickson et al., 2004). By acting both as a donor and acceptor of Zn and Cu, MT-I+II may control Zn- and Cu-dependent proteins, enzymes, and transcription factors in the cells (Ghoshal and Jacob, 2001; Kelly and Palmiter, 1996; Kelly et al., 1996).

Moreover, leucocyte studies suggest that MT-I+II affect immune cells like monocytes and lymphocytes. Thus, MT-I+II induction in monocytes inhibit monocytic activation including cell adhesion, IL-1 accumulation, and the oxidative burst (Koropatnick and Zalups, 1997), while decreased MT-I+II levels enhance monocyte activation such as their adherence, invasion and the respiratory burst (Leibbrandt and Koropatnick, 1994; Leibbrandt et al., 1994). Moreover, MT-I+II inhibit macrophage-induced T cell proliferation (Youn et al., 1995) and immune responses of cytotoxic T cells (Youn and Lynes, 1999) and antigen-specific humoral responses (Canpolat and Lynes, 2001; Lynes et al., 1993).

In the 1980's, in vitro data showed that MT-I is an efficient ROS scavenger, which can inhibit ROS-induced cyto- and nuclear toxicity more effectively than proteins 10-50-times its molecular weight (Thornalley and Vasak, 1985). Other in vitro studies support the antioxidant effects of MT-I+II (Viarengo et al., 2000), which protect against ROS-induced DNA degradation with much higher molar efficiency than glutathione (Abel and de Ruiter, 1989; Maret, 1995; Miura et al., 1997), as well as MT-I+II may protect against ionizing radiation, toxic agents and electrophilic anticancer drugs (Aschner, 1997; Cai et al., 2004; Sato and Kondoh, 2002). In yeast, increased MT-I+II levels were suggested to functionally compensate for deficiency of Cu/Zn-super oxide dismutase (Cu/Zn-SOD) in the defense against oxidative stress (Tamai et al., 1993). Accordingly, studies of genetically modified cells showed that MT-I overexpression protects against oxidative stress and associated apoptotic cell death; and vice versa in MT-I+II deficient cell cultures (Klaassen et al., 1999; Kondo et al., 1997; Suzuki et al., 2000; Wanpen et al., 2004).

As presented here, it soon became clear that MT-I+II also inhibit oxidative stress and ROS-induced neuron death in vivo after brain injury and toxicity.

Even though thousands of MT-I+II studies were published within the first four decades after the discovery of MT-I+II in 1957, and although they described the MT-I+II structure, chemical characteristics, regulation, expression, distribution, degradation and the consequences of reducing or increasing MT-I+II in cells, the precise functions of MT-I+II were not clarified. Besides, genetic studies of MT-KO mice showed that during basal physiological (healthy) settings, MT-I+II are not essential as MT-KO mice develop and reproduce normally. Hence, the ubiquitous MT-I+II may not be essential for a normal healthy life, or a back-up system may exist to compensate for the absent MT-I+II. Still, in the 1990s the possible functions of MT-I+II during diseases and pathology were not established; and particularly, the in vivo significance of MT-I+II during brain disorders remained enigmatic.

Another appealing aspect was the evolution of MT-I+II: Why would evolutionary forces conserve multiple MT-I+II genes so well and favor their ubiquitous expression throughout species, if they did not provide selective advantages in life?

In order to elucidate the in vivo significance of MT-I+II in CNS disorders, we performed a multitude of neuropathological experiments, of which 8 publications were selected for this doctoral thesis. Some of these research data were the first to pinpoint the in vivo MT-I+II neuroprotective functions.

OBJECTIVE

The brain research presented here was initiated in 1993 in order to determine the MT-I+II expression and roles during CNS disorders.

The aim of this thesis is to bring together and review a series of data including the expression, functions and therapeutic relevance of MT-I+II during neuropathological conditions.

Accordingly, medical and scientific brain research conducted at the University of Copenhagen will be presented in its latest version, in order to be shared not only with scientific researchers and doctors, but with anybody taking an interest in brain research.

METHODOLOGY

ANIMALS

Rats

Neonatal (day 0) rat pups (I) were used to study MT-I+II expression in developing CNS after brain injury (cryogenic lesion in cerebral cortex). Similar studies were done in adult rats (Penkowa et al., 2001b).

Mice

Adult mice (II-VIII) were used in order to study cerebral expression, regulation and roles of MT-I+II during brain injury (cryogenic lesion in cortex) and 6-AN-induced toxicity in brain stem.

Both normal (wildtype) mice and genetically modified mice were used (II-VIII) as in parallel experiments (Carrasco et al., 2000b; Giralt et al., 2002a, b; Molinero et al., 2003; Penkowa et al., 1999a, 2003b, 2004).

The genetically modified mice included different genotypes, such as:

1. Mice with genetic IL-6 deficiency (IL-6 knock-out (IL-6KO) mice) (II)
2. Mice with astrocyte-targeted transgenic IL-6 overexpression (GFAP-IL6 mice) (III, V, VI).
3. Mice with genetic MT-I+II deficiency (MT-I+II knock-out (MT-KO) mice) (IV, V, VII).
4. Mice with transgenic, cerebral MT-I overexpression (TgMT mice) (VI, VIII).
5. Mice with simultaneous IL-6 overexpression and MT-I+II deficiency (GFAP-IL6/MT-KO mice) (V).
6. Mice with simultaneous IL-6 and MT-I overexpression (GFAP-IL6/TgMT mice) (VI).

Further details on their generation, genetic background, choice of wildtype controls, genotyping and characterization are described elsewhere (II-VIII; Campbell et al., 1993, 2001; Kopf et al., 1994; Masters et al., 1994; Michalska and Choo, 1993; Palmiter et al., 1993; Penkowa et al., 2001a, c, 2004).

EXPERIMENTAL PROCEDURES

Traumatic brain injury

Animals were subjected to a primary injury, which was produced by extracranial application of dry ice to the skull (I-VI). This results in a cryogenic (freeze) lesion in cerebral cortex with immediate necro-

sis and blood-brain barrier (BBB) disruption, which are followed by inflammatory responses. The model is a simple and reproducible method for creating a localized injury, which is easily identified as a cortical necrotic cavity (with no viable cells) surrounded by unlesioned parenchyma (Carrasco et al., 1999, 2003; Penkowa et al., 1999a, 2001b). KO mice with genetic deficiency and their wildtype controls could tolerate such lesioning for 50 seconds, while transgenic overexpressors endured 60 seconds and rats 90 seconds. Lesioned animals and unlesioned controls were simultaneously processed and fixed by transcardial perfusion by different time points such as 1-90 days post-lesioning (dpl), after which organs were removed for histopathological analyses (I-VI).

Brain stem toxicity induced by 6-AN

Mice were injected i.p. with niacin antagonist 6-AN (VII-VIII), which inhibits the hexose monophosphate pathway used preferentially by protoplasmic astrocytes as well as rapidly dividing cells like bone marrow cells (Krum, 1995, 1996; Penkowa and Hidalgo, 2000a). Hence, 6-AN induces acute astroglial toxicity, edema and cell death; which are seen mainly in the brain stem grey matter areas, whether or not these have projections outside the CNS (e.g. cranial nerve nuclei, pontine nuclei, oliva inferior nucleus, red nucleus). This 6-AN-induced primary damage is followed by inflammation, delayed neurodegeneration and clinical symptoms like motor impairments and paralyses (Penkowa et al., 1997, 2002). Mice were injected with 6-AN (10 mg/kg) (Sigma-Aldrich) and 1-3 days post-injection (dpi), these mice and their healthy controls were fixed by transcardial perfusion and organs were removed for histopathological analyses.

TISSUE PROCESSING

The CNS and immunologically relevant organs such as bone marrow and spleen were collected from the animals and processed for either paraffin embedding with microtome sectioning or cryo-sectioning (I-VIII). Sections were prepared for histochemistry, immunohistochemistry (IHC), in situ detection of DNA fragmentation, immunofluorescence and fluorescence histochemistry; which included antigen retrieval techniques, blocking of endogenous peroxidase and nonspecific background as well as specific blocking of endogenous mouse IgG (I-VIII; Carrasco et al., 1999; Giralt et al., 2002a, b; Penkowa et al., 2001a-c, 2003a-c).

MT-I+II EXPRESSION

Expression of MT-I+II was detected by using IHC and immunofluorescence. Hence, sections were incubated with rabbit anti-rat MT-I+II or mouse anti-horse MT-I+II (I-VIII), followed by biotinylated secondary antibodies and streptavidin-biotin-peroxidase complex and visualization by 3,3-diaminobenzidine-tetrahydrochloride (DAB) with H_2O_2 (Penkowa et al., 1997, 1999a,b, 2001a-c). Also, anti-MT-I+II antibodies were detected by immunofluorescence. Control sections were incubated in absence of primary or secondary antibody; and IHC for MT-I+II was also evaluated in MT-KO mice (IV, VII, VIII).

IL-6 IN MT-I+II REGULATION

The roles for IL-6 in MT-I+II regulation were studied by using IL-6KO (II; Penkowa et al., 1999a) and GFAP-IL6 (III, VI) genotypes along wildtype controls following the cryogenic lesion. The cerebral MT-I+II expression was detected by using IHC (as above) by 1-20 dpl. Also, in a blindly manner MT-I+II expressing cells were quantified from matched brain areas for statistical evaluation (see below). IHC for IL-6 was also performed and was absent in IL-6KO mice (II, III; Penkowa et al., 1999a).

MT-I+II ROLES DURING CNS PATHOLOGY

Genetic approach

In order to study the roles for MT-I+II during CNS disorders, the MT-KO (IV, V, VII) and TgMT (VI, VIII) genotypes were studied along their wildtype controls. Clinical symptoms and histopathological responses were examined and compared following brain injury and 6-AN-induced toxicity. The CNS histopathology was estimated by using various stainings for inflammatory responses, cytokines, growth factors and their receptors, neurotrophins, oxidative stress, antioxidants, neurodegeneration, cell death and repair mechanisms including glial scarring, angiogenesis and growth cones (IV-VIII). For this purpose, a range of different markers, antibodies and detection systems were applied (see list below).

To identify the roles of IL-6 versus MT-I+II, various genotypes were applied: MT-I+II deficient GFAP-IL6 (GFAP-IL6/MT-KO) mice; MT-I+II heterozygous GFAP-IL6 mice (GFAP-IL6-MT+/- mice); double-transgenic IL-6 and MT-I overexpressors (GFAP-IL6/TgMT mice) (V, VI; Giralt et al., 2002a; Molinero et al., 2003; Penkowa et al., 2004). Comparing GFAP-IL6 mice with either deficient, heterozygous, normal or increased MT-I+II expression can be used to separate the roles of IL-6 versus MT-I+II. Brain lesioned GFAP-IL6/TgMT mice along with TgMT and GFAP-IL6 genotypes were also included (VI). Since MT-I+II inhibit IL-6 mRNA and protein in the brain, TgMT mice display low IL-6 levels and thus provide a model for studying MT-I+II actions without IL-6 interference.

