Metallothionein, an Endogenous Antioxidant, Protects against Retinal Neuron Damage in Mice

Shinsuke Suemori,1,2 Masamitsu Shimazawa,1 Kazubide Kawase,2 Masabiko Satoh,3 Hisamitsu Nagase,5 Tetsuya Yamamoto,2 and Hideaki Hara1

PURPOSE. To clarify the functional role of metallothionein (MT) in retinal damage in mice deficient in both MT-I and -II (MT-I/-II-deficient mice [C57BL/6 background]) and wild-type (C57BL/6J) mice and MT induction (zinc sulfate [ZnSO4] and II– deficient mice [C57BL/6J background]) and wild-type NMDA injection. To examine the involvement of induced ret-recognizes both proteins) 12 and 24 hours after intravitreous reverse-transcription-PCR of total retinal RNA from eyes injected or not injected with NMDA. In wild-type mice, MT-I and -II immunohistochemistry was performed (with antibody that recognizes both proteins) 12 and 24 hours after intravitreous NMDA injection. To examine the involvement of induced retinal MT, ZnSO4 (10 nmol/eye) or Vit. D3 (0.2 or 2 ng/eye) was intravitreously injected 24 hours before NMDA injection in wild-type or MT-I/-II-deficient mice, and ganglion cell layer (GCL) cell loss and inner plexiform layer (IPL) thinning were evaluated 7 days after the NMDA injection. The protective effect of Vit. D3 was assessed against the RGC-5 cell death induced by oxidative stress (using buthionine sulfoximine [BSO] to deplete glutathione in combination with glutamate to inhibit cystine uptake).

RESULTS. In wild-type mice, MT-II mRNA expression was time-dependently elevated by NMDA (5.9 and 7.4 times versus the nontreated control at 4 and 12 hours, respectively, after injection), with the normal level being regained within 24 hours. In contrast, MT-I and -II showed persistent decreases (to <50% control) from 4 to 24 hours. In wild-type mice, MT-like immunoreactivity was increased in the inner retina (GCL and IPL) 12 and 24 hours after NMDA injection. At 7 days after NMDA injection in MT-I/-II-deficient mice (versus wild-type mice), GCL cell loss was increased, but IPL thickness was not different. Pretreatment with ZnSO4 or Vit. D3 increased inner retinal MT-like immunoreactivity 24 hours after NMDA injection and significantly attenuated NMDA-induced GCL cell loss in wild-type mice, but ZnSO4 pretreatment did not protect against such cell loss in MT-I/-II-deficient mice. In vitro, Vit. D3 pretreatment (100 nM) reduced BSO+glutamate-induced RGC-5 cell death.

CONCLUSIONS. These findings suggest that MT, especially MT-II, protects against retinal neuron damage, by acting as an endogenous antioxidant. (Invest Ophthalmol Vis Sci. 2006;47: 3975–3982) DOI:10.1167/iows.06-0275

Retinal ganglion cell death is a common feature of ophthalmic disorders such as glaucoma and central artery and vein occlusion. In humans and monkeys, glaucoma is associated with a significant elevation in the vitreous glutamate concentration.1 Retinal ganglion cells are exquisitely sensitive to the effects of both glutamate and its analogue N-methyl-D-aspartate (NMDA), which produces a dose-dependent cell loss both in vivo and in vitro, and glutamate toxicity has been implicated in the pathophysiology of glaucoma.2 NMDA receptor-mediated neurotoxicity has been reported to depend in part on the generation of nitric oxide and superoxide anions, which react to form peroxynitrite.3 Thus, oxidative stress, leading to the formation of free radicals, has been implicated as part of the final common pathway for neurotoxicity in a wide variety of acute and chronic neurodegenerative diseases.4–6

In contrast, all aerobic cells possess an antioxidant defense system that has enzymatic and nonenzymatic components. The enzymatic component comprises three major enzymes: superoxide dismutase, glutathione peroxidase, and catalase. The nonenzymatic component includes vitamins A and E and also glutathione (GSH), a key component of the cell-defense system.10 Abnormally low levels of GSH have been reported in the lens7 and whole blood of patients with age-related cataract10 and in the blood of patients with glaucoma,11 proliferative diabetic retinopathy (PDR), proliferative viretiretinopathy (PVR),12 or age-related macular degeneration (ARMD).13 Thus, in several retinal diseases, decreases in GSH could reduce the scavenging capacity against reactive oxygen species, causing an increased vulnerability of neurons.

