Assessment of Neuroprotective Effects of Glutamate Modulation on Glaucoma-Related Retinal Ganglion Cell Apoptosis In Vivo

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Purpose. To assess the neuroprotective effects of different glutamate modulation strategies, with a nonselective (MK801) and a selective (ifenprodil) NMDA receptor antagonist and a metabotropic glutamate receptor agonist (mGluR Group II, LY354740), in glaucoma-related in vivo rat models of retinal ganglion cell (RGC) apoptosis.

Methods. RGC apoptosis was induced in Dark Agouti (DA) rats by staurosporine (SSP) treatment. Single agents MK801, ifenprodil, or LY354740, or MK801 and LY354740 combined, were administrated intravitreally at different doses. Eyes were imaged in vivo using a recently established technique and the results confirmed histologically. The most effective combined therapy regimen of MK801 and LY354740 was then assessed in a chronic ocular hypertension (OHT) rat model with application at 0, 1, and 2 weeks after OHT surgery and the effects assessed as described before.

Results. All strategies of glutamate modulation reduced SSP-induced-RGC apoptosis compared with the control, in a dose-dependent manner: MK801 ($R^2 = 0.8863$), ifenprodil ($R^2 = 0.4587$), and LY354740 ($R^2 = 0.9094$), with EC_{50}s of 0.074, 0.0138, and 19 nanomoles, respectively. The most effective combination dose of MK801 and LY354740 was 0.06 and 20 nanomoles ($P < 0.05$), respectively, and the optimal timing of the therapy was 0 weeks after OHT surgery ($P < 0.05$).

Conclusions. This novel SSP model was validated as a useful tool for screening neuroprotective strategies in vivo. Group II mGluR modulation may be a useful treatment for RGC death. Combination therapy optimized to limit neurotoxic effects of MK801 may be an effective neuroprotective approach in retinal degenerative disease. Furthermore, treatments that minimize secondary RGC degeneration may be most useful in glaucoma. (*Invest Ophthalmol Vis Sci.* 2006;47:626–633) DOI: 10.1167/iovs.05-0754

Glaucoma is a major cause of worldwide irreversible blindness. Vision loss is attributed to retinal ganglion cell (RGC) death—a hallmark of glaucoma. Glaucomatous RGC death has been shown to involve the apoptosis pathway,[1,2] and RGC apoptosis is one of the earliest signs of the disease process in glaucoma.[1,3] Excessive activation of glutamate receptors from the release of glutamate from injured RGCs is heavily implicated in this process.[4] Glutamate is the principal excitatory neurotransmitter in the central nervous system (CNS) and the retina and has been found to be increased in glaucoma.[4–6] Inhibition or blockade of glutamate activity by modulation of its receptors—in particular, modulating NMDA (*N*-methyl-D-aspartate)-type glutamate receptors— has been advocated as an important strategy for neuroprotection in glaucoma.

In the CNS and the retina, glutamate mediates excitatory neurotransmission via ion channel-associated (ionotropic) and G protein-coupled (metabotropic) receptors.[7,8] The ionotropic (iGlu) receptors include NMDA, AMPA (α-animo-3-hydroxy-5-methyl-4-isoxazolepropionate), and KA (kainate) subtypes.[7,8] A variety of NMDA antagonists, including memantine, MK801 (dizocilpine), dextromethorphan, flupirtine, and eliprodil, have been shown to ameliorate ischemia-induced insults to the retina. in vivo[9–12] and in vitro,[9,13] and to prevent or delay RGC death in several different models.[9–11] NMDA receptors are thought to be heteromeric ion channel complexes that consist of two NR1 subunits and two NR2 subunits that can be either of the NR2A, -2B, -2C, or -2D type. In the rat retina, RGCs express both NR2A and -2B subunits, and it is thought that cells have a combination of different NMDA receptor types.[14]

Glutamate release has been implicated as a mechanism of RGC death in glaucoma,[4,15–18] particularly with regard to secondary RGC degeneration.[19–22] In addition, it has been heavily implicated in IOP-induced ischemia.[23] It is very much the basis of several experimental glaucoma treatment studies, including those involving the NMDA antagonists MK801 in a rat ocular hypertensive model[24] and memantine in rat and primate models,[25–27] in which NMDA antagonists were shown to be neuroprotective. However, all these studies have relied on the quantification of RGC loss histologically and have not looked at the effects of agents on levels of RGC apoptosis.