Medical approach

The potential therapeutic use of MT-I and MT-II was tested after having determined the optimum doses and treatment schedule. In a first set of experiments, freeze lesioned and 6-AN injected mice received MT-II treatment (rabbit native Zn-MT-II, Sigma-Aldrich) in a dose of 0.5 mg/kg/day i.p. The i.p. injected MT-II reaches the CNS extracellular space in 15-45 minutes by passing through disruptions in the BBB (Penkowa and Hidalgo, 2000b). The control treatments consisted of saline and ZnCl. The latter control is indispensable, as MT-II carry Zn, which could have biological effects *per se*. Rather surprisingly, the exogenous rabbit-derived MT-II induced the same effects during neuropathology in mice as those of endogenous (murine) MT-I overexpression (VI, VIII; Giralt et al., 2002b). Thus, to investigate if MT-I and MT-II from different mammalian and non-mammalian species could have effects in brain injured mice, series of lesioned wildtype and MT-KO mice along unlesioned mice were injected with MT-I or MT-II derived from different species. The mice received daily treatment (0.5 mg/kg/day i.p.) with human native MT-II (MT-IIa); human recombinant MT-II (MT-IIa); horse native MT-I; rabbit native MT-II; mouse recombinant MT-I; and drosophila recombinant MT from the day of lesioning and until sacrifice at 3 dpl. Unlesioned controls also received 3 days of treatment. Except from the drosophila MT, the proteins used were homologous mammalian MTs. The drosophila MT (MTN) contains 40 amino acids including 10 cysteine residues (Valls et al., 2000). MTN is not comparable to the mammalian MTs, and it has been suggested to represent a primeval form in MT-I+II evolution. All the MTs used were Zn-MTs with a half-life around 19-25 hours (Klaassen et al., 1999); however, the horse MT-I also contained small amounts of Cd, but it did not cause toxic effects in this short-term experiment. The human native MT-II was kindly provided by Professor Milan Vasak, Institute of Biochemistry at the University of Zurich. The recombinant MTs were kindly provided by Dr. Silvia Atrian, Department of Genetics at the Autonomous University of Barcelona. The rabbit MT-II and horse MT-I proteins were commercially available (Sigma-Aldrich). As an extra control to MT-I+II, the isoform MT-III/GIF was used (human recombinant MT-III/ GIF, 0.5 mg/kg/day i.p). MT-III/GIF was kindly provided by Professor Milan Vasak.

Staining methods and techniques

After brain injury and 6-AN-damage, a range of different staining methods and techniques were used to determine the actions of MT-I+II. These included histochemistry, IHC, immunofluorescence,

fluorescence histochemistry and in situ detection of DNA fragmentation (IV-VIII).

Histochemistry

Sections from MT-KO and TgMT genotypes (IV-VIII) were incubated with tomato lectin from the Lycopersicon Esculentum (a marker for resting and activated microglia, macrophages and blood vessels). Also, CNS sections were stained for Hematoxylin-Eosin (HE). Bone marrow and spleen histology was examined by toluidine blue and Schiff's stainings according to standard procedures.

IHC

The histopathology in MT-KO mice and MT-I overexpressors was studied by IHC (IV-VIII), which is performed routinely in our lab and the details have already been extensively described (Carrasco et al., 1999, 2000a,b, 2003; Ceballos et al., 2003; Espejo et al., 2001a,b, 2002; Giralt et al., 2001, 2002a,b; Molinero et al., 2003; Penkowa and Hidalgo, 2000a,b, 2001, 2003; Penkowa et al., 1997, 1999a,b, 2001a-c, 2002, 2003a-c, 2004). Thus, the IHC procedures are only outlined here in very short and refer to the Abbreviation List as well as the publications.

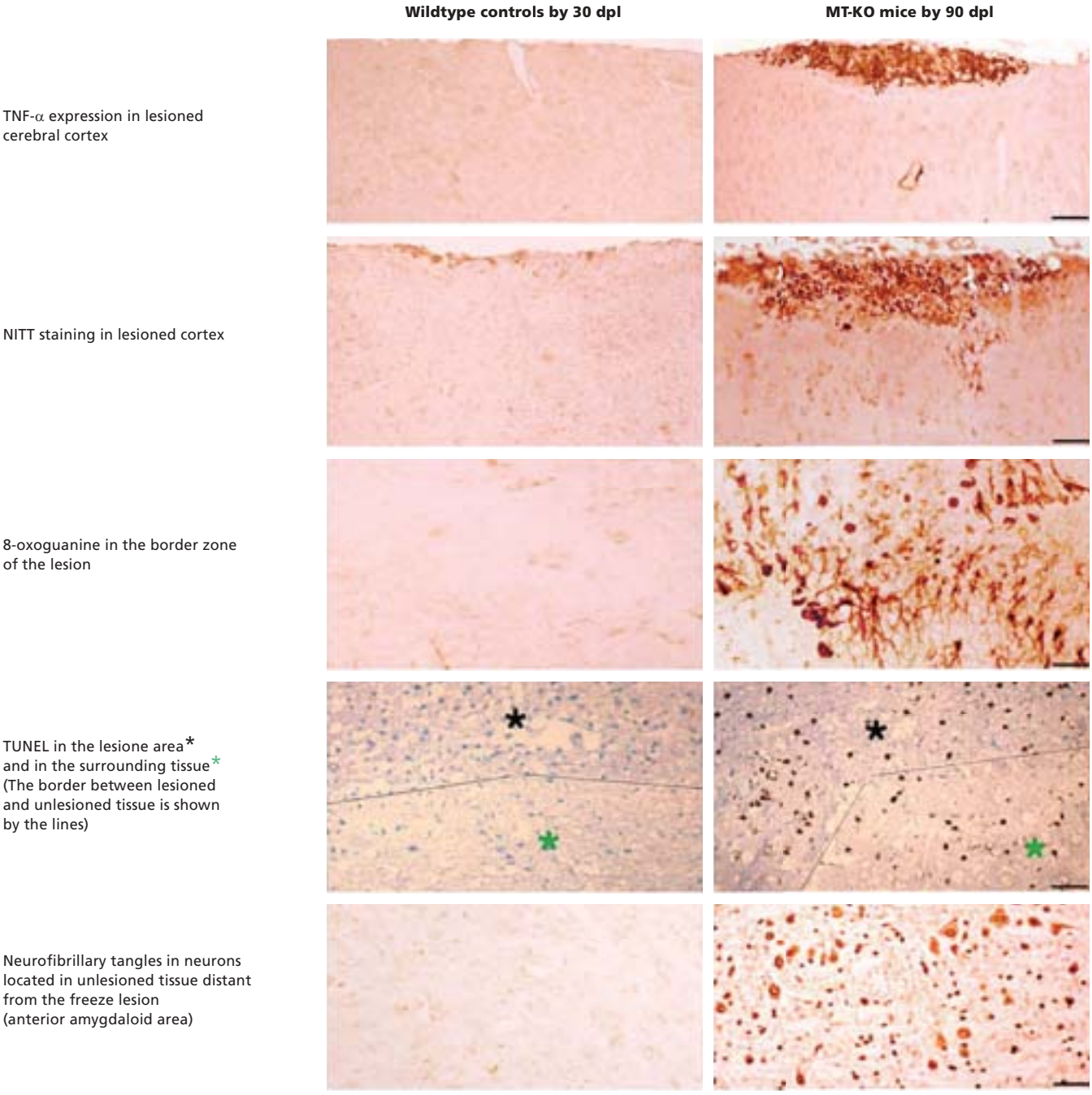


Figure 1. In the lesioned wildtype controls, TNF-α expression and the amounts of NITT, 8-oxoguanine and TUNEL have returned to basal levels by 30 dpl, where the lesion-induced histopathology in cerebral cortex basically had ended. After cryogenic injury, normal neurons were only found in unlesioned (intact) brain tissue distant from the lesioned area. These neurons were rarely containing neurofibrillary tangles in the wildtype mice. In MT-KO mice by 90 dpl, a manifest lesion wound with increased levels of TNF-α, oxidative stress (NITT and 8-oxoguanine) and TUNEL+ cell death could still be seen. TUNEL+ apoptosis was seen inside the lesion but also in the surrounding brain parenchyma of MT-KO mice. Also in distant (unlesioned) brain tissue, which is far away from the injured region and thereby theoretically intact (such as the amygdaloid area), MT-KO mice showed ongoing histopathology and neurodegeneration (intraneuronal neurofibrillary tangles) by 90 dpl.

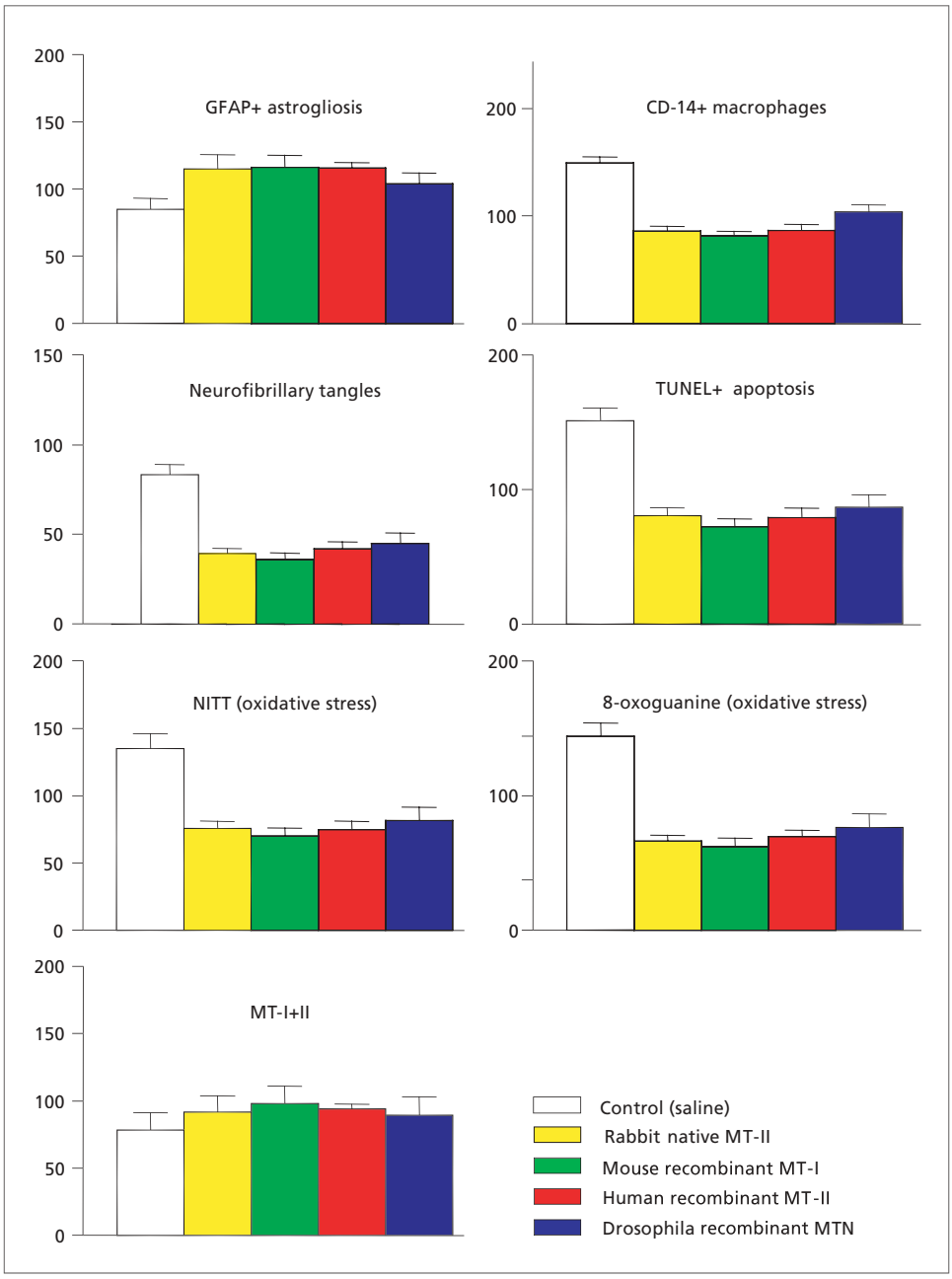


Figure 2. Lesioned wildtype mice by 3 dpl and responses to MT-I and MT-II treatment. Cell counts (mean ± SE) were carried out for GFAP+ astroglia, CD14+ macrophages, degenerating neurons (intraneuronal neurofibrillary tangles), TUNEL+ apoptotic cells, cells suffering oxidative stress (NITT and 8-oxoguanine) and cells containing MT-I+II. The number of macrophages and the levels of brain tissue damage (oxidative stress, neurodegeneration and cell death) increased after the brain injury, and such increases were reduced significantly by MT-I and MT-II treatment relative to placebo ($p<0.001$). The GFAP+ astrocytes were mildly stimulated by MT-I+II; however, this did not reach significance ($p=0.055$). MT-I and MT-II treatment did not significantly alter the number of cells with intracellular MT-I+II.