Metallothionein (MT) is a class consisting of four low-molecular-weight (6000–7000), metal-binding proteins with a high cysteine content.14 These MT isoforms are known as MT-I, -II, -III, and -IV,15–17 and they serve as important regulators of metal homeostasis and as a source of the zinc incorporated into proteins, including transcription factors.18 Several studies have found that MT has a free-radical–scavenging function in tissues subjected to oxidative stress and that it affords cytoprotection via this radical-scavenging ability.19–22 Of interest, it has been reported that the expressions of MT-I and -II, but not that of MT-III, increase after cerebral ischemia and that MT-I isoform-overexpressing transgenic mice have a reduced infarct size after cerebral ischemia.23–24 These findings suggest that MT may be an important neuroprotective substance for stroke and other acute neurodegenerative diseases. However, little is known about the functional role of MT in retinal diseases.

In the present study, we monitored the time course of changes in the expressions of MT isoforms (MT-I, -II, and -III mRNAs) and in the distribution of MT proteins during retinal damage in mice. In addition, we used mice deficient in both MT-I and -II and two inducers of MT (zinc and 1α, 25-dihydroxyvitamin D3) to clarify the role played by MT in retinal neurons.

FROM THE DEPARTMENTS OF 1 BIOFUNCTIONAL MOLECULES AND 2 HYGIENOLOGY, GIFU PHARMACEUTICAL UNIVERSITY, GIFU, JAPAN; AND THE 3 DEPARTMENT OF OPHTHALMOLOGY, GIFU UNIVERSITY GRADUATE SCHOOL OF MEDICINE, GIFU, JAPAN.

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Corresponding author: Hideaki Hara, Department of Biofunctional Molecules, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan; hidehara@gifu-pu.ac.jp.

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MATERIALS AND METHODS

Animals

Male metallothionein-I and-II (MT-I/II)–deficient \(^{25}\) and wild-type mice weighing 20 to 25 g were used in the present study. The background strain of mice was C57BL/6J, and this strain was used as the wild-type control. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Intravitreal Injection

Mice were anesthetized with 3.0% isoflurane, then maintained using 1.5% isoflurane in 70% \(\text{N}_2\text{O} \) and 30% \(\text{O}_2 \) using an animal general anesthesia machine (Soft Lander, Sin-Ei Industry Co. Ltd., Saitama, Japan). Intravitreal injections (2 \(\mu\)L) were performed using a 32-gauge needle 0.5 mm behind the limbus in the inferior region of the globe, through the conjunctiva and sclera. Eyes were routinely injected with 2 \(\mu\)L of 20 mM NMDA (40 nmol/eye; Sigma-Aldrich, St. Louis, MO). Any mice that exhibited postoperative complications, such as retinal hemorrhage, vitreous hemorrhage, or retinal detachment, were excluded from the analysis.

In another series of experiments, zinc sulfate (\(\text{ZnSO}_4 \), 10 mmol/eye; Wako, Osaka, Japan) or 1 \(\mu\)g 25-dihydroxvitamin D\(_3\) (Vit. D\(_3\), 2 ng/eye; Biomol, Plymouth Meeting, PA), each of which induces MT expression (or vehicle; and identical dose of sulfuric acid or ethanol in saline served as the respective control) was intravitreally injected 24 hours before the NMDA injection in wild-type or MT-I/II deficient mice. The time point for administration of MT inducers chosen to be 24 hours before the NMDA injection, because the intravitreal injection of Vit. D\(_3\) (2 ng/eye) sufficiently increased MT-like immunoreactivity in retina after 24 hours, as mentioned later.

Histology

The mice were euthanatized by inhalation of diethyl ether at the end of the experiment. Each eye was enucleated and postfixed overnight in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) at 4°C and embedded in paraffin. Six paraffin-embedded sections (thickness, 4 \(\mu\)m) cut through the optic disc of each eye were prepared in a standard manner and stained with hematoxylin and eosin. Retinal damage was evaluated as described previously, \(^{20}\) with three sections from each eye being used for the morphometric analysis. Light microscope images were photographed, and the cell counts in the ganglion cell layer (GCL) at a distance between 350 and 650 \(\mu\)m from the optic disc, and the thickness of the inner plexiform layer (IPL) were measured on the micrographs in a masked fashion by a single observer (SS). Data from three sections (selected randomly from the six sections) were averaged for each eye and used to evaluate the GCL cell count and the IPL thickness.