G protein-coupled glutamate receptors are called metabotropic (mGlu) receptors because they couple to intracellular second messengers.[28,29] Eight mGlu receptor subtypes have been identified so far, and these have been classified into three groups.[28,29] The $mGluR_1$ and $mGluR_5$ are coupled positively to phospholipase C, and both are included in group I, whereas the others are coupled negatively to adenylate cyclase and belong to group II ($mGluR_2$ and $mGluR_3$) and group III ($mGluR_4$, $mGluR_6$, $mGluR_7$, and $mGluR_8$).[28,29] mGluRs can modulate excitatory and inhibitory synaptic transmission through various transduction pathways. There is evidence that

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activation of group I mGluRs increases neuronal excitation, whereas that of group II and III mGluRs reduces synaptic transmission[29]; therefore, group I mGluR antagonists and group II and III mGluR agonists can be thought to be neuroprotective.[29] Various studies have shown expression of mRNA and/or receptor proteins for all mGluRs in the retina.[30–34] Furthermore, it has been recently shown that expression of some mGluRs is stimulated in ocular hypertension (OHT) rodent glaucoma models,[35] although the effects of a combination of group I mGluR antagonists and group II and III mGluR agonists were not found to be protective of RGC death in an axotomy and NMDA excitotoxic model.[36] Although group II mGluR agonists by themselves have been reported to be neuroprotective against apoptotic neuronal death,[29] until now, specific and targeted modulation of group II mGluRs has not been assessed in retinal apoptosis or glaucoma models.

In this study, we sought to assess the effects of the broad-spectrum NMDA antagonist MK801 in our recently described model of staurosporine (SSP)-induced RGC apoptosis.[3] To assess the relative contributions of NR2B-containing NMDA receptors in this apoptotic process, we also studied the effects of the NR2B-selective antagonist ifenprodil. As activation of group II mGluRs is neuroprotective through a different mechanism than that of NMDA antagonism, using this same model we investigated the actions of the group II agonist LY354740, and compared these effects with blockade of NMDA receptors. Finally, we assessed the effects of these agents in the OHT model of rodent glaucoma. All agents were investigated with our novel technique of in vivo RGC apoptosis imaging, which involves the correlation of the level of histologically confirmed RGC apoptosis to the effectiveness of neuroprotection.[3]

Methods

Animals

Adult male Dark Agouti (DA) rats weighing 150 to 200 g were treated with procedures approved by the U.K. Home Office and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Glaucoma-Related Animal Models

RGC apoptosis was induced by either SSP treatment or surgical elevation of IOP, using methods and models we have described and characterized.[2,3,37] All animals were given intraperitoneal ketamine (37.5%)/medetomidine (25% Domitor; Pfizer Animal Health, Exton, PA) solution (0.75 mL ketamine, 0.5 mL medetomidine, and 0.75 mL sterile water) at 0.2 mL/100 g.[3]

Briefly, to establish the SSP model, we injected the rats intravitreally with SSP (2.14 nanomoles) in PBS, using a 30-gauge needle attached to a 10-μL syringe (Hamilton, Reno, NV) that was connected with a syringe pump (UMP2; World Precision Instruments, Sarasota, FL). The needle was inserted through the sclera superiorly, 1 mm behind the limbus, at an angle of 45°.[3]

For the OHT model,[2,3,37] the IOP was elevated in the left eye of each animal by injection of 50 μL of hypertonic saline solution (1.80 M) into the episcleral vein,[38] using the syringe pump (60 μL/min; UMP2, World Precision Instruments).[2] Contralateral, nonsurgical eyes acted as the control. The IOP of both eyes in each rat was measured and calculated. Peak IOP, defined as the maximum elevation in IOP attained at any time during the study period, was recorded in all animals after surgery. In each animal, the integral IOP, defined as the integral of IOP elevation over time, was calculated from the area under the curve, as previously described.[2,3] The animals were killed at 3 weeks after IOP elevation.

Identification of RGCs

For identification of normal RGCs, a subgroup of rats (n = 12) had RGCs retrogradely labeled by the application of DiAsp4 4-(4-(didecylamino)styryl)-*N*-methylpyridinium iodide (4-Di-10-Asp; Molecular Probes-Cambridge Biosciences, Cambridge, UK) to both superior colliculi, by a method we have described.[2,3] Ten days after the DiAsp4 labeling, the rats underwent either SSP treatment or surgery, to elevate IOP.

