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# 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; Pérez-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; Christine 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 $\beta$ 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).

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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 genomic library (Palmiter et al., 1992). A disruption vector was constructed by replacing a 2.2 kb *Xho*I–*Nde*I region containing the promoter and exons 1 and 2 with a neomycin resistance cassette and inserting thymidine kinase genes at *Bam*HI and *Bst*EII sites in the 5′ and 3′ flanking regions, respectively. Electroporation and selection of AB1 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 *Sst*I by using a 0.6 kb *Bst*EII–*Nde*I 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 germline, 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 $S_1$ nuclease; the $S_1$-resistant complexes were collected on Whatman GF/C filters after precipitation with trichloroacetic acid. Oligonucleotide 350 (5′-CACACAGTCCTTGGCACACTTCTC-3′), complementary to a unique sequence in exon 3 of MT-III, oligonucleotide 57 (5′-GGGACAAATGATTTGGGGGCAAAAG-3′), complementary to the 3′ untranslated region of MT-I, and oligonucleotide JE01 (5′-GGTCATTGAGCTCCATCATCTCTGC-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/$\mu$g 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 $\mu$l was incubated with 50 $\mu$l of a $CdSO_4$ solution containing 1000 ppm Cd and $2.5 \times 10^6$ cpm of $^{109}$Cd for 10 min at room temperature. Then the sample was placed in boiling water for 1.5 min, cooled on ice, and centrifuged at 20,000 × *g* for 20 min. The supernatant was loaded onto a 1 × 40 cm Sephadex G-75 superfine column equilibrated with 20 mM Tris-HCl and 10 mM NaCl, pH 8; 0.85 ml fractions were collected and assayed for $^{109}$Cd.

*Histochemistry and immunostaining.* Mice were killed by $CO_2$ inhalation and cardiac-perfused with 4 ml of 10% neutral-buffered formalin (NBF). Brains were removed and placed in NBF for up to 1 week before being embedded in paraffin. Coronal sections (7 $\mu$m) 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 (Dakopatts, 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 Jarrel–Ash 955 spectrometer.

*Detection of histochemically reactive zinc.* Timm's stain was performed as described (Sloviter, 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 $\mu$m 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 × 47 × 30 cm (L × W × 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 × 12 cm squares was placed over the video monitor, and the number of squares entered was recorded for each mouse.

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 × 16.5 × 21 cm, separated by a door controlled by the investigator. One chamber was lined on all surfaces 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.

The Morris water maze consisted of a steel circular pool (1.15 m in diameter) with white walls that was partially filled with water (~24°C) and four gallons of whole milk. A white escape platform (10 cm in diameter) was placed in the middle of one of four quadrants at a depth of 1 cm below the surface of the liquid. Prominent visual cues were mounted on the walls and ceiling of the room. A video camera was mounted above the pool, allowing an investigator to monitor each mouse by video and to record the trials. A training trial involved placing a mouse in the pool at one of four start sites, which varied from trial to trial, and allowing the mouse to search for the platform. The time required for the mouse to climb onto the platform was recorded. If a mouse failed to find the platform in 60 sec, it was placed on the platform. Three trials separated by ~1.5 hr were conducted each day for seven consecutive days in the case of young adult mice and 10 d in the case of 2-year-old mice. A probe trial was performed the day after the last day of training. In this trial, the escape platform was removed from the pool, and the mouse was allowed to search for 60 sec. Recordings of the probe trial were viewed to determine the time spent in each quadrant and the number of times the site where the platform had been located was crossed.

*Zinc and cadmium toxicity.* Eight-week-old mice ($n = 6$ for each genotype) were injected subcutaneously for 30 consecutive days with 10 $\mu$mol Cd/kg body weight, and 7-d-old mice ($n = 6$ for each genotype) were given a single subcutaneous injection at the same dose. The injection solution consisted of 2 mM $CdCl_2$ (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 $ZnCl_2$ (Sigma). On days 1, 2, and 3, mice received 200, 300, and 400 $\mu$mol Zn/kg body weight, respectively, with a 100 mM $ZnCl_2$ solution. On days 4 and 5, mice received 600 $\mu$mol Zn/kg body weight from a 200 mM $ZnCl_2$ solution, and on days 6 and 7 they received 800 $\mu$mol Zn/kg body weight. This regimen provides exposure to zinc that is close to the maximum that mice can tolerate on a chronic basis (Kelly et al., 1996). Another group of mice was injected concurrently with similar volumes of PBS. One-half of the surviving mice were killed 24 hr after the last injection for biochemical and neuropathological analyses; the other one-half were tested for possible behavioral impairments.

