Disruption of the Metallothionein-III Gene in Mice: Analysis of Brain Zinc, Behavior, and Neuron Vulnerability to Metals, Aging, and Seizures

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Metallothionein-III (MT-III), a brain-specific member of the metallothionein family of metal-binding proteins, is abundant in glutamatergic neurons that release zinc from their synaptic terminals, such as hippocampal pyramidal neurons and dentate granule cells. MT-III may be an important regulator of zinc in the nervous system, and its absence has been implicated in the development of Alzheimer's disease. However, the roles of MT-III in brain physiology and pathophysiology have not been elucidated. Mice lacking MT-III because of targeted gene inactivation were generated to evaluate the neurobiological significance of MT-III. MT-III-deficient mice had decreased concentrations of zinc in several brain regions, including hippocampus, but the pool of histochemically reactive zinc was not disturbed. Mutant mice exhibited normal spatial learning in the Morris water maze and were not sensitive to systemic zinc or cadmium exposure. No neuropathology or behavioral deficits were detected in 2-year-old MT-III-deficient mice, but the age-related increase in glial fibrillary acidic protein expression was more pronounced in mutant brain. MT-III-deficient mice were more susceptible to seizures induced by kainic acid and subsequently exhibited greater neuron injury in the CA3 field of hippocampus. Conversely, transgenic mice containing elevated levels of MT-III were more resistant to CA3 neuron injury induced by seizures. These observations suggest a potential role for MT-III in zinc regulation during neural stimulation.

Key words: metallothionein; zinc; seizures; kainic acid; hippocampus; cadmium; Morris water maze; glial fibrillary acidic protein; Alzheimer's disease; zinc-containing neurons

Zinc is an essential element with multiple functions in the CNS. Most zinc is tightly bound to metalloproteins that use it for catalytic activity or structural stability (Frederickson, 1989; Vallee and Falchuck, 1993). In addition, zinc is concentrated in secretory vesicles in the presynaptic terminals of a subset of glutamatergic neurons (Haug, 1967; Perez-Clausell and Danscher, 1985; Frederickson, 1989), so-called zinc-containing neurons, and is released during high frequency neuronal firing (Assaf and Chung, 1984; Howell et al., 1984; Charton et al., 1985; Sloviter, 1985; Aniksztejn et al., 1987). Zinc released from neuron terminals may serve a neuromodulatory function, as suggested by the multiple selective actions of zinc on synaptic function (Khulusi et al., 1986; Forsythe et al., 1988; Xie and Smart, 1991; Buhl et al., 1996) and the potency of zinc ions to modulate numerous neurotransmitter receptors and ion channels (Westbrook and Mayer, 1987; Christie and Choi, 1990; Draguhn et al., 1990; Rassendren et al., 1990; Hollman et al., 1993; Li et al., 1993; Harrison and Gibbons, 1994).

Zinc is implicated in the pathophysiology of several neurological disorders. A role for zinc in epilepsy is suggested by its modulatory actions on neuron activity (Wright, 1986; Reece et al., 1994; Buhl et al., 1996), the prominence of zinc-containing pathways in seizure-prone brain regions (Frederickson, 1989), abnormalities of cerebral zinc in epileptic animals (Chung and Johnson, 1983; Kasarskis et al., 1987; Fukahori et al., 1988; Feller et al., 1991), and the effects of zinc manipulations on seizure susceptibility (Pei and Koyama, 1986; Wright, 1986; Fukahori and Itoh, 1990; Mitchell and Barnes, 1993). Zinc is also neurotoxic (Yokoyama et al., 1986; Choi et al., 1988; Lees et al., 1990; Duncan et al., 1992; Freund and Reddig, 1994), and its intracellular accumulation may contribute to neuron death caused by seizures (Frederickson et al., 1989; Weiss et al., 1993) and transient cerebral ischemia (Koh et al., 1996). In addition, zinc ions promote aggregation of the amyloid β protein (Bush et al., 1994), an effect that could facilitate the development of Alzheimer's disease (AD) neuropathology. Despite the importance of zinc in brain physiology and disease, zinc regulation in the CNS is poorly understood.

Metallothioneins (MTs) are small, cysteine-rich proteins that bind zinc with high affinity and capacity and function in metal ion regulation and detoxification (Kägi and Kojima, 1987; Suzuki et al., 1993). Four MT isoforms exist in the mouse (Palmiter et al., 1993). MT-I and MT-II are expressed coordinately in all tissues (Palmiter, 1987), MT-III is expressed specifically in the CNS (Palmiter et al., 1992; Tsuji et al., 1992), and MT-IV is found in stratified squamous epithelia (Quaife et al., 1994). MT-III is expressed by zinc-containing neurons and is most abundant in dentate granule cells, the mossy fiber projections of which contain the highest concentration of zinc in the brain, and in hippocampal pyramidal neurons (Masters et al., 1994b). MT-III might provide zinc-containing neurons, or neurons exposed to high concentrations of zinc, with a means of distributing, recycling, or buffering zinc. In support of a role in brain zinc regulation, MT-III binds zinc in vivo (Masters et al., 1994b) and confers resistance to zinc cytotoxicity in cultured cells (Palmiter, 1995).
To elucidate the neurobiological significance of MT-III and its role in zinc neurophysiology, we have manipulated its expression in mice. We previously reported that transgenic mice expressing high levels of MT-III had elevated concentrations of brain zinc but did not exhibit overt behavioral or morphological abnormalities (Erickson et al., 1995). Here we report the generation of mice lacking MT-III because of targeted gene disruption. Brain chemistry, morphology, and behavior were examined in MT-III-deficient mice under normal conditions, after metal exposure, after kainate treatment, and in old age. The most striking discovery was that MT-III-deficient mice were more sensitive to seizure-induced injury to CA3 hippocampal neurons, whereas mice expressing elevated levels of MT-III were more resistant to this damage.