Single-Agent Neuroprotective Treatment in an SSP Model

Rats were randomly divided into three different treatment groups that received treatment with MK801 (n = 37), ifenprodil (n = 34), or LY354740 (n = 23; kindly donated by Ann Kingston, Lilly Research Laboratories). Each drug was dissolved in sterilized water and administrated in a range of doses of 0 to 3 nanomoles for MK801 and ifenprodil, and 0 to 50 nanomoles for LY354740, with a vehicle-only (sterilized water) treatment (n = 11) used as the control. Agents were administered intravitreally at the same time as SSP and annexin V-labeled Alexa Fluor 488 (Molecular Probes), so that a final total volume of 5 μL containing all agents was given to all eyes.

Combined-Agent Neuroprotective Treatment in an SSP Model

We next investigated the effects of MK801 and LY354740 combined, with optimal doses chosen from the results of the single-agent experiments. Combined doses of MK801/LY354740 were: 0.03/1, 0.03/20, 0.06/20, and 3/50 nanomoles, respectively (n = 3 per group). Animals underwent the same protocol as for the single-agent treatment, and the results were compared to vehicle treated control (n = 11).

Combined-Agent Neuroprotective Treatment in an OHT Model

Based on the foregoing results, we used our established OHT rat model[2,3,37] to assess the best combined regimen of low-dose MK801 (to minimize toxicity) and LY354740. The optimal dose was found to be 0.06 nanomoles of MK801 with 20 nanomoles of LY354740. Rats were randomly divided into four groups (n = 4 per group), with the control (no treatment/OHT, no treatment/no IOP elevation) and combined treatment at 0, 1, or 2 weeks after IOP elevation, with intravitreal injections administered daily for 3 days.

In Vivo Imaging of RGC Apoptosis

Eyes were imaged at 2 hours after SSP treatment, which we have shown to be the time of peak RGC apoptosis in this model, with a prototype confocal laser scanning ophthalmoscope (cSLO; Carl Zeiss Meditec, Inc., Dublin, CA) using annexin V-Alexa Fluor 488 (Molecular Probes) to detect RGC apoptosis in vivo, as we have described elsewhere.[3] The retinal images were collected, and a retinal montage was constructed for each eye. For the OHT model, all animals were assessed at 3 weeks after IOP elevation, the time point of peak RGC apoptosis, using our technique of in vivo RGC apoptosis imaging.[3]

Confocal Histologic Assessment of RGC Apoptosis

The animals were killed immediately after cSLO imaging. The eyes were enucleated and fixed in 4% fresh paraformaldehyde overnight. The retinas were dissected and whole flat retinas were mounted as we have described.[2,3] The flat retinas were examined with the aid of confocal laser scanning microscopy software (CLSM 510 META; Carl Zeiss Meditec, Inc.). Using ×16 magnification, we assessed 81 adjacent microscopic fields (each measuring 0.329 mm^2) radiating outward from the optic nerve head in the rat and accounting for 40% of the whole retina. A retinal montage was then made for each whole retina.[2,3] The number of apoptotic RGCs (labeled with annexin V) was

counted manually with image-analysis software (MetaMorph; Universal Imaging Corp., West Chester, PA) by observers masked to treatment protocols.[2,3] Surviving RGCs labeled by DiAsp4 were also assessed.

Statistics

Mean values were calculated with 95% confidence intervals (CIs) for the counts and the percentage reduction of RGC apoptosis after treatments with the different agents. All results were computed as a percentage reduction of RGC apoptosis compared with the vehicle-treated control eyes. Dose–response curves were constructed to fit one-site competition EC_{50} (SigmaPlot; Systat Software, Inc., Point Richmond, CA). All treatment groups were compared to each other and the control by ANOVA ($P < 0.05$).

Results

Effect of a Single Application of Neuroprotective Agents on RGCs in an SSP Model

All treatment groups were assessable for RGC apoptosis in vivo with our new technique (Fig. 1). In vivo findings were validated by histologic assessment with confocal microscopy (Fig. 2).

Histologic RGC apoptotic counts in all animals were computed as the percentage reduction from the vehicle-treated control eyes. All treatments reduced the rate of SSP-induced RGC apoptosis (Fig. 3). The nonselective NMDA antagonist MK801 was found to be more effective than the selective NR2B antagonist ifenprodil (Figs. 3A, 3C). The MK801 EC_{50} was 0.074 nanomoles (Fig. 3E) and that of ifenprodil 0.0138 nanomoles, respectively. The group II mGluR agonist LY354740 showed a dose-dependent neuroprotective effect (Figs. 3B, 3D, 3F), with an EC_{50} of 19 nanomoles.