Real-time RT-PCR

Retinas were obtained from wild-type mice before and at 4, 12, and 24 hours after the NMDA injection. Total RNA was isolated from these retinas using an SV total RNA isolation system (Promega, Madison, WI). Quantitative real-time PCR, after reverse transcription, was performed to determine the time-course of changes in the gene expression of MT isoforms (MT-I, -II, and -III). Briefly, 1 \(\mu\)g total RNA was used for first-strand cDNA synthesis (SuperScript II reverse transcriptase; Invitrogen, Carlsbad, CA) with Oligo (dT) 12 to 18 primer (Invitrogen), in accordance with the manufacturer’s instructions. Real-time PCR was performed in 50 \(\mu\)L of reaction mixture containing each primer (see Table 1), template cDNA, and supermix (SYBR Green; Bio-Rad, Hercules, CA) using a single-color real-time PCR detection system (MxIQ; Bio-Rad) according to the manufacturer’s instructions. Reactions were performed in 40 cycles consisting of 15 seconds’ denaturation (94°C) and 60 seconds’ elongation with annealing (60°C). \(\beta\)-Actin was used as the reference standard, and relative levels of MT isoforms compared with that of \(\beta\)-actin were calculated.

Immunohistochemistry

To detect MT protein expression in the retina, MT immunostaining was performed. A total of 24 animals was used and divided into four groups: nontreated in wild-type mice (\(n = 6\)), nontreated in MT-I/II-deficient mice (\(n = 6\)), 12 hours after NMDA (40 nmol/eye) injection in wild-type mice (\(n = 6\)), and 24 hours after NMDA (40 nmol/eye) injection in wild-type mice (\(n = 6\)). Eyes were then postfixed overnight in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) at 4°C, and embedded in paraffin. Horizontal sections (4 \(\mu\)m) through the optic nerve were obtained from the paraffin-embedded eyes. Such sections were deparaffinized with xylene and dehydrated through a graded ethanol series. Immunohistochemical staining was performed in accordance with the protocol provided for a commercially available immunohistologic staining kit (Histomouse-Plus Kits; Zymed Laboratories Inc., South San Francisco, CA). Briefly, tissue sections were washed in a 0.01 M phosphate-buffered saline solution (PBS) for 10 minutes, and then endogenous peroxidase was quenched by treating the sections with 3% hydrogen peroxide in absolute methanol for 10 minutes. After the sections were washed with PBS, they were incubated with blocking solutions A and B for 30 and 10 minutes, respectively, to eliminate nonspecific background. After the sections were washed with PBS, a mouse monoclonal antibody against MT-I and -II proteins (clone E9; Zymed Laboratories Inc.) was added at a dilution of 1:100, and incubation was allowed to proceed for 60 minutes. After the sections were washed with PBS, a biotinylated secondary antibody was added, and the sections were incubated for 30 minutes. After the sections were washed with PBS, streptavidin-peroxidase was added, incubated for 20 minutes, and visualized as a red deposit by addition of a chromogen solution substrate. Omission of primary antibodies served as a negative control. The level of MT-like immunoreactivity in retina was estimated compared with that in corneal endothelium in each specimen.

For induction of MT protein in the retina, \(\text{ZnSO}_4 \) (10 nmol/eye), Vit. D\(_3\) (0.2 ng and 2 ng/eye; or vehicle [see above] as control) was intravitreally injected in wild-type mice. Twenty-four hours later, the upregulation of MT protein was detected using the immunostaining described earlier. The concentrations achieved in the vitreous body before intravitreous injections of \(\text{ZnSO}_4 \) and low- and high-dose Vit. D\(_3\) were estimated to be 1 \(\mu\)M, 20 \(\mu\)g/mL, and 200 \(\mu\)g/mL, respectively. The doses of these inducers used in this study were chosen by reference to previous studies in which \(\text{ZnSO}_4 \) and Vit. D\(_3\) were reported to induce MT-I and -II mRNA expressions. \(^{27,28}\)

