# Activation of Matrix Metalloproteinase-9 via Neuronal Nitric Oxide Synthase Contributes to NMDA-Induced Retinal Ganglion Cell Death

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**Purpose.** Understanding the mechanism of neuronal cell death in retinal diseases like glaucoma is important for devising new treatments. One factor involves excitatory amino acid stimulation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, excessive $Ca^{2+}$ influx, and formation of nitric oxide (NO) via neuronal NO synthase (nNOS). Another factor is the abnormal activation of matrix metalloproteinases (MMPs), in particular MMP-9, which triggers an extracellular signaling cascade leading to apoptosis. This study was designed to investigate the mechanism of excitotoxic retinal ganglion cell (RGC) death in vivo and its relationship to MMP activation.

**Methods.** NMDA and glycine were injected into the vitreous of the eye in rats and in nNOS-deficient mice ($nNOS^{-/-}$) versus control. Gelatinolytic activity of MMP-9 and MMP-2 by zymography and cellular localization by immunohistochemistry were examined, and the effect of MMP inhibition on NMDA-induced RGC death was tested.

**Results.** NMDA was found to upregulate the proform of MMP-9 in the retina and to increase MMP-9 gelatinolytic activity. Retrograde labeling with aminostilbamidine to identify RGCs confirmed that MMP activity occurred only in these retinal neurons and not in glial or other retinal cell types after excitotoxic insult. Deconvolution fluorescence microscopy revealed that MMP activity colocalized with immunoreactive *S*-nitrosylated protein. NMDA-induced MMP activation was diminished in the retina of $nNOS^{-/-}$ mice, implying that *S*-nitrosylation of MMP had indeed occurred. In addition, the broad-spectrum MMP inhibitor GM6001 protected RGCs after intravitreal NMDA injection.

**Conclusions.** These findings suggest that an extracellular proteolytic pathway in the retina contributes to RGC death via NO-activated MMP-9. (*Invest Ophthalmol Vis Sci.* 2005;46: 4747–4753) DOI:10.1167/iovs.05-0128

Neuronal injury and death play a critical role in the pathogenesis of neurodegenerative disorders, including those affecting the retina.[1–4] Understanding the mechanism of neuronal cell death in retinal diseases such as glaucoma is important for devising new treatments. Glutamate is a major excitatory neurotransmitter in the retina as well as in other regions of the central nervous system. Elevated levels of endogenous glutamate and/or excessive activation of glutamate receptors are thought to contribute to a variety of acute and chronic neurologic disorders, including hypoxic-ischemic brain injury (stroke), trauma, seizures, and various forms of dementia and neurodegeneration, as well as several retinal diseases, including retinal artery occlusion and glaucoma.[5–8] One mechanism of neuronal injury and death involves excessive *N*-methyl-D-aspartate (NMDA) receptor stimulation, leading to excessive $Ca^{2+}$ influx, which in turn triggers formation of nitric oxide (NO) via neuronal NO synthase (nNOS).[9–13] In animal models, intravitreal injection of NMDA induces retinal ganglion cell (RGC) apoptosis, thinning of the inner retina, and visual dysfunction.[12,14–16] Pharmacological studies in the retina with specific inhibitors have shown that mild insults with NMDA stimulate several intracellular transduction pathways, for example, with activation of the p38 mitogen-activated protein kinase (MAPK) pathway contributing to the apoptotic-like death of RGCs.[16] As an intracellular signaling molecule, NO modulates the activity of various proteins that contribute to apoptosis and other biological processes, including p38 MAPK activation and mitochondrial activity.[17–23] Pharmacological inhibition of NOS protects cultured RGCs from anoxia and excitatory amino acids.[24] In addition, mice with nNOS deficiency are protected from NMDA or arterial occlusion–induced RGC death.[10] It remains unknown, however, whether NO regulates extracellular signaling events involved in NMDA-induced retinal cell death.

Matrix metalloproteinases (MMPs) are extracellular or membrane-bound endopeptidases that modulate cell-cell and cell-extracellular matrix (ECM) interactions.[25,26] MMPs have been implicated in the pathogenesis of retinal and other neurodegenerative disorders, including glaucoma, stroke, trauma, Alzheimer's disease, HIV dementia, and multiple sclerosis.[27–29] MMP-9 (gelatinase B, EC 3.4.24.35) in particular is significantly elevated in humans after stroke.[30] Additionally, MMP-2 levels are acutely increased in the brains of baboons after stroke.[31] Our group recently demonstrated a novel extracellular proteolytic cascade in which excitotoxin-induced *S*-nitrosylation and subsequent oxidation activates MMP-9, leading to cortical neuronal apoptosis.[32] To investigate the mechanism of RGC excitotoxicity in vivo in relation to this extracellular proteolytic pathway, we injected NMDA and glycine into the vitreous humor of rats. We found that NMDA activated MMP-9, contributing to RGC apoptosis, while nNOS deficiency or MMP inhibition attenuated these effects.