*Kainic acid treatment.* Kainic acid (Sigma) was dissolved in PBS and

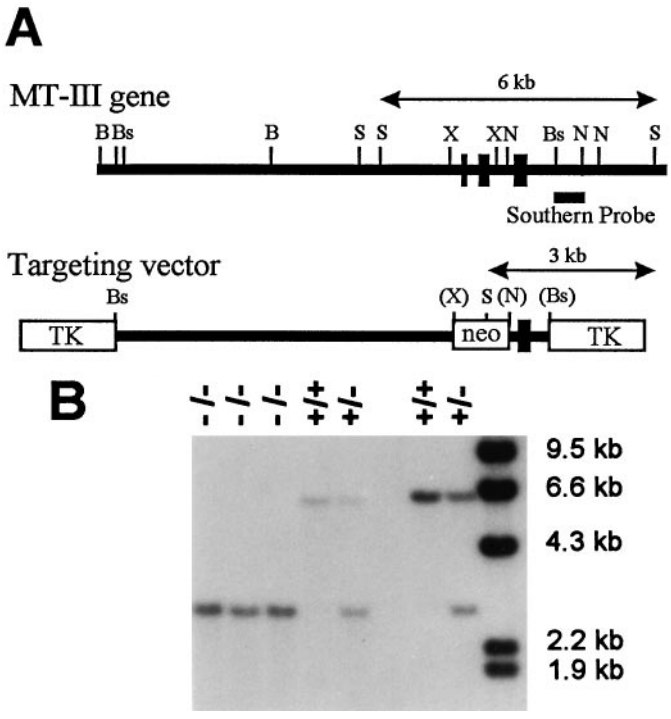


*Figure 1.* Targeted disruption of the MT-III gene. *A*, Map of the murine MT-III gene and the targeting vector. Abbreviations include the following: *B*, *Bam*HI; *Bs*, *Bst*E2; *N*, *Nde*I; *S*, *Sst*I; *X*, *Xho*I. Restriction sites in *parentheses* were destroyed. *B*, Southern blot of tail DNA digested with *Sst*I and probed with a 0.6 kb *Bst*EII–*Nde*I fragment not present in the targeting vector. +/+, Wild-type; +/−, heterozygote; −/−, mutant.

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 d 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 1*A*. The gene was inactivated by deleting the proximal promoter and exons 1 and 2 by homologous recombination in embryonic stem cells (Fig. 1*A*). 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. 1*B*).

Brain MT-III mRNA was abundant in wild-type mice, reduced

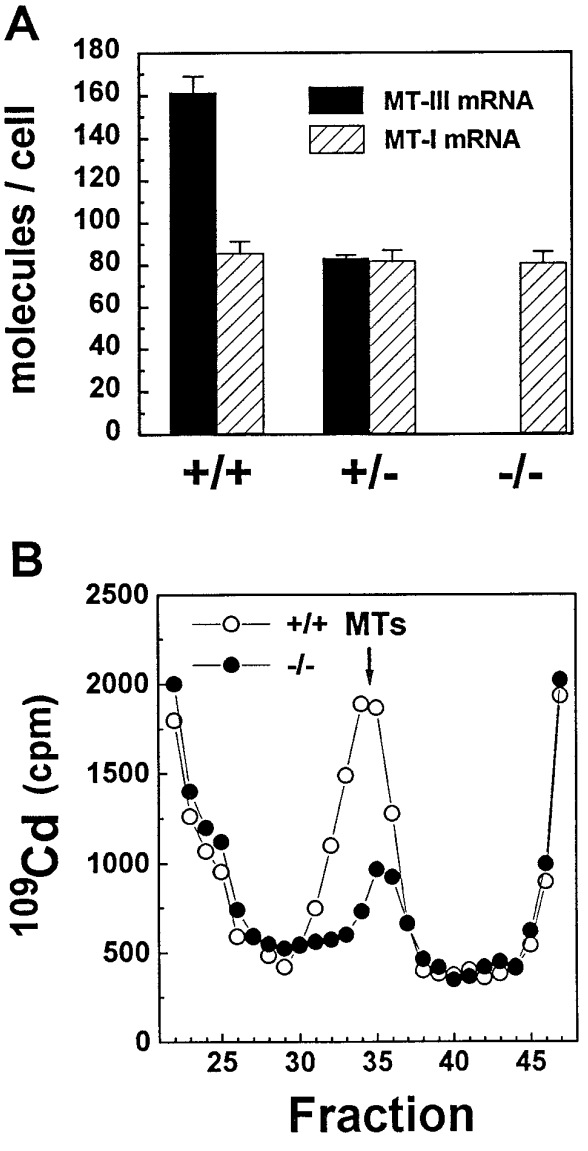


*Figure 2.* *A*, MT-III and MT-I mRNA levels in brain of wild-type mice (+/+) and MT-III-deficient mice (−/−) as measured by solution hybridization. Values are mean ± SEM; $n = 4$. *B*, Total MT protein in brain. Brain extracts were saturated with $^{109}Cd$ and fractionated on a Sephadex G-75 column. Representative chromatographic profiles of MT-III-deficient brain (−/−) and wild-type brain (+/+) are shown. *Arrow* indicates MT peak.

by ~50% in heterozygotes, and undetectable in mutant mice, as determined by quantitative solution hybridization (Fig. 2*A*). MT-I mRNA levels in brains of mutants and heterozygotes were unchanged, as compared with controls (Fig. 2*A*), 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. 2*B*), 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-

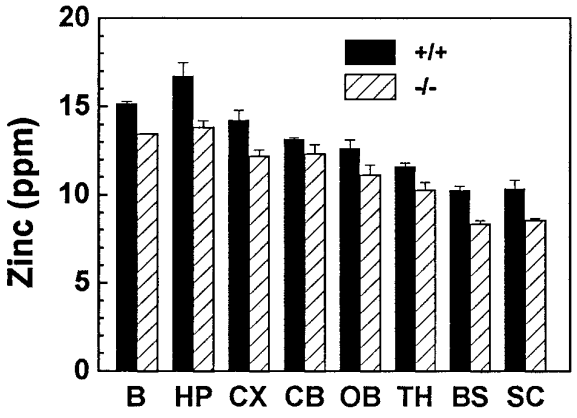


*Figure 3.* Zinc levels are decreased in multiple CNS regions of MT-III-deficient mice. Significant differences between wild-type mice (+/+) and MT-III-deficient mice (−/−) were observed in brain, hippocampus, cortex, brainstem, and spinal cord. Values are mean ± SEM; $n = 5$; $p < 0.05$, unpaired $t$ test. *B*, Whole brain; *BS*, brainstem; *CB*, cerebellum; *CX*, cortex; *HP*, hippocampus; *OB*, olfactory bulb; *TH*, thalamus; *SC*, spinal cord.