The primary antibodies were detecting:

CNS resident and recruited cells: NF and NSE (neuron-specific markers); GFAP (marks astroglia); F4/80 (marks all microglia/macrophages); CD14 (marks blood monocytes/macrophages); MOMA-1 (marks spleen, lymph node and blood monocytes/macrophages); MOMA-2 (marks all peripheral macrophages); CD34 (marks myeloid and lymphoid hematopoietic progenitor cells); CD3 (marker of T cells); CD4 (marks T-helper cells); CD8 (marks cytotoxic T cells); CD19 and CD20 (B cell markers); CD35 (marks bone marrow myelomonocytes).

Proinflammatory cytokines: IL-1β, IL-6, IL-12, TNF-α.

Haematopoietic factors: M-CSF, GM-CSF, GM-CSF-Receptor (GM-CSF-R), IL-3.

Oxidative stress: iNOS (inducible NO synthase), MDA (malondialdehyde), NITT (Nitrotyrosine), 8-oxoguanine.

Antioxidants: Cu/Zn-SOD, Mn-SOD, Catalase.

Neurodegeneration: Neurofibrillary Tangles, Amyloid Precursor Protein Frameshift Mutant (APP).

Apoptosis: Activated/cleaved caspase-3, caspase-1, cytoplasmic cytochrome-c, single stranded DNA (ssDNA).

Growth factors/neurotrophins, angiogenesis and repair: TGFβ, TGFβ-R, bFGF, bFGF-R, NT-3, NT-4/5, NGF, VEGF, GAP-43 (marks neuronal growth cones), P-40 (marks growth cones), angiopoietin-1/4 and CD34 (marks proliferating vascular endothelial cells besides the hematopoietic progenitors).

BBB properties: Albumin, EBA (EBA: Endothelial Barrier Antigen that marks intact BBB-endothelium).

The primary antibodies were detected by biotinylated secondary antibodies and streptavidin-biotin-peroxidase complex, followed by amplification by using tyramide and streptavidin-peroxidase complex (NEN, Life Science Products) and visualized by DAB as chromogen. Control sections were incubated in absence of primary or secondary antibody or after preabsorption of the primary antibody

with its corresponding antigen (I-VIII; Carrasco et al., 2003; Penkowa and Hidalgo, 2000a; Penkowa et al., 2001a-c, 2003a, 2004). Results were considered only if these controls were negative (as illustrated in V and VIII).

In situ detection of DNA fragmentation

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL) was performed as previously described, including negative and positive control sections (II-IV; Penkowa et al., 2001b,c). The results obtained by TUNEL were also judged by stainings for ssDNA and apoptotic signaling (caspases and mitochondrial leakage of cytochrome-c to the cytoplasm). The morphological criteria for apoptosis were also included (VI; Penkowa and Hidalgo, 2001; Penkowa et al., 2001a).

MT-I+II colocalization

By using single, double and triple fluorescence stainings (immunofluorescence and fluorescence histochemistry including fluorophore-linked TUNEL and fluorophore-linked lectin staining) as well as IHC in thin, consecutive sections, MT-I+II expression was colocalized to different cell types; as well as MT-I+II were spatiotemporally correlated to cytokine production; neurotoxicity; oxidative stress; neurodegeneration; apoptotic cell death; expression of growth factors/neurotrophins and their receptors, other antioxidants; angiogenesis; and astroglial scar tissue formation (I-VIII; Carrasco et al., 1999, 2000a,b; Espejo et al., 2001a,b, 2002; Giralt et al., 2001, 2002a,b; Molinero et al., 2003; Penkowa and Hidalgo, 2000a,b, 2001, 2003; Penkowa et al., 1999a,b, 2001a-c, 2002, 2003a-c).

Cell counts

Positively stained cells were counted in a blinded manner from matched CNS areas for statistical evaluation of the results. Positively stained cells were defined as those cells with cytoplasmic staining. TUNEL+ and ssDNA+ cells were those with nuclear staining. Representative identifications of positively versus negatively stained cells are shown in the publications (III: Figure 2; V: Figure 5). In case of traumatic brain injury (II-VI), cells were counted from both unlesioned hemispheres (contralateral cortex) and lesioned hemispheres (ipsilateral cortex). When counting from lesioned hemispheres, matched areas were chosen at the border zone of the deeper parts of the lesion in all the mice (shown in publication IV: Figures 1, 2 and 4). In the 6-AN experiments (VIII), cells were counted from unilateral brain stem at the border of the damaged grey matter areas (border of facial nucleus). Cell counts were performed in at least 2-3 sections per brain, and brains were taken from at least 3 different mice per group. A mean value for each animal is calculated and used for overall quantifications. Counts were always done blinded to animal identity.

Statistical analysis

The results obtained by cellular countings were evaluated by two-way analysis of variance ANOVA.

In the cryo-lesion studies (II-VI) the main factors were the genotype and freeze lesion. Separate sets of two-way ANOVA were carried out for the ipsilateral and contralateral hemispheres. When cryo-lesioned mice received medical treatment with MTs versus placebo, the main factors were freeze lesion and treatment. In the 6-AN studies (VIII), main factors were genotype and 6-AN-toxicity as well as MT-II treatment and 6-AN-toxicity. When only two groups were compared, the Student "t" test was used.

ETHICS

All the animals were kept under standardized conditions under constant temperature with free access to food and water ad libitum. Animal welfare was observed in compliance with the European Community regulations on this subject, and all animal experiments have adhered to the standards of the National Research Council's Guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering, keep animals pathogen-free and to reduce the number of animals used (Implementation of Refinement, Reduction, Replacement).

RESULTS

MT-I+II EXPRESSION IN CNS PATHOLOGY

MT-I+II expression in genetically normal animals

During physiological, healthy conditions of embryonic, neonatal and adult rats and wildtype mice, MT-I+II are expressed in low amounts in meninges, choroid plexus, ependymal cells, blood vessel endothelium and in a few scattered astrocytes (I-VIII; Penkowa et al., 1999b).

During CNS pathology such as traumatic brain injury and 6-AN-toxicity in brain stem, expression of MT-I+II increases significantly (I-VIII), and as shown elsewhere both MT-I+II mRNA and proteins increase (Giralt et al., 2002b; Penkowa et al., 1999a, 2004). After cryogenic brain injury in neonatal and adult rats (I; Penkowa et al., 2001b) and in wildtype mice (II-VI) increased MT-I+II expression is seen in reactive astroglia and activated microglia/macrophages. MT-I+II expression peaks by 3-10 dpl, by which inflammatory reactions are most pronounced. By 20-30 dpl, MT-I+II expression has returned to basic levels. However, the lesioned neonatals show a slightly different MT-I+II response relative to adults, because in neonatals, only reactive astrocytes increase MT-I+II; which transiently peak by 3 dpl, cease from 6 dpl and is absent by 14 dpl.

After 6-AN-toxicity in the brain stem, MT-I+II proteins are significantly increased by 1-3 days around the damaged grey matter areas (VII, VIII). The main cell source of increased MT-I+II after 6-AN is reactive astroglia, but a few activated microglia/macrophages also contain MT-I+II.

In general, endogenous MT-I+II expression is seen intracellularly in non-neuronal cells and is located in the cytoplasm and often also in the nucleus during neuropathology (I-VIII; Penkowa et al., 2001b,c, 2002). Thereby, significant amounts of extracellular MT-I+II in CNS are only detected following exogenous administration of MT-I+II (Penkowa and Hidalgo, 2000b).

MT-I+II expression in genetically modified mice

IL-6KO mice

Brain injured IL-6KO mice show reduced MT-I+II expression relative to wildtypes by 1-20 dpl (II). As previously shown, IL-6 deficiency decreases both MT-I+II mRNA and proteins in the contra- and ipsilateral hemisphere following the lesion (Penkowa et al., 1999a). Even if MT-I+II were radically reduced in IL-6KO mice versus wildtypes, the cell sources of MT-I+II were comparable as were the degree of primary injury (II). In all lesioned mice, the MT-I+II expression is seen in surviving cells whereas apoptotic markers and MT-I+II are not colocalizing. MT-I+II levels are also reduced in unlesioned IL-6KO mice relative to wildtype controls.

GFAP-IL6 mice

In both lesioned and unlesioned GFAP-IL6 genotypes, MT-I+II are significantly increased (III, VI). Hence, even in the normal, undamaged CNS, IL-6 overexpression increases MT-I+II. After the lesion, MT-I+II expression is significantly increased by 1-20 dpl in astrocytes and microglia/macrophages of GFAP-IL6 mice relative to wildtypes (III, VI). Moreover, the MT-I+II levels by 1-20 dpl are inversely related to the degree of secondary brain damage such as oxidative stress and apoptosis.

MT-KO and TgMT mice

Both healthy and brain damaged TgMT mice (VI, VIII) show increased MT-I+II throughout the CNS relative to wildtype controls, while the cellular sources of MT-I+II are similar in these genotypes. TgMT mice significantly increase their MT-I+II levels after pathol-

ogy relative to healthy control conditions. Thus, even by 20 dpl the lesioned TgMT mice show significantly increased MT-I+II, which in wildtypes have returned to normal levels by then. Along increased MT-I+II proteins, TgMT genotypes also show higher mRNA levels than wildtypes (Giralt et al., 2002b; Molinero et al., 2003). As expected, the MT-KO mice do not show positive MT-I+II immunostainings (IV, V, VII).

MT-I+II ROLES DURING CNS PATHOLOGY

MT-I+II roles during brain injury

The freeze lesion model in normal adults

The lesion produces a primary injury with immediate necrosis and BBB breakdown. By 1-6 dpl, genetically normal, adults (both rats and mice) show pronounced inflammatory responses at the lesion site, which is surrounded and invaded by reactive astrocytes, activated microglia/macrophages/monocytes and lymphocytes expressing proinflammatory cytokines; and alongside, oxidative stress and apoptotic cell death occur (II-VI; Penkowa et al., 1999a, 2001b). By 10 dpl, these responses have generally decreased; and after 20-30 dpl, normal adult animals usually have replaced the necrotic lesion cavity with a glial scar tissue, vessels and extracellular components. Thus, the freeze lesion is initially a necrotic cavity depleted of viable cells, which only appear in the surrounding, unlesioned (intact) tissue. After the lesion wound has been reorganized and substituted by glial scar tissue, it can still be easily identified, as it contains no normal neurons or neuronal cell bodies.

MT-I+II roles

The MT-I+II roles during brain injury were initially examined by using lesioned MT-KO and TgMT mice along wildtype controls (IV-VI). These experiments show consistently that MT-I+II protect the brain following injury. Hence, MT-I+II modulate inflammatory responses; decrease delayed (secondary) tissue damage (such as oxidative stress, neurodegeneration and apoptosis); and stimulate tissue repair mechanisms including glial scar formation and angiogenesis.

Accordingly, MT-I+II reduce activation and recruitment of microglia/macrophages/monocytes and lymphocytes including their expression of proinflammatory cytokines IL-1β, IL-6, TNF-α. MT-I+II reduce macrophages systemically, while lymphocyte recruitment is inhibited locally in the CNS (IV-VI; Giralt et al., 2002b; Penkowa and Hidalgo, 2000b). Also, MT-I+II decrease the number of hematopoietic progenitor cells recruited from bone marrow to the CNS. In addition, MT-I+II are antioxidant and antiapoptotic factors reducing oxidative stress and cell death after injury (IV-VI). Thus, MT-I+II counter cerebral levels of iNOS, protein nitration (NITT), lipid peroxidation (MDA), oxidative DNA alterations (8-oxoguanine), DNA fragmentation, caspase-1 levels, caspase-3 cleavage and mitochondrial leakage of cytochrome-c. Thereby oxidative stress and apoptosis after the lesion are significantly reduced in MT-I overexpressing mice and increased in MT-KO mice relative to the wildtype controls.