**MATERIALS AND METHODS**

**Generation of MT-III-deficient mice.** The mouse MT-III gene was isolated from a 129Sv genomics library (Palmiter et al., 1992). A disruption vector was constructed by replacing a 2.2 kb XhoI–NdeI region containing the promoter and exons 1 and 2 with a neomycin resistance cassette and inserting thymidine kinase genes at the XhoI–NdeI sites in the 5' and 3' flanking regions, respectively. Electroporation and selection of ABI embryonic stem cells was performed as described (Thomas et al., 1995). Colonies were screened for targeting by PCR and confirmed by Southern blot analysis of DNA digested with SsrI by using a 0.6 kb BstEII–NdeI fragment as a probe. Eight positive clones were identified from 840 clones screened. Four clones were used to generate chimeric mice as described (Thomas et al., 1995), but only one clone transmitted through the germ line, and it was used to establish a line of mice carrying the disrupted allele. F2 and F3 generation C57Bl/129Sv hybrid mice of both sexes were used in all experiments. Congenic 129Sv mice were used for kainic acid experiments in addition to hybrid mice. Transgenic mice expressing human MT-III (Erickson et al., 1995) and their wild-type littermates also were used for kainic acid experiments.

**Quantitation of specific mRNA levels in brain.** MT-III, MT-I, and glial fibrillary acidic protein (GFAP) mRNA levels in brain were measured by solution hybridization as described (Durnam and Palmiter, 1983; Townes et al., 1985). Briefly, total nucleic acids were isolated and hybridized overnight with end-labeled oligonucleotide probes and treated with S1 nuclease; the S1-resistant complexes were collected on Whatman GF/C filters after precipitation with trichloroacetic acid. Oligonucleotide 350 (5'-CACAGCTCCTTGACGACACTTCT-3'), complementary to a unique sequence in exon 3 of MT-III, oligonucleotide 57 (5'-GGGCAAAATGATTGCGGGCAGAG-3'), complementary to the 3' untranslated region of MT-I, and oligonucleotide 220 (5'-GGTACTGAGGTCCCTCATCTCTGC-3'), complementary to exon 1 of GFAP, were used. Standards with known amounts of MT-III or MT-I mRNA were used to determine the amount of mRNA in each sample. Values are presented as molecules mRNA/cell, assuming 6.4 pg DNA/cell. A standard was not available for GFAP, so values are presented as cpm/μg total nucleic acid.

**Determination of MT protein levels.** Brain homogenates (5% w/v) were prepared in 20 mM Tris-HCl and 10 mM NaCl, pH 8.6; 950 μl of fraction was collected and assayed for 109Cd, prepared in 20 mM Tris-HCl and 10 mM NaCl, pH 8.6; 950 μl of fraction was collected and assayed for 109Cd. 3H-inserting thymidine kinase genes at the SsrI–NdeI sites in the 5' and 3' flanking regions, respectively. Electroporation and selection of ABI embryonic stem cells was performed as described (Thomas et al., 1995). Colonies were screened for targeting by PCR and confirmed by Southern blot analysis of DNA digested with SsrI by using a 0.6 kb BstEII–NdeI fragment as a probe. Eight positive clones were identified from 840 clones screened. Four clones were used to generate chimeric mice as described (Thomas et al., 1995), but only one clone transmitted through the germ line, and it was used to establish a line of mice carrying the disrupted allele. F2 and F3 generation C57Bl/129Sv hybrid mice of both sexes were used in all experiments. Congenic 129Sv mice were used for kainic acid experiments in addition to hybrid mice. Transgenic mice expressing human MT-III (Erickson et al., 1995) and their wild-type littermates also were used for kainic acid experiments.

**Histology and immunostaining.** Mice were killed by CO2 inhalation and cardiac-perfused with 4% paraformaldehyde solution. Brains were removed and placed in NBF for up to 1 week before being embedded in paraffin. Coronal sections (7 μm) were cut and used for conventional staining and immunohistochemistry. Immunostaining was performed according to a standard streptavidin-peroxidase procedure using a 1:1000 dilution of rabbit antiserum to bovine GFAP (Dukopatts, Glostrup, Denmark) and a 1:1000 dilution of monoclonal anti-MAP2 antibody (Sigma, St. Louis, MO).

**Measurement of tissue metal concentrations.** Dissected brain regions were weighed, placed in acid-washed glass flasks, digested in 3 ml of ultrapure nitric acid (J. T. Baker, Phillipsburg, NJ), evaporated to dryness, and resuspended in 2 ml of 2.5% (v/v) nitric acid. The concentration of 20 elements was determined by inductively coupled plasma emission spectrometry with a Jarrell–Ash 955 spectrometer.

Detection of histochemically reactive zinc. Timm's stain was performed as described (Slomvier, 1982). Briefly, mice were perfused with 0.37% sodium sulfide in 86 mM phosphate buffer, pH 7.2, for 5 min, followed by NBF for 5 min. Brains were removed, placed in NBF for ~6 hr, embedded in paraffin, and cut into 10 μm coronal sections. Deparaffinized sections were incubated in the dark for 90 min at room temperature in 0.1% silver lactate and 0.85% hydroquinone in 30% citrate-buffered, arabic gum. TS-Q (Molecular Probes, Eugene, OR) histofluorescence was performed as described (Frederickson et al., 1987; Erickson et al., 1995).