Statistical analysis showed significant effects of LY354740 ($R^2 = 0.9094$) and MK801 ($R^2 = 0.8863$) on the reduction of SSP-induced RGC apoptosis compared with that in vehicle-treated, control eyes.

Effect of Combined Application of Neuroprotective Agents on RGCs in an SSP model

To investigate possible synergistic interactions between different neuroprotective mechanisms, we used combined applications of low-dose MK801 with LY354740 in the SSP model. Again, in vivo imaging (Fig. 1) was confirmed by histology. Figure 4 shows the effect of the different dose combinations on RGC apoptosis in SSP-treated eyes compared with MK801 alone at the same doses. All treatments resulted in a significant reduction compared with the control ($P < 0.05$). The most effective combined regimen with low-dose MK801 was 0.06/20 (MK801/LY354740) nanomoles which resulted in a maximum percentage reduction in number of apoptotic RGCs to 72 ± 9 compared with MK801 alone at 64 ± 12.

The efficacy of MK801 at low doses (0.03 and 0.06 nanomoles) was significantly increased on combination with LY354740 ($P < 0.05$). Of interest, the reverse was true at high doses (i.e., at 3 nanomoles, MK801 alone was superior to combination therapy with LY354740; $P < 0.05$). There was no significant difference in efficacy when LY354740 was given in combination with MK801 compared with LY354740 alone, at any regimen.

IOP Elevation in Surgical Eyes

Surgery produced an increase of IOP in all eyes, with mean peak IOP elevation of 30.8 ± 3.5 mm Hg and a mean IOP integral of 238.6 ± 34.2 mm Hg days. The duration of raised IOP was 21 days in all animals (Table 1).

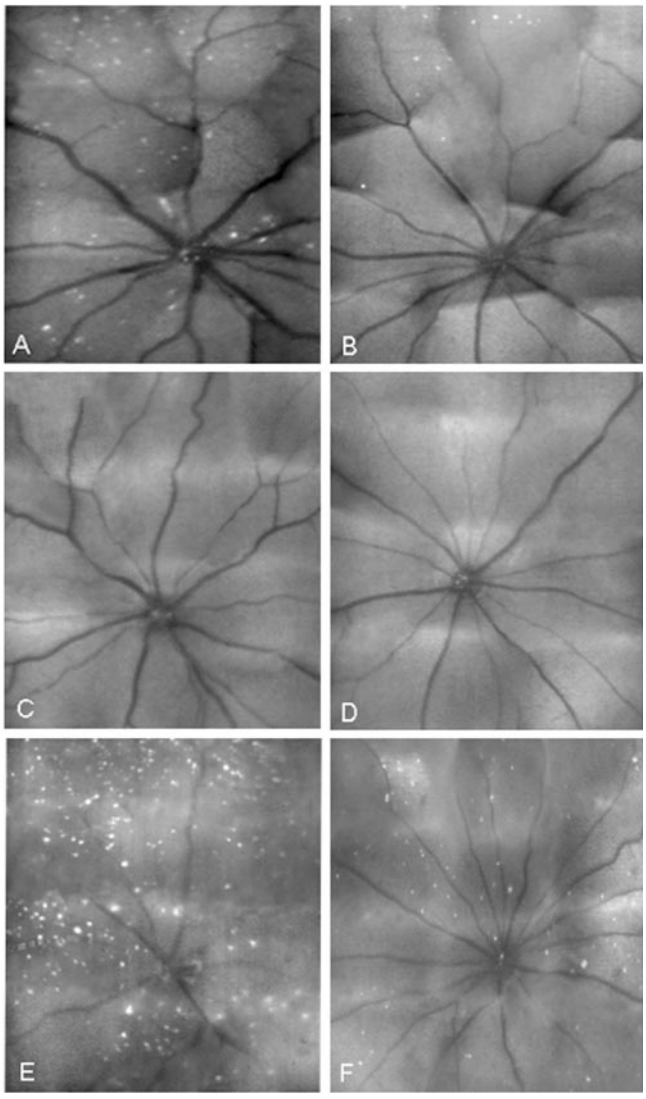

Figure 1. Visualization of RGC apoptosis using SLO imaging of in vivo glaucoma-related models. Staurosporine (SSP)-induced RGC apoptosis, appearing as white spots (**A**), was reduced by ifenprodil (0.6 nanomoles, **B**), MK801 (0.6 nanomoles, **C**), and LY354740 (20 nanomoles, **D**) at 2 hours after treatment. Similarly, RGC apoptosis at 3 weeks in a rat OHT model (**E**) was significantly reduced by treatment with a combined low dose of MK801/ LY354740 (0.06/20 nanomoles, **F**).