## Materials and Methods

### Retrograde Labeling of Retinal Ganglion Cells

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For most experiments, adult male Long-Evans rats weighing 200 to 250 g were

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obtained from a local breeder and housed under a 12-hour light–dark cycle with access to food and water ad libitum. For other experiments, nNOS-deficient mice were used, as described below. Animals were anesthetized with 1% to 2% isoflurane and 70% $N_2O$ for all experimental manipulations. Retrograde labeling was achieved by injection of 5% aminostilbamidine (FluoroGold; Molecular Probes, Eugene, OR) into the superior colliculus to allow quantification of RGC bodies, as previously described.[16,33]

## Drug Application

Four days after injection of aminostilbamidine, intravitreal injections were performed using a 33-gauge needle attached to a 5-$\mu$l syringe (MS NE05; ITO Corp., Fuji, Japan) after pupil dilation with 1% atropine sulfate. Hydroxyethylcellulose drops (SCOPISOL 15; Senju Pharmaceutical Co. Ltd., Osaka, Japan) were applied to the cornea, and a small cover glass was then placed on the cornea for intraocular visualization under stereomicroscopy. The tip of the needle was inserted into the vitreous just above the retina through the dorsal limbus of the eye. Intravitreal injections were performed over a 3-minute period using one of several doses of NMDA, 10 nmol glycine, and either 5 nmol GM6001 (Ilomastat; Chemicon, Temecula, CA), an MMP inhibitor, or an equal volume of vehicle (dimethyl sulfoxide [DMSO]). Although a rare event, any animal with visible lens damage and/or vitreal hemorrhage after the injection was euthanatized and not included in the analysis.

## Quantification of Surviving RGCs

One day after intravitreal injection, rats or mice were euthanatized with an overdose of pentobarbital, and the eyes were removed. Eyecups were prepared by removing anterior segments in phosphate-buffered saline (PBS) solution and fixing in 4% paraformaldehyde for 20 minutes. Then each retina was carefully dissected from the eye, prepared as a flat whole-mount in PBS, mounted on a glass slide, and examined by epifluorescence microscopy to visualize RGCs. The number of surviving RGCs in experimental and control retinas was determined by counting aminostilbamidine-labeled neurons in three standard areas in each retinal quadrant at one-sixth, one-half, and five-sixths of the retinal radius, for a total area of 2.25 $mm^2$, as previously described.[16,33] RGC survival for each group of animals was assessed from the density (RGC/$mm^2$; mean ± SEM, $n = 6$ retinas). Statistical comparisons were performed using a Student's $t$-test with values of $P < 0.05$ considered significant.

## Gelatin Zymography

The presence of a specific MMP protein in retinal homogenates was determined by gelatin zymography, as previously described.[32,34] This technique detects both the latent proenzyme and active enzyme because SDS activates the proenzyme, thus allowing both the latent and active forms of the MMP to degrade the gelatin matrix; however, the difference in size of the latent and active forms allows them to be differentiated on the gel. In brief, retinas from two eyes were homogenized in 400 $\mu$L lysate buffer, containing 1% Triton X-100, 100 $\mu$M phenylmethylsulfonylfluoride, and protein inhibitor cocktail (Roche, Mannheim, Germany) in TBS (50 mM Tris-HCl [pH 7.6], 5 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij35 [Sigma, St. Louis, MO], 0.02% $NaN_3$). The supernatant was collected, and the samples were assayed immediately or stored at −80°C before use. Protein concentrations were measured with an assay kit (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL) using albumin as the standard. Aliquots containing 1.5 mg protein were added to 40 $\mu$L gelatin-conjugated sepharose beads (Gelatin Sepharose 4B; Amersham Pharmacia Biotech AB, Uppsala, Sweden) for affinity precipitation, and incubated overnight at 4°C in a rotator. The beads were rinsed 3 times with 500 $\mu$L TBS, transferred to 50 $\mu$L TBS in 10% DMSO, and incubated 30 minutes at 4°C. The supernatants were then collected after centrifugation (1 minute at 200$g$). Each sample was mixed with an equal volume of Tris-Glycine SDS sample buffer (LC2676; Invitrogen, Carlsbad, CA), incubated for 10 minutes at room temperature (RT), then separated on a 10% gelatin zymogram gel (Invitrogen). Gels were soaked in 1× zymogram renaturing buffer for 30 minutes at RT, incubated in 1× zymogram developing buffer for 2 days at 37°C, stained with Coomassie blue (0.25% in a mixture of methanol, $H_2O$, and acetic acid at a ratio of 9:9:2) for 5 hours, destained for 2 hours, and then dried.