**Behavioral tests.** Testing was done during the light phase on 10- to 16-week-old C57Bl/129Sv hybrid mice unless otherwise stated. Mutant mice and wild-type mice were littermates. All procedures were approved by the University of Washington Animal Care Committee.

Locomotor activity in an open-field environment was measured by placing a mouse in the center of a brightly illuminated Plexiglass container 88 x 47 x 30 cm (L x W x H), lined with white paper, and allowing it to move freely for 5 min. The mouse was observed on a video monitor via a camera mounted above the field. A grid dividing the field into 12 x 12 cm squares was placed over the video monitor, and the number of squares entered by each mouse was counted.

Passive inhibitory avoidance testing was performed with a Coulbourn Solid State Shocker and Model E10.10 Modular Test Cage (Lehigh, PA) consisting of two chambers, each 17.5 x 16.5 x 21 cm, separated by a door controlled by the investigator. One chamber was lined on all sides with black plastic to make it nearly dark, and the other chamber was illuminated with a 6 W light. Training consisted of placing a mouse in the lighted chamber and timing its latency to fully enter the dark chamber. Then the door was closed and a 0.05 mA shock was delivered to the grid floor. Testing was identical to training except that no shock was delivered after the mouse entered the dark chamber. Mice not entering the dark chamber after 5 min were removed and given a latency score of 5 min.

**Zinc and cadmium toxicity.** Eight-week-old mice (n = 6 for each genotype) were injected subcutaneously for 30 consecutive days with 10 μmol Cd/kg body weight, and 7-old mice (n = 6 for each genotype) were given a single subcutaneous injection at the same dose. The injection solution consisted of 2 mM CdCl2 (Sigma) and was given in a volume of ~0.1 ml for adults and ~0.025 ml for newborns. Biochemical and histological analyses were conducted with tissues isolated 1 d after the last injection.

Mice 12–14 weeks old were injected subcutaneously for seven consecutive days with increasing doses of ZnCl2 (Sigma). On days 1, 2, and 3, mice received 200, 300, and 400 μmol Zn/kg body weight, respectively, with a 100 mM ZnCl2 solution. On days 4 and 5, mice received 600 μmol Zn/kg body weight from a 200 mM ZnCl2 solution, and on days 6 and 7 they received 800 μmol Zn/kg body weight. This regimen provides exposure to zinc that is close to the maximum that mice can tolerate on a diet, but did not exhibit overt behavioral or morphological abnormalities (Erickson et al., 1995). Here we report the generation of mice lacking MT-III because of targeted gene disruption. Brain chemistry, morphology, and behavior were examined in MT-III-deficient mice under normal conditions, after metal exposure, after kainate treatment, and in old age. The most striking discovery was that MT-III-deficient mice were more sensitive to seizure-induced injury to CA3 hippocampal neurons, whereas mice expressing elevated levels of MT-III were more resistant to this damage.
injected intraperitoneally into 12- to 16-week-old mice. Hybrid mice (C57Bl/129Sv) were given a dose of 25 mg/kg body weight, and congenic mice (129Sv) were given a dose of 35 mg/kg body weight. Preliminary experiments revealed that these doses were sufficient to produce seizures in the majority of mice. Each mouse was observed continuously for 2 hr, and the occurrence and duration of limb clonus and generalized tonic-clonic convulsions were recorded. Typically, seizures occurred between 10 and 90 min after injection. The occurrence of other seizure-related behaviors such as head bobbing, rearing, falling, and death also was noted. The total time each mouse exhibited limb clonus or tonic-clonic convulsions was used as an index of seizure activity.

Brains were obtained for histological analysis 3 da after kainate treatment as described above. Coronal sections through hippocampus were stained with hematoxylin and eosin. One section from three different levels of dorsal hippocampus was examined for each mouse. The extent of damage in the CA3 and CA1 pyramidal cell layers was estimated by assigning a score from 0 to 3 to each region. A score of 0 was assigned when no pyknotic cells were observed, a score of 1 when occasional pyknotic cells were present, a score of 2 when ~20–50% of the cells were pyknotic, and 3 when >50% of the cells were pyknotic. The left and right sides of each brain section were scored separately. The CA3 and CA1 injury scores used for each mouse were the average values from three sections (i.e., 6 different scores per region). All sections were scored by the same investigator who was blind to genotype.

**RESULTS**

**Generation of MT-III-deficient mice**

The murine MT-III gene consists of three exons, depicted as solid rectangles in Figure 1A. The gene was inactivated by deleting the proximal promoter and exons 1 and 2 by homologous recombination in embryonic stem cells (Fig. 1A). A single line of mice carrying the disrupted MT-III allele was generated from one clone. The mutation was confirmed by Southern blot analysis of tail DNA (Fig. 1B).

Brain MT-III mRNA was abundant in wild-type mice, reduced by ~50% in heterozygotes, and undetectable in mutant mice, as determined by quantitative solution hybridization (Fig. 2A). MT-I mRNA levels in brains of mutants and heterozygotes were unchanged, as compared with controls (Fig. 2A), suggesting that MT-I and MT-II, which are expressed coordinately, are not up-regulated in response to MT-III deficiency. Total MT protein levels were reduced by ~70% in mutant brain (Fig. 2B), with the remaining MT protein consisting of MT-I and -II.