Effect of Combined Application of MK801 and LY354740 on RGCs in an OHT Model

With combined doses of 0.06 nanomoles of MK801 with 20 nanomoles of LY354740, all three treatment groups showed reduced OHT-induced RGC apoptosis. However, the most effective timing of the treatment application was at the time of OHT surgery at 0 weeks, compared with 1 or 2 weeks, and the difference was statistically significant. ($P < 0.05$; Fig. 5).

Discussion

We have demonstrated for the first time that it is possible to assess potential glaucoma neuroprotective strategies using our recently developed model of SSP-induced RGC apoptosis.[3] We have compared different glutamate-modulation strategies and

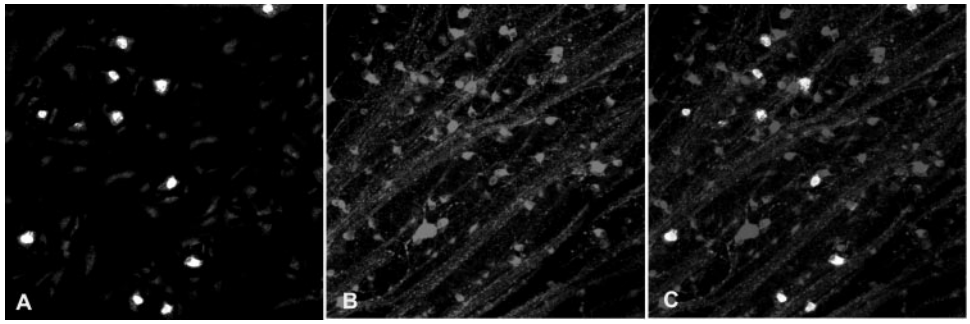

FIGURE 2. Histologic confirmation that apoptotic cells (**A**, labeled with Annexin V/Alexa-488, *white*) were localized to RGCs (**B**, retrograde labeled with DiAsp4, *gray*), as shown in the combined micrograph (**C**), in an SSP model.

shown that the efficacy of low doses of MK801 is increased when given in combination with the group II mGluR agonist LY354740. We found the broad-spectrum NMDA receptor antagonist MK801 to be more effective than the NR2B-selective NMDA receptor antagonist ifenprodil. Furthermore, we have demonstrated for the first time that group II mGluR modulation is useful in prevention of RGC death. Finally, we have shown that our SSP-induced RGC apoptosis model may be used to identify and screen neuroprotective strategies that can then be successfully applied to the rat OHT model.

NMDA antagonists have been demonstrated to be effective in preventing neuronal degeneration in neurologic disorders such as Alzheimer's disease.[39,40] They have also been investigated in the eye, and NMDA receptors have been shown to be expressed in RGCs.[41] Overstimulation of NMDA receptors by intravitreal injection of glutamate or NMDA induces RGC