In lesioned wildtype controls, inflammation, oxidative stress and apoptosis are transient and have remitted by 20-30 dpl, by which

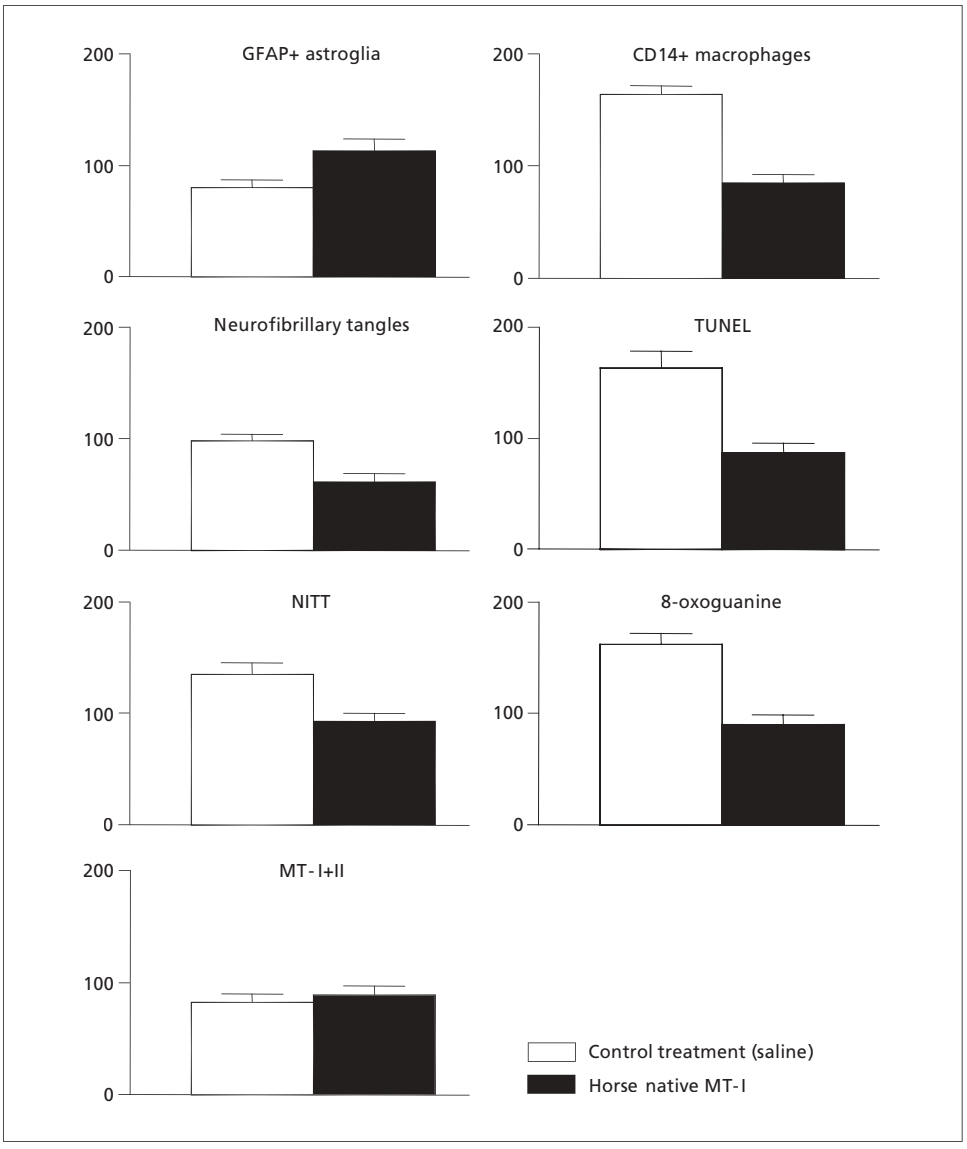


Figure 3. Lesioned wildtype mice by 3 dpl and treatment responses to MT-II versus controls. Cell counts (mean ± SE) were carried out for GFAP+ astroglia, CD14+ macrophages, degenerating neurons (showing intraneuronal neurofibrillary tangles), TUNEL+ apoptosis, oxidative stress (NITT and 8-oxoguanine) and cells containing MT-I+II. Treatment with horse MT-I affected significantly the responses to brain injury relative to control treatment (saline). Horse MT-I reduced post-lesional CD14+ macrophages ($p=0.0001$), NITT ($p=0.01$), 8-oxoguanine ($p<0.001$), neurofibrillary tangles ($p=0.004$) and TUNEL+ apoptosis ($p<0.002$). The horse MT-I also increased GFAP+ astrogliosis ($p=0.03$). However, horse MT-I treatment had no significant effects upon the number of cells with intracellular MT-I+II.

repair mechanisms have replaced the lesion cavity with a glial scar tissue flanked by increased levels of growth/trophic factors (bFGF, TGFβ?, NT-3-5, VEGF), and their receptors (TGFβ-R and bFGF-R), as well as increased angiogenesis and expression of angiopoietin-1/4 are seen. By 90 dpl, a more premorbid cytoarchitecture is seen in the scar tissue of wildtypes, although neuronal cell bodies remain absent. All 3 types of glia, neo-vascularization and extracellular matrix are seen in the scar.

In contrast, the MT-KO mice display pronounced inflammation and histopathology by 20-30 dpl and still after 60-90 dpl, the MT-I+II deficient brains show a manifest cortical lesion filled with activated immune cells (V); although, the lesion size is diminished by 60-90 dpl relative to 20-30 dpl. In this context, MT-KO mice display decreased astrogliosis, glial scar formation, angiogenesis and repair-associated molecules like bFGF, TGFβ, TGFβ-R, bFGF-R, VEGF, angiopoietin-1/4 and NT-3-5 relative to wildtypes. As shown here for the first time, the levels of proinflammatory cytokines, oxidative stress and cell death are still significantly increased by 90 dpl in MT-KO mice (Figure 1). Hence, by 60-90 dpl several inflammatory cells are expressing IL-1β, IL-12 and TNF-α at the lesion site of MT-KO mice, which also showed increased NITT, MDA and 8-oxoguanine levels along with numerous TUNEL+, caspase-3+ apoptotic cells inside and around the lesioned area. These responses of MT-KO mice were not only seen at the lesion site, but also in uninjured tissue like in striatum, thalamus and amygdaloid areas that are located far away from the lesion wound. Such uninjured brain tissue is quite easily distinguished from the once lesioned parenchyma, since the latter contains no normal neuronal cell bodies; as judged by neuron-specific stainings including neurodegeneration markers. The latter revealed that MT-I+II deficiency causes significant and prolonged neurodegeneration in uninjured brain tissue like thalamus, striatum and amygdaloid areas located far away from the lesion. This neurodegeneration was judged by intraneuronal occurrence of neurofibrillary tangles and APP, which are pronounced in MT-KO mice by 60-90 dpl, while the wildtype controls are devoid of neurodegeneration in uninjured areas (Figure 1).

All the data from lesioned MT-KO mice suggest that MT-I+II are protective during a brain injury, as MT-I+II deficient mice develop more severe and chronic brain damage with persistent neuron loss, as seen in both the directly injured (lesioned) cortex and in the distant, unlesioned tissue that was supposed to be intact.

Quite the opposite responses are seen in lesioned TgMT mice, which showed shorter and less intense histopathology and enhanced tissue restoration and wound healing (VI). The brain injured TgMT mice show reduced CNS inflammation with macrophages/microglia, lymphocytes and proinflammatory cytokines, and decreased oxidative stress levels (NITT, MDA and 8-oxoguanine) and apoptotic cell death (TUNEL and cleaved caspase-3) relative to wildtype

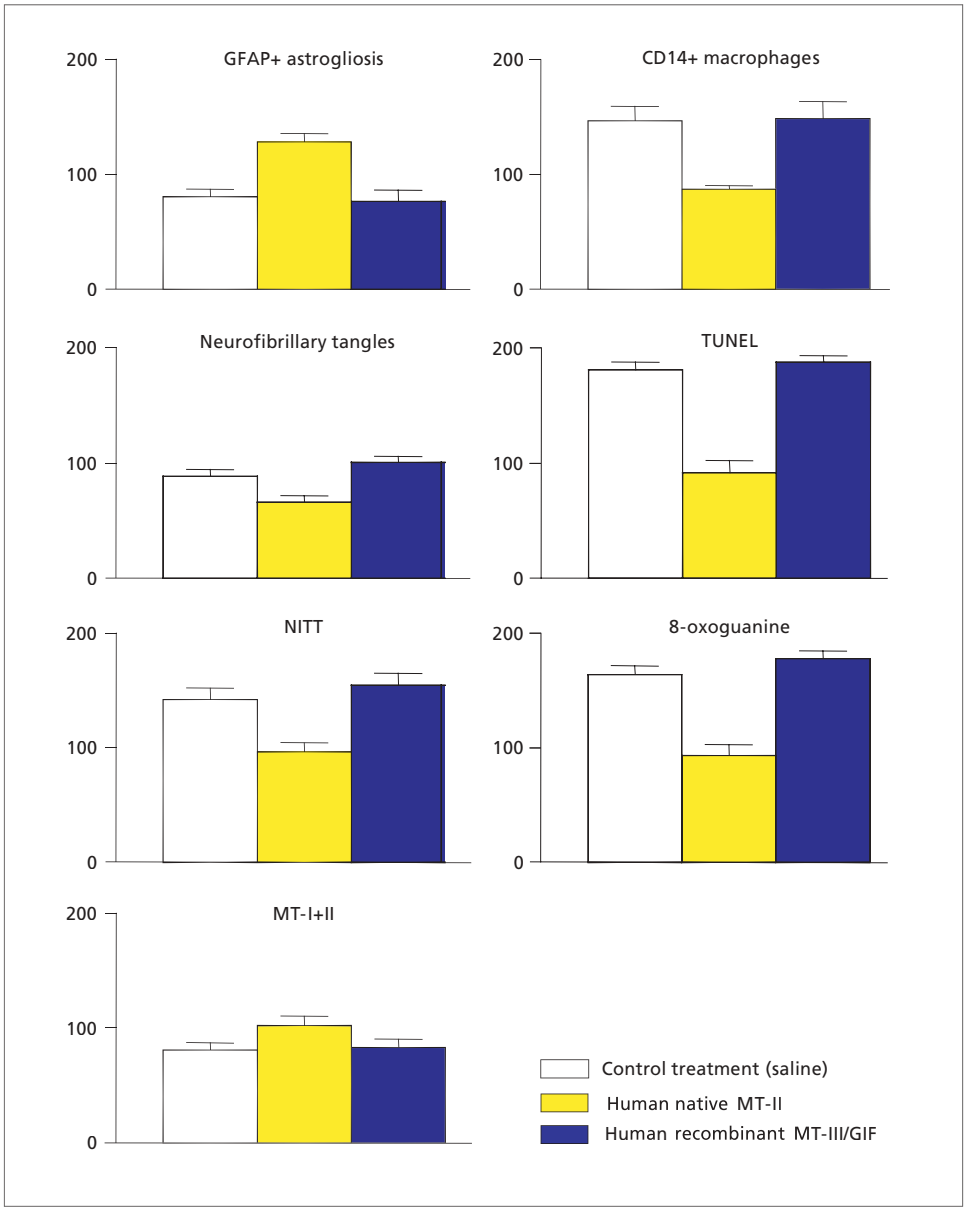


Figure 4. Lesioned wildtype mice by 3 dpl and treatment responses to MT-II versus controls. Cell counts (mean ± SE) were carried out for GFAP+ astroglia, CD14+ macrophages, degenerating neurons (with intraneuronal neurofibrillary tangles), TUNEL+ apoptotic cells, cells suffering oxidative stress (NITT and 8-oxoguanine) and MT-I+II containing cells. Treatment with human MT-II had significant effects relative to the two control treatments (consisting of saline or the MT isoform MT-III/GIF). Thus, human MT-II reduced CD14+ macrophages ($p = 0.004$), NITT ($p = 0.003$), 8-oxoguanine ($p < 0.0001$), neurofibrillary tangles ($p = 0.003$) and TUNEL+ apoptosis ($p < 0.0001$). Also, MT-II treatment increased GFAP+ astrocytosis ($p = 0.001$) after the lesion. However, human MT-II treatment did not significantly change the number of cells with intracellular MT-I+II.