RGC-5 Culture

 Cultures of RGC-5 cells were maintained in Dulbecco’s modified Eagle’s medium (D-MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Valeant, Costa Mesa, CA), 100 U/mL penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan), and 100 \(\mu\)g/mL streptomycin (Meiji Seika Kaisha, Ltd.) in a humidified atmosphere of 95% air and 5%
CO₂ at 37°C. The RGC-5 cells were passaged by trypsinization every 3 days, as in our previous report.26

Cell Mortality Assay after Oxidative Stress

For induction of oxidative stress in an RGC-5 cell culture in vitro, buthionine sulfoximine (BSO) and a high dose of glutamate (10 mM) were added to the cell culture medium, as previously described by Maher and Hanneken.29 BSO, a glutamate-cysteine ligase inhibitor, inhibits GSH synthesis, resulting in a depletion of intracellular GSH, whereas high-dose glutamate inhibits the uptake of cystine, a ratelimiting amino acid in GSH synthesis. Such coadministration of BSO and glutamate induces cell death through an oxidative-stress pathway.

RGC-5 cells were plated at a density of 1000 cells/well in 96-well culture plates (3072; Falcon; BD Biosciences, Bedford, MA). Cells were washed twice with D-MEM, then with the same medium supplemented with 1% FBS/H₁₁₀₀₁ Vit. D₃ at 10 or 100 nM or with vehicle (0.1% ethanol). One hour later, BSO (500 μM; Wako) plus 10 mM glutamate (Nakarai Tesque, Inc., Kyoto, Japan) or vehicle (D-MEM), was added to each medium. Forty-eight hours after this addition, cell viability was measured using nuclear staining methods. Briefly, cell death was assessed on the basis of combination-staining with fluorescent dyes (Hoechst 33342; Invitrogen, Eugene, OR; and YO-PRO-1; Invitrogen), by inverted epifluorescence microscope (IX70; Olympus, Tokyo, Japan). At the end of the culture period, Hoechst 33342 and YO-PRO-1 dyes were added to the culture medium at 8 and 0.1 μM, respectively, for 30 minutes. Images were collected with a cooled charge-coupled device (CCD) camera (Olympus). In a blind manner, at least 400 cells per condition were counted with image-processing software (Image-J ver. 1.33f; National Institutes of Health). Cell mortality was quantified by determining the percentage of YO-PRO-1-positive cells to Hoechst 33342–positive cells.

Statistical Analysis

Data are presented as the mean ± SEM. Statistical comparisons were made by using a Student’s t-test or Dunnett test (Stat View ver. 5.0; SAS Institute Inc., Cary, NC). P < 0.05 was considered to indicate statistical significance.

**Figure 1.** Distribution of MT-like immunoreactivity in mouse retina after NMDA injection. Retinal cross sections were labeled with antibody against MT. (A) Hematoxylin and eosin staining, (B) control (nontreated normal retina), (C) 12 hours after intravitreous injection of NMDA in wild-type mice, (D) 24 hours after intravitreous injection of NMDA in wild-type mice. MT-like immunoreactivity was increased in the inner retina at both 12 and 24 hours after intravitreous injection of NMDA at 40 nmol/eye. Scale bar, 50 μm.

**Figure 2.** Time-course data for changes in MT-I, -II, and -III mRNAs in mouse retina after NMDA injection. Expressions of MT mRNAs in the retina were measured using a quantitative real-time reverse-transcriptase PCR assay at 4, 12, and 24 hours after intravitreous injection of NMDA (40 nmol) in wild-type mice. Data are the mean ± SEM of results in four animals. (A) MT-I, (B) MT-II, (C) MT-III. *P < 0.05, **P < 0.01 versus control (nontreated control retina Dunnett test).
GCL

Retinal damage was evaluated by counting the number of cells in the GCL (Fig. 1A for reference. Normal retina stained with hematoxylin and eosin is shown in A and B). MT-like immunoreactivity was observed in the retina of MT-I/- (Figs. 6G), although no significant difference in IPL thickness was observed between MT-I/-II– deficient (19.8 ± 0.3 μm, n = 9) and wild-type mice (19.8 ± 0.4 μm, n = 9). In contrast, there were no significant differences in the number of GCL cells or IPL thickness between MT-I/-II– deficient (97.9 ± 1.7 cells/mm for GCL and 37.7 ± 0.5 μm for IPL, n = 9) and wild-type mice in retinas obtained from nontreated control eyes (97.3 ± 1.5 cells/mm for GCL and 36.7 ± 0.7 μm for IPL, n = 9).