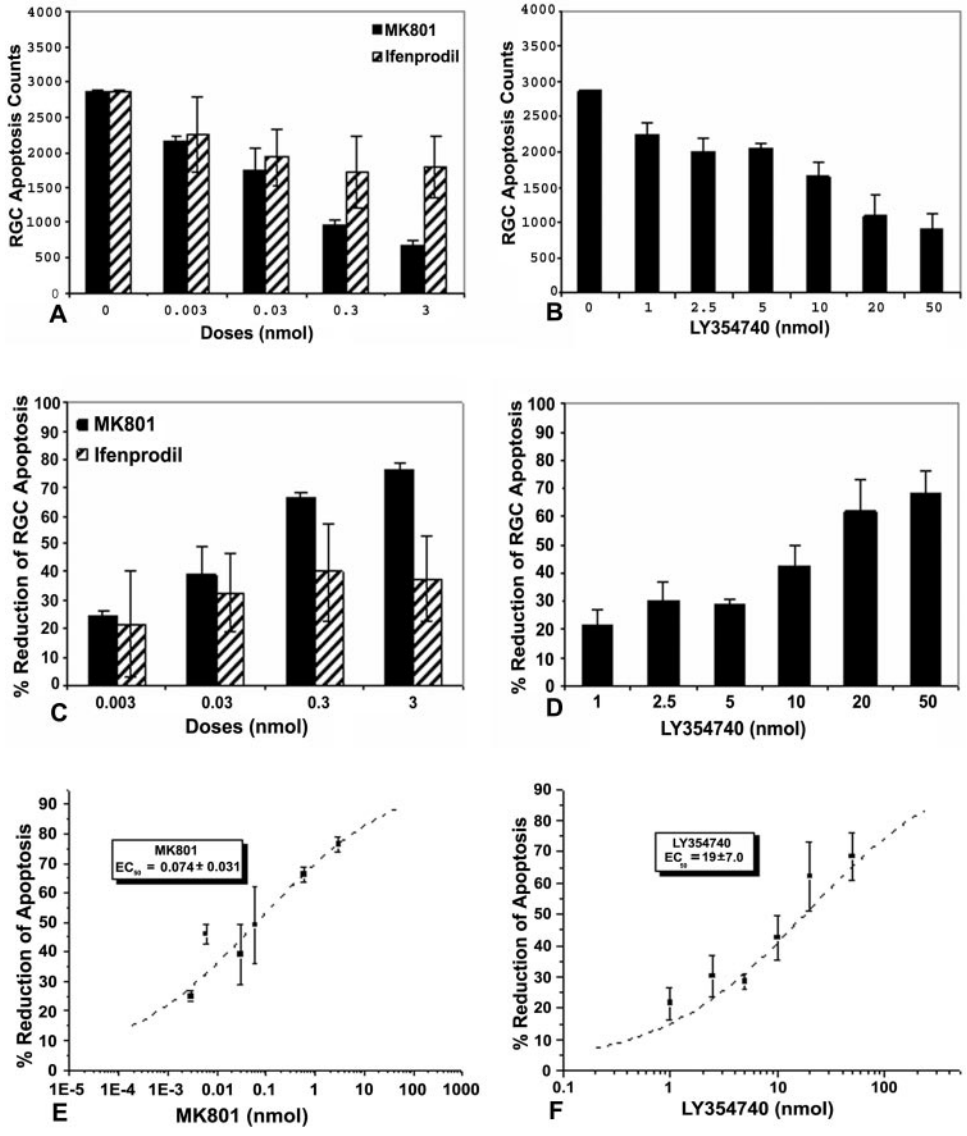

FIGURE 3. Effect of single neuroprotective agents on RGC apoptosis in an SSP rat model. All treatments induced a dose-dependent reduction of RGC apoptosis (**A**, **C** MK801 and ifenprodil; **B**, **D** LY354740). The nonselective NMDA receptor antagonist MK801 was more effective than the selective antagonist ifenprodil. The EC_{50}s of MK801 (**E**) and LY354740 (**F**) were 0.074 and 19 nanomoles with R^2 regression of 0.8863 and 0.9094, respectively.

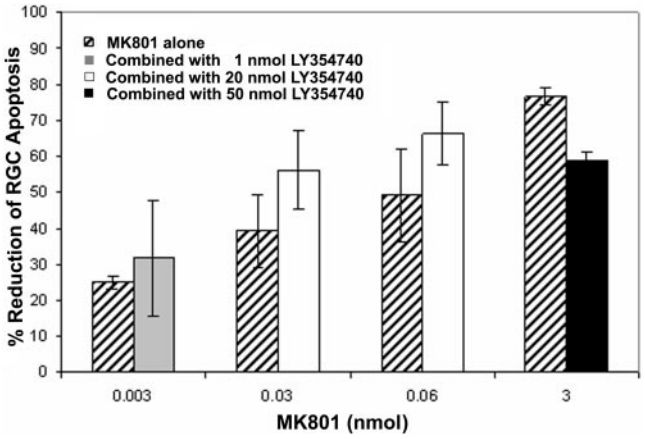

Figure 4. The effect of MK801 alone and with different dose combinations of LY354740 on RGC apoptosis in SSP-treated eyes. Results are shown as a percentage reduction of histologic RGC apoptosis compared with the vehicle-treated control. All treatments resulted in significant reduction of RGC apoptosis compared with the control ($P < 0.05$). The most effective combined regimen with low-dose MK801 was 0.06/20 (MK801/LY354740) nanomoles.

death,[42,43] and NMDA antagonists, such as MK801, dextromethorphan, flupirtine, and eliprodil, have been shown to be preventive against RGC damage, both in vivo and in vitro.[9–11] MK801, a nonselective NMDA antagonist, in particular, has been shown to protect retinal neurons from NMDA-induced toxicity,[9] and has also been demonstrated to be neuroprotective in OHT in the rat.[10,24] However, both these studies based their results on RGC loss and not RGC apoptosis.