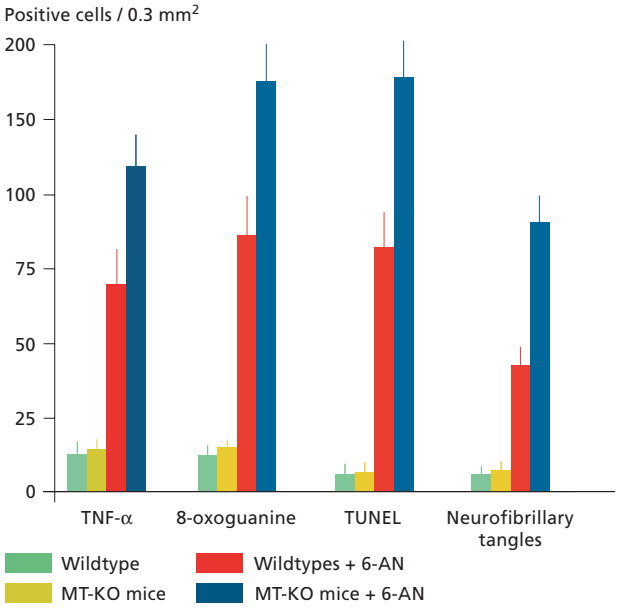


Figure 5. Cell counts (mean ± SE) were carried out for cells showing TNF-α, 8-oxoguanine, TUNEL and neurofibrillary tangles in brain stem of uninjected and 6-AN injected wildtype and MT-KO mice. By 3 days after 6-AN, both genotypes showed increased TNF-α expression, oxidative stress (8-oxoguanine), TUNEL+ apoptotic cells and degenerating neurons (with intraneuronal neurofibrillary tangles). However, these neuropathological responses were significantly increased in MT-KO mice relative to wildtypes. Results were evaluated with two-way ANOVA with 6-AN treatment and strain as main factors. Both were significant ($p<0.001$) for TNF-α, 8-oxoguanine, TUNEL and neurofibrillary tangles.

controls. Double and triple stainings revealed that neurons and glia suffer from oxidative stress and apoptotic death, and these cells are devoid of MT-I+II, which are confined to surviving cells (III-VI; Penkowa et al., 2003c).

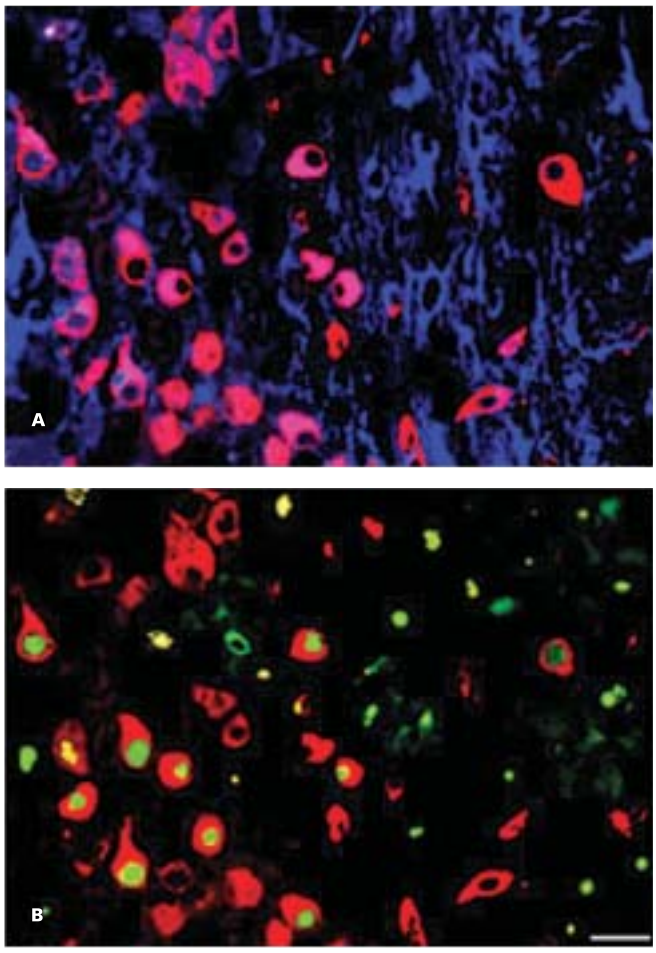
In addition, MT-I overexpression after brain injury stimulates reactive astrogliosis, expression of growth/trophic factors (bFGF, TGFβ?, NT-3-5, VEGF) and their receptors (TGFβ-R and bFGF-R), as well as TgMT mice show increased angiogenesis and levels of pro-angiogenic factors relative to controls. Accordingly, the TgMT mice show enhanced cortical wound healing and repair including astrogliotic scar formation and vascular remodeling relative to wildtype controls (VI). As judged by GAP-43 and P-40 stainings, MT-I overexpression also enhances growth cone formation and process outgrowths from surviving neurons situated outside the lesioned area. Initially, these growth cones and outgrowths are only seen surrounding the lesion; but after 10-20 dpl they have extended and are then seen at the borderzone of the injured tissue.

In this experiment (VI), lesioned wildtype mice show a slight delay in their wound healing relative to that seen in previous experiments (II, IV, V), which is due to variations in the lesioning procedures being of either 50 or 60 seconds. Consequently, wildtype controls lesioned for 50 seconds generally show a completed glial scar tissue by 20 dpl, while lesioning for 60 seconds would delay this till 30 dpl.

As shown (III-VI), both MT-I+II and IL-6 induce angiogenesis after brain injury. The angiogenic actions of MT-I+II are further supported by comparing various genotypes such as GFAP-IL6 mice; MT-I+II deficient GFAP-IL6 (GFAP-IL6/MT-KO) mice; MT-I+II heterozygous GFAP-IL6 mice (GFAP-IL6-MT+/- mice); double-transgenic IL-6 and MT-I overexpressors (GFAP-IL6/TgMT mice); and TgMT mice (V,VI). The IL-6 overexpressors are suitable for studying angiogenesis as they spontaneously develop proliferative angiopathy. This IL-6-induced angiogenesis is severely obstructed by simultaneous MT-I+II deficiency and enhanced by MT-I overproduction. Thus, the mice showing most brain injury-induced angiogenesis were the MT-I overexpressors, whether their IL-6 levels were low or high. As MT-I+II inhibit IL-6 mRNA and protein in CNS, the TgMT mice display low IL-6 expression and provide a model for studying MT-I+II actions without IL-6 interference. In fact, the TgMT mice show practically as much angiogenesis after brain injury as the double-transgenic GFAP-IL6/TgMT mice, which display maximum responses (VI). In agreement with this, the GFAP-IL6/MT-KO mice show drastically reduced angiogenesis relative to GFAP-IL6 mice (V).

Moreover, as demonstrated in genetically modified mice (II-V; Giralt et al., 2002b), both IL-6 and MT-I+II may have some neuroprotective functions. In order to separate the roles of IL-6 versus MT-I+II, double-transgenic GFAP-IL6/TgMT mice were freeze lesioned along with TgMT and GFAP-IL6 genotypes (VI). The results demonstrate that IL-6 exerts neuroprotection, but this is very likely due to IL-6-induced MT-I+II increases. Hence, the delayed damage occurring by 1-20 dpl was neither related to the neuroinflammation nor to IL-6 but instead to MT-I+II: The higher the MT-I+II levels, the less brain damage and cell death (VI). In this set-up, it is again relevant that TgMT mice have low IL-6 expression, as it allows examination of the MT-I+II cerebral roles without IL-6 interference.

Furthermore, our studies show that both endogenous (murine) MT-I overexpression and exogenous (rabbit-derived) MT-II treatment can induce rather identical CNS effects in mice (VI, VIII;



Scale bar: 40 μm (A, B).

Figure 6. Consecutive brain stem sections from MT-KO mice 3 days after 6-AN injection, in which oxidative stress, neurodegeneration and apoptotic cell death were co-localized. **A:** Shows double immunofluorescence for neurofibrillary tangles (red) and 8-oxoguanine (blue). Many degenerating neurons containing neurofibrillary tangles are also suffering oxidative stress (pink). Immunostaining for 8-oxoguanine is also seen in cells devoid of neurofibrillary tangles. **B:** Shows TUNEL (green) and neurofibrillary tangles (red). Neuronal apoptotic death and degeneration co-localized after 6-AN, as seen in cells with nuclear TUNEL and cytoplasmic neurofibrillary tangles. After 6-AN toxicity, apoptosis was also seen in other cells without neurofibrillary tangles.

Giralt et al., 2002b). Hence, TgMT and MT-II treated mice show comparable reductions in CNS inflammation, oxidative stress and cell death alongside similar increases in repair processes relative to controls. To study the functional similarity between different MT-I and MT-II proteins, we examined whether native or recombinant MT-I or MT-II derived from different species could induce neuroprotection in lesioned wildtype and MT-KO mice. The latter are suitable, as the effects of exogenous MT-I and MT-II can be identified without interference from endogenous MT-I+II expression. It is hereby revealed for the first time, that treatments with native or recombinant MT-I or MT-II proteins derived from various non-mammalian and mammalian species result in comparable and significant protective effects in lesioned mice relative to control (placebo) therapy (Figures 2-4).

First, lesioned mice were treated with recombinant human MT-II or mouse MT-I or drosophila MT or with native rabbit MT-II, which consistently induce neuroprotective effects by 3 dpl relative to control treatments.

Thus, MT-I and MT-II injections inhibit CNS recruitment of monocytes/macrophages and T cells; and reduce neurodegeneration, oxidative stress and apoptotic cell death after injury (Figure 2). As shown, these MT-I and MT-II proteins decrease significantly intraneuronal neurofibrillary tangles and the number of CNS cells containing NITT, 8-oxoguanine and TUNEL. Besides, the TUNEL+ apoptosis was confirmed by stainings for ssDNA, caspase-1 and -3 and cytoplasmic cytochrome-c; while degenerating neurons were also positive for APP (not shown). Also, MT-I and MT-II increase astrogliosis relative to placebo treatment. In subsequent experiments, freeze lesioned mice received treatment with native horse MT-I (Figure 3) or native human MT-II (Figure 4); which both induce similar therapeutic effects as those of recombinant human MT-II, mouse MT-I, drosophila MT and native rabbit MT-II.

Thus, both exogenous/extracellular and endogenous/intracellular MT-I+II induce significant protective effects after brain injury in wildtype and MT-KO mice. These neuroprotective actions are similarly induced by MT-I and MT-II proteins derived from different mammalian and non-mammalian species. In fact, even drosophila MT induces the characteristic (mammalian) MT-I+II-actions after brain injury. This indicates independence of species of origin as far as the biological functions of MT-I+II are concerned, at least this is the case in mice, and it suggests that MT-I+II could have rather universal actions during tissue damage.

The observed treatment effects of MT-I and MT-II are similar in lesioned wildtypes versus MT-KO mice, although the latter in general develop more severe histopathology and neuronal damage, which is a response pattern of MT-KO mice that is already described (IV, V; Giralt et al., 2002b). Interestingly, the MT-I+II treatment effects could not be induced by another member of the MT family, the isoform MT-III/GIF (Figure 4), which resembles MT-I+II structurally and was used as a further control against MT-I+II. Hence, when freeze lesioned mice received MT-III/GIF treatment, no significant effects were detected upon gliosis, macrophages, lymphocytes, oxidative stress, degeneration or apoptotic cell death. Thus, MT-III/GIF is likely to have different functions than MT-I+II during brain injury, which is in agreement with other studies (Carrasco et al., 2003).

No significant treatment effects of the exogenous MTs are detected in controls without cerebral pathology. After the freeze lesion, the i.p. injected MT-I and MT-II proteins enter the CNS only because the BBB is disrupted; which is in agreement with other MT-II treatment experiments (VIII; Penkowa and Hidalgo, 2000b). Hence, MT-I+II are increased in the extracellular space of the lesioned brain within 15-45 minutes after the i.p. injection and after 6-8 hours, MT-I+II are seen intracellularly in CNS cells.

This thesis shows for the first time that medical treatment with native or recombinant MT-I or MT-II after a brain injury induces comparable and significant neuroprotective effects that are functionally independent of species of origin.