More than 200 MT-III-deficient mice were generated. Mutant mice were born at expected frequency, exhibited normal growth, and were physically indistinguishable from wild-type littermates throughout their approximately 2-year lifespan. MT-III-deficient mice reproduced without any apparent deficit in fertility, litter size, or litter survival. Weight and gross morphology of brains from adult MT-III-deficient mice were normal, and no abnormal-
Averagemossyfiberhistofluorescenceintensitiesweresimilarforwild-typemice (+/+) andMT-III-deficientmice (−/−) observed in brain, hippocampus, cortex, brainstem, andspinal cord. Values are mean ± SEM; n = 5; p < 0.05, unpaired t test. B, Wholebrain; BS, brainstem; CB, cerebellum; CX, cortex; HP, hippocampus; OB,olfactory bulb; TH, thalamus; SC, spinal cord.

### Zinc is decreased in the CNS of MT-III-deficient mice

The concentrations of zinc and other metals were measured in whole brains and dissected brain regions of adult mice. As shown in Figure 3, MT-III-deficient brain contained ~12% less zinc than normal brain. Zinc levels were significantly lower in hippocampus, cortex, brainstem, thalamus, and spinal cord of mutant mice, with hippocampus showing the greatest decrease (Fig. 3). The concentration of zinc in cerebellum, a region that expresses MT-III at relatively low levels and has virtually no vesicular zinc, was not significantly different between mutants and controls, nor was it altered in MT-III-deficient brain or spinal cord (data not shown).

To determine whether loss of MT-III perturbs the pool of histochemically reactive or “free” zinc, which consists primarily of the zinc sequestered in synaptic vesicles, we stained brain sections with Timm’s reagent (Fig. 4A,B). No changes in the intensity or distribution of Timm’s product were observed in MT-III-deficient brain. Frozen sections also were stained with the zinc-sensitive fluorescent dye TS-Q (Fig. 4C,D), and the fluorescence signal was quantitated in the zinc-rich mossy fiber region of hippocampus. Average mossy fiber histofluorescence intensities were similar for wild-type mice, 1342 ± 104 arbitrary units (n = 3), and MT-III-deficient mice, 1158 ± 52 arbitrary units (n = 3). Thus, although total zinc levels were reduced in brains of MT-III-deficient mice, the pool of histochemically reactive zinc was not significantly affected under steady-state conditions.

### MT-III-deficient mice exhibit normal learning and memory

The behavior of adult MT-III-deficient mice was evaluated by a battery of tests. First, spontaneous locomotor activity was measured in an open-field environment. Movement of mutant mice was similar to that of wild-type mice, with mutants (n = 18) entering 112 ± 9.2 squares and controls (n = 16) entering 107 ± 9.7 squares during a 5 min trial.

The ability of MT-III-deficient mice to learn and remember a simple association was tested in a passive avoidance task. In the first trial, the latency to enter the dark side of a shuttle box was 13.9 ± 2.9 sec for wild-type mice (n = 9) and 15.4 ± 2.6 sec for mutant mice (n = 8). Immediately after the mice entered the dark chamber, a brief electric shock was delivered. When the mice were tested the next day, the latency to enter was significantly longer for both wild-type mice, 117 ± 31 sec, and mutant mice, 114 ± 28 sec, indicating that both groups associated the dark chamber with the aversive shock. The increased latencies for both groups persisted when tested again 5 d later.

The ability of MT-III-deficient mice to use distant spatial cues to locate a hidden platform, a hippocampal-dependent task, was tested in the Morris water maze (Morris et al., 1982). No differences were seen between mutant mice and littermate controls in the acquisition phase, as indicated by similar escape latencies for the two groups on each of the 7 d of training (Fig. 5A). The day after the last training trial, the escape platform was removed, and each mouse was allowed to search for 60 sec. During this probe trial, mutant mice and control mice selectively searched the quadrant where the platform had been located during training (Fig. 5B), and both groups crossed the site where the platform had been located more often than an equivalent site in the opposite quadrant (Fig. 5C). No differences were observed between the two groups in this trial, indicating that MT-III-deficient mice learned the spatial relations between distal cues and the hidden platform as effectively as controls. Another probe trial was performed 7 d after the last day of training, and again both groups showed a strong preference for the quadrant and the site where the platform had been located (data not shown). To test the ability of MT-III-deficient mice to unlearn a spatial pattern and acquire a new one, we retrained the same mice with the platform in the quadrant opposite to its previous location. Performance of MT-III-deficient mice after reversal of the platform location was similar to that of controls during the training period and in subsequent probe trials (data not shown).

Transgenic mice expressing elevated levels of MT-III also were evaluated in the Morris maze, passive avoidance task, and open-field locomotor activity tests, but no abnormalities were observed (data not shown).
MT-III is not required for protection against systemic cadmium or zinc exposure

To test whether MT-III is required for protecting the CNS against the toxic metal cadmium, we gave adult MT-III-deficient and wild-type mice (n = 6 for each group) cadmium injections for 30 consecutive days with a dose that MT-I and -II deficient mice cannot tolerate (Masters et al., 1994a). All mice survived the treatment regimen, and none displayed obvious signs of neurological dysfunction, although both groups showed generalized signs of mild toxicity, such as modest weight loss and poor grooming. Histological examination of brains from cadmium-treated mice revealed mild pericapillary edema in cerebral cortex of both mutants and controls (data not shown). No other neuropathology was observed. Brain cadmium levels, which are undetectable under normal conditions, were similar in cadmium-treated wild-type mice (0.67 ppm) and mutant mice (0.63 ppm); MT-I mRNA levels in brain were elevated approximately twofold in both treated groups. In contrast, liver accumulated massive amounts of cadmium in treated mice, with an average concentration of 207 ppm in wild-type mice and 217 ppm in mutant mice, as compared with <1 ppm in untreated mice; hepatic MT-I mRNA was induced ~20-fold.