## Evaluation of Retinal Thickness

Morphometric analysis was carried out in a manner similar to that described previously.[9] After enucleation, eyes were immersed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 24 hours at 4°C, then embedded in mounting compound (Tissue-Tek OCT; Sakura Finetechnical Co., Ltd., Tokyo, Japan) on dry ice. Transverse sections (8 $\mu$m thick) were cut through the optic disc on a cryostat, mounted onto glass slides coated with poly-L-lysine, and stained with hematoxylin and eosin. We measured the thickness of the inner plexiform layer (IPL), inner nuclear layer (INL), and outer nuclear layer (ONL) at a distance 1.0 mm temporal from the optic disc using image analysis software (NIH Image, version 1.61; National Institutes of Health, Bethesda, MD). The data from three sections were averaged for each eye. Data are presented as means ± SEM.

## In Situ Zymography and Immunohistochemistry

The in situ zymography technique identified cells in tissues that manifest proteinase activity using a fluorogenic substrate (DQ-gelatin-FITC; Molecular Probes), which emits a fluorescent signal when cleaved by MMPs, as previously described.[32] Unfixed cryostat sections on poly-L-lysine-coated glass slides were dried at RT for 10 minutes, then incubated for 30 to 60 minutes at 37°C with 50 $\mu$g/mL DQ-gelatin-FITC in TBS. For simultaneous immunohistochemistry, sections were fixed in 4% paraformaldehyde and 30% sucrose in PBS (pH 7.4) for 10 minutes, then incubated with blocking solution composed of 10% normal goat serum (NGS) in PBS for 1 hour, followed by overnight incubation at 4°C in either anti–glial fibrillary acidic protein (GFAP, 1:100; Sigma) to identify astrocytes, anti–microtubule-associated protein-2 (MAP-2, 1:100; Sigma) to identify neurons, or anti–*S*-nitrosocysteine protein (SNO-P, 1:100; Calbiochem, La Jolla, CA). The sections were then incubated in secondary antibody conjugated with Alexa Fluor 594 (1:200; Molecular Probes) in TBS with 10% NGS and 2 $\mu$M 4′,6-diamidino-2-phenylindole (DAPI) at RT for 1 hour. Finally, sections were washed 3 times in 10 mM PBS, exposed to 1 drop of antifade solution (Gel/Mount; Biomeda Corp., Foster City, CA), mounted on glass coverslips, and visualized under deconvolution microscopy. The specificity of the anti–SNO-P antibody was verified as described previously.[22]

## In Situ Zymography of nNOS-Knockout Mice

For experiments assessing the involvement of NO in MMP-9 activation after intravitreal NMDA injection, we used $nNOS^{-/-}$ mice from Jackson Laboratory (Bar Harbor, ME). Wild-type littermate mice ($nNOS^{+/+}$) were used as controls. Intravitreal injection of NMDA and glycine was performed in a manner similar to that detailed above for the rat experiments: 10 nmol each of NMDA and glycine were injected in 2 $\mu$L PBS into the vitreous of $nNOS^{-/-}$ and $nNOS^{+/+}$ mice, and eyes were enucleated 6 hours after the injection. The procedures for anesthesia, tissue preparation, and in situ zymography were similar to those described above for the rat experiments.

# Results

## NMDA Insult Activation of Retinal MMP-9

NO-activated MMP-9 has been implicated in the pathogenesis of excitotoxic damage in the cerebral cortex.[32] In the present study, we initially examined MMP-9 activation by NO in the rat retina after NMDA insult. Gelatin zymography revealed an increase in the expression of both proform of MMP-9