MT-I+II roles in brain stem toxicity

The 6-AN model

6-AN induces acute (within 24h) astroglial toxicity and cell death in grey matter areas of the brain stem whether or not these have projections outside the CNS (VII, VIII; Penkowa and Hidalgo, 2000a; Penkowa et al., 1997, 2002). For instance, 6-AN depletes astroglia in the facial nucleus, pontine reticular nucleus, cuneate nucleus, hypoglossal nucleus, sensory and motor trigeminal nucleus, red nucleus, substantia nigra, and vestibular nuclei, oliva inferior nucleus, para- and periolivary nuclei. After 3 dpi, reactive astrocytes surround the damaged areas that are infiltrated by macrophages and lymphocytes expressing proinflammatory cytokines and alongside the BBB is disrupted. Also, secondary (delayed) damage is seen after 3 days including oxidative stress, neurodegeneration and apoptosis (VIII; Penkowa et al., 2003a, 2004).

MT-I+II roles

The roles for MT-I+II during 6-AN-induced toxicity were at first studied by using MT-KO mice (VII) and afterwards, TgMT and MT-II treated mice were applied (VIII).

The acute (primary) brain stem toxicity induced directly by 6-AN is comparable in MT-KO and wildtype mice as seen by 1 dpi, where the mice also show similar neurological symptoms. After 3 days, 6-AN injected MT-KO mice display reduced reactive astrogliosis around the damaged areas relative to wildtypes. Also, activation and recruitment of microglia/macrophages and lymphocytes are clearly decreased in MT-KO mice; but this is very likely an epiphenomenon resulting from enhanced 6-AN-induced bone marrow depression in these mice. Thus, in MT-KO mice 6-AN causes more severe suppression of haematopoietic cells such as myeloid and lymphoid progenitor cells, myelomonocytes and macrophages, whereby the CNS infiltration by these cells is hampered (VII). The bone marrow depression is determined by using lectin, toluidine blue and Schiff's stainings, and IHC for CD34, CD35, F4/80, CD14, IL-6, MOMA-1 and -2. The haematopoietic factors IL-3, M-CSF, GM-CSF and the GM-CSF-R are also reduced in both brain stem and bone marrow of MT-KO mice relative to wildtypes after 6-AN.

This thesis shows for the first time that despite a reduced brain stem inflammation, 6-AN injected MT-KO mice suffer from enhanced secondary CNS damage (Figures 5 and 6). Hence, by 3 dpi MT-KO mice display increased oxidative stress (NITT, MDA and 8-oxoguanine), neurodegeneration (intraneuronal neurofibrillary tangles) and apoptosis in the brain stem, when compared to those of wildtypes (Figure 5). In general, the degenerating neurons as well as some astrocytes by 3 dpi are suffering from simultaneous oxidative stress and apoptotic cell death (Figure 6); responses that are increased in MT-KO mice relative to wildtypes.

Furthermore, both exogenous and endogenous MT-I+II induce immunomodulatory and neuroprotective actions after 6-AN, as seen in TgMT and MT-II treated mice (VIII).

The 6-AN-induced brain stem toxicity raises mortality in mice receiving placebo treatment but not in mice treated with exogenous MT-II. However, in the experiment with TgMT and wildtype mice, no changes in mortality are detected after 6-AN. The 6-AN-induced motor impairments are comparable in all mice.

In control groups (wildtype mice +/- placebo treatment) the primary 6-AN-toxicity is pronounced in brain stem grey matter areas, which show reduced numbers of viable cells as judged by HE stainings. In TgMT genotypes and MT-II treated mice, brain stem grey matter areas show diminutive 6-AN-toxicity relative to controls. Thus, the grey matter astrocytes of TgMT and MT-II treated mice are rather unaffected by 6-AN, and alongside, the reactive astrogliosis is increased in these mice by 3 dpi. Subsequent to 6-AN, TgMT and MT-II treated mice also display increased expression of growth/trophic factors (TGFβ, bFGF, VEGF, NGF, NT-3-5) and formation of neuroglial growth cones (GAP-43 and P-40 stainings). Along this, MT-I overexpression and MT-II treatment reduce the in-

flammatory responses of microglia/macrophages/monocytes, T and B lymphocytes and expression of IL-1β, IL-6, IL-12 and TNF-α relative to controls (VIII; Penkowa et al., 2004). This MT-I+II-inhibition of CNS macrophages during 6-AN-damage may be due to diminished recruitment of peripheral haematogenous cells, which in the controls are the main source of brain macrophages after 6-AN.

By 3 dpi, oxidative stress (iNOS, MDA, NITT and 8-oxoguanine stainings), neurodegeneration (intraneuronal neurofibrillary tangles and APP) and apoptotic cell death (TUNEL, caspases, cytochrome-c and ssDNA) are significantly reduced by MT-I overexpression and MT-II treatment relative to controls (VIII; Penkowa et al., 2004). Oxidative stress and apoptosis are observed in most cell types, but primarily in astroglia and neurons located inside and closely around the grey matter areas.

If mice are not injected with 6-AN, no clinical or histological differences can be detected between the different genotypes or treatment groups, except from the MT-I+II expression levels (VII, VIII).

IMPLICATIONS AND PERSPECTIVE

MT-I+II expression increases significantly in response to brain damage, during which IL-6 is a major inducer of MT-I+II mRNA and proteins (I-VIII; Penkowa and Hidalgo, 2000a; Penkowa et al., 1999a, 2001c, 2003a). The IL-6 mechanisms involve signal transducers and activators of transcription (STAT-1 and -3) that induce transcription by binding to the MT-I promoter (Haq et al., 2003; Lee et al., 1999); however, IL-6 also affects post-transcriptional processes (Miles et al., 2000).

This thesis demonstrates that in CNS, MT-I+II have immunomodulatory effects reducing inflammatory cells (microglia/macrophages/monocytes, T and B lymphocytes and haematopoietic precursers) and expression of proinflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α. This is consistently observed during CNS pathology in different genotypes and treatment groups. However, as 6-AN-injected MT-KO mice show severe bone marrow toxicity and haematopoietic depression, the pool of leucocytes in these mice is depleted leading to impaired recruitment of inflammatory cells to the CNS. The thesis also shows that MT-I+II inhibit delayed (secondary) tissue damage such as oxidative stress, degeneration and apoptotic cell death after brain injury and 6-AN-toxicity (IV-VIII). Exogenous MT-II treatment also reduces the primary (acute) CNS damage induced directly by 6-AN and improve the mortality. During the brain injury and 6-AN-toxicity, MT-I+II also increase astrogliosis, expression of growth/trophic molecules (TGFβ, TGFβ-R, bFGF, bFGF-R, VEGF, NT-3-5 and NGF) and cerebral repair including glial scar formation, angiogenesis and neuronal growth cones.

The MT-I+II angiogenic roles in cerebral repair are validated by using various genetically modified mice such as GFAP-IL6 mice with either deficient, heterozygous, normal or increased MT-I+II expression, by which the roles of IL-6 versus MT-I+II can be separated. The IL-6 overexpression results in spontaneous cerebral angiogenesis, which is severely inhibited by MT-I+II deficiency and moderately inhibited by MT-I+II heterozygosity, while MT-I overexpression enhances the angiogenesis. In fact, MT-I+II exert significant pro-angiogenic functions in CNS, since TgMT mice that have low IL-6 levels show practically as much angiogenesis as the GFAP-IL6/TgMT mice, which show the maximum responses. The angiogenic actions of MT-I+II can contribute to the improved brain tissue recovery seen in mice with increased MT-I+II (IV-VI; Giralt et al., 2002b; Penkowa, 2002). Recently, independent researchers have confirmed and reproduced some of these data (Chung and West, 2004; Chung et al., 2003; Swartz et al., 2001). For instance, Chung et al. (2003) confirmed that MT-II treatment induces wound healing and brain repair after a traumatic injury.

As shown here, the freeze lesioned wildtype mice have replaced the necrotic lesion cavity with an astrogliotic scar tissue by either 20 or 30 dpl, depending on the lesioning procedures being of either 50 or 60 seconds. Thus, wildtypes lesioned for 50 seconds generally show a completed glial scar tissue by 20 dpl, while lesioning for 60 seconds would delay this till 30 dpl.

Furthermore, the cerebral actions of MT-I+II can be equally induced by endogenous MT-I overexpression and exogenous treatment with various MT-I and MT-II proteins, which induced comparable effects independent of the species of origin. Accordingly, native or recombinant MT-I and MT-II proteins derived from different mammalian and non-mammalian species induce analogous immunomodulatory, antioxidant, antidegenerative and antiapoptotic effects in brain injured mice. This MT-I+II independence of species of origin is described for the first time in this doctoral thesis, and it indicates that the evolutionary highly conserved MT-I+II proteins have rather universal roles during tissue injury. In line with this, both human MT-I and yeast MT can be inserted in bacterial cells (*Escherichia coli*), which then express these eukaryotic MTs as full-length proteins without instability or degradation (Sousa et al., 1998). The same cell culture study shows that human and yeast MT proteins maintain their ability to bind metals after they are inserted in *Escherichia coli*. Likewise, the in vitro ROS scavenging properties of mollusc (oyster) MT are comparable to those of mammal MT-I+II obtained from rabbits (Anderson et al., 1999). Thus, MT-I+II proteins appear to function uniformly without phylogenetic constraints or dependency on species of origin.

The immunoregulatory, antioxidant and neuroprotective functions of MT-I+II shown here could provide new therapeutic strategies to ameliorate CNS disorders. Even though immunological activation is a normal host defense reaction, inflammatory responses in the immune-privileged brain may ultimately have detrimental actions. CNS inflammation is a main feature of most acute and chronic neurological diseases; during which sustained inflammation may contribute directly to neuronal dysfunction and death. Thus, high levels of IL-1β, IL-6, IL-12 and TNF-α induce spontaneous inflammation, demyelination and neuron loss; and these cytokines are involved in various autoimmune, demyelinating and neurodegenerative brain diseases (Allan and Rothwell, 2003; Villoslada and Genain, 2004; Wang et al., 2002). In addition, inflammation results in formation of ROS that oxidize essential cellular components such as DNA, proteins and lipids (Floyd et al., 1999; Mhatre et al., 2004); and such oxidative stress is a major inducer of neuronal degeneration, dysfunction and cell loss (Barnham et al., 2004; Rego and Oliveira, 2003). Consequently, the MT-I+II-inhibition of proinflammatory responses and ROS can counteract neuronal degeneration and cell death. In agreement with this, endogenous and exogenous MT-I+II inhibit CNS histopathology and clinical symptoms during brain autoimmune disorders as shown in animals with EAE and human tissue from MS patients (Penkowa and Hidalgo, 2000b, 2001, 2003; Penkowa et al., 2001a, 2003b,c). Hence, MT-I+II significantly impede EAE-mediated macrophages, lymphocytes, proinflammatory cytokines, demyelination and axonal transection as well as MT-I+II improve the EAE tissue recovery, clinical symptoms and mortality.

Besides the antiinflammatory actions in CNS, MT-I+II are shown to affect the peripheral immune cells like blood and bone marrow monocytes/macrophages, lymphocytes, their bone marrow progenitors and haematopoietic factors (IL-3, M-CSF, GM-CSF and GM-CSF-R). This is supported by other data showing that MT-KO mice are 10 times more vulnerable to anemia (reduced erythrocytes, hemoglobin and hematocrit) relative to wildtypes (Liu et al., 1999). The same study shows that MT-KO mice are less resistant than wildtypes to changes in peripheral leucocytes, cytokine levels, splenic and thymic pathology.