A likely explanation for MT-III-deficient mice being resistant to cadmium is that the blood–brain barrier is sufficient for detoxifying this metal (Arvidson and Tjalve, 1986; Valois and Webster; 1987). Therefore, 7-d-old mice, which lack a mature blood–brain barrier and are susceptible to CNS damage induced by systemic cadmium (Webster and Valois, 1981; Valois and Webster, 1987), were given a single cadmium injection. This treatment produced profound histological changes characterized by extensive cortical necrosis, edema, and hemorrhage (data not shown). However, no clear differences in the extent or severity of damage were observed between MT-III-deficient mice and control mice. No damage was observed in mice of either genotype when given one-half as much cadmium, suggesting that the threshold for cadmium-induced brain damage also was unaffected by the loss of MT-III.

The response of MT-III-deficient mice to high doses of systemic zinc was examined by injecting 11 adult MT-III-deficient mice and 11 adult wild-type mice for seven consecutive days with increasing amounts of zinc. All mice appeared moribund, and one mouse of
learning was observed in zinc-treated mice of either genotype
by systemic zinc overload. However, no impairment of spatial
exposed to zinc, most likely reflecting the general malaise induced
mice (Fig. 5)
activity was depressed although their spatial preference was weaker than that of young
groups. Noneuropathology was detected. Open-field locomotor
mRNA in brain was induced approximately twofold in both
or brain MT-IIImRNA levels in wild-type mice (data not shown).
MT-III-deficient mice did not influence brain MT-ImRNA levels in either group (Fig. 6)
with aging, was unaffected by the loss of MT-III. Old MT-III-
deficient mice were also similar to old wild-type mice in the
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with an antibody to MAP-2, a marker of neuronal processes, but
sections also were immunostained
conventional stains, Bielschowsky silver stain was used to highlight
Pathological changes associated with aging. Old mice were
mice revealed normal morphology and cellularity, with no appreciable loss of neurons in cortex or hippocampus. In addition to
the hippocampus. No differences in neurofilament staining were observed between old mutant and wild-type mice. Sections also were immunostained with an antibody to MAP-2, a marker of neuronal processes, but
in staining pattern were apparent in aged MT-III-
deficient mice.
GFAP, an intermediate filament protein, is a marker of fibrillary astrocytes (Federoff and Vernadakis, 1986), and its levels in
brain increase with advanced age (Goss et al., 1991; O’Callaghan and Miller, 1991; Kohama et al., 1995). As expected, GFAP
mRNA was elevated significantly in old mice, as compared with young mice (Fig. 6). However, the age-related increase in GFAP
expression was more pronounced in MT-III-deficient mice, with old mutant mice having approximately twofold higher levels of
GFAP mRNA than old wild-type mice (Fig. 6). In contrast, aging did not influence brain MT-I mRNA levels in either group (Fig. 6)
or brain MT-III mRNA levels in wild-type mice (data not shown).

Table I. Response of wild-type (+/+), MT-III-deficient (−/−), and MT-III transgenic (TG) mice to kainic acid

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genetic background</th>
<th>Percent seizing</th>
<th>Latency (min)</th>
<th>Total convulsion time (sec)</th>
<th>Mortality (%)</th>
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<tr>
<td>+/+</td>
<td>hybrid</td>
<td>74</td>
<td>29.3 ± 3.0</td>
<td>121 ± 18</td>
<td>18</td>
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<tr>
<td>−/−</td>
<td>hybrid</td>
<td>86</td>
<td>21.5 ± 4.1</td>
<td>221 ± 25**</td>
<td>43*</td>
</tr>
<tr>
<td>TG</td>
<td>hybrid</td>
<td>71</td>
<td>31.5 ± 3.5</td>
<td>115 ± 20</td>
<td>11</td>
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<tr>
<td>+/+</td>
<td>129 Sv</td>
<td>76</td>
<td>30.6 ± 4.0</td>
<td>107 ± 25</td>
<td>0</td>
</tr>
<tr>
<td>−/−</td>
<td>129 Sv</td>
<td>88</td>
<td>20.9 ± 3.0</td>
<td>184 ± 30</td>
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Hybrid mice were given 25 mg of kainate/kg body weight and congenic mice (129 Sv) were given 35 mg of kainate/kg body weight. Latencies and convulsion times are the means ± SEM, n = 24 for each group. *Significantly greater than +/+ hybrid mice; p < 0.05, χ² analysis. **Significantly greater than +/+ hybrid mice; p < 0.01, unpaired t test.

MT-III-deficient mice express higher levels of GFAP in old age
A group of 27 MT-III-deficient mice and 18 wild-type mice was maintained for two years to determine whether MT-III influences the neurological changes associated with aging. Old mice were tested first in the Morris water maze. Two-year-old mice required ~10 d of training to locate the hidden platform effectively, but no differences in acquisition were observed between mutant and wild-type mice (Fig. 5D). A probe trial revealed that old mice of both genotypes preferentially searched the quadrant and the site where the platform had been located during training (Fig. 5E,F), although their spatial preference was weaker than that of young mice (Fig. 5B,C). No differences between old mutant and wild-type mice were observed in the probe trial, indicating that the ability to store and recall spatial information, although impaired with aging, was unaffected by the loss of MT-III. Old MT-III-deficient mice were also similar to old wild-type mice in the passive inhibitory avoidance task and open-field activity tests (data not shown).

Histological examination of brains from aged MT-III-deficient mice revealed normal morphology and cellularity, with no appreciable loss of neurons in cortex or hippocampus. In addition to conventional stains, Bielschowsky silver stain was used to highlight neurofilaments and test for the presence of neurofibrillary tangles. No differences in neurofilament staining were observed between old mutant and wild-type mice. Sections also were immunostained with an antibody to MAP-2, a marker of neuronal processes, but no changes in staining pattern were apparent in aged MT-III-deficient mice.