RESULTS

Expression of Metallothioneins

To clarify the changes in MT protein in the retina after retinal injury, MT expression was examined before and after an intravitreal injection of NMDA. For this, we used immunostaining with a specific antibody recognizing both MT-I and -II proteins. Normal retina stained with hematoxylin and eosin is shown in Figure 1A for reference.

In the normal retina of wild-type mice before NMDA injection, MT-like immunoreactivity was localized to the retinal nerve fiber layer (RNFL) and inner plexiform layer (IPL), but was only slight (Fig. 1B) compared with that in the cornea. No MT-like immunoreactivity was observed in the retina of MT-I/-II– deficient mice (data not shown). Intravitreal injection of NMDA at 40 nmol/eye increased the MT-like immunoreactivity in the inner retina at 12 and 24 hours after the injection (Figs. 1C, 1D). Marked increases in MT-like immunoreactivity were observed in RNFL and in cells in the ganglion cell layer (GCL).

The time course of changes in the mRNA expressions for MT isoforms (MT-I, -II, and -III) after NMDA injection was examined in the retina of wild-type mice by real-time RT-PCR (Fig. 2). The MT-II mRNA level was time-dependently elevated 5.9- and 7.4-fold (P < 0.05) versus the nontreated control at 4 and 12 hours, respectively, after the NMDA injection (Fig. 2B), and it returned to the normal level by 24 hours. In contrast, MT-I and -III mRNA showed persistent decreases (to less than half the relevant control) from 4 to 24 hours (Figs. 2A, 2C).

Exacerbated NMDA-Induced Retinal Damage in MT-I/-II–Deficient Mice

The participation of MT in retinal cell damage was examined by comparing MT-I/-II–deficient mice with the wild-type mice. As shown in Figure 3, MT-I/-II–deficient mice showed a smaller number of cells in the GCL (22.1 ± 1.1 cells/mm, n = 9) at 7 days after the NMDA injection than in wild-type mice (31.6 ± 1.0 cells/mm, n = 9) (P < 0.01). However, no significant difference in IPL thickness was observed between MT-I/-II–deficient (19.8 ± 0.3 μm, n = 9) and wild-type mice (19.8 ± 0.4 μm, n = 9). In contrast, there were no significant differences in the number of GCL cells or IPL thickness between MT-I/-II–deficient (97.9 ± 1.7 cells/mm for GCL and 37.7 ± 0.5 μm for IPL, n = 9) and wild-type mice in retinas obtained from nontreated control eyes (97.3 ± 1.5 cells/mm for GCL and 36.7 ± 0.7 μm for IPL, n = 9).

Protective Effect of Increases in MT-I/II Induced by Zinc Ion or Vit. D₃ against NMDA-Induced Retinal Damage

To clarify the protective effects of MT against retinal injury, MT was induced either by zinc sulfate (ZnSO₄) or by Vit. D₃. Twenty-four hours after intravitreal injection of ZnSO₄ at 10 nmol/eye or of Vit. D₃ at 0.2 or 2 ng/eye in wild-type mice, MT-like immunoreactivity was markedly elevated in the inner retina (versus the vehicle-treated retina; Figs. 4B, 4C, 5). In contrast, no immunoreactivity was observed in retinas treated without the primary antibody (Fig. 4A).

The effects of ZnSO₄ on retinal damage in MT-I/-II–deficient mice and wild-type mice are summarized in Figure 6. In wild-type mice, treatment with ZnSO₄ at 24 hours before the NMDA injection significantly protected against cell loss in the GCL (versus the vehicle-treated NMDA control group; Figs. 6A–C, 6G), although no significant difference in IPL thickness was observed between ZnSO₄-treated (22.2 ± 1.0 μm, n = 5) and vehicle-treated groups (21.6 ± 0.9 μm, n = 8). In contrast, no protective effect of ZnSO₄ was evident in MT-I/-II–deficient mice (Figs. 6D–6G). The effects of Vit. D₃ on retinal damage in wild-type mice are shown in Figure 7. Treatment with Vit. D₃ at 24 hours before the NMDA injection significantly protected against cell loss in GCL (versus the vehicle-treated NMDA control group; Figs. 7A–D), although no significant difference in IPL thickness was observed between the Vit. D₃-pretreated (20.0 ± 0.3 μm, n = 10) and vehicle-treated groups (19.4 ± 0.4 μm, n = 11; Figs. 7B, 7C, 7E).