Accordingly, MT-I+II may have immunomodulatory functions during peripheral diseases. Consistent with this, MT-I+II exert immunosuppression during experimential rheumatoid arthritis (Youn et al., 2002). Thus, MT-I+II treatment reduces the disease incidence and severity as well as lymphocyte activation and expression of

proinflammatory TNF-α and cyclooxygenase-2; whereas anti-inflammatory TGFβ is increased by MT-I+II. Moreover, human patients with autoimmune diseases including rheumatoid arthritis show depletion of MT-I+II in serum as their disease aggravates; after which MT-I+II levels can be fully replenished by cortisone administration, which is followed by significant clinical improvement (Miesel and Zuber, 1993). Supporting this, human patients with autoimmunity and Group III allergy show (auto)antibodies to MT-I+II (Jin et al., 2003), indicating that human immunological dysregulation may be related to MT-I+II malfunctioning or depletion. In that case, administration of effective MT-I+II inducers may result in clinical improvement. In fact, steroid-induced immunosuppression could be mediated by steroid-induced MT-I+II, given that glucocorticoid-treated humans show significant MT-II increases in peripheral lymphocytes concomitant with treatment effects (Knutsson et al., 1995). In vitro, dexamethasone-induced MT-II expression can be used as an indicator of glucocorticoid sensitivity (Bronnegard et al., 1991), which may also be relevant during CNS damage, as cerebral MT-I+II are also induced by glucocorticoids (Hidalgo et al., 1997a,b). If a direct relation exists between steroid-induced therapeutic effects and MT-I+II levels, the latter might be used as a prognostic marker and/or an indicator of the patient responsiveness to treatment.

Although the molecular mechanisms of action are elusive, many studies have demonstrated that MT-I+II are directly involved in activation of monocytes/macrophages, T and B lymphocytes. In monocytes, reduced MT-I+II levels lead to increased ROS production, cell adherence and invasiveness, while increased MT-I+II levels inhibit this activation (Koropatnick and Zalups, 1997; Leibbrandt and Koropatnick, 1994; Leibbrandt et al., 1994). MT-I+II also reduce antigen-specific humoral responses, immunoglobulin levels in the circulation and lymphoproliferative responses (Crowthers et al., 2000). Exogenous MT-I+II bind specifically to the cell membranes of macrophages, T and B cells leading to reduced activity of these cells (Canpolat and Lynes, 2001; Lynes et al., 1993; Youn and Lynes, 1999; Youn et al., 1995), which indicates that MT-I+II can inhibit inflammation by interfering with cell-cell interactions.

MT-I+II could also be important for proper functioning of phagocytes. Hence, removal of the lesion necrosis and debris and subsequent wound healing are impaired in MT-KO mice, although they display increased macrophage numbers at the lesion site. In support of this, MT-I+II deficient macrophages show several functional defects including impaired phagocytosis and antigen-presentation (Sugiura et al., 2004). Thus, it is likely that on one side, MT-I+II inhibit the macrophage numbers, but on the other hand MT-I+II may stimulate phagocytosis and macrophage-induced resolution of inflammation.

In addition, MT-I+II significantly alter the cerebral expression of several pro- and antiinflammatory factors that also may contribute to the observed immunoregulation by MT-I+II (for further discussion see: V-VIII; Penkowa et al., 2003b, 2004).

Accordingly, MT-I+II support the CNS immune privilege, even if the precise MT-I+II mechanisms are still not fully elucidated. To address this, we are currently investigating MT-I+II signaling and transduction in neuronal and glial cell cultures. Also, the DNA-binding abilities of MT-I+II will be investigated in order to determine if MT-I+II might be involved in gene activation or interact with transcription factors.

Furthermore, this thesis shows that MT-I+II protect the brain tissue by exerting in vivo antioxidant and antiapoptotic functions. MT-I+II are directly involved in ROS scavenging and besides, MT-I+II exert antioxidant activity due to redox capabilities, sulfhydryl nucleophilicity, sequestration of redox sensitive transition metals (Sato and Kondoh, 2002; Viarengo et al., 2000). MT-I+II scavenge ROS more effectively than proteins 10-50 times its molecular weight (Thornalley and Vasak, 1985) and have higher antioxidative capacity inhibiting ROS-induced DNA degradation than other common antioxidants, for instance glutathione (Abel and De Ruiter, 1989; Cai and Cherian, 2003). This thesis presents some of the first in vivo studies showing MT-I+II-inhibition of CNS oxidative stress and apoptosis after brain injury and 6-AN-toxicity. Apoptotic cells are generally devoid of MT-I+II that are confined to surviving cells, indicating that MT-I+II may alter the pro-apoptotic signaling inside cells. As shown here, MT-I+II inhibited pro-apoptotic signals (caspases and mitochondrial leakage of cytochrome-c) along with DNA fragmentation. Also, MT-I+II affects NFκB levels and cellular localization after brain injury (Penkowa et al., 2001b) and excitotoxicity (Carrasco et al., 2000b). By specific interaction with the p50 subunit of NFκB, MT-I+II transactivate NFkB and are involved in its DNA-binding; and this has been suggested as possible mechanisms of MT-I+II actions (Abdel-Mageed and Agrawal, 1998; Butcher et al., 2004). Also, MT-I+II interact with proto-oncogenes and tumor suppressor protein p53 that are critical in the steps regulating apoptosis (Abdel-Mageed and Agrawal, 1997; Kondo et al., 1997). MT-I+II could accomplish such roles in the cell nucleus, as karyophilic MT-I+II can sequester and release Zn leading to either de- or increased Zn-dependent gene expression and activity of zinc finger-containing molecules (Coyle et al., 2002; Ghoshal and Jacob, 2001; Ogra and Suzuki, 2000). Due to these roles in cell survival and death, it is not surprising that MT-I+II have been associated with malignancies. By potent cytoprotective actions, MT-I+II might endow certain tumors with resistance to radio- and chemotherapy. However, in some tissues, MT-I+II are correlated with less malignancy and better outcome (Simpkins, 2000; Theocharis et al., 2004) and thus, it remains to be elucidated if MT-I+II may truly influence carcinogenesis.

The significance of MT-I+II in CNS cell death is evident in the MT-KO mice, which continue to show increased neurodegeneration and apoptosis by 60-90 days after brain injury. In contrast, wildtype mice have replaced the necrotic lesion cavity with a glial scar by 20-30 dpl; and TgMT mice resolve the necrotic wound some days earlier (IV-VI; Giralt et al., 2002b). Other researchers have independently confirmed these protective actions of MT-I+II after brain injury (Chung et al., 2003).

MT-I+II also inhibit neuronal damage during many other pathological conditions like epileptic seizures, EAE, nerve crush and inflammatory encephalopathies (Ceballos et al., 2003; Hidalgo et al., 2002; Penkowa, 2002), and other groups have added that MT-I+II protect against ischemia and motor neuron disease. After focal cerebral ischemia MT-KO mice develop larger infarcts, more brain edema, higher mortality and worse neurological outcome relative to controls (Trendelenburg et al., 2002); whereas the TgMT mice show entirely opposite responses (Van Lookeren Campagne et al., 1999). In addition, MT-I+II deficiency deteriorates familial ALS in mice by advancing clinical disease and brain cell death (Nagano et al., 2001); and dopaminergic neurotoxicity in substantia nigra is enhanced in MT-KO mice relative to wildtypes (Asanuma et al., 2002). Moreover, endogenous and exogenous MT-I+II directly inhibit dopaminergic and hippocampal neuron death (Køhler et al., 2003; Sharma and Ebadi, 2003); as well as MT-I+II counter neurotoxicity after intake of recreational drugs like ecstasy and ethanol (Aschner, 1998; Xie et al., 2004).

The actual mechanisms through which MT-I+II inhibit cell death and increase CNS recovery remain elusive, but many possibilities are likely. First, the MT-I+II antioxidant and antiinflammatory effects could reduce CNS apoptosis. Besides MT-I+II stimulate astrocytes, which are important for maintaining a normal CNS microenvironment. Astrocytes endow the brain with antioxidants, energy substrates and trophic/growth factors as well as they promote neurogenesis, neuron survival and regeneration (Liberto et al., 2004; Takuma et al., 2004). Hence, ablation of astroglia during CNS injury results in extensive degeneration, demyelination, cell death, clinical dysfunction and inflammatory activation of leucocytes (Faulkner et al., 2004). Moreover, the MT-I+II-induced increases in growth factors, their receptors and neurotrophins (TGFβ, TGFβ-R, bFGF,

bFGF-R, VEGF, NT-3-5 and NGF) as well as the pro-angiogenic actions of MT-I+II can contribute to improved CNS cell survival and tissue recovery (Hidalgo et al., 2001; Penkowa, 2002). Furthermore, MT-I+II could inhibit CNS cell loss by being essential for the tissue regulation of metal ions, which in exceeding amounts are neurotoxic (Frederickson et al., 2004). An abnormal Zn or Cu homeostasis in the CNS or disrupted metal-binding proteins may cause oxidative stress and neuronal death (Barnham et al., 2004). Hence, the MT-I+II regulation of metals in the normal and injured CNS is likely to protect the cerebral tissue.

According to this, MT-I+II may protect the brain through a variety of different pathways and actions.

The MT-I+II neuroprotective functions can be induced by two separate mechanisms. One possibility is that MT-I+II influence neurons indirectly through effects upon astroglia, inflammatory reactions including cytokines, growth factors/neurotrophins and oxidative stress. On the other hand, MT-I+II have direct effects on CNS neurons (Køhler et al., 2003). In primary, dopaminergic and hippocampal neuron cultures devoid of immune and glial cells, MT-II treatment induces neuronal differentiation, growth and survival. Likewise, in vitro studies show that MT-II treatment promotes CNS neurite elongation, sprouting and postinjury axonal growth (Chung et al., 2003) as well as MT-I+II increase nerve growth and regeneration (Ceballos et al., 2003). This is intriguing, since MT-I+II are almost never detected inside neurons but primarily in non-neuronal glia. This indicates that neuronal injury is followed by MT-I+II increases in neighbouring glial cells, which could then release MT-I+II that may induce neuroprotection and improved outcome through immunomodulation and antioxidation as well as by direct neuronal actions. Indeed, MT-I+II may have extracellular roles since the protective effects seen after brain pathology could be induced by both endogenous/intracellular MT-I+II and exogenous/extracellular MT-I or MT-II treatment. This needs further attention, as the dogma otherwise states that MT-I+II are intracellular proteins. However, it is currently unknown whether MT-I+II function through binding to a specific cell surface receptor or via intracellular molecules. Even though, this thesis is first to show that in the injured brain, exogenous MT-I and MT-II exert comparable in vivo functions that are independent of species of origin.

As shown here, IL-6 also induces neuroprotection and brain tissue repair after the freeze injury, and these actions are quite similar to those induced by MT-I+II. Studying various genotypes with altered IL-6 and MT-I+II genes (as described above), it has become clear that MT-I+II are major neuroprotective factors in the injured CNS (II-VIII; Giralt et al., 2002a; Molinero et al., 2003; Penkowa et al., 2003a, 2004). Although GFAP-IL6 mice show decreased neuropathology relative to wildtypes, such effects could be mediated by IL-6-induced MT-I+II increases. Thus, inflammation, neuronal degeneration and cell death are inversely associated with MT-I+II levels and not with IL-6. In support of this, mice with transgenic MT-I overproduction (TgMT and GFAP-IL6/TgMT mice) show diminutive CNS histopathology (VI; Molinero et al., 2003) and are protected against the primary damage induced directly by 6-AN (VIII; Penkowa et al., 2004), even though the TgMT mice show low IL-6 mRNA and protein expression.

By the time of writing this thesis, we are raising mice with overexpression of MT-I and simultaneous IL-6 deficiency in order to study pathology without IL-6 effects. However, these mice might not necessarily provide us with new knowledge, in that the TgMT mice already contain very low levels of IL-6. Besides, even if IL-6 induces specific neuroprotection, it is a multifunctional cytokine that can induce various unfavorable actions involving the immune, hematopoietic and nervous system. Accordingly, GFAP-IL6 mice show spontaneous inflammation, demyelination, chronic neurodegeneration, cognitive decline and physiological impairments (Giralt et al., 2002a; Molinero et al., 2003; Samland et al., 2003). Thereby, IL-6 is not an optimal candidate for a new therapeutic approach.