GFAP, an intermediate filament protein, is a marker of fibrillary astrocytes (Federoff and Vernadakis, 1986), and its levels in brain increase with advanced age (Goss et al., 1991; O’Callaghan and Miller, 1991; Kohama et al., 1995). As expected, GFAP mRNA in brain was induced approximately twofold in both groups. No neuropathology was detected. Open-field locomotor activity was depressed ~50% in both mutant and wild-type mice exposed to zinc, most likely reflecting the general malaise induced by systemic zinc overload. However, no impairment of spatial learning was observed in zinc-treated mice of either genotype when tested in the Morris water maze (data not shown).

MT-III-deficient mice are more susceptible to seizures induced by kainic acid and subsequently exhibit increased neuron injury in the CA3 field of hippocampus
Seizure susceptibility of MT-III-deficient mice was examined by administration of kainic acid, a structural analog of the excitatory transmitter, glutamate, which elicits limbic system seizures (Schwob et al., 1980; Ben-Ari et al., 1981). In hybrid (C57Bl/129Sv) mice, the total duration of motor convulsions and the mortality because of severe seizures were significantly greater for mutant mice (p < 0.01 and p < 0.05, respectively; Table 1). In addition, the percentage of mice exhibiting convulsions was greater for mutants, and the latency to seizure onset was shorter for mutants, but these differences were not significant (Table 1). A trend toward increased seizure susceptibility also was seen in inbred MT-III-deficient mice (Table 1).

Brains of kainate-treated mice were examined for neuropathol-
Pyknotic neurons were apparent in cortex, hippocampus, and other brain regions in a pattern characteristic of kainate-induced damage (Schwob et al., 1980; Ben-Ari et al., 1981), with mutant and wild-type mice exhibiting a qualitatively similar distribution of damage. The CA3 pyramidal cell layer of hippocampus was analyzed in detail because this region receives a dense input from zinc-containing terminals and both the presynaptic and postsynaptic neurons express the highest levels of MT-III (Masters et al., 1994b). Pyknotic CA3 neurons were apparent in 53% of wild-type mice and 76% of mutant mice ($p < 0.05$, $\chi^2$ analysis). The severity of damage in the CA3 region was also greater in MT-III-deficient mice (Fig. 7A).

The greater damage in the CA3 pyramidal cell layer of MT-III-deficient mice likely reflects the increased severity of seizures in these mice. However, loss of MT-III also might result in increased neuron vulnerability, thereby compounding the injury inflicted by seizures. To address this possibility, we compared the neuropathology of mutant and wild-type mice that exhibited similar seizures, as assessed by behavioral criteria. After relatively mild seizures characterized by cumulative convulsion durations of <100 sec, 68% of mutant mice contained extensive numbers of pyknotic neurons in the CA3 layer, but only 16% of wild-type mice exhibited damage in this region ($p < 0.01$, $\chi^2$ analysis). Moreover, CA3 neuron damage was clearly more severe in mutant (Figs. 7B, 8C) than in wild-type mice (Figs. 7B, 8H) after mild seizures. In contrast, CA3 neuron injury was similar in mutant (Figs. 7B, 8D,E) and wild-type mice (Figs. 7B, 8I,J) after more extensive convulsing.

We also treated transgenic mice containing approximately five-fold higher levels of MT-III and ~50% greater levels of brain zinc, with kainate. Although equally excitable as control mice (Table 1), only 29% of kainate-treated MT-III transgenic mice contained pyknotic CA3 neurons 3 d after treatment ($p < 0.05$ compared with wild-type mice; $p < 0.01$ compared with MT-III-deficient mice, $\chi^2$ analysis). The average injury score for MT-III transgenic mice was also lower than that for wild-type and MT-III-deficient mice (Fig. 7A). The neuroprotective effect of elevated MT-III was most apparent in mice having seizures characterized by intermediate convulsion durations (Figs. 7B, 8M,N). However, after extensive convulsions, MT-III transgenic mice exhibited damage in the CA3 layer similar to that seen in wild-type and MT-III-deficient mice (Figs. 7B, 8O).

A clear correlation between seizure severity and neuron injury also was observed in the CA1 region of hippocampus (Fig. 7C). However, in contrast to the CA3 region, the frequency and severity of CA1 neuron damage was not significantly different among wild-type, MT-III-deficient, and MT-III transgenic mice after similar convulsion durations (Fig. 7C).

**DISCUSSION**

A role for MT-III in the regulation of brain zinc was suggested by previous work demonstrating an anatomic correlation between vesicular zinc and MT-III (Masters et al., 1994b). Consistent with MT-III playing a role in cerebral zinc regulation, MT-III-deficient mice exhibited a selective reduction in brain zinc content. Assuming that each molecule of MT-III binds seven zinc ions (Sewell et al., 1995), the reduction in brain zinc can be accounted for quantitatively by the absence of zinc bound to MT-III. Despite a reduction in tissue zinc levels, histochemically reactive zinc was unaffected in the brains of mutant mice, as determined by both TS-Q histofluorescence and Timm’s staining, suggesting that MT-III is not critical for the regulation of this pool of zinc under normal physiological conditions. However, MT-III selectively protects CA3 neurons from seizure-induced death. A, Average semiquantitative neuron injury scores (mean ± SEM) in the CA3 hippocampal field of MT-III transgenic mice (TG, $n = 38$), wild-type mice (+/+, $n = 114$), and MT-III-deficient mice (−/−, $n = 45$) 3 d after kainate treatment. $p < 0.02$, as compared with wild-type mice; $p < 0.01$, as compared with MT-III transgenic mice, unpaired $t$ test. B, CA3 neuron injury scores are plotted in relation to seizure intensity, as assessed by cumulative convulsion time. Values are mean ± SEM, $n \geq 9$ for mutant mice; $n \geq 14$ for wild-type mice; $n \geq 7$ for MT-III transgenic mice. CA3 neuron damage scores for MT-III-deficient mice were significantly greater than for both wild-type and MT-III transgenic mice after <100 sec of convulsing; $p < 0.05$ and $p < 0.01$, respectively, unpaired $t$ test. CA3 neuron injury scores in MT-III-deficient mice and wild-type mice were greater than in MT-III transgenic mice after moderate seizures characterized by 100–300 sec of convulsions. C, Neuron injury in the CA1 hippocampal field of the same mice was not significantly affected by MT-III level.