Glutamate and NMDA excitotoxicity are believed to contribute to RGC death in glaucoma.[4] The mechanisms of glutamate activity in the development of cell death have been well documented: excessive activation of NMDA receptors, induced by the high concentration of extracellular glutamate,[7,8] leads to a large amount of Ca^{2+} influx into cells, which causes inappropriate activation of the complex cascades of nucleases, proteases, and lipases, resulting in cell death.[7,8] The NMDA receptor antagonists are believed to inhibit the influx of excessive amounts of Ca^{2+} into cells. Paradoxically, overinhibition of the glutamate receptors can disturb their normal physiological activity, which can also induce cell death.[44]

Table 1. The IOP Profile of Individual OHT Animals

Rat	Peak IOP (mm Hg)	Duration (d)	Integral IOP (mm Hg days)
1	36.2	21	213.3
2	36.4	21	225.4
3	22.7	21	313.2
4	22.8	21	242.5
5	31.5	21	184.4
6	30.8	21	172.8
7	25.2	21	285.0
8	20.4	21	306.1
9	29.3	21	214.0
10	37.9	21	194.2
11	37.3	21	195.4
12	26.0	21	295.6
13	35.0	21	248.1
14	35.5	21	227.2
15	25.7	21	287.5
16	39.5	21	212.3
Average	30.8	21	238.6
n	16	16	16.0
95% CI	3.5	0	34.2

Ifenprodil is a NR2B subunit-selective NMDA antagonist, and a recent study has suggested that it is selective against SSP-induced cell death.[45] Although ifenprodil reduced RGC apoptosis in our SSP rat model, it was less effective than the broad-spectrum NMDA antagonist MK801. Because rat RGCs express both NR2B and -2A subunits, and receptors are composed as NR1/NR2A, NR1/NR2B, and to a lesser extent NR1/NR2A/NR2B types,[14] it is perhaps not surprising that ifenprodil is less effective than MK801. However, this indicates that, although NR2B-containing receptors are involved in apoptosis, it is not possible to account for the whole of the apoptotic process with these receptors and that activation of NR2A-containing receptors is also necessary.

LY354740 is a highly potent and selective group II mGluR agonist and can be applied orally.[46] It has been shown to be neuroprotective against NMDA- or SSP-induced neuronal death in rat cortical neuronal cultures and to be more effective against excitotoxic death in mixed glial–neuronal cultures than in pure neuronal culture.[47] Systemic application of LY354740 is neuroprotective in the ischemic rat brain.[48] Our data demonstrate that single intravitreal injections of LY354740 were effective in preventing SSP-induced RGC apoptosis. This was confirmed in vivo by our novel imaging technique and also histologically.

Our results are different from those published by Kermer et al.,[36] who concluded that modulation of mGluRs was not neuroprotective to RGC. This may be attributable, first, to their use of agents with combined group I mGluR and group II or III mGluR activity—LY354740 is a much more specific and potent pharmacological agent with pure group II effects; second, to their assessment of efficacy by RGC loss as opposed to RGC apoptosis; and finally, to their study of different models of ON transection or NMDA excitotoxicity.

Several potential mechanisms of neuroprotection by group II mGluR agonists have been proposed. One possibility is that they inhibit glutamate release at the presynaptic level.[29] Although controversial,[41] ischemia-induced increases in glutamate levels in glaucoma have been documented and may explain, at least in part, RGC death in this disease.[4,49] The reduction in glutamate release by LY354740 may therefore

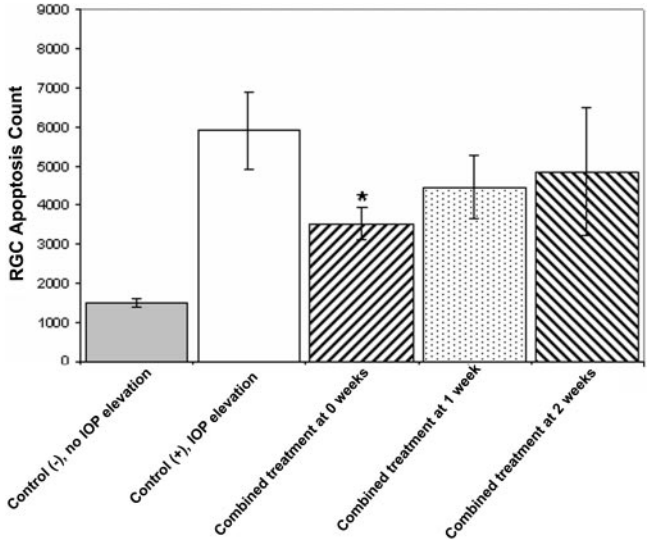

Figure 5. Effect of combined application of MK801/LY354740 (0.06/20 nanomoles) on RGC apoptosis at 3 weeks in an OHT model, when administrated at different times after surgery. All three treatment groups resulted in a reduction in RGC apoptosis. However, the most effective timing of the treatment application was at 0 weeks (at the time of OHT surgery), compared with 1 or 2 weeks ($^*P < 0.05$).