Instead, the MT-I+II proteins could represent new drug targets for the treatment of CNS diseases. However, further studies are necessary to thoroughly address the mechanism of MT-I+II actions; but regardless of the exact mechanisms, the data presented here suggest that MT-I+II can protect the brain. As emerging data support MT-I+II protective functions in the human brain and in neurological patients, MT-I+II seem to demonstrate potentials as new therapeutic strategies for treatment of brain disorders.

CONCLUSIONS

The fact that evolutionary forces conserved MT-I+II genes so well and favor their ubiquitous expression throughout species, may underline their biological significance. Though MT-I+II may not be essential for a normal healthy life, these proteins seem to be essential during diseases not only in CNS but also in other organs.

Within the CNS, MT-I+II have a potential as pharmaceutical targets, provided that they can be delivered to their site of action. MT-I+II are proteins with an unusual cysteine abundance and high metal content, which are unlikely to enter the CNS through an intact BBB. Accordingly, only during neuropathology with BBB breakdown, peripherally administered MT-I+II proteins enter the CNS. However, some CNS diseases are relapsing-remitting and the BBB will likely be intact during remissions. Thereby, the MT-I+II proteins have to be modified if they shall be used as pharmaceuticals, and one option could be to encapsulate MT-I or MT-II in lipophilic nanocapsules that will likely cross the BBB; or cerebral MT delivery can be facilitated by coupling to a vector-mediated peptide-drug delivery system and/or a molecule that undergoes receptor-mediated transcytosis through the BBB. Based on the results presented here, it may also be feasible to use only a fragment or a single domain of MT-I or MT-II that might be small enough to cross the intact BBB, as the drosophila MT (MTN consisting of 40 amino acids) has the same treatment effects as mammal MT-I+II (consisting of 61 amino acids). To this end, we have developed some new MT-I and MT-II compounds that exert the usual MT-I+II functions and are able to cross the intact BBB. This quality gives these new molecules a pharmaceutical advantage over native MT-I+II proteins. These compounds are being tested at the moment.

Moreover, the specific MT-I+II mechanisms of action are being investigated by using different in vitro systems such as primary cell cultures of neurons or different glial cell types in order to determine the MT-I+II molecular pathways and signal transduction. Besides, we are studying the functional significance of MT-I+II in human cells obtained from neurological patients. The ambition is to develop novel neuroprotective paradigms and therapeutic drugs, of which neurological patients may benefit.

Obviously, we do not hold all the pieces of the puzzle, and many aspects need to be addressed before we will understand the precise MT-I+II functions during human brain diseases. However, MT-I+II seem to demonstrate potentials as new therapeutic targets for treatment of brain disorders.

Despite the fact that drugs for neurological diseases constitute the fastest-growing pharmaceutical market, no truly effective curative or prophylactic drugs exist. Thus, it is of major interest to discover novel neuroprotective paradigms for the future treatment of various brain diseases.

SUMMARY

This doctoral thesis describes the expression and roles of Metallothioneins I and II (MT-I+II) during CNS pathology. MT-I+II expression increases significantly in reactive astrocytes and microglia/macrophages after traumatic brain injury (cryogenic lesion in cerebral cortex) and brain stem toxicity induced by the gliotoxin

6-aminonicotinamide (6-AN). Interleukin-6 (IL-6) is a key inducer of brain MT-I+II, as judged by using genetic IL-6 deficient mice (IL-6 knock-out (IL-6KO) mice) and IL-6 overexpressing (GFAP-IL6) mice subjected to brain injury. These studies also indi-

cate that IL-6 and/or IL-6-induced MT-I+II exert neuroprotective functions.

To study MT-I+II roles in CNS, genetically modified mice with MT-I+II deficiency (MT-I+II knock-out (MT-KO) mice) or transgenic MT-I overexpression (TgMT mice) were applied along wildtype controls; as well as wildtype, and MT-KO mice receiving exogenous MT-I or MT-II treatment were examined. Both MT-KO and TgMT genotypes and mice receiving exogenous MT were studied after cryogenic brain injury and 6-AN-induced toxicity in brain stem. These experiments overall show that MT-I+II have significant immunomodulating, antioxidant and neuroprotective roles. Accordingly, MT-I+II reduce CNS activation of macrophages and lymphocytes including expression of proinflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α. Moreover, MT-I+II are antioxidant and antiapoptotic factors counteracting reactive oxygen species (ROS)/oxidative stress, neurodegeneration and apoptotic cell death, whereby the delayed (secondary) tissue damage was inhibited after brain injury and 6-AN-toxicity. MT-I+II also diminish the primary CNS toxicity caused directly by 6-AN and the clinical outcome (mortality). Additionally, MT-I+II stimulate astrogliosis; expression of growth factors, their receptors and neurotrophins (TGFβ, TGFβ-Receptor, bFGF, bFGF-Receptor, VEGF, NT-3, NT-4/5, NGF); angiogenesis; and growth cone formation. Hence, MT-I+II enhance CNS tissue repair as seen clearly after the cryogenic injury, after which MT-I+II promote substitution of the necrotic lesion cavity with a glial scar tissue including revascularization. In the surrounding tissue, MT-I+II stimulate growth cone formation and outgrowths of surviving neurons situated outside the lesioned area; while inside the lesion, normal neurons or neuronal cell bodies were never seen.

The angiogenic actions of MT-I+II were supported by comparing various genotypes like GFAP-IL6 mice; MT-I+II deficient GFAP-IL6 (GFAP-IL6/MT-KO) mice; MT-I+II heterozygous GFAP-IL6 mice (GFAP-IL6-MT+/- mice); double-transgenic IL-6 and MT-I overexpressors (GFAP-IL6/TgMT mice); MT-KO mice; and TgMT mice. The IL-6 overexpressors are suitable for this as they spontaneously develop proliferative angiopathy/angiogenesis, which is inhibited by MT-I+II deficiency and enhanced by MT-I overexpression. In fact, angiogenesis was highest in the MT-I overexpressing TgMT and GFAP-IL6/TgMT mice, even though the TgMT mice show quite low IL-6 levels as MT-I+II inhibit brain IL-6 mRNA and protein.

Moreover, GFAP-IL6/TgMT and TgMT and GFAP-IL6 genotypes were lesioned along wildtype controls in order to identify the roles of IL-6 versus MT-I+II. Although IL-6 induces CNS protection, this could be due to IL-6-induced MT-I+II, as neuroprotection and recovery are associated with MT-I+II levels only and not with IL-6 or inflammation: The more MT-I+II, the less neuropathology.

The MT-I+II actions seen after brain injury and 6-AN-toxicity are induced by both endogenous MT-I overexpression and exogenous MT-II treatment. As presented here for the first time, analogous therapeutic effects are obtained after brain injury by using native or recombinant MT-I or MT-II derived from diverse non-mammalian and mammalian species like drosophila, mouse, rabbit, horse and human. Treatment with these MT-I and MT-II proteins significantly reduce inflammation, oxidative stress, neurodegeneration and apoptotic cell death after brain injury, while astroglia is stimulated. This indicates that MT-I+II function independently of species of origin.

Previously, we showed that MT-I+II also ameliorate autoimmune, excitotoxic and inflammatory CNS disorders, and independent groups have confirmed this and have in fact added that MT-I+II are also major neuroprotective factors during ischemia and motor neuron disease. As emerging data validate these functions in human tissue and neurological patients, MT-I+II seem to demonstrate potentials as therapeutic targets for the treatment of CNS disorders.

ABBREVIATIONS

AD: Alzheimer Disease.
ALS: Amyotrophic Lateral Sclerosis.
6-AN: 6-Aminonicotinamide.
ANOVA: Analysis Of Variance.
BBB: Blood-Brain Barrier.
bFGF: Basic Fibroblast Growth Factor.
bFGF-R: bFGF-Receptor.
Cd: Cadmium.
CD: Cluster of Differentiation.
CNS: Central Nervous System.
CSF: Colony-stimulating factor.
GM-CSF: Granulocyte-Macrophage CSF.
M-CSF: Macrophage CSF.
Cu: Copper.
DAB: 3,3-Diaminobenzidine-Tetrahydrochloride.
DNA: DeoxyRiboNucleic Acids.
Dpi: Days Post-Injection.
Dpl: Days Post-Lesioning.
EAE: Experimental Autoimmune Encephalomyelitis.
GFAP: Glial Fibrillary Acidic Protein.
GFAP-IL6 mice: Transgenic, astrocyte-targeted IL-6 overexpressing mice.
GFAP-IL6-MT+/- mice:-GFAP-IL6 mice heterozygous for MT-I+II.
GFAP-IL6/MT-KO mice: GFAP-IL6 mice with MT-I+II deficiency.
GFAP-IL6/TgMT mice: GFAP-IL6 mice with simultaneous overexpression of MT-I (double-transgenic overexpressors).
HE: Hematoxylin-Eosin. Hg: Mercury.
Hg: Mercury.
HRP: Horse Radish Peroxidase.
Ig: Immunglobulin.
IL: Interleukin.
IL-6KO mice: IL-6 knock-out mice (genetic IL-6 deficient mice).
IHC: ImmunoHistoChemistry.
iNOS: Inducible Nitric-Oxide Synthase.
IFN: Interferon.
i.p.: IntraPeritoneally.
kDa: KiloDalton.
KO: Knock-Out.
MDA: MalonDiAldehyde.
Mn: Manganese.
MOMA: MOnocyte-MAcrophage (marker).
mRNA: Messenger RiboNucleic Acids.
MS: Multiple Sclerosis.
MT: Metallothionein.
MT-KO mice: MT-I+II knock-out mice (genetic MT-I+II deficiency).
MTN: Drosophila MT protein.
MT-III/GIF: Metallothionein III/Growth inhibitory factor.
NF: NeuroFilament.
NFκB: Nuclear Factor-kappa-B.
NGF: Nerve Growth Factor.
NITT: Nitrotyrosine.
NSE: Neuron Specific Enolase.
NT: NeuroTrophin.
PD: Parkinsons Disease.
ROS: Reactive Oxygen Species.
SOD: Superoxide Dismutase.
ssDNA: Single Stranded DNA.
STAT: Signal Transducer and Activator of Transcription.
TGFβ: Transforming Growth Factor-β.
TGFβ-R: TGFβ-Receptor.
TgMT mice: Mice with transgenic MT-I overexpression in CNS.
TNF-α: Tumor Necrosis Factor-α.
TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-digoxigenin Nick End Labeling.
VEGF: Vascular Endothelial Growth Factor.
Zn: Zinc.

THE PRESENT THESIS IS BASED ON THE FOLLOWING ARTICLES

I. Penkowa M and Moos T (1995) Disruption of the blood-brain interface in neonatal rat neocortex induces a transient expression of metallothionein in reactive astrocytes. GLIA. 13:217-227.

II. Penkowa M, Giralt M, Carrasco J, Hadberg H, and Hidalgo J (2000) Impaired inflammatory response and increased oxidative stress and neurodegeneration after brain injury in interleukin-6-deficient mice. GLIA. 32:271-285.

III. Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernandez J, Molinero A, Campbell IL, and Hidalgo J (2003) Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp Neurol. 181:130-148.

IV. Penkowa M, Carrasco J, Giralt M, Moos T, and Hidalgo J (1999) CNS wound healing is severely depressed in metallothionein I- and II-deficient mice. J Neurosci 19:2535-2545.

V. Penkowa M, Carrasco J, Giralt M, Molinero A, Hernández J, Campbell IL, and Hidalgo J (2000) Altered central nervous system cytokine-growth factor expression profiles and angiogenesis in metallothionein-I+II deficient mice. J Cereb Blood Flow Metab 20:1174-1189.

VI. Penkowa M, Camats J, Giralt M, Molinero A, Hernandez J, Carrasco J, Campbell IL, and Hidalgo J (2003) Metallothionein-I overexpression alters brain inflammation and stimulates brain repair in transgenic mice with astrocyte-targeted interleukin-6 expression. GLIA. 42:287-306.

VII. Penkowa M, Giralt M, Moos T, Thomsen PS, Hernandez J, and Hidalgo J (1999) Impaired inflammatory response to glial cell death in genetically metallothionein-I- and -II-deficient mice. Exp Neurol 156:149-164.

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