**Figure 7.** MT-III selectively protects CA3 neurons from seizure-induced death. A, Average semiquantitative neuron injury scores (mean ± SEM) in the CA3 hippocampal field of MT-III transgenic mice (TG, $n = 38$), wild-type mice (+/+, $n = 114$), and MT-III-deficient mice (−/−, $n = 45$) 3 d after kainate treatment. $p < 0.02$, as compared with wild-type mice; $p < 0.01$, as compared with MT-III transgenic mice, unpaired $t$ test. B, CA3 neuron injury scores are plotted in relation to seizure intensity, as assessed by cumulative convulsion time. Values are mean ± SEM, $n \geq 9$ for mutant mice; $n \geq 14$ for wild-type mice; $n \geq 7$ for MT-III transgenic mice. CA3 neuron damage scores for MT-III-deficient mice were significantly greater than for both wild-type and MT-III transgenic mice after <100 sec of convulsing; $p < 0.05$ and $p < 0.01$, respectively, unpaired $t$ test. CA3 neuron injury scores in MT-III-deficient mice and wild-type mice were greater than in MT-III transgenic mice after moderate seizures characterized by 100–300 sec of convulsions. C, Neuron injury in the CA1 hippocampal field of the same mice was not significantly affected by MT-III level.
steady-state conditions. These results are consistent with previous observations that elevated levels of MT-III in transgenic mice produced an increase in total brain zinc but did not perturb histochemically reactive zinc (Erickson et al., 1995). MT-III might play a significant role in the regulation of “free” zinc under conditions not examined in this study, such as during nutritional zinc deficiency or seizures.

Because MT-III is most abundant in neurons of hippocampus and dentate gyrus, it was conceivable that its absence might impair hippocampal-dependent processes. This possibility was supported by the observation that zinc levels were reduced in hippocampus of MT-III-deficient mice more than other brain regions. Also, perturbations in brain zinc can exert profound effects on hippocampal-dependent behaviors (Hesse et al., 1979; Golub et al., 1983; Frederickson et al., 1990). However, open-field activity, passive avoidance conditioning, and spatial learning were normal in mutant mice, suggesting that MT-III and zinc bound to MT-III are not required for proper hippocampal function.

The lack of phenotypic abnormalities in MT-III-deficient mice under normal conditions might be attributed to compensation by MT-I and MT-II. Several observations argue against this possibility. First, MT-I mRNA levels, which are also indicative of MT-II mRNA levels, were not higher in MT-III-deficient brain under any condition examined. Second, total MT protein levels and tissue zinc concentrations were reduced significantly in MT-III-deficient brain. Finally, although there is overlap in the distribution of MTs in the CNS, MT-I and MT-II are expressed primarily in glial cells (Young et al., 1991; Nishimura et al., 1992; Blauwegers et al., 1993; Masters et al., 1994b; Nakajima and Suzuki, 1995), whereas MT-III is found predominantly in neurons in mice (Masters et al., 1994b). Alternatively, MT-III might be physiologically important only under certain conditions as is the case for MT-I and MT-II (Michalska and Choo, 1993; Masters et al., 1994a; Kelly et al., 1996; Kelly and Palmer, 1996). In an attempt to reveal a functional role of MT-III under “extreme” conditions, we challenged MT-III-deficient mice with metals, old age, and kainate.

Treatment of MT-III-deficient mice with zinc and cadmium demonstrated that MT-III does not constitute a primary defense against peripheral exposure to these metals, although previous
work showed that it can confer resistance to both metals in vitro (Palmiter, 1995). The doses of cadmium and zinc administered to MT-III-deficient mice were near the lethal doses for normal mice and were identical to those that revealed hepatic and pancreatic sensitivity in mice lacking MT-I and MT-II (Masters et al., 1994a; Kelly et al., 1996). Because neither zinc nor cadmium readily penetrate the blood–brain barrier (Kasarskis, 1984; Arvidson and Tjalve, 1986; Valois and Webster, 1987; Frederickson, 1989; Franklin et al., 1992; Takeda et al., 1994), the absence of metal-induced neurotoxicity in MT-III-deficient adult mice was not surprising. Even when cadmium entry into brain was increased by treating immature mice, the absence of MT-III did not enhance toxicity, probably because the primary damage was cerebral capillary endothelial cell necrosis rather than direct neuronal injury (Webster and Valois, 1981). These experiments do not rule out the possibilities that MT-III protects central neurons from zinc released by nerve terminals or from neurotoxic metals that readily access the CNS, such as methylmercury (Atchison and Hare, 1994), lead (Winship, 1989), and trimethyltin (Chang, 1986).