Effect of Vit. D₃ on Retinal Cell Death Induced by Oxidative Stress

Representative fluorescence nuclear staining using Hoechst 33342 and YO-PRO-1 dyes are shown in Figure 8A. In vehicle-treated control cells, we observed normal nuclear morphology and negative staining with YO-PRO-1 dye (which stains early apoptotic and later-stage cells). Cells treated for 48 hours with a combination of BSO (which inhibits glutamate cysteine ligase) and glutamate (which inhibits the uptake of cystine) revealed shrinkage and condensation of their nuclei and posit...
tive staining with both YO-PRO-1 and Hoechst 33342 dyes. The number of cells exhibiting YO-PRO-1 fluorescence was counted, and positive cells were expressed as the percentage of YO-PRO-1-positive to Hoechst 33342-positive cells (Fig. 8B). The combination of 500 μM BSO and 10 mM glutamate induced significant cell death, the resulting percentage of YO-PRO-1-positive cells being 57.1% ± 3.2% (n = 8), whereas in the vehicle-treated control group, it was 0.9% ± 0.2% (n = 8). Pretreatment with Vit. D₃ at 100 nM, but not at 10 nM, significantly inhibited the cell death (approximately 20% inhibition) induced by the combination of BSO and glutamate. In contrast, treatment with Vit. D₃ alone at 100 nM had no effect on cell viability (versus the vehicle-treated control group; Fig 8B).

DISCUSSION

The present study, performed to assess the functional roles played by MT in retinal damage, used MT-I/-II--deficient mice and two inducers of MT. Intravitreal injection of NMDA increased the MT-like immunoreactivity in the inner retina of wild-type mice at both 12 and 24 hours (Figs. 1C, 1D). In particular, marked increases in MT-like immunoreactivity were observed in RNFL, in some of the GCL cells, and in the inner nuclear layer. In contrast, no MT-like immunoreactivity was observed in the retina in MT-I/-II--deficient mice.

Activation of the NMDA receptor leads to an intracellular Ca²⁺ influx and to an increase in reactive oxygen species (ROS) that may be detrimental to cell viability.34 Furthermore, activation of this receptor depletes intracellular GSH, which could decrease the intracellular capacity for inactivation of ROS.34 Thus, oxidative stress, leading to the formation of ROS, has been implicated as a stage in the final common pathway for neurotoxicity in a wide variety of acute and chronic neurologic diseases such as stroke, Alzheimer’s, and Parkinson’s diseases. In such pathologic conditions, an increase in MT could compensate for the decreased radical-scavenging ability. Because we used an antibody that recognizes both MT-I and -II proteins in this study, we could not tell whether the increased MT-like immunoreactivity in the retina after NMDA injection represented MT-I or -II (or both). To clarify which MT isoform(s) might be increased after retinal damage, the time-course of the changes in the mRNA levels for MT isoforms (MT-I, -II, and -III) occurring after NMDA injection were examined in the retina of wild-type mice. The MT-II mRNA level was time-dependently elevated at 4 and 12 hours after NMDA injection, and it had returned to normal level by 24 hours (Fig. 2). In contrast, for MT-I and -III there were persistent decreases (to less than half control) from 4 to 24 hours. These findings suggest that the increased MT-like immunoreactivity we observed was derived from MT-II protein. In contrast, in studies of cerebral ischemia: (1) persistent increases in the expressions of MT-I and -II mRNA, but not of MT-III mRNA, in the infarcted cortex have been found after 72 hours cerebral ischemia reperfusion in mice and rats, and (2) increases in MT-I and -II mRNAs and MT-protein have been detected in the endothelial cells of microvessels and in astrocytes in the infarcted cortex (by in situ hybridization and immunocytochemistry).35,36 Our findings differ from these previous reports in that the MT-I mRNA level showed a persistent decrease in the retina after NMDA injection. This discrepancy may reflect differences in experimental conditions and/or in the target tissue examined.