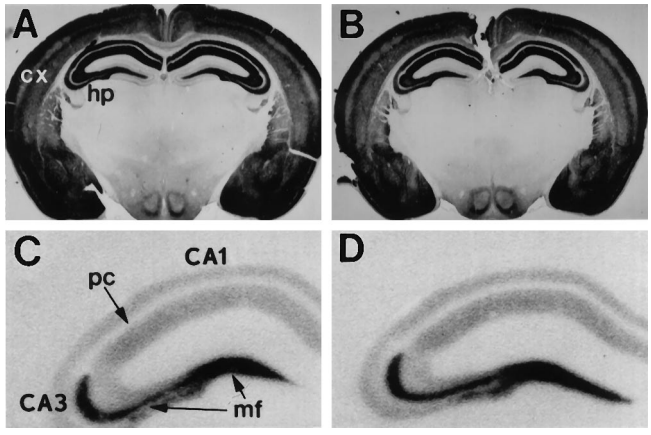


*Figure 4.* Histochemically reactive zinc is not perturbed in MT-III-deficient mice. Shown are Timm's-stained coronal brain sections from a wild-type mouse (*A*) and a mutant mouse (*B*). *cx*, Cortex; *hp*, hippocampus. Also shown are computer-generated images of TS-Q histofluorescence in hippocampus of a wild-type mouse (*C*) and a mutant mouse (*D*). Regions of intense fluorescence are indicated by *black*, intermediate intensity by *gray*, and low intensity by *white*. *mf*, Mossy fibers; *pc*, pyramidal cell layer.

ities in cellularity or microscopic anatomy were detected on examination of brain sections stained with hematoxylin and eosin, cresyl violet, Nissl stain, or Bielschowsky silver stain.

### 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. Concentrations of other elements, including copper and calcium, were not 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. 4*A,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. 4*C,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. 5*A*). 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. 5*B*), and both groups crossed the site where the platform had been located more often than an equivalent site in the opposite quadrant (Fig. 5*C*). 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).

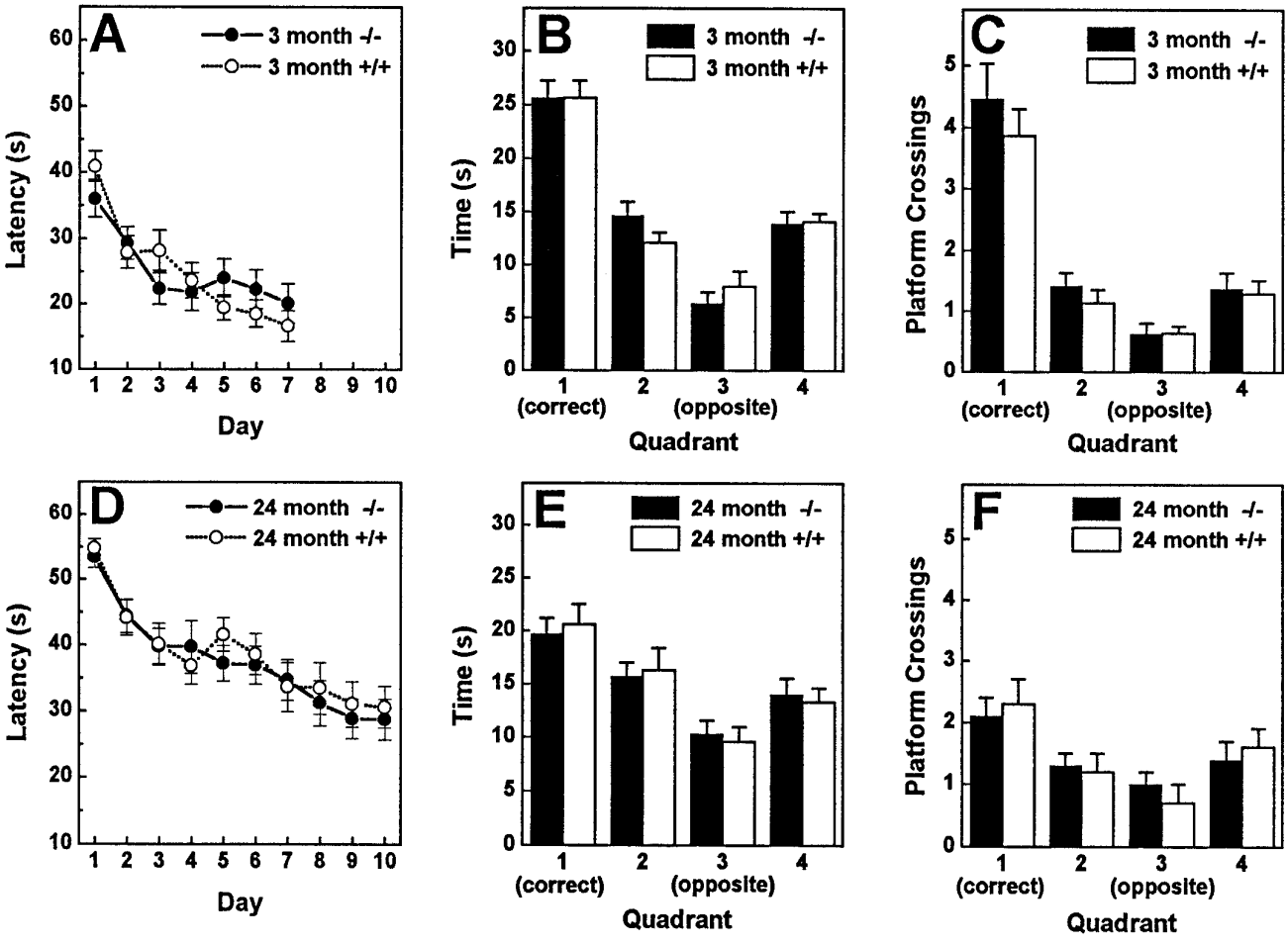


*Figure 5.* MT-III-deficient mice exhibit normal spatial learning in the hidden platform version of the Morris water maze. *A*, Escape latencies of 3-month-old wild-type mice (+/+, $n = 28$) and MT-III-deficient mice (−/−, $n = 24$) in the acquisition phase. The average latency of three trials is shown for each day of training. *B*, Quadrant preferences of 3-month-old mice 24 hr after the last day of training. The times spent in each quadrant during a 60 sec probe trial in which the escape platform was removed are shown. *C*, Number of times the platform site and equivalent sites in the wrong quadrants were crossed by 3-month-old mice during the probe trial. *D*, Escape latencies of 2-year-old wild-type mice ($n = 18$) and MT-III-deficient mice ($n = 27$) in the acquisition phase. *E*, Quadrant preferences of 2-year-old mice in the probe trial. *F*, Platform site crossings of 2-year-old mice in the probe trial. Testing conditions were identical for young and old mice, except that old mice were trained for 10 d instead of 7 d. All values are mean ± SEM. There were no significant differences between wild-type and mutant mice.