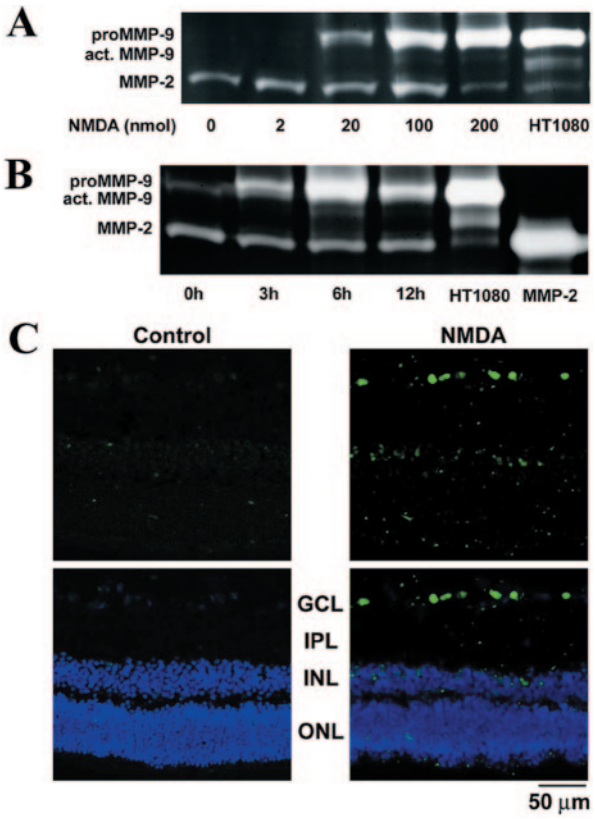


**FIGURE 1.** Increased proMMP-9 expression and MMP gelatinolytic activity in retina of rats treated with intravitreal injection of NMDA and glycine. (**A**) Gelatin zymography of NMDA-induced MMP-9 activity in the retina. Expression of MMP-2 was relatively unchanged. Conditioned medium from the fibroblast cell line HT1080, containing proMMP-9, activated MMP-9 (act. MMP-9), and MMP-2 served as a control. (**B**) Time course of MMP-9 upregulation. Expression of proMMP-9 and activated MMP-9 was maximal 6 hours after injection of 200 nmol NMDA plus 10 nmol glycine. HT1080-conditioned medium and recombinant MMP-2 were used as positive controls. (**C**) In situ zymography revealed an increase in MMP activity in the retina within 6 hours of exposure to NMDA (*green; upper right panel*) relative to vehicle (PBS and glycine; *upper left panel*). Nuclear staining with DAPI (*blue*) was performed in each sample, and the merged images are shown in the *lower panels*. Each experiment was performed three times with similar results.

(proMMP-9) and activated MMP-9 in a dose-dependent manner after injection of ≥ 20 nmol NMDA into the vitreous body (Fig. 1A). Activation of MMP-9 reached a maximum 6 hours after excitotoxin injection and decreased gradually thereafter (Fig. 1B). In contrast, MMP-2 did not change throughout this time period after intravitreal injection of NMDA and glycine and thus served as a loading control for the gelatin zymograms. In addition, using in situ zymography we found an increase in MMP gelatinolytic activity in rat retinas exposed to NMDA relative to PBS-treated controls (Fig. 1C). The increased MMP gelatinolytic activity was observed in the ganglion cell layer (GCL), IPL, and INL.

## MMP-9 Activation in Retinal Neurons but Not in Glial Cells

To determine what cell types express activated MMP-9, we performed double-labeling experiments using in situ zymography for protease activity and immunohistochemistry with anti-GFAP or anti-MAP-2 antibodies to distinguish astrocyte-like cells from neurons, respectively. MMP activity did not colocalize with GFAP immunoreactivity (seen at low magnification in Figs. 2A–D and high magnification in Figs. 2E–H), suggesting that MMP-9 was not activated in the glial cell population. On the other hand, we detected colocalization of MMP activity and MAP-2 immunoreactivity, indicating that MMP-9 was activated in a subpopulation of neurons. To confirm this observation, we performed in situ zymography using rat retinal sections in which RGCs were retrogradely prelabeled with aminostilbamidine. Colocalization of aminostilbamidine and MMP activity was seen in the GCL (Figs. 2M–O). Taken together, we conclude that MMP-9 is activated on retinal neurons, including RGCs, but not glial cells after excitotoxic stimulation.

## Involvement of nNOS in MMP Activation through *S*-Nitrosylation

To determine whether NO is required for MMP-9 activation, we performed in situ zymography on the retina of $nNOS^{-/-}$ versus $nNOS^{+/+}$ mice 6 hours after intravitreal injection of 10 nmol NMDA plus 10 nmol glycine. As a control, injection of PBS and glycine in $nNOS^{+/+}$ mice did not induce MMP activation (Fig. 3A–C). In contrast, intravitreal injection of NMDA and glycine activated MMPs in the GCL, IPL, and INL of the $nNOS^{+/+}$ mouse retina (Fig. 3D–F), similar to the aforementioned rat experiments, while far less MMP activation was observed in $nNOS^{-/-}$ mice (Fig. 3G-I).

Next, to assess whether MMPs are *S*-nitrosylated after NMDA/glycine injection, we performed double labeling to co-visualize MMP activity (via in situ zymography) and *S*-nitrosothiol formation (via immunohistochemistry using a specific

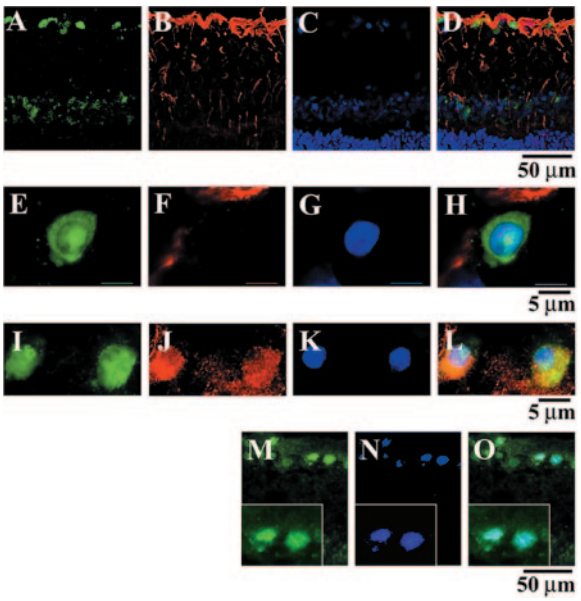


**FIGURE 2.** In situ zymography visualized under deconvolution microscopy revealed an increase of MMP activity in RGCs but not in glial cells 6 hours after treatment of rats with intravitreal injection of 200 nmol NMDA plus 10 nmol glycine. (**A–D**) MMP gelatinolytic activity (*green*; **A**) did not colocalize with GFAP marker for astrocytes (*red*, **B**) and nuclear DAPI staining (*blue;* **C**) in the merged image (**D**). (**E–H**) Higher magnification of the GCL, corresponding to the panels above (**A–D**, respectively). (**I–L**) Double labeling of MMP activity and anti-MAP-2 immunoreactivity to identify neurons. MMP activity (*green*; **I**) colocalized with anti-MAP-2 antibody (*red*; **J**) and nuclear DAPI staining (*blue*; **K**) in the merged image (**L**). (**M–O**) In situ zymography of retrogradely labeled RGCs with aminostilbamidine (*blue*; **N**). Insets (*bottom*) show RGCs at higher magnification. MMP gelatinolytic activity (*green*; **M**) is seen in RGCs in the merged image (**O**). Illustrations are representative of three separate experiments.

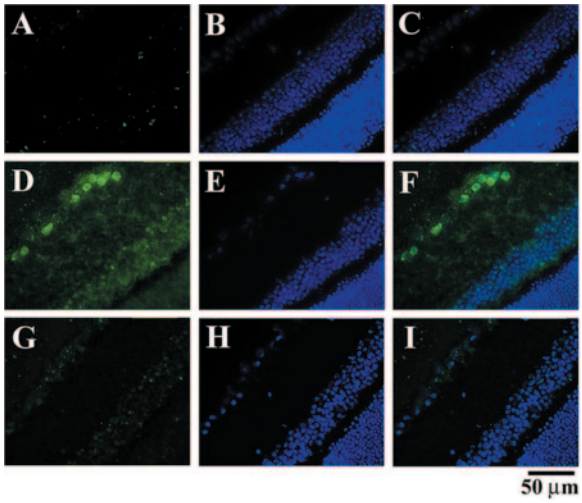


**FIGURE 3.** In situ zymography of retinas from nNOS-knockout and wild-type littermate mice 6 hours after NMDA/glycine exposure. (**A**, **D**, **G**) in situ zymography of MMP gelatinolytic activity (*green*) visualized under deconvolution microscopy; (**B**, **E**, **H**) DAPI nuclear staining (*blue*); (**C**, **F**, **I**) merged images ($n$ = 3/experiment). MMP activity increased after NMDA/glycine injection in nNOS wild-type littermate mice (**D–F**) compared to vehicle-treated controls (**A–C**). The increased MMP activity was abrogated in nNOS-knockout mice (**G–I**).

anti–SNO-P antibody). Initially, we determined the specificity of the SNO-P antibody by immunoblotting *S*-nitroso-BSA and by immunohistochemistry of tissue sections exogeneously treated with an NO donor, as described previously.[22] We observed a substantial increase in SNO-P immunoreactivity in retinas of NMDA/glycine-exposed rats compared to controls, and MMP activity colocalized with this immunoreactivity (Fig. 4). Taken together, these findings suggest that excitotoxins induce MMP activation via *S*-nitrosylation in the retina, similar to previous observations in the cerebral cortex.[32]

### Effects of MMP-9 Inhibition on Tissue Elimination and Cell Survival

To analyze the contribution of activated MMP-9 to NMDA-induced retinal toxicity, we intravitreally injected NMDA and glycine in the presence or absence of 5 nmol GM6001, a broad-spectrum MMP inhibitor. After NMDA/glycine injection, we observed reduced thickness of the IPL and INL in the rat retina (Figs. 5A, 5B), consistent with previous reports.[12] Simultaneous injection of GM6001 prevented thinning of the IPL 3 to 7 days after exposure to excitotoxin (Fig. 5C). However, the effect of GM6001 on INL thinning did not reach significance (Fig. 5D), implying that the neuroprotective effect was more effective on neuronal dendritic processes than on cell bodies. Next, we investigated whether inhibition of MMPs specifically affects RGC survival. Intravitreal injection of NMDA and glycine resulted in a substantial loss of RGCs, identified by retrograde labeling (Fig. 6A). Simultaneous application of GM6001 significantly reduced RGC loss (Figs. 6B, 6C), indicating that inhibition of MMP activity protects RGCs from excitotoxic damage.

## DISCUSSION

This study was designed to test the hypotheses that MMPs contribute to NMDA-induced neuronal death in the retina and that MMP activation is mediated through nNOS.

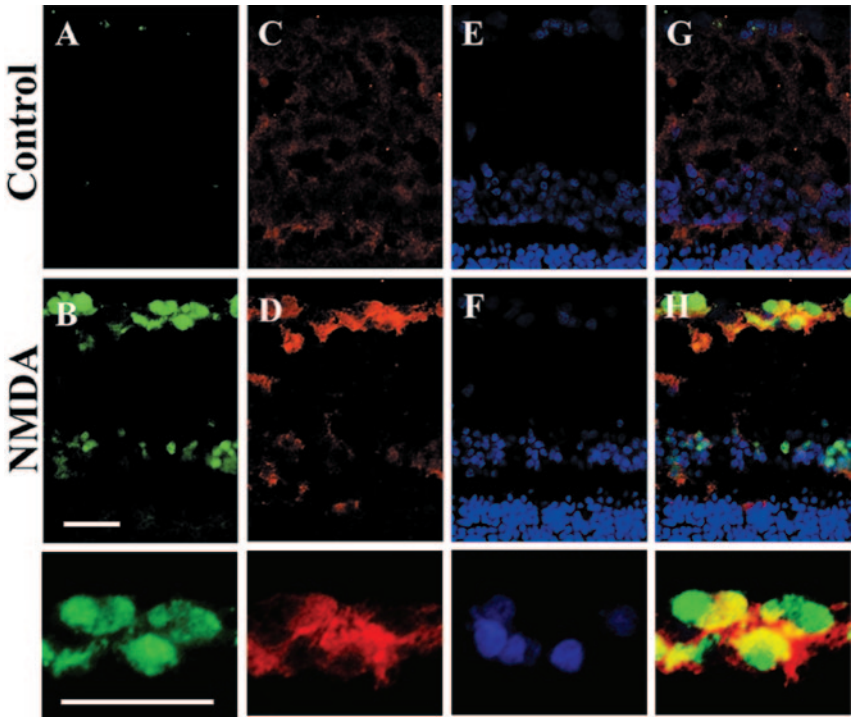


**FIGURE 4.** SNO-P immunoreactivity and MMP activity under deconvolution microscopy. (**A**, **C**, **E**, **G**) retina of rats exposed to vehicle control (PBS and glycine); (**B**, **D**, **F**, **H**) retina of rats exposed to treatment (200 nmol NMDA plus 10 nmol glycine); (**E**, **F**) nuclear DNA counterstaining with DAPI (*blue*). MMP activity (*green*; **A**, **B**) and SNO-P immunoreactivity (*red*; **C**, **B**) dramatically increased in retinas of NMDA-exposed rats compared to the PBS/glycine-treated controls. The increased SNO-P immunoreactivity colocalized with MMP activity in the RGC layer, as shown in the merged images (*yellow*; **G**, **H**). Insets (*bottom row*) show higher magnification of MMP and SNO-P activities. Scale bars, 25 μm.

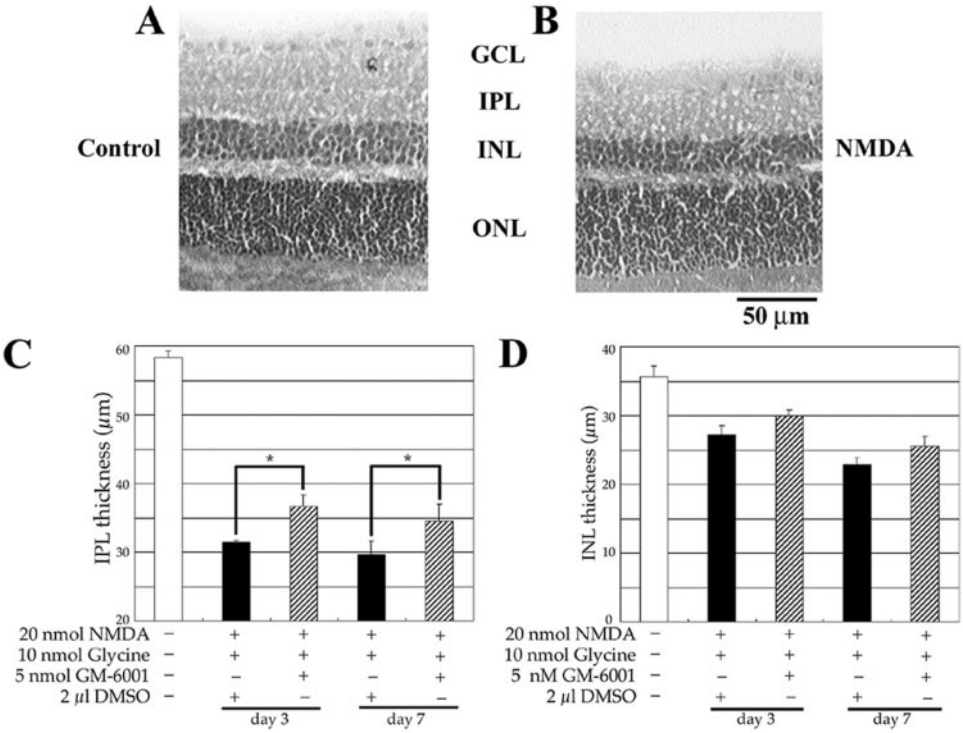


**FIGURE 5.** Protective effects of the broad-spectrum MMP inhibitor GM6001 on NMDA/glycine-induced retinal damage in rats. (**A**, **B**) Hematoxylin and eosin staining revealed cell loss in the GCL in addition to thinning in both the IPL and INL 3 days after intravitreal injection of 20 nmol NMDA plus 10 nmol glycine (**B**) compared to the retina of PBS-treated control rats (**A**). (**C**, **D**) Quantitative effect of GM6001 on the IPL (**C**) and INL (**D**) after NMDA injection. GM6001 significantly prevented thinning of the IPL: $^*P < 0.05$ ($n = 6$/group).

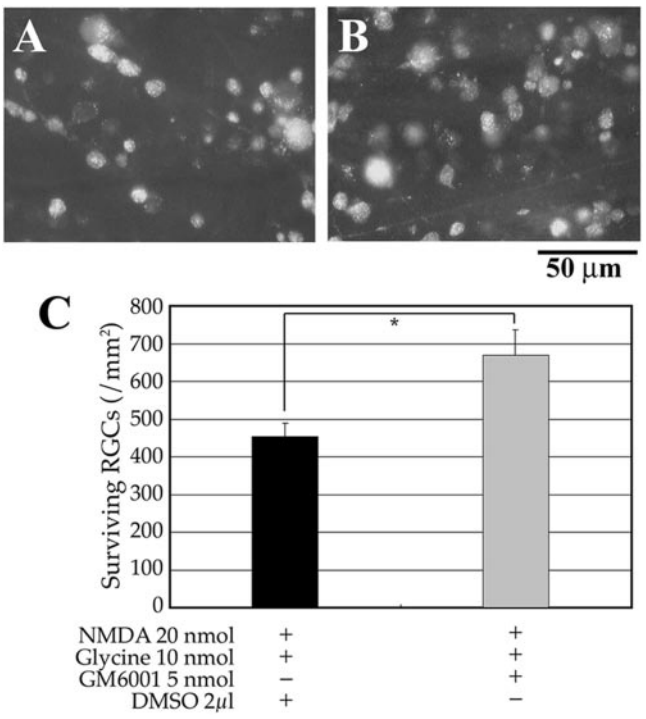


**FIGURE 6.** Neuroprotective effects of the MMP inhibitor GM6001 on RGCs in rats treated with intravitreal injection of NMDA and glycine. (**A**, **B**) Fluorescence microscopic photographs of aminostilbamidine-labeled RGCs 1 day after intravitreal injection of 20 nmol NMDA and 10 nmol glycine in the absence (**A**) or presence (**B**) of 5 nmol GM6001. (**C**) Quantification of surviving RGCs in rat retinas treated as in the panels above (**A**, **B**). GM6001 significantly protected RGCs: $^*P < 0.05$; Student's *t*-test; ($n = 6$/group). DMSO, the diluent for GM6001, was used as a control.

## Effects of MMP-9 Activation on NMDA Neurotoxicity in the Retina

Excessive NMDA receptor (NMDAR) stimulation causes retinal neuron death that is apoptotic in character if the insult is relatively mild but necrotic if more severe.[12,16] Similarly, overactivation of NMDARs can contribute to neuronal cell death in a large number of neurodegenerative disorders that can be either apoptotic or necrotic.[5–8,15] Previously, we and others showed that intravitreal injection of nanomoles of NMDA and glycine induced RGC death in vivo in a dose-dependent manner via apoptosis, triggering $Ca^{2+}$-dependent activation of nNOS and p38 MAPK pathways within 6 hours of the insult.[16] In the present study, we found using gelatin zymography that exposure to NMDA and glycine led to increased expression of both the pro and active forms of MMP-9 in a dose-dependent manner (Fig. 1A). A positive-feedback mechanism linking MMP-9 activity with the efficiency of *mmp-9* gene transcription may explain the increased levels of proMMP-9 in NMDA-exposed animals.[35] MMP-9 was maximally activated by 6 hours after exposure to NMDA and glycine. In addition, histologic examination showed that MMP activity was localized primarily on RGCs and was not observed on glia. These findings suggest that MMP-9 is activated on RGCs at a time when it may contribute to NMDA-induced retinal cell death. In further support of this hypothesis, we found that the specific MMP inhibitor, GM6001, reduced RGC death as well as retinal thinning in the IPL after NMDA/glycine injection. These results are consistent with the notion that MMP activity contributes to NMDA-induced retinal injury and death. Along similar lines, Chintala and colleagues[36] recently reported that excessive activation of AMPA/kainate-type ionotropic glutamate receptors upregulate expression of MMP-9 in the retina and promote retinal degeneration.

There are at least three possible explanations for the pathologic activation of MMP-9 in this setting. One is a neurotoxic effect of MMP-9, with the proteinase directly contributing to the signal transduction machinery that leads to cell death. Another is the phenomenon known as anoikis, in which cells

detached from ECM undergo apoptotic death. In this case, MMP-9 could indirectly cause cell death by degrading ECM, thereby interfering with cell attachment and integrin-mediated survival signaling.[29,32,37] As a third possibility, MMP-9 might cleave inactive precursor proteins to their pathologically active, death-promoting forms. For example, tumor necrosis factor (TNF)-$\alpha$, transforming growth factor-$\beta$, interleukin-6, TNF receptors, L-selectin, and Fas ligand are all synthesized as precursors that require processing by MMP-related enzymes for maturation.[38]

### NMDA Induction of NO and Activation of MMP-9

NMDA-induced RGC death is partially mediated by formation of NO via nNOS.[10] Because, similar to MMPs, NO influences synaptic plasticity, neurite outgrowth, and apoptosis,[18,19,21,39,40] we hypothesized that the effects of NO in the retina might in part be mediated by MMP activation. Along similar lines, we recently reported that NO could activate MMP-9 in the cerebrocortex by regulating the enzyme's "cysteine switch" via *S*-nitrosylation.[32] Supporting the hypothesis that NMDA-induced RGC death is mediated by nNOS through MMP-9 activation, we found that MMPs were far less active in $nNOS^{-/-}$ mice after exposure to NMDA and glycine compared with wild-type littermate mice. To provide a more direct link between MMP activation and NO, we performed double labeling experiments in which MMP activity was visualized by in situ zymography and *S*-nitrosylation by SNO-P immunolabeling. We found that immunoreactivity for SNO-P increased in NMDA-exposed retinas and colocalized with MMP activity in the ganglion cell layer. These experiments strongly suggest that MMP-9 is at least partially activated by NO through *S*-nitrosylation. Moreover, the fact that both NOS inhibition and MMP inhibition partially ameliorate NMDA-induced retinal cell death is consistent with the notion that NO-activated MMPs contribute to the mechanism of retinal excitotoxicity.

In conclusion, the present study showed that MMP-9 is activated on RGCs after exposure to NMDA and glycine. The inhibitor studies coupled with in situ localization suggest that MMP-9 contributes to NMDA-induced RGC death after NO generated from nNOS activates the MMP via *S*-nitrosylation. Because excessive NMDAR activity has been linked to a number of retinal disorders, further elucidation of signaling pathway downstream from NMDAR stimulation may lead to more effective strategies for treating retinal neurodegenerative diseases, including glaucoma, retinal artery occlusion, and ischemic optic neuropathy.

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