Previous studies suggested that reduced levels of MT-III in the brains of humans might contribute to the formation of neurofibrillary tangles in AD (Uchida et al., 1991; Tsuji et al., 1992). A potential role for MT-III in AD is bolstered by other studies implicating zinc in the pathophysiology of this disorder (Constantinidis, 1990; Wenstrup et al., 1990; Samudralwar et al., 1995; Tully et al., 1995). However, the brains of MT-III-deficient mice did not exhibit obvious neuronal loss and were void of neurofibrillary tangles up to 2 years of age. Moreover, learning and memory in old mice lacking MT-III closely resembled that of old wild-type mice, as evaluated by behavioral tests that can detect impairments in transgenic mice with Alzheimer’s-like neuropathology (Hsiao et al., 1996). The lack of overt neuropathology and the absence of accelerated cognitive deficits in aged MT-III-deficient mice, in combination with our previous observation that MT-III was not decreased in most AD brains (Erickson et al., 1994), casts doubt on a primary role of MT-III in the pathogenesis of AD.

Nevertheless, a role for MT-III in the response of the nervous system to aging was suggested by the observation that old MT-III-deficient mice contained approximately twofold higher levels of GFAP mRNA in brain than old wild-type mice. In contrast, GFAP mRNA levels in young mutant and wild-type mice were similar, indicating that MT-III specifically influences age-related changes in brain GFAP expression. The increase in GFAP mRNA was probably not attributable to global enhancement of glial cell gene expression, because MT-I mRNA, which also is expressed predominantly in astrocytes, was not affected by aging. GFAP levels and the number of GFAP-positive astrocytes in brain are known to increase with aging (Goss et al., 1991; O’Callaghan and Miller, 1991; Kohama et al., 1995) and in conditions associated with neuronal injury (Eng and Ghirnikar, 1994; O’Callaghan et al., 1995). The modest enhancement of GFAP expression in old MT-III-deficient mice might, therefore, reflect an astrocytic response to senescent changes in neuronal vulnerability that were not apparent by conventional histology.

MT-III-deficient mice were more susceptible to seizures induced by kainic acid and subsequently developed more pronounced damage in the CA3 region of hippocampus. The extent of neuron injury resulting from kainate treatment correlated with the severity of seizure activity, as determined by behavioral criteria. However, the relationship between convulsion duration and neuron injury was different in MT-III-deficient and MT-III transgenic mice, as compared with wild-type mice; loss of MT-III exacerbated CA3 neuron injury resulting from mild seizures, whereas increased levels of MT-III attenuated the injury associated with mild and moderately severe seizures. In contrast, injury to the CA1 region of hippocampus was unaffected by the level of MT-III. These results suggest that MT-III provides selective and limited protection to CA3 neurons during seizures. Whether this neuroprotective effect of MT-III is attributable to a selective attenuation of CA3 neuron activity during seizures or to an increase in the resistance of CA3 neurons to excitotoxic insults is an important question that cannot be answered with the current data.

The increased susceptibility to seizures and the apparent sensitivity to seizure-induced CA3 neuron damage in mice lacking MT-III may reflect a role for MT-III in the regulation of hippocampal zinc under stimulated conditions. The effects of zinc on neuron activity are complex, with zinc reportedly having both proconvulsant (Itoh and Ebadi, 1982; Pei and Koyama, 1986; Reece et al., 1994; Buhl et al., 1996) and anticonvulsant properties (Kulusi et al., 1986; Peters et al., 1987; Lees et al., 1990; Morton et al., 1990; Frederickson and Moncrieff, 1994). During high frequency firing, zinc is released from the mossy fiber terminals of dentate granule cells, which densely innervate CA3 neurons (Sloviter, 1985; Aniksztejn et al., 1987; Frederickson et al., 1988). Secreted zinc subsequently may inhibit the activity of postsynaptic CA3 neurons, thereby delaying seizure development, as suggested by the proconvulsant effects of chelating zinc (Mitchell et al., 1990; Mitchell and Barnes, 1993; Xu and Mitchell, 1993) and the reduced mossy fiber zinc content of seizure-susceptible mice (Fukahori et al., 1988; Feller et al., 1991). An attractive possibility is that MT-III is important for maintaining sufficient amounts of vesicular zinc during sustained neuronal firing by facilitating the recycling of zinc or serving as a reserve depot of zinc. Without MT-III, vesicular zinc in the mossy fibers or in other zinc-containing terminals may decline rapidly during prolonged stimulation, resulting in a reduced capacity to prevent seizure activity.

Zinc also is implicated in the pathogenesis of seizure-induced neuron death. Zinc is neurotoxic (Yokoyama et al., 1986; Choi et al., 1988; Lees et al., 1990; Duncan et al., 1992; Koh et al., 1996), especially when cells are depolarized (Weiss et al., 1993; Freund and Reddig, 1994), and zinc pretreatment potentiates kainate neurotoxicity in vivo (Nave and Connor, 1993). Moreover, kainate-evoked seizures produce a selective translocation of zinc from mossy fiber terminals to CA3 hippocampal somata, an event that precedes neuron death (Frederickson et al., 1989). Because MT-III confers resistance to zinc toxicity when expressed in tissue culture cells (Palmiter, 1995), it is tempting to speculate that MT-III selectively protects CA3 neurons during seizures by chelating potentially toxic influxes of zinc. This hypothesis presumes that there is either a pool of apo-MT-III available to bind excess zinc or that MT-III facilitates the redistribution of incoming zinc to locations where it is less toxic. By virtue of its high cysteine content, MT-III also may protect cells from reactive oxygen species generated during intense excitation (Coyle and Puttfarcken, 1993). Additional studies will be required to establish the mechanism by which the absence of MT-III facilitates seizure development, exacerbates seizure-induced neuron death, and enhances age-associated increases in GFAP.

REFERENCES


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