### 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

**Table 1. Response of wild-type (+/+), MT-III-deficient (−/−), and MT-III transgenic (TG) mice to kainic acid**

| Genotype | Genetic background | Percent seizing | Latency (min) | Total convulsion time (sec) | Mortality (%) |
|---|---|---|---|---|---|
| +/+ | hybrid | 74 | 29.3 ± 3.0 | 121 ± 18 | 18 |
| −/− | hybrid | 86 | 21.5 ± 4.1 | 221 ± 25** | 43* |
| TG | hybrid | 71 | 31.5 ± 3.5 | 115 ± 20 | 11 |
| +/+ | 129 Sv | 76 | 30.6 ± 4.0 | 107 ± 25 | 0 |
| −/− | 129 Sv | 88 | 20.9 ± 3.0 | 184 ± 30 | 13 |

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, $n \geq 24$ for each group. *Significantly greater than +/+ hybrid mice; $p < 0.05$, $\chi^2$ analysis. **Significantly greater than +/+ hybrid mice; $p < 0.01$, unpaired $t$ test.

each genotype did not survive the treatment. The day after the last injection, zinc levels in brain were elevated ~15%, and MT-I 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 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. 5*D*). 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. 5*E,F*), although their spatial preference was weaker than that of young mice (Fig. 5*B,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 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).

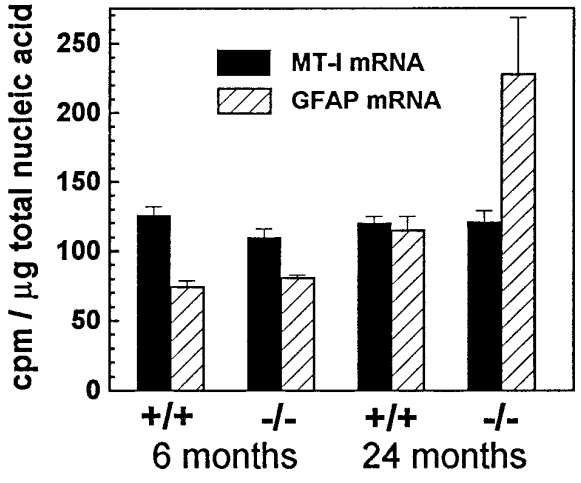


*Figure 6.* MT-III-deficient mice exhibit an enhanced increase in GFAP expression associated with aging. GFAP mRNA and MT-I mRNA in brains of wild-type mice (+/+) and MT-III-deficient mice (−/−) were quantitated by solution hybridization. Values are mean ± SEM. GFAP mRNA levels were significantly higher in 24-month-old mutant mice ($n = 11$), as compared with 24-month-old wild-type mice ($n = 7$). $p < 0.05$, unpaired $t$ test. GFAP mRNA levels were also significantly higher in 24-month-old mice of both genotypes, as compared with 6-month-old wild-type mice ($n = 4$) and 6-month-old mutant mice ($n = 5$). $p < 0.05$. No significant differences in MT-I mRNA levels were detected among any group.

Immunostaining for GFAP revealed greater numbers of positive astrocytes in gray matter of 2-year-old mice, as compared with 6-month-old mice, with old MT-III-deficient mice displaying appreciably more GFAP-immunoreactive cells than old wild-type mice (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-

ogy 3 d later. 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. 7*A*).

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. 7*B*, 8*C*) than in wild-type mice (Figs. 7*B*, 8*H*) after mild seizures. In contrast, CA3 neuron injury was similar in mutant (Figs. 7*B*, 8*D,E*) and wild-type mice (Figs. 7*B*, 8*I,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. 7*A*). The neuroprotective effect of elevated MT-III was most apparent in mice having seizures characterized by intermediate convulsion durations (Figs. 7*B*, 8*M,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. 7*B*, 8*O*).

A clear correlation between seizure severity and neuron injury also was observed in the CA1 region of hippocampus (Fig. 7*C*). 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. 7*C*).

## 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

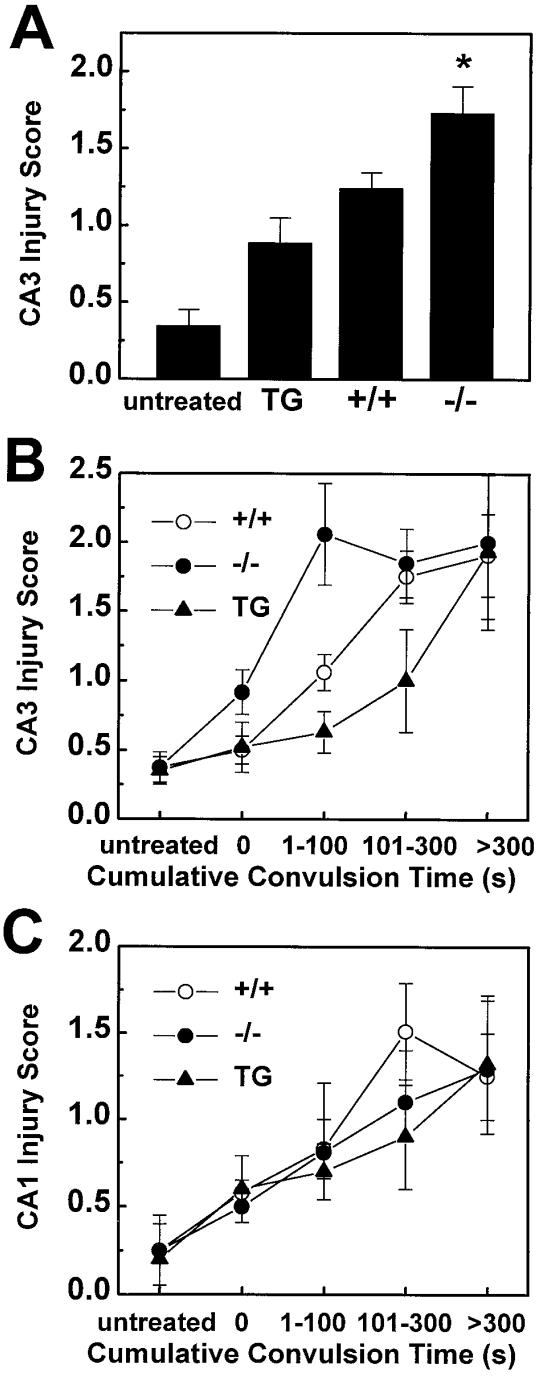


*Figure 7.* MT-III selectively protects CA3 neurons from seizure-induced death. *A*, Average semiquantitative neuron injury scores (mean ± SEM) in the CA3 hippocampal field of MT-III transgenic mice (*TG*, $n = 38$), wild-type mice (+/+, $n = 114$), and MT-III-deficient mice (−/−, $n = 45$) 3 d after 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.

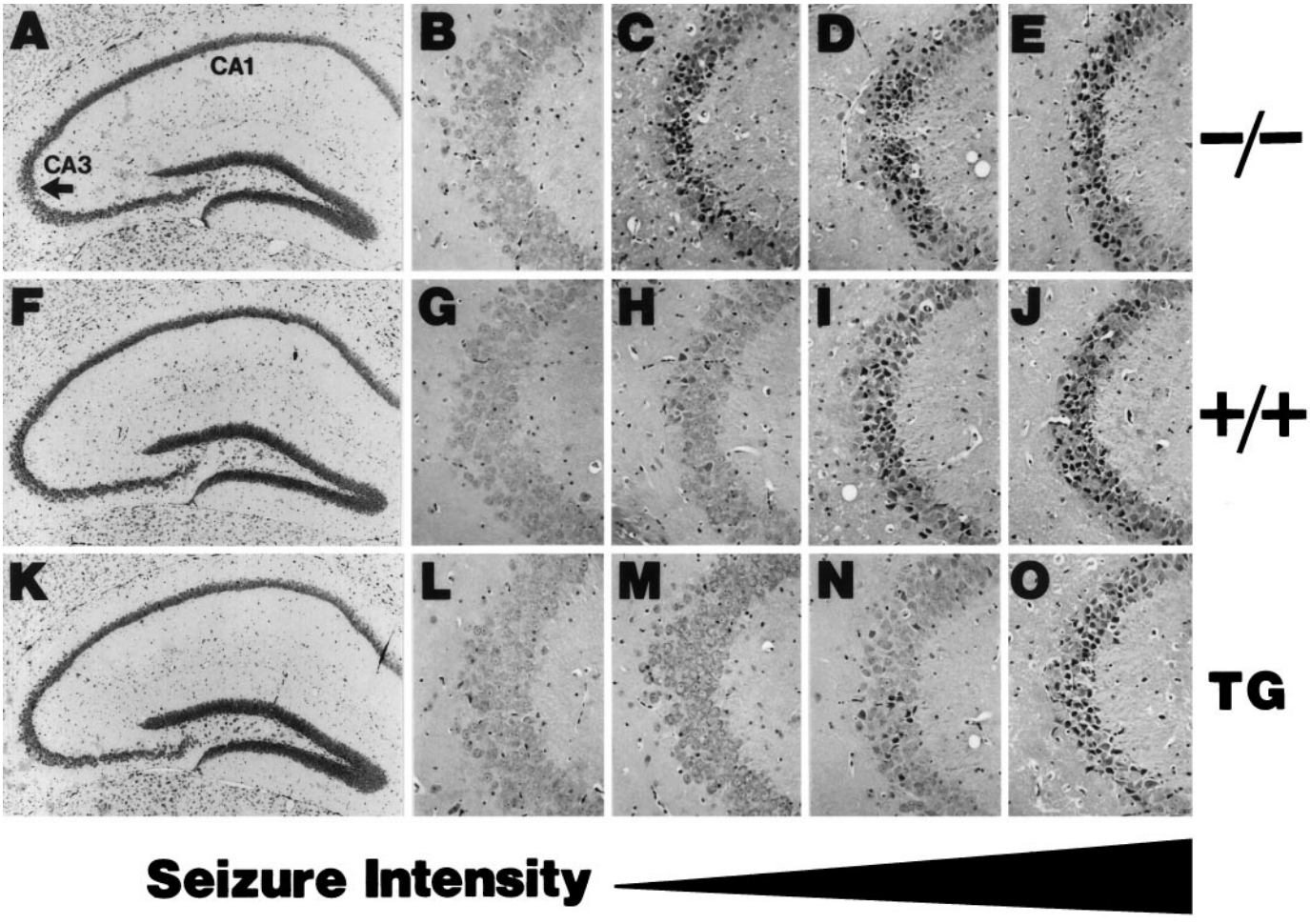


*Figure 8.* Representative photomicrographs of hippocampus from MT-III-deficient mice (−/−), wild-type mice (+/+), and MT-III transgenic mice (*TG*) under normal conditions and 3 d after kainate treatment. *A*, *F*, *K*, Low magnification view of cresyl violet-stained sections of hippocampus under normal conditions. All other photomicrographs are higher magnification views of the CA3 pyramidal neuron layer (*arrow* in *A*) stained with hematoxylin and eosin. *B*, *G*, *L*, CA3 layer of mice not treated with kainate. *C*, *H*, *M*, CA3 pyramidal neurons in mice that had convulsions lasting less than a combined 100 sec. Pyknotic neurons, appearing as shrunken dark cells, are present only in the MT-III-deficient mouse (*C*). *D*, *I*, *N*, CA3 pyramidal layer from mice that had moderate seizures characterized by cumulative convulsion times lasting 100–300 sec. Degenerating cells are abundant in the MT-III-deficient mouse (*D*) and the wild-type mouse (*I*), but not in the MT-III transgenic mouse (*N*). *E*, *J*, *O*, Pyknotic neurons are present in mice of all genotypes after extensive convulsions.

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; Blaauwgeers 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 Palmiter, 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 viability 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 (Khulusi 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

Aniksztejn L, Charton G, Ben-Ari Y (1987) Selective release of endogenous zinc from hippocampal mossy fibers *in situ*. Brain Res 404:58–64.

Arvidson B, Tjalve H (1986) Distribution of 109-cadmium in the nervous system of rats after intravenous injection. Acta Neuropathol (Berl) 69:111–116.

Assaf SY, Chung SH (1984) Release of endogenous $Zn^{2+}$ from brain tissue during activity. Nature 308:734–736.

Atchison WD, Hare MF (1994) Mechanisms of methylmercury-induced neurotoxicity. FASEB J 8:622–629.

Ben-Ari Y, Tremblay E, Riche D, Ghilini G, Naquet R (1981) Electrographic, clinical, and pathological alterations following systemic administration of kainic acid, bicuculline, or pentetrazole: metabolic mapping using the deoxyglucose method with special reference to the pathology of epilepsy. Neuroscience 6:1361–1391.

Blaauwgeers HGT, Sillevis Smitt PAE, De Jong JMBV, Troost D (1993) Distribution of metallothionein in the human central nervous system. Glia 8:62–70.

Buhl EH, Otis TS, Mody I (1996) Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. Science 271:369–373.

Bush AI, Pettingell WH, Multhaup G, Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE (1994) Rapid induction of Alzheimer A$\beta$ amyloid formation by zinc. Science 265:1464–1467.

Chang LW (1986) Neuropathology of trimethyltin: a proposed pathogenetic mechanism. Fundam Appl Toxicol 6:217–232.

Charton G, Rovira C, Ben-Ari Y, Leviel V (1985) Spontaneous and evoked release of endogenous $Zn^{2+}$ in the hippocampal mossy fiber zone of the rat *in situ*. Exp Brain Res 58:202–205.

Choi DW, Yokoyama M, Koh J (1988) Zinc neurotoxicity in cortical cell culture. Neuroscience 24:67–79.

Christine CW, Choi DW (1990) Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. J Neurosci 10:108–116.

Chung SH, Johnson MS (1983) Divalent transition-metal ions ($Cu^{2+}$ and $Zn^{2+}$) in the brains of epileptogenic and normal mice. Brain Res 280:323–334.

Constantinidis J (1990) Alzheimer's disease and the zinc theory. Encephale 16:231–239.

Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. Science 262:689–695.

Draguhn A, Verdorn TA, Ewert M, Seeburg PH, Sakmann B (1990) Functional and molecular distinction between recombinant rat $GABA_A$ receptor subtypes by $Zn^{2+}$. Neuron 5:781–788.

Duncan MW, Marini AM, Watters R, Kopin IJ, Markey SP (1992) Zinc, a neurotoxin to cultured neurons, contaminates cycad flour prepared by traditional guamanian methods. J Neurosci 12:1523–1537.

Durnam DM, Palmiter RD (1983) A practical approach for quantitating specific mRNAs by solution hybridization. Anal Biochem 131:385–393.

Eng LF, Ghirnikar RS (1994) GFAP and astrogliosis. Brain Pathol 4:229–237.

Erickson JC, Sewell AK, Jensen LT, Winge DR, Palmiter RD (1994) Enhanced neurotrophic activity in Alzheimer's disease cortex is not associated with down-regulation of metallothionein-III. Brain Res 649:297–304.

Erickson JC, Masters BA, Kelly EJ, Brinster RL, Palmiter RD (1995) Expression of human metallothionein-III in transgenic mice. Neurochem Int 27:35–41.

Federoff S, Vernadakis A (1986) Astrocytes, Vol 3, Cell biology and pathology of astrocytes. Orlando: Academic.

Feller DJ, Tso-Olivas DY, Savage DD (1991) Hippocampal mossy fiber zinc deficit in mice genetically selected for ethanol withdrawal seizure susceptibility. Brain Res 545:73–79.

Forsythe ID, Westbrook GL, Mayer ML (1988) Modulation of excitatory synaptic transmission by glycine and zinc in cultures of mouse hippocampal neurons. J Neurosci 8:3733–3741.

Franklin A, Pullen RGL, Hall GH (1992) Blood-brain exchange routes and distribution of $^{65}Zn$ in rat brain. Neurochem Res 17:767–771.

Frederickson CJ (1989) Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol 31:145–238.

Frederickson CJ, Moncrieff DW (1994) Zinc-containing neurons. Biol Signals 3:127–139.

Frederickson CJ, Kasarskis EJ, Ringo D, Frederickson RE (1987) A quinoline fluorescence method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in the brain. J Neurosci Methods 20:91–103.

Frederickson CJ, Hernandez MD, Goik SA, Morton JD, McGinty JF (1988) Loss of zinc staining from hippocampal mossy fibers during kainic acid-induced seizures: a histofluorescence study. Brain Res 446:383–386.

Frederickson CJ, Hernandez MD, McGinty JF (1989) Translocation of zinc may contribute to seizure-induced neuron death. Brain Res 480:317–321.

Frederickson RE, Frederickson CJ, Danscher G (1990) *In situ* binding of bouton zinc reversibly disrupts performance on a spatial memory task. Behav Brain Res 38:25–33.

Freund WD, Reddig S (1994) AMPA/$Zn^{2+}$-induced neurotoxicity in rat primary cortical cultures: involvement of L-type calcium channels. Brain Res 654:257–264.

Fukahori M, Itoh M (1990) Effects of dietary zinc status on seizure susceptibility and hippocampal zinc content in the *El* (epilepsy) mouse. Brain Res 529:16–22.

Fukahori M, Itoh M, Oomagari K, Kawasaki H (1988) Zinc content in discrete hippocampal and amygdaloid areas of the epilepsy (*El*) mouse and normal mice. Brain Res 455:381–384.

Golub MS, Gershwin ME, Vijayan VK (1983) Passive avoidance performance of mice fed marginally or severely zinc-deficient diets during postembryonic brain development. Physiol Behav 30:409–413.

Goss JR, Finch CE, Morgan DG (1991) Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain. Neurobiol Aging 12:165–170.

Harrison NL, Gibbons SJ (1994) $Zn^{2+}$: an endogenous modulator of ligand and voltage-gated ion channels. Neuropharmacology 33:935–952.

Haug FMS (1967) Electron microscopic localization of the zinc in hippocampal mossy fiber synapses by a modified sulphide silver procedure. Histochemie 8:355–368.

Hesse GW, Frank Hesse KA, Catalanotto FA (1979) Behavioral characteristics of rats experiencing chronic zinc deficiency. Physiol Behav 22:211–215.

Hollman M, Boulter J, Maron C, Beasley L, Sullivan J, Pecht G, Heinemann S (1993) Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. Neuron 10:943–954.

Howell GA, Welch MG, Frederickson CJ (1984) Stimulation-induced uptake and release of zinc in hippocampal slices. Nature 308:736–738.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, A$\beta$ elevation, and amyloid plaques in transgenic mice. Science 274:99–102.

Itoh M, Ebadi M (1982) The selective inhibition of hippocampal glutamic acid decarboxylase in zinc-induced epileptic seizures. J Neurochem 41:823–829.

Kägi JHR, Kojima Y (1987) Chemistry and biochemistry of metallothionein. Experientia [Suppl] 52:25–80.

Kasarskis EJ (1984) Zinc metabolism in normal and zinc-deficient rat brain. Exp Neurol 85:114–127.

Kasarskis EJ, Forrester TM, Slevin JT (1987) Amygdalar kindling is associated with elevated zinc concentration in the cortex and hippocampus of rats. Epilepsy Res 1:227–233.

Kelly EJ, Palmiter RD (1996) A murine model of Menkes disease reveals a physiological function of metallothionein. Nat Genet 13:219–222.

Kelly EJ, Quaife CJ, Froelick GJ, Palmiter RD (1996) Metallothionein-I and -II protect against zinc deficiency and zinc toxicity. J Nutr 126:1782–1790.

Khulusi SS, Brown MW, Wright DM (1986) Zinc and paired-pulse potentiation in the hippocampus. Brain Res 363:152–155.

Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 272:1013–1016.

Kohama SG, Goss JR, Finch CE, McNeill TH (1995) Increases of glial fibrillary acidic protein in the aging female mouse brain. Neurobiol Aging 16:59–67.

Lees GJ, Lehman A, Sandberg M, Hamberger A (1990) The neurotoxicity of zinc in the rat hippocampus. Neurosci Lett 120:155–158.

Li C, Peoples RW, Li Z, Weight FF (1993) $Zn^{2+}$ potentiates excitatory action of ATP on mammalian neurons. Proc Natl Acad Sci USA 90:8264–8267.

Masters BA, Kelly EJ, Quaife CJ, Brinster RL, Palmiter RD (1994a) Targeted disruption of metallothionein-I and -II genes increases sensitivity to cadmium. Proc Natl Acad Sci USA 91:584–588.

Masters BA, Quaife CJ, Erickson JC, Kelly EJ, Froelick GJ, Zambrowicz BP, Brinster RL, Palmiter RD (1994b) Metallothionein-III is expressed in neurons that sequester zinc in synaptic vesicles. J Neurosci 14:5844–5857.

Michalska AE, Choo KH (1993) Targeting and germ-line transmission of

a null mutation at the metallothionein I and II loci in mouse. Proc Natl Acad Sci USA 90:8088–8092.
Mitchell CL, Barnes MI (1993) Proconvulsant action of diethyldithiocarbamate in stimulation of the perforant path. Neurotoxicol Teratol 15:165–171.
Mitchell CL, Barnes MI, Grimes LM (1990) Diethyldithiocarbamate and dithizone augment the toxicity of kainic acid. Brain Res 506:327–330.
Morris RGM, Garrud P, Rawlins JNP, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681–683.
Morton JD, Howell GA, Frederickson CJ (1990) Effects of subcutaneous injections of zinc chloride on seizures induced by noise and by kainic acid. Epilepsia 31:139–144.
Nakajima K, Suzuki K (1995) Immunocytochemical detection of metallothionein in brain. Neurochem Int 27:73–87.
Nave JM, Connor JD (1993) Influence of $ZnCl_2$ pretreatment on behavioral and histological responses to kainic acid in rats. Brain Res 604:298–303.
O'Callaghan JP, Miller DB (1991) The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. Neurobiol Aging 12:171–174.
Nishimura N, Nishimura H, Ghaffer A, Tohyama C (1992) Localization of metallothionein in the brain of rat and mouse. J Histochem Cytochem 40:309–315.
O'Callaghan JP, Jensen KF, Miller DB (1995) Quantitative aspects of drug and toxicant-induced astrogliosis. Neurochem Int 26:115–124.
Palmiter RD (1987) Molecular biology of metallothionein gene regulation. Experientia [Suppl] 52:63–80.
Palmiter RD (1995) Constitutive expression of metallothionein-III (MT-III), but not MT-I, inhibits growth when cells become zinc deficient. Toxicol Appl Pharmacol 135:139–146.
Palmiter RD, Findley SD, Whitmore TE, Durnam DM (1992) MT-III, a brain-specific member of the metallothionein gene family. Proc Natl Acad Sci USA 89:6333–6337.
Palmiter RD, Sandgren EP, Koeller DM, Findley SD, Brinster RL (1993) Metallothionein genes and their regulation in transgenic mice. In: Metallothionein III: biological roles and medical implications (Suzuki KD, Imura N, Kimura M, eds), pp 399–406. Basel: Birkhauser Verlag.
Pei YQ, Koyama I (1986) Features of seizures and behavioral changes induced by intrahippocampal injection of zinc sulfate in the rabbit: a new experimental model of epilepsy. Epilepsia 27:183–188.
Pérez-Clausell J, Danscher G (1985) Intravesicular location of zinc in rat telencephalic boutons. A histochemical study. Brain Res 337:91–98.
Peters S, Koh J, Choi DW (1987) Zinc selectively blocks the action of *N*-methyl-D-aspartate on cortical neurons. Science 236:589–593.
Quaife CJ, Findley SD, Erickson JC, Froelick GJ, Kelly EJ, Zambrowicz BP, Palmiter RD (1994) Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. Biochemistry 33:7250–7259.
Rassendren FA, Lory P, Pin JP, Nargeot J (1990) Zinc has opposite effects on NMDA and non-NMDA receptors expressed in *Xenopus* oocytes. Neuron 4:733–740.
Reece LJ, Dhanjal SS, Chung SH (1994) Zinc induces hyperexcitability in the hippocampus. NeuroReport 5:2669–2672.
Samudralwar DL, Diprete CC, Ni BF, Ehman WD, Markesbery WR (1995) Elemental imbalances in the olfactory pathway in Alzheimer's disease. J Neurol Sci 130:139–145.
Schwob JE, Fuller T, Price JL, Olney JW (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. Neuroscience 5:991–1014.
Sewell AK, Jensen LT, Erickson JC, Palmiter RD, Winge DR (1995) Bioactivity of metallothionein-3 correlates with its novel $\beta$ domain sequence rather than metal binding properties. Biochemistry 34:4740–4747.
Sloviter RS (1982) A simplified Timm stain procedure compatible with formaldehyde fixation and routine paraffin embedding of rat brain. Brain Res Bull 8:771–774.
Sloviter RS (1985) A selective loss of hippocampal mossy fiber Timm stain accompanies granule cell seizure activity induced by perforant path stimulation. Brain Res 330:150–153.
Suzuki KD, Imura N, Kimura M (1993) Metallothionein III: biological roles and medical implications. Basel: Birkhauser Verlag.
Takeda A, Akiyama T, Sawashita J, Okada S (1994) Brain uptake of trace metals, zinc and manganese, in rats. Brain Res 640:341–344.
Thomas SA, Matsumoto AM, Palmiter RD (1995) Noradrenaline is essential for mouse fetal development. Nature 374:643–646.
Townes TM, Lingrel JB, Chen HY, Brinster RL, Palmiter RD (1985) Erythroid-specific expression of human $\beta$-globin genes in transgenic mice. EMBO J 7:1715–1723.
Tsuji S, Kobayashi H, Uchida Y, Ihara Y, Miyatake T (1992) Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. EMBO J 11:4843–4850.
Tully CL, Snowden DA, Markesbery WR (1995) Serum zinc, senile plaques, and neurofibrillary tangles: findings from the Nun study. NeuroReport 6:2105–2108.
Uchida Y, Takio K, Titani K, Ihara Y, Tomonaga M (1991) The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. Neuron 7:337–347.
Vallee BL, Falchuck KH (1993) The biochemical basis of zinc physiology. Physiol Rev 73:79–118.
Valois AA, Webster WS (1987) Retention and distribution of cadmium in the mouse brain: an autoradiographic and gamma counting study. Neurotoxicology 8:463–470.
Webster WS, Valois AA (1981) The toxic effects of cadmium on the neonatal mouse CNS. J Neuropathol Exp Neurol 40:247–257.
Weiss JH, Hartley DM, Koh J, Choi DW (1993) AMPA receptor activation potentiates zinc neurotoxicity. Neuron 10:43–49.
Wenstrup D, Ehman WD, Markesbery WR (1990) Trace element imbalances in isolated subcellular fractions of Alzheimer's disease brains. Brain Res 533:125–131.
Westbrook GL, Mayer ML (1987) Micromolar concentrations of $Zn^{2+}$ antagonize NMDA and GABA responses in hippocampal neurons. Nature 328:640–643.
Winship KA (1989) Toxicity of lead: a review. Adverse Drug React Acute Poisoning Rev 8:117–152.
Wright DM (1986) Effect of zinc on neuronal activity in the rat forebrain. Adv Exp Med Biol 203:599–609.
Xie X, Smart TG (1991) A physiological role for endogenous zinc in rat hippocampal synaptic neurotransmission. Nature 349:521–524.
Xu H, Mitchell CL (1993) Chelation of zinc by diethyldithiocarbamate facilitates bursting induced by mixed antidromic plus orthodromic activation of mossy fibers in hippocampal slices. Brain Res 624:162–170.
Yokoyama M, Koh J, Choi DW (1986) Brief exposure to zinc is toxic to cortical neurons. Neurosci Lett 71:351–355.
Young JK, Garvey JS, Huang PC (1991) Glial immunoreactivity for metallothionein in rat brain. Glia 4:602–610.