account for the decrease in RGC apoptosis in this study. An alternative explanation could be that group II mGluR agonists alter the adenylate cyclase production postsynaptically.[29] Increased levels of cAMP are found in the hippocampus after transient ischemia[50] and traumatic brain injury[51] and have been linked to the enhanced levels of Ca^{2+} in salamander RGC cells.[52] A third possibility is that stimulation of mGluRs evokes postsynaptic interactions that modulate the activity of NMDA and AMPA receptors.[28,53] In addition, there is evidence that group II mGluR agonists promote synthesis and release of neurotrophic factors—in particular, TGF-β1 and TGF-β2.[54] We have demonstrated that TGF-β2 is significantly downregulated in the retinal ganglion cell layer (RGCL) in OHT rats and this change correlated significantly with increasing RGC apoptosis.[2] It is interesting to note that the maximum degree of neuroprotective effects exerted by LY354740 is comparable to those conferred by MK801, perhaps because both of these compounds exert their effects at different points in the same pathway, as discussed earlier—for example, reduction of glutamate release by LY354740 and blockade of postsynaptic glutamate receptors by MK801.

There is accumulating evidence that MK801 is neurotoxic and induces acute neuronal vacuolization histologically.[55,56] MK801 has been also reported to cause a behavioral clinical syndrome of hyperactivity, hyperreactivity, and motor dysfunction in a dose-dependent manner.[57] This problem is believed to occur due to its high-affinity for the NMDA receptor channel and its slow off rate,[58] resulting in its accumulating in the channels and blocking critical normal functions.[44] For this reason, MK801 has not reached advanced stages of clinical trials, although it remains a useful tool for probing potential NMDA mechanisms. To minimize drug doses but still take advantage of the neuroprotective properties, we investigated the effects of combined MK801 and LY354740. We demonstrated this combination to be most effective in preventing RGC apoptosis in our SSP model compared with application of either agent alone. This finding is similar to previous ones in traumatic neuronal injury, when combined application of MK801 with a group II mGluR agonist elicited significantly more neuroprotection than the administration of individual drugs alone.[51] The mechanism of the effective combination may be attributed to their different pharmacological properties in modulating glutamate excitatory transmission, as discussed earlier.

SSP, a protein kinase inhibitor, is one of the most potent inducers of neuronal apoptosis known. In this study, we have demonstrated that our in vivo model of SSP-induced RGC apoptosis is a useful tool in the assessment of neuroprotective strategies, with strong data attainable within a relatively short time. Using these results, we were able to identify the most effective combined dose of MK801 with LY354740, which we have applied to our OHT rat model.

Because in our OHT model the peak rate of RGC apoptosis is at 3 weeks after IOP elevation,[3] we assessed the neuroprotective effect of all combination regimens at this time point, with treatment given at 0, 1, and 2 weeks after surgery. We demonstrated that all three regimens reduced RGC apoptosis, but the most effective timing of the treatment application was at the time of OHT surgery at 0 weeks.

RGC apoptosis in optic neuropathies such as glaucoma is believed to occur as a result of primary neuronal damage caused by an initial insult and secondary degeneration—a process in which RGCs that survive the primary insult are subsequently injured by the toxic effects of the primary degenerating neurons.[19–22] This effect has been attributed to the release of excitatory amino acids, and glutamate release in particular has been strongly implicated in the secondary RGC degeneration described in glaucoma.[19–22] Our study strongly supports the involvement of glutamate in glaucoma,[15–17] because both MK801 and LY354740 are glutamate modulators. An interesting finding, however, was that application of these agents at the time of the OHT surgery was most effective. This is a finding to similar to those in Chaudhary et al.[24] and Lam et al.[10] who showed MK801 was more effective in the episcleral cauterization OHT rat model when given 1 day before, as opposed to 2 days after IOP elevation. A possible explanation for this may be that administration of neuroprotective therapy at this time point, the time of the primary insult, significantly inhibited glutamate release from primary injured RGCs, resulting in the prevention of secondary degeneration. It would be interesting to investigate the role of glutamate transporters such as GLT-1, GLAST, and EAAT, