Divergent NMDA Signals Leading to Proapoptotic and Antiapoptotic Pathways in the Rat Retina

Shin-ichi Manabe and Stuart A. Lipton

Purpose. To investigate the involvement of the p38 mitogen-activated protein (MAP) kinase and phosphatidylinositol-3 (PI-3) kinase-Akt signaling pathways after pathologic stimulation by *N*-methyl-D-aspartate (NMDA) receptors of retinal neurons in vivo.

Methods. NMDA (2–200 nmol), SB203580 (0.2–10 nmol, an inhibitor of p38 MAP kinase), LY294002 (6 nmol, an inhibitor of PI-3 kinase), or control solution was injected into the vitreous of Long-Evans rats. To assess retinal ganglion cell (RGC) death quantitatively, we labeled RGCs retrogradely by injecting aminostilbamidine (FluoroGold) into the superior colliculus and subsequently counting fluorescently labeled RGCs in retinal wholemounts. Phosphorylation of p38 and Akt was assessed by immunoblot of whole retinal lysates, and activity was measured with in vitro kinase assays. To localize phospho-p38 and phospho-Akt, immunohistochemistry was performed. TUNEL staining coupled with morphologic assessment was performed to assess apoptotic cell death.

Results. Intravitreous injection of more than 10 nmol NMDA induced RGC death. Before death, NMDA-stimulated retinas manifested increased phospho-p38 and phospho-Akt in the ganglion cell and inner nuclear layers. Subsequently, pyknotic, TUNEL-positive cells were also localized to these regions. SB203580 partially rescued RGCs, whereas LY294002 enhanced death of RGCs due to 10 nmol NMDA. SB203580 and LY294002 specifically inhibited the activity of p38 MAP kinase and Akt, respectively.

Conclusions. The p38 MAP kinase and PI-3 kinase-Akt pathways are involved in signal transduction after excessive stimulation of NMDA receptors in the retina. These inhibitor studies suggest that the p38 MAP kinase pathway is proapoptotic, whereas the PI-3 kinase-Akt pathway is antiapoptotic in RGC death induced by NMDA. (*Invest Ophthalmol Vis Sci.* 2003;44:385–392) DOI:10.1167/iovs.02-0187

Glutamate, a major excitatory neurotransmitter in the vertebrate retina, has long been known to exert neurotoxic actions on various populations of neurons in the inner retina.[1] The effects of glutamate are mediated by ionotropic receptors that have an associated ion channel—*N*-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors—and by metabotropic receptors that are coupled to G-proteins.[2] Among these receptors, NMDA receptors, which are activated by the coagonists NMDA (or glutamate) and glycine, are known to be predominantly involved in neuronal cell death in the retina and elsewhere in the brain.[3–6] In fact, there are several reports that glutamate is involved in retinal diseases, including glaucoma,[7] ischemia,[8–10] and optic neuropathy.[11]

NMDA may also protect against death of immature neurons in vitro.[12–14] In these events, glutamate is thought to play a role in development and innervation. However, it is not known whether these opposing effects of NMDA on neuronal death occur in adult neurons in vivo.

Mitogen-activated protein (MAP) kinases are serine-threonine kinases and play an instrumental role in signal transduction from the cell surface to the nucleus. p38 represents a group of enzymes in the MAP kinase family that are activated and phosphorylated on a Thr-Gly-Tyr motif by environmental stress, such as hyperosmolarity, exposure to UV radiation, proinflammatory cytokines, and endotoxin.[15] p38 MAP kinase is thought to participate in one of the signaling pathways mediating apoptosis in several cell types in various species.[16–19] Glutamate signaling through the NMDA receptor also induces phosphorylation and activation of MAP kinases in primary neuronal cultures.[20,21] Regarding retinal neurons in these events, we previously reported the following: Axotomy of the optic nerve induces apoptotic death of retinal ganglion cells (RGCs) in vivo; p38 MAP kinase is activated and phosphorylated in RGCs after axotomy; inhibition of p38 rescues RGCs from death after axotomy; MK-801, an antagonist of NMDA receptors, attenuates activation of p38 after axotomy in vivo; MK-801 protects RGCs from death after axotomy in vivo; and inhibition of p38 activity protects cultured RGCs from NMDA-induced apoptosis in vitro.[22]

Conversely, a number of factors in the survival of retinal and nonretinal neurons have been identified, including serum, insulin-like growth factor-1, and neurotrophins.[14,23–29] These factors activate the phosphatidylinositol-3 (PI-3) kinase pathway, which is one of several signal transduction pathways implicated in the survival of neurons. Then, PI-3 kinase phosphorylates and activates Akt, some of whose targets have been proposed to play a role in the regulation of cell survival, including glycogen synthase kinase (GSK)-3β, Bcl-2 antagonist of cell death (BAD), forkhead transcription factors, and caspase-9.[30–33] Although NMDA has been reported to prolong the survival and outgrowth of developing cerebellar granule cell neurons,[34] this type of prosurvival event has not been studied in detail in RGCs. In addition, to date, there have been no studies of the PI-3 kinase-Akt pathway in the retina exposed to NMDA.

In this study, we show that NMDA causes neuronal death after activating and phosphorylating p38 in rat retinas in vivo. However, NMDA also activates Akt. We demonstrate that the p38 pathway is proapoptotic and the PI-3 kinase-Akt pathway is antiapoptotic for RGCs.

Materials and Methods

All experiments were performed in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All antibodies for immunoblotting, kinase assays, and immunohistochemistry

From the Center for Neuroscience and Aging, The Burnham Institute, La Jolla, California.

Supported by National Eye Institute Grant R01 EY05477 and a grant from Allergan, Inc., Irvine, California.

Submitted for publication February 25, 2002; revised July 10, 2002; accepted July 19, 2002.

Commercial relationships policy: F, C, P.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Stuart A. Lipton, Center for Neuroscience and Aging, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037; slipton@burnham.org.

were obtained from Cell Signaling Technology Inc. (Beverly, MA), with the exception of the mouse anti-phospho-p38 antibody used in immunohistochemistry, which was obtained from Sigma Chemical Co. (St. Louis, MO).

Retrograde Labeling of RGCs

Adult male Long-Evans rats weighing 200 to 250 g were obtained from a local breeder and housed in a 12-hour light–dark cycle with access to food and water ad libitum. 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 (Fluoro-Gold; Molecular Probes, Eugene, OR) into the superior colliculus to allow quantification of cell bodies, as previously described.[22,35,36]

Drug Application

Four days after the injection of aminostilbamidine, intravitreous injections were performed with a 33-gauge needle attached to a 5-μL syringe (MS NE05; Ito Corp., Fuji, Japan) after pupil dilation with 1% atropine sulfate. Hydroxyethyl cellulose (Scopisol 15; Senju Pharmaceutical Co. Ltd., Osaka, Japan) was dropped onto the cornea, and a small cover glass was placed 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. Injections were completed over a period of 3 minutes. Intravitreous injections were performed with several doses of NMDA and 10 nmol glycine, with SB203580 (Calbiochem, San Diego, CA), LY294002 (Calbiochem), or control solutions (an equal volume of dimethyl sulfoxide [DMSO]). Any animal with visible lens damage and/or vitreous hemorrhage was not included in the analysis.

Quantification of RGC Survival

At various time points, rats were killed with an overdose of pentobarbital, and the eyes were removed. Eyecups were prepared by removing anterior segments in phosphate-buffered saline (PBS) solution and fixed in a 4% paraformaldehyde solution for 20 minutes. Then the retina was carefully dissected from the eye, prepared as a flatmount in PBS solution, mounted on glass slides, 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 of 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 by Kikuchi et al.[22] RGC survival for each group of animals was assessed from the mean density (RGCs/mm^2) ± SEM for three to nine retinas.

Immunohistochemistry

After enucleation, the eyes were immersed overnight in fixative composed of 4% paraformaldehyde in PBS (pH 7.4) at 4°C, followed by cryoprotection by soaking in 30% sucrose overnight at 4°C. Eyes were frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetechnical Co., Ltd., Tokyo, Japan) on dry ice, and 8-μm-thick cryostat sections were cut, thaw mounted onto glass slides coated with poly-L-lysine, and air dried overnight at 4°C. Sections were treated for 15 minutes at room temperature with Tris-buffered saline (pH 7.4, TBS) containing 0.1% Triton X-100 (TBST) to increase membrane permeability, followed by 0.3% hydrogen peroxide for 10 minutes to block intrinsic peroxidase activity. After they were rinsed, sections were incubated with TBS containing 10% normal goat serum (NGS) for 1 hour. Sections were incubated overnight at 4°C in TBS with 10% NGS and either 1:100 anti-phospho-p38 (Thr180/Tyr182) or 1:200 anti-phospho-Akt (Ser 473; imunohistochemistry-specific). The sections were then incubated in TBS with 10% NGS and 1:100 biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 1 hour. After another rinse, the sections were incubated in avidin-biotin complex (ABC) reagent (Vectastain ABC Kit; Vector Laboratories, Inc.) at room temperature for 30 minutes, according to the manufacturer's instructions. Color development was performed with diaminobenzidine. Finally, counterstaining was performed with 1% methyl green.

For double-staining of phospho-p38 and phospho-Akt, sections were incubated with 1:100 mouse anti-phospho-p38 antibody and 1:100 rabbit anti-phospho-Akt antibody overnight at 4°C. The sections were then incubated in TBS with 10% NGS, 1:200 anti-mouse IgG (Alexa Fluor 594), 1:200 anti-rabbit IgG (Alexa Fluor 488), and 2 μM 4′,6-diamidino-2-phenylindole (DAPI; all from Molecular Probes) at room temperature for 1 hour. Finally, sections were washed three times in 10 mM PBS, exposed to 1 drop of anti-fade solution (Gel/Mount; Biomeda Corp., Foster City, CA), mounted on glass coverslips, and visualized with epifluorescence microscopy.

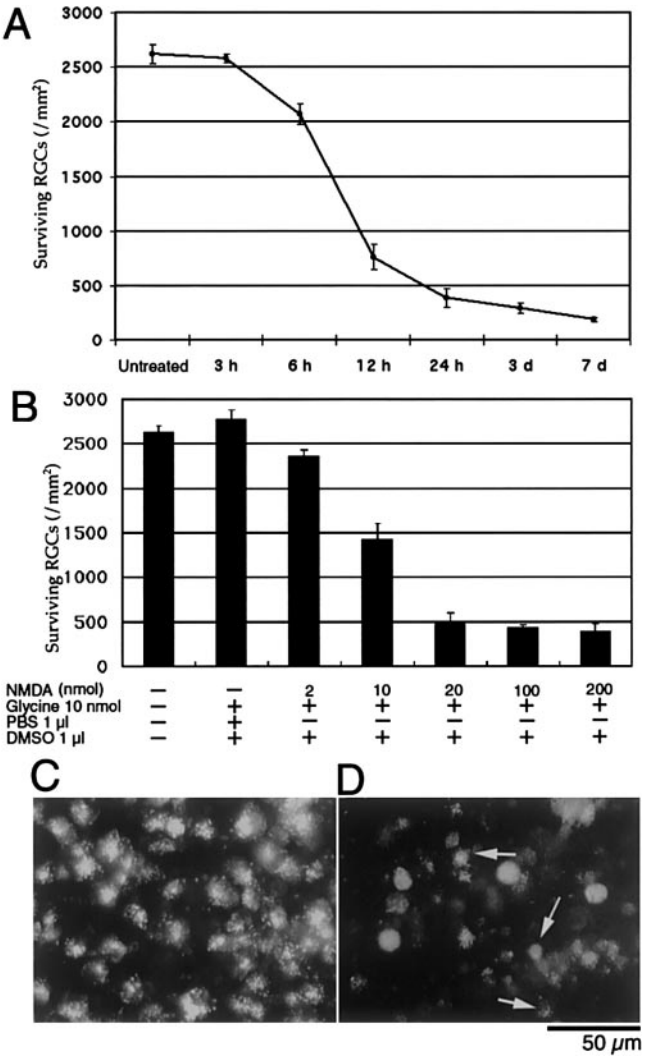

FIGURE 1. (**A**) Time course of surviving RGCs after intravitreous injection of 200 nmol NMDA and 10 nmol glycine. (**B**) Dose-dependent RGC death induced by various doses of NMDA and 10 nmol glycine 1 day after injection. Low doses of NMDA (2 nmol) did not kill RGCs. In contrast, 10 nmol NMDA killed approximately 50% of the RGCs at 1 day, whereas 20 nmol or more NMDA killed approximately 80% of the RGCs. (**C**, **D**) Representative micrographs of flatmounted aminostilbamidine-labeled RGCs in corresponding regions of an untreated control retina (**C**; 2677 RGCs/mm^2) and 1 day after injection of 200 nmol NMDA and 10 nmol glycine (**D**; 425 RGCs/mm^2). *Arrows*: pyknotic RGCs. In this and subsequent figures, data are expressed as mean ± SEM (n = 3 to 9 for each group).

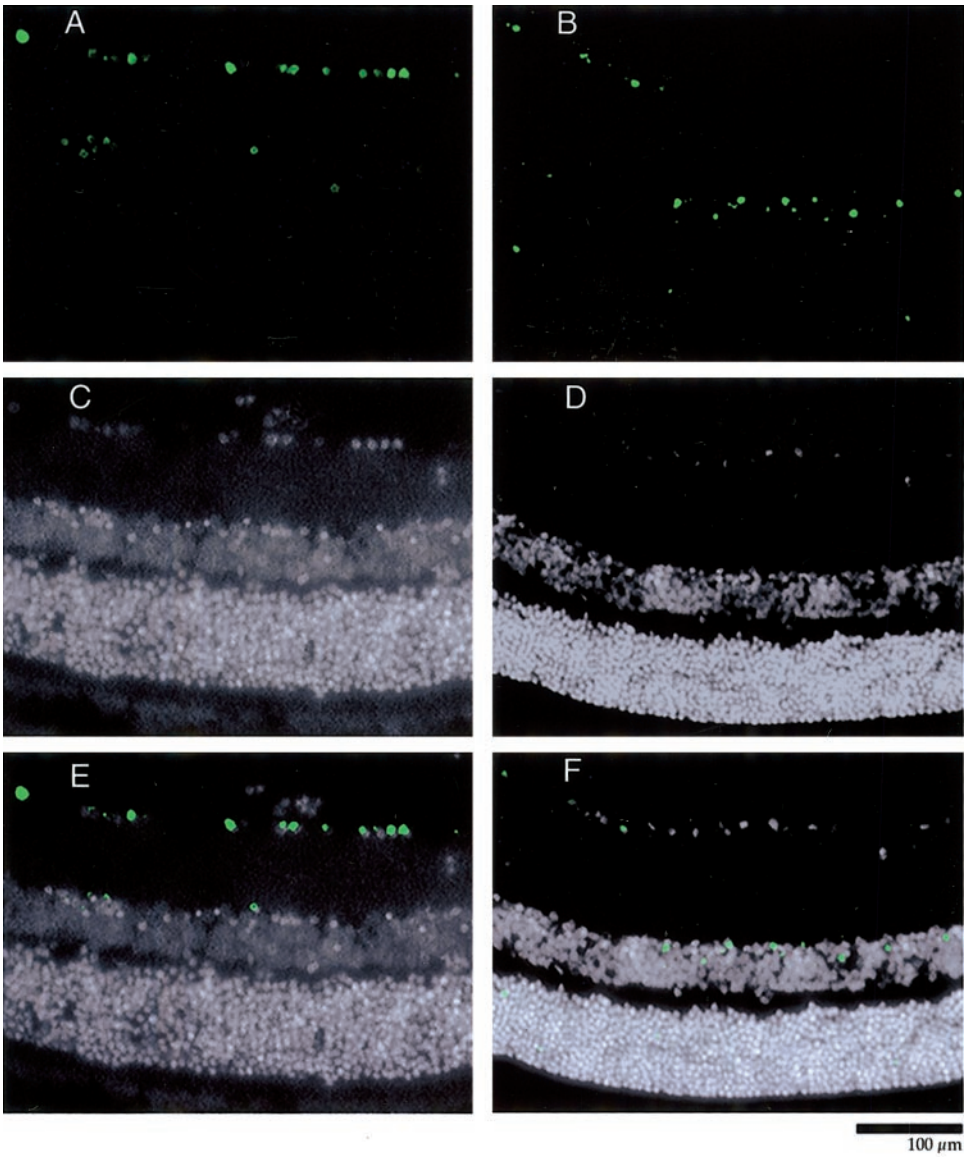

FIGURE 2. TUNEL staining of retinas after injection of 10 nmol NMDA. Six hours after injection, most TUNEL-positive cells were located in the GCL (**A**, TUNEL; **C**, DAPI; **E**, merged). TUNEL-positive cells were located in both the GCL and the INL by 12 hours after injection of excitotoxin (**B**, TUNEL; **D**, DAPI; **F**, merged).

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining was performed with a fluorescein apoptosis detection system (Promega Corp., Madison, WI), according to the manufacturer's instructions. In brief, after cryosections were rinsed in PBS and reacted with 20 μg/mL proteinase K for 10 minutes at room temperature, they were incubated with terminal dUTP transferase enzyme and a mix of nucleotides in equilibration buffer for 60 minutes at 37°C in a moist chamber. After termination of the reaction by immersing in 2× SSC for 15 minutes, the samples were washed three times with PBS for 5 minutes each. For nuclear staining, samples were incubated in 2 μM DAPI solution for 5 minutes.

Immunoblot Analysis

Retinas were homogenized in buffer containing 10 mM HEPES, 2 mM EDTA, 0.1% 3-([3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), 0.35 mg/mL phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL pepstatin A, 10 μg/mL aprotinin, 20 μg/mL of leupeptin, and 10 mM sodium orthovanadate (pH 7.2), and centrifuged. The supernatant was collected, and the samples were used immediately or stored at −80°C before use. Protein concentrations were measured with a BCA protein assay reagent kit (Pierce, Rockford, IL), with albumin used as the standard. Aliquots containing 50 μg protein were added to 4× SDS sample buffer (NuPAGE; Invitrogen, Carlsbad, CA), boiled for 10 minutes, separated, and then transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech Inc., Piscataway, NJ). The membranes were blocked with TBST containing 10% NGS for 2 hours at room temperature and probed with the following antibodies: 1:1000 anti-p38, 1:1000 anti-phospho-p38, 1:1000 anti-Akt, or 1:1000 anti-phospho-Akt (Ser473). After rinsing with TBST, membranes were incubated in TBST containing 1:2000 horseradish peroxidase-linked anti-rabbit IgG for 1 hour at room temperature. Immunoblots were visualized with chemiluminescence detection (LumiGLO; Cell Signaling Technology). The intensity of each band was measured by densitometric analysis using image analysis software (NIH Image, ver. 1.61; NIH Image; W. Rasband, National Institutes of Health; available by ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, Springfield, VA, catalog number PB95-500195GEI).

Kinase Activity Assay

We measured p38, Akt, and extracellular signal-regulated kinase (ERK) activities according to the instructions from relevant kits (Cell Signaling Technology) with slight modification. In brief, retinas were incu-

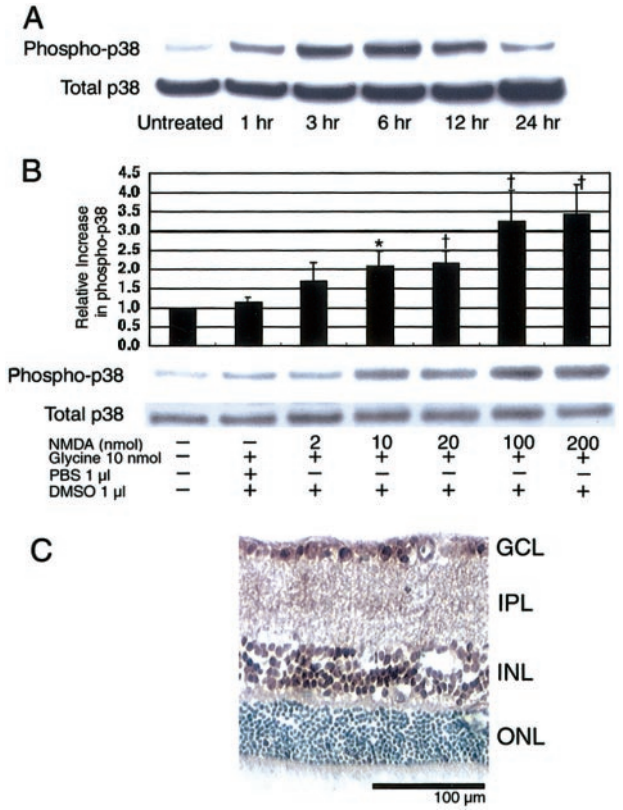

FIGURE 3. Phosphorylation of retinal p38 (MAP kinase) after exposure to NMDA. (**A**) Time course of phosphorylation of p38 after injection of 200 nmol NMDA and 10 nmol glycine. Phosphorylation of p38 was first detected at 1 hour, reached a peak at 3 to 6 hours, and decreased thereafter. (**B**) Immunoblot and densitometric analyses at 6 hours after treatment show that NMDA produced dose-dependent phosphorylation of p38. Only doses of NMDA that subsequently produced neuronal apoptosis (≥10 nmol) resulted in significant phosphorylation of p38. (**C**) Localization of phosphorylated p38 immunoreactivity 6 hours after injection of 10 nmol NMDA and 10 nmol glycine. Immunoreactivity of phosphorylated p38 was seen in the GCL, IPL, and INL. ONL, outer nuclear layer. The immunoblots are representative of four experiments (*$P < 0.05$; †$P < 0.01$, compared with vehicle-treated control).

bated in ice-cold cell lysis buffer plus 1 mM PMSF for 10 minutes, homogenized, and then centrifuged. The supernatant was collected, and the samples were used immediately or stored at −80°C before use. Protein concentrations were measured, and aliquots containing 150 μg protein were immunoprecipitated with anti-phospho-p38, anti-phospho-Akt, or anti-phospho-ERK immobilized antibodies. The immune complexes were collected by centrifugation and incubated for 30 minutes at 30°C in 50 μL kinase buffer supplemented with 200 μM adenosine triphosphate (ATP) and the corresponding substrate protein (2 μg activating transcription factor [ATF]-2 for p38, 1 μg GSK-3 for Akt, or 2 μg Elk-1 for ERK). The supernatant was transferred to a new tube containing 20 μL of 4× SDS sample buffer (NuPAGE, Invitrogen) and 4 μL of 1 M DTT to stop the reaction. Phospho-ATF-2, phospho-GSK3∂/β, or phospho-Elk-1 was detected by immunoblot analysis, and, as an index of kinase activity, the intensity of each band was determined by densitometric analysis. The relative activity of each sample was normalized to that of untreated samples for each experimental series.

Statistical Analysis

Statistical significance of the data was determined by an ANOVA followed by a post hoc Dunnett test.

RESULTS

Effects of NMDA on RGCs and Other Retinal Neurons

RGCs labeled retrogradely with aminostilbamidine displayed a characteristically fine-dotted pattern of fluorescence in the perikarya. NMDA-induced RGC death was both time- and dose-dependent within 7 days of injection of 200 nmol NMDA (Figs. 1A, 1B). In the untreated retinas the mean RGC density was 2620 ± 92 RGCs/mm^2 (±SEM; a representative image is shown in Fig. 1C), whereas RGC density decreased to 385 ± 88 RGCs/mm^2 at 1 day after injection of 200 nmol NMDA and 10 nmol glycine (representative in Fig. 1D). The appearance of most affected RGCs was shrunken and pyknotic. Very few RGC perikarya were swollen after any dose of NMDA. In contrast, injection of glycine alone or vehicle (PBS or DMSO) did not kill RGCs (data not shown).

TUNEL Staining

TUNEL-positive cells were observed in the ganglion cell layer (GCL) and the inner nuclear layer (INL) as early as 6 hours after injection of NMDA (Figs. 2A, 2C, 2E). At this time point, most of the TUNEL-positive cells were located in the GCL. In contrast, at 12 hours after injection TUNEL-positive cells were located in both the GCL and INL (Figs. 2B, 2D, 2F).

Activation of p38 in Retinal Neurons after Injection of NMDA

Immunoblot analysis and densitometry revealed that phosphorylated p38 was first detected 1 hour after injection of 10 nmol or more NMDA, reached a maximum at 3 to 6 hours and decreased gradually thereafter (Figs. 3A, 3B). Phosphorylation occurred in an NMDA dose-dependent manner (Fig. 3B),

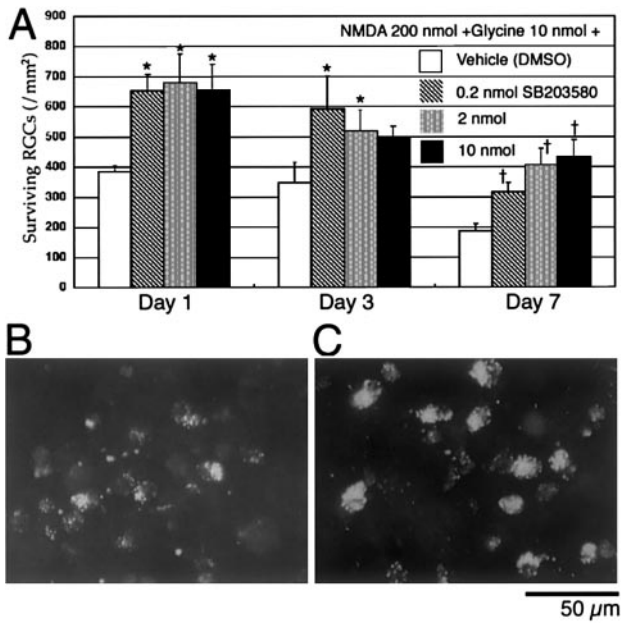

FIGURE 4. (**A**) Quantitative assessment of the neuroprotective effect of the p38 inhibitor SB203580 on RGC death induced by NMDA. At doses as low as 0.2 nmol, SB203580 partially protected RGCs exposed to 200 nmol NMDA and 10 nmol glycine (*$P < 0.05$, †$P < 0.01$, compared with the vehicle-treated control). (**B**, **C**) Representative micrographs of RGCs 7 days after injection of 200 nmol NMDA, 10 nmol glycine, and 1 μL DMSO (**B**; 212 RGCs/mm^2) versus 200 nmol NMDA, 10 nmol glycine, and 10 nmol SB203580 dissolved in DMSO (**C**; 473 RGCs/mm^2).

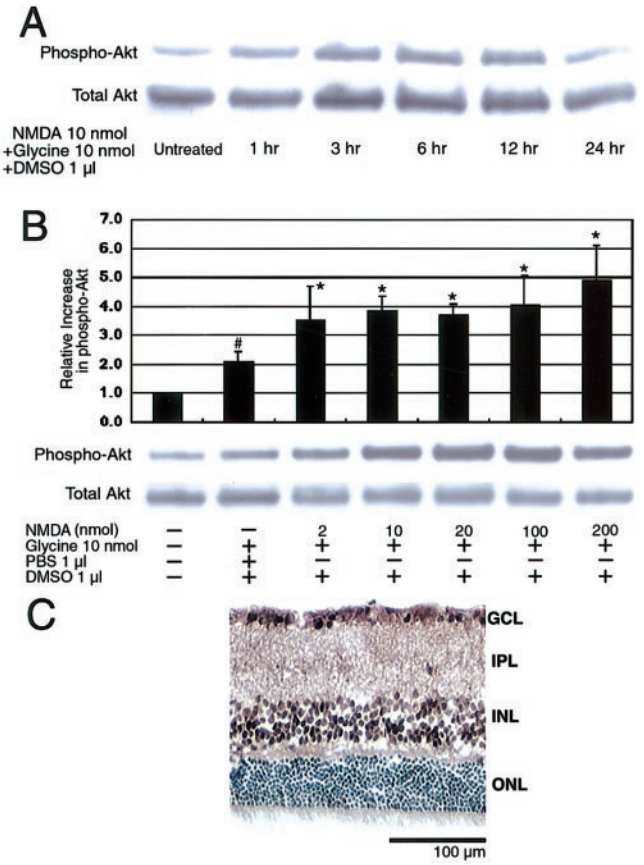

FIGURE 5. Phosphorylation of retinal Akt after exposure to NMDA. (**A**) Time course of phosphorylation of Akt after injection of 10 nmol NMDA and 10 nmol glycine. Phosphorylation of Akt was detected within 1 hour of the injection, peaked by 6 hours, and returned to baseline by 24 hours. (**B**) Immunoblot and densitometric analyses at 6 hours after injection (#$P < 0.05$, compared with the untreated group; *$P < 0.05$, compared with vehicle-treated control). (**C**) Localization of immunoreactivity of phosphorylated Akt was observed 6 hours after injection of 10 nmol NMDA and 10 nmol glycine. Immunoreactivity of phosphorylated Akt was observed in the GCL, IPL, and INL. Immunoblots are representative of four experiments. ONL, outer nuclear layer.

whereas no phosphorylation was observed after a sublethal dose of NMDA or injection of control solution. In another series of experiments, we investigated the localization of phosphorylated p38 by immunohistochemistry. Phospho-p38 immunoreactivity was observed in the GCL, the inner plexiform layer (IPL), and the INL within 6 hours of excitotoxin injection (Fig. 3C).

Effect of p38 Inhibitors on Survival of RGCs after Injection of NMDA

We examined whether treatment with SB203580, a relatively specific inhibitor of p38, prevents NMDA-induced RGC death. SB203580 was administered intravitreously concurrently with NMDA and glycine. SB203580 increased the number of surviving RGCs 1, 3, and 7 days after injection at a concentration as low as 0.2 nmol (approximately 1.6 μM in the vitreous; Fig 4). We also examined the effect of repeat administration of SB203580 at 6 hours after the initial injection, but additional protection against RGC death was not observed (data not shown).

Activation of Akt in Retinal Neurons after Injection of NMDA

Immunoblot analysis and densitometry revealed that phosphorylation of Akt at Ser473 increased within 1 hour of injection of NMDA, reached a peak at 6 hours, and returned to the basal level by 24 hours (Fig. 5A). When analyzed 6 hours after injection, NMDA resulted in a dose-dependent increase in phosphorylation of Akt (Fig. 5B). A smaller increase in phosphorylation of Akt was also observed 6 hours after injection of the control solution containing glycine but not NMDA. Immunohistochemistry demonstrated that anti-phospho-Akt labeling was observed in the GCL, the IPL, and the INL (Fig. 5C).

Effect of Inhibition of PI-3 Kinase on Survival of RGCs after Injection of NMDA

Next, we investigated whether LY294002, an inhibitor of PI-3 kinase, would affect NMDA-induced death of RGCs . LY294002 (6 nmol) was administered intravitreously along with NMDA and glycine. LY294002 enhanced RGC death induced by 10 nmol, but not by 200 nmol NMDA. In addition, a second administration of LY294002 6 hours after the initial injection further increased RGC death (Fig. 6).

In Vitro Kinase Activity Assays

In kinase assays, we found that the activity of p38 MAP kinase and Akt increased 6 hours after injection of 10 nmol NMDA and glycine (Fig. 7). SB203580 significantly inhibited activity of p38 MAP kinase without affecting the activity of Akt. Similarly, LY294002 specifically inhibited the activity of Akt. In addition we measured the activity of ERK as a control kinase to confirm the specificity of the inhibitors. Neither inhibitor affected the activity of ERK at the concentration used (data not shown).

Double-Labeling of Phospho-p38 and Phospho-Akt

We performed double-labeling of phospho-p38 and phospho-Akt to determine whether both p38 and Akt could be activated in the same cells. Some cells in the GCL and INL were labeled by both antibodies (Fig. 8), whereas others were labeled by only one or neither antibody.

DISCUSSION

It is well known that glutamate causes cell death in retinal neurons.[1,5,37–39] However, most reports have focused only on the potential death-promoting signaling pathways and not on neuroprotective pathways triggered by glutamate. The hypothesis that NMDA activates neuroprotective pathways concomitantly with death-promoting pathways prompted us to investigate various signal-transduction pathways downstream of the NMDA receptor.

Injection of NMDA into the vitreous resulted in death that appeared to be apoptotic, because dying RGCs became pyknotic. Furthermore, our findings with TUNEL staining supported this notion and are consistent with previous reports.[37] The distribution of TUNEL-positive cells varied temporally and spatially. We speculate that regional differences in retinal thickness may account for this phenomenon.

Within 1 day of injection of 20 nmol NMDA, more than 80% of the RGCs died, and little additional damage was observed after 200 nmol of NMDA. In contrast, 2 nmol NMDA did not cause significant cell death. After 10 nmol NMDA, approximately 50% of the RGCs died. These results are similar to the results in a previous report[5] and show that 10 nmol NMDA causes reproducible but subtotal death of RGCs.

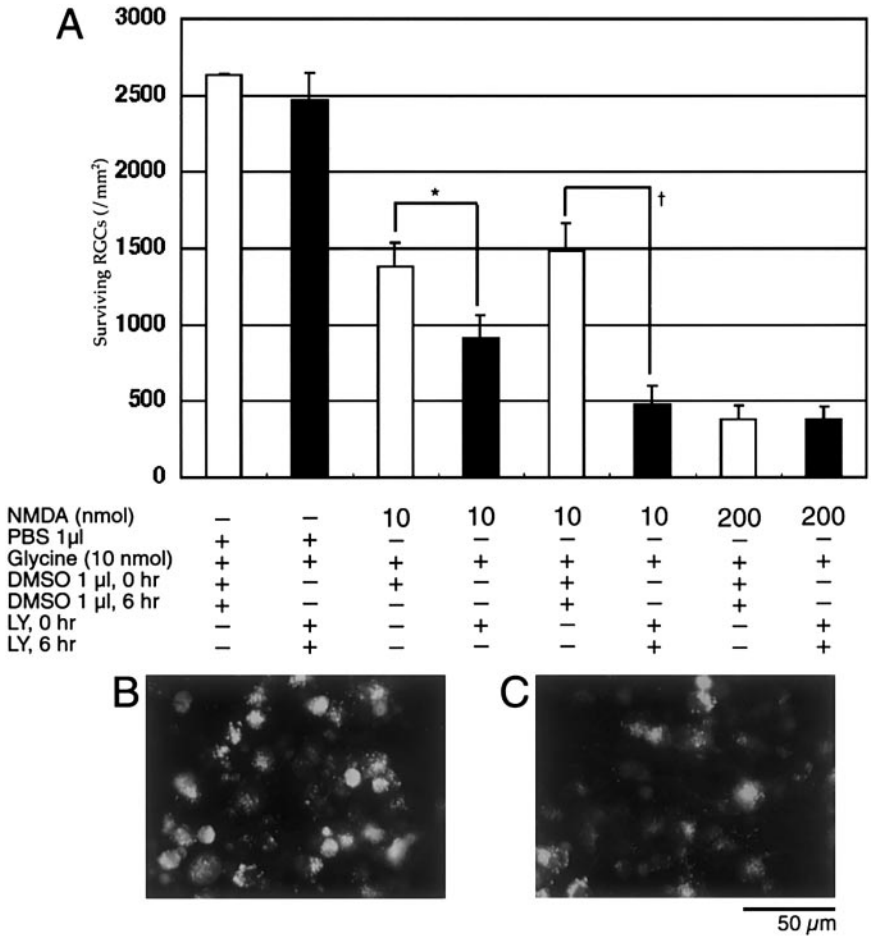

FIGURE 6. Quantitative assessment of the effect of the PI-3 kinase inhibitor, LY294002 (LY), on NMDA-induced death of RGCs. (**A**) LY294002 enhanced RGC death induced by 10 nmol NMDA monitored 1 day after injection but manifested no effect by itself. A second injection of LY294002 6 hours after the first injection further increased NMDA-induced RGC death. In contrast, LY294002 had no further effect on RGC death induced by 200 nmol NMDA monitored 1 day after injection ($^*P < 0.05$, $\dagger P < 0.01$). (**B**, **C**) Representative micrographs of RGCs 1 day after injection of 10 nmol NMDA, 10 nmol glycine, and 1 μL DMSO (**B**; 1381 RGCs/mm^2) versus NMDA, glycine, and two injections of 6 nmol LY294002 (**C**; 531 RGCs/mm^2).

We detected p38 phosphorylation after exposure to 10 nmol or more NMDA with glycine. Phosphorylation of p38 was detected within 1 hour of injection of an excitotoxin and continued for 24 hours, whereas TUNEL-positive cells began to appear at 6 hours. This temporal difference indicates that activation of p38 preceded apoptosis. It interested us that p38 was phosphorylated only after lethal doses of NMDA, consistent with the notion that p38 is involved in a death-promoting pathway. In further support of this hypothesis, SB203580, a p38 inhibitor, partially protected RGCs from NMDA-induced death. The effective dose of 0.2 nmol SB203580 corresponds to a concentration of 1.6 μM in the vitreous (the volume of rat vitreous is ~120 μL).[40] At 1 to 2 μM, SB203580 has been reported to be relatively specific for p38.[41] In fact, in our studies this concentration of SB203580 inhibited p38 MAP kinase activity both significantly and specifically, in that in control experiments it did not affect Akt or ERK activity. Taking all findings together, we suggest that the protective effect of SB203580 on RGCs is caused by inhibition of p38, although we acknowledge that another mode of action of the drug cannot be completely eliminated.

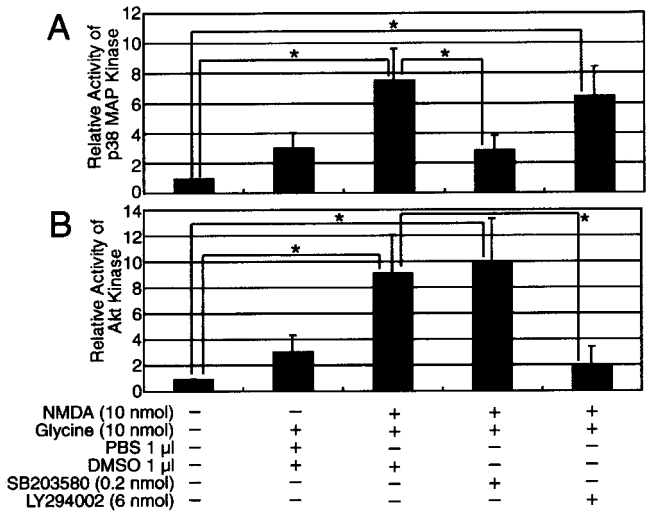

FIGURE 7. Activity of p38 MAP kinase and Akt after exposure to 10 nmol NMDA and glycine. Activity of (**A**) p38 MAP kinase and (**B**) Akt increased significantly after exposure to excitotoxin. SB203580 and LY294002 significantly and specifically inhibited activity of p38 and Akt, respectively ($^*P < 0.05$).

Similar to p38, we also found that NMDA-glycine induced phosphorylation and activation of Akt. However, even sublethal doses of NMDA (2 nmol) produced significant phosphorylation of Akt. In fact, a slight increase in phosphorylation of Akt was observed after injection of glycine alone in the absence of NMDA. Recently, a novel type of NMDA receptor was cloned that responds to glycine alone,[42] and it is possible that this receptor or the conventional inhibitory glycine receptor was involved in this response. Alternatively, the injection into the vitreous itself, with consequent osmotic and inflammatory changes, could have triggered the increase in Akt phosphorylation. Whatever the reason, however, the degree of phosphorylation of Akt in the absence of injected NMDA (onefold increase) was slight compared with the effect of NMDA (fivefold increase).

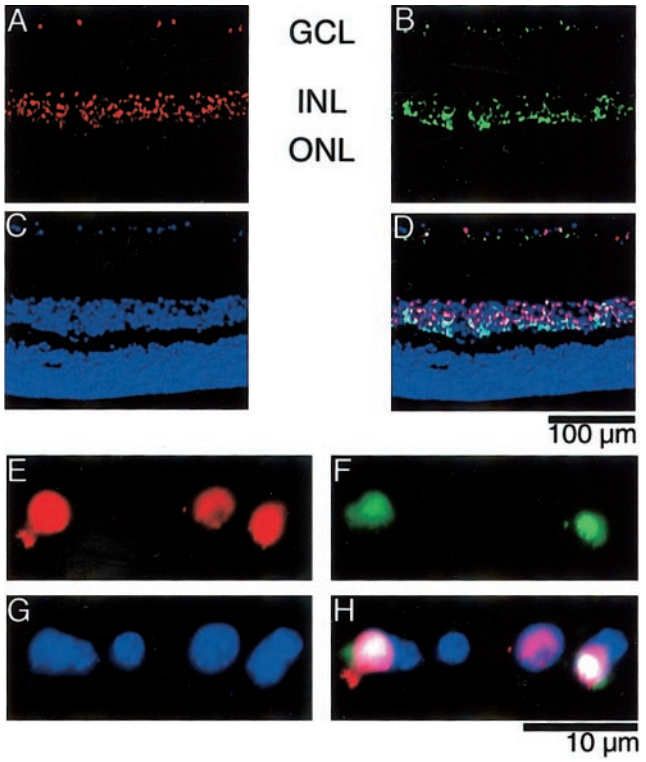

FIGURE 8. Double-labeling with anti-phospho-p38 and anti-phospho-Akt in retinas 6 hours after injection of 10 nmol NMDA and glycine. (**A**, **E**) phospho-p38; (**B**, **F**) phospho-Akt; (**C**, **G**) DAPI; (**D**, **H**) merged. (**E**–**H**) GCL at higher magnification. Both p38 and Akt were activated in some cells.

To investigate whether activation of the PI-3 kinase–Akt pathway is involved in RGC survival, we administered the PI-3 kinase inhibitor LY294002 to eyes injected with NMDA. LY294002 appeared to inhibit the activity of Akt both significantly and specifically at the concentration used in our studies, in that it did not affect the activity of p38 or ERK in control experiments. Simultaneous administration of 6 nmol LY294002 (corresponding to 50 μM in the vitreous) enhanced RGC death in retinas injected with 10 nmol NMDA. Furthermore, unlike SB203580, a second administration of LY294002 had an additional detrimental effect on RGC survival after injection with 10 nmol NMDA. However, LY294002 did not increase RGC death due to 200 nmol NMDA, although a ceiling effect may have been reached, with 85% of the RGCs dying under these conditions. The results with LY294002 and 10 nmol NMDA suggest that the PI-3 kinase-Akt pathway is antiapoptotic in RGCs.

Recently, memantine, an uncompetitive open-channel blocker of the NMDA receptor, has entered advanced clinical trials for treatment of neurodegenerative diseases, including glaucoma.[43–46] However, blockade of the NMDA receptor may inhibit neuroprotective pathways (e.g., Akt) as well as death-promoting pathways (e.g., p38), because we showed that at least some cells displayed activation of both Akt and p38 after exposure to an excitotoxin. Additional effectiveness could be gained by blocking only the proapoptotic pathways or by enhancing the antiapoptotic pathways in some other manner.

In conclusion, we show that the p38 MAP kinase and PI-3 kinase–Akt pathways are both activated after stimulation of NMDA receptors in the retina in vivo. Our inhibitor studies suggest that the p38 MAP kinase pathway is proapoptotic, whereas the PI-3 kinase–Akt pathway is antiapoptotic in RGCs. The elucidation of these divergent signaling pathways after stimulation of NMDA receptors may lead to more effective strategies for treating neurodegenerative diseases, including glaucoma, retinal ischemic disease, and optic neuropathy, in that excessive activity of NMDA receptors has been linked to these ophthalmic disorders.

Acknowledgments

The authors thank Masashi Kikuchi, Murat Digicaylioglu, Shu-ichi Okamoto, Marcus Kaul, and Ella Bossy-Wetzel for helpful discussions.

References

1. Lucas DR, Newhouse JP. The toxic effect of sodium L-glutamate on the inner layers of the retina. *Arch Ophthalmol.* 1957;58:193–201.
2. Nakanishi S. Molecular diversity of glutamate receptors and implications for brain function. *Science.* 1992;258:597–603.
3. Levy DI, Lipton SA. Comparison of delayed administration of competitive and uncompetitive antagonists in preventing NMDA receptor-mediated neuronal death. *Neurology.* 1990;40:852–855.
4. Lam TT, Siew E, Chu R, Tso MO. Ameliorative effect of MK-801 on retinal ischemia. *J Ocul Pharmacol Ther.* 1997;13:129–137.
5. Siliprandi R, Canella R, Carmignoto G, et al. N-methyl-D-aspartate-induced neurotoxicity in the adult rat retina. *Vis Neurosci.* 1992; 8:567–573.
6. Matini P, Moroni F, Lombardi G, Faussone-Pellegrini MS. Ultrastructural and biochemical studies on the neuroprotective effects of excitatory amino acid antagonists in the ischemic rat retina. *Exp Neurol.* 1997;146:419–434.
7. Dreyer EB, Zurakowski D, Schumer RA, Podos SM, Lipton SA. Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. *Arch Ophthalmol.* 1996;114:299–305.
8. Delbarre G, Delbarre B, Calinon F, Ferger A. Accumulation of amino acids and hydroxyl free radicals in brain and retina of gerbil after transient ischemia. *J Ocul Pharmacol.* 1991;7:147–155.
9. Neal MJ, Cunningham JR, Hutson PH, Hogg J. Effects of ischaemia on neurotransmitter release from the isolated retina. *J Neurochem.* 1994;62:1025–1033.
10. Perlman JI, McCole SM, Pulluru P, et al. Disturbances in the distribution of neurotransmitters in the rat retina after ischemia. *Curr Eye Res.* 1996;15:589–596.
11. Yoles E, Schwartz M. Elevation of intraocular glutamate levels in rats with partial lesion of the optic nerve. *Arch Ophthalmol.* 1998;116:906–910.
12. Balazs R, Jorgensen OS, Hack N. N-methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience.* 1988;27:437–451.
13. Rocha M, Martins RA, Linden R. Activation of NMDA receptors protects against glutamate neurotoxicity in the retina: evidence for the involvement of neurotrophins. *Brain Res.* 1999;827:79–92.
14. Bhave SV, Ghoda L, Hoffman PL. Brain-derived neurotrophic factor mediates the anti-apoptotic effect of NMDA in cerebellar granule neurons: signal transduction cascades and site of ethanol action. *J Neurosci.* 1999;19:3277–3286.
15. Woodgett JR, Avruch J, Kyriakis J. The stress activated protein kinase pathway. *Cancer Surv.* 1996;27:127–138.
16. Castagne V, Clarke PG. Inhibitors of mitogen-activated protein kinases protect axotomized developing neurons. *Brain Res.* 1999; 842:215–219.
17. Horstmann S, Kahle PJ, Borasio GD. Inhibitors of p38 mitogen-activated protein kinase promote neuronal survival in vitro. *J Neurosci Res.* 1998;52:483–490.
18. Kawasaki H, Morooka T, Shimohama S, et al. Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J Biol Chem.* 1997;272:18518–18521.
19. Kummer JL, Rao PK, Heidenreich KA. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J Biol Chem.* 1997;272:20490–20494.
20. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science.* 1995;270:1326–1331.

21. Bading H, Greenberg ME. Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science.* 1991;253:912–914.
22. Kikuchi M, Tenneti L, Lipton SA. Role of p38 mitogen-activated protein kinase in axotomy-induced apoptosis of rat retinal ganglion cells. *J Neurosci.* 2000;20:5037–5044.
23. D'Mello SR, Borodezt K, Soltoff SP. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3- kinase in IGF-1 signaling. *J Neurosci.* 1997;17:1548–1560.
24. Dudek H, Datta SR, Franke TF, et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science.* 1997; 275:661–665.
25. Miller TM, Tansey MG, Johnson EM Jr, Creedon DJ. Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization- and insulin-like growth factor I-mediated survival of cerebellar granule cells. *J Biol Chem.* 1997;272:9847–9853.
26. Parrizas M, Saltiel AR, LeRoith D. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3′-kinase and mitogen-activated protein kinase pathways. *J Biol Chem.* 1997; 272:154–161.
27. Philpott KL, McCarthy MJ, Klippel A, Rubin LL. Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. *J Cell Biol.* 1997;139:809–815.
28. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science.* 1995;267:2003–2006.
29. Kermer P, Klocker N, Labes M, Bahr M. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 In vivo. *J Neurosci.* 2000;20:2–8.
30. Pap M, Cooper GM. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. *J Biol Chem.* 1998;273:19929–19932.
31. Cardone MH, Roy N, Stennicke HR, et al. Regulation of cell death protease caspase-9 by phosphorylation. *Science.* 1998;282:1318–1321.
32. Datta SR, Dudek H, Tao X, et al. Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. *Cell.* 1997;91:231–241.
33. Kops GJ, Burgering BM. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J Mol Med.* 1999;77: 656–665.
34. Pearce IA, Cambray-Deakin MA, Burgoyne RD. Glutamate acting on NMDA receptors stimulates neurite outgrowth from cerebellar granule cells. *FEBS Lett.* 1987;223:143–147.
35. Vorwerk CK, Simon P, Gorla M, et al. Pilocarpine toxicity in retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 1999;40:813–816.
36. Vorwerk CK, Gorla MS, Dreyer EB. An experimental basis for implicating excitotoxicity in glaucomatous optic neuropathy. *Surv Ophthalmol.* 1999;43(suppl 1):S142–S150.
37. Lam TT, Abler AS, Kwong JM, Tso MO. N-methyl-D-aspartate (NMDA)–induced apoptosis in rat retina. *Invest Ophthalmol Vis Sci.* 1999;40:2391–2397.
38. el-Asrar AM, Morse PH, Maimone D, Torczynski E, Reder AT. MK-801 protects retinal neurons from hypoxia and the toxicity of glutamate and aspartate. *Invest Ophthalmol Vis Sci.* 1992;33: 3463–3468.
39. Olney JW, Price MT, Fuller TA, et al. The anti-excitotoxic effects of certain anesthetics, analgesics and sedative-hypnotics. *Neurosci Lett.* 1986;68:29–34.
40. Hughes A. A schematic eye for the rat. *Vision Res.* 1979;19:569–588.
41. Harada J, Sugimoto M. An inhibitor of p38 and JNK MAP kinases prevents activation of caspase and apoptosis of cultured cerebellar granule neurons. *Jpn J Pharmacol.* 1999;79:369–378.
42. Chatterton JE, Awobuluyi M, Premkumar LS, et al. Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature.* 2002;415:793–798.
43. Naskar R, Vorwerk CK, Dreyer EB. Saving the nerve from glaucoma: memantine to caspases. *Semin Ophthalmol.* 1999;14: 152–158.
44. Gu Z, Yamamoto T, Kawase C, et al. Neuroprotective effect of N-methyl-D-aspartate receptor antagonists in an experimental glaucoma model in the rat [in Japanese]. *Nippon Ganka Gakkai Zasshi.* 2000;104:11–16.
45. Dreyer EB, Grosskreutz CL. Excitatory mechanisms in retinal ganglion cell death in primary open angle glaucoma (POAG). *Clin Neurosci.* 1997;4:270–273.
46. Hare W, WoldeMussie E, Lai R, et al. Efficacy and safety of memantine, an NMDA-type open-channel blocker, for reduction of retinal injury associated with experimental glaucoma in rat and monkey. *Surv Ophthalmol.* 2001;45(suppl 3):S284–S289; discussion S295–S296.