To investigate the functional role of MT we used MT-I/-II--deficient mice, which have inactivated MT-I and -II genes.37 Such mice possess a reduced radical-scavenging capability and an increased sensitivity to cadmium.38,39 Furthermore, they have been reported to exhibit not only a poor neurologic outcome after stroke (versus wild-type animals), but also increased infarct volumes.32 In the present study, the cell loss in GCL was significantly greater in MT-I/-II--deficient mice than in wild-type mice at 7 days after an intravitreal injection of NMDA (Fig. 3). In contrast, no significant difference in IPL thickness was observed between MT-I/-II--deficient and wild-type mice. These results seem consistent with our observations of marked increases in MT-like immunoreactivity in RNFL and in GCL cells, but not in IPL. Taken together, our results indicate that MT may exert an important neuroprotectant influence over retinal ganglion cells.

Next, we examined whether the upregulation of MT induced by zinc sulfate (ZnSO₄) or Vit. D₃ would protect against cell death in the NMDA-damaged retina in vivo and/or against

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4.** Effect of ZnSO₄ on MT-like immunoreactivity of mouse retina. Retinal cross sections were labeled with a primary antibody against MT in wild-type mice. Twenty-four hours after intravitreous injection of vehicle (A) without or (B) with the primary antibody; (C) 24 hours after intravitreous injection of ZnSO₄ (10 nmol). Scale bar, 50 μm.

![FIGURE 5](https://example.com/figure5.png)

**FIGURE 5.** Effect of Vit. D₃ on MT-like immunoreactivity in mouse retina. Retinal cross sections were labeled with a primary antibody against MT in wild-type mice 24 hours after intravitreous injection of (A) vehicle, (B) Vit. D₃ (0.2 ng/eye: low dose), or (C) Vit. D₃ (2 ng/eye: high dose). Scale bar, 50 μm.
oxidative stress-induced cell death in vitro. It has been reported that transcription of MT genes is upregulated in response to zinc, and that oral administration of Vit. D₃ to mice results in increased levels of MT mRNA in the liver, kidney, and skin. In our study, intravitreous injection of ZnSO₄ at 10 nmol/eye or of Vit. D₃ at 0.2 or 2 ng/eye in wild-type mice markedly elevated MT-like immunoreactivity in the inner retina, especially in RNFL and GCL cells. Moreover, pretreatment
with ZnSO₄ protected against the cell loss in GCL induced by NMDA in wild-type mice, and this effect was not apparent in MT-I/-II– deficient mice (Fig. 6). However, in this study comparisons were based on small sample sizes in the different subgroups (n = 5–8)—a possible limitation of the study. Pretreatment with Vit. D₃ also showed evidence of protective effects in wild-type mice (Fig. 7). Previous studies have found (1) that zinc pretreatment significantly reduces the increased levels of thiobarbituric acid-reactive substance (a marker of oxidative status) and of conjugated diene during ischemia-reperfusion and also increases metallothionein levels (versus saline injection), and (2) that Vit. D₃ inhibits oxygen-mediated ultraviolet injury in mouse skin indicating a protective effect of Vit. D₃ via induced MT. In the present experiment on RGC-5 in vitro, Vit. D₃ also showed evidence of protective effects in wild-type mice (Fig. 7). Previous studies have found (1) that zinc pretreatment significantly reduces the increased levels of thiobarbituric acid-reactive substance (a marker of oxidative status) and of conjugated diene during ischemia-reperfusion and also increases metallothionein levels (versus saline injection), and (2) that Vit. D₃ inhibits oxygen-mediated ultraviolet injury in mouse skin indicating a protective effect of Vit. D₃ via induced MT. In the present experiment on RGC-5 in vitro, Vit. D₃ inhibited the oxidative stress-related cell death caused by depletion of GSH and inhibition of cystine uptake (induced by treatment with a combination of buthionine sulfoximine and glutamate; Fig. 8). Taken together, these findings suggest that the protective effects of ZnSO₄ and Vit D₃ may each be attributable to an upregulation of MT. However, we did not examine the effect of Vit D₃ in MT-I/-II–deficient mice in this study. Therefore, we could not exclude the possibility that the protective effect of Vit. D₃ is derived from other mechanisms, although MT protein was markedly increased in retina after the intravitreous injection of Vit. D₃.

In conclusion, we have demonstrated that MT-II mRNA is upregulated in the injured murine retina and that MT-I/-II knockdown exacerbates retinal damage. Hence, our findings suggest that MT, especially MT-II, plays a central role as a neuroprotectant against retinal neuronal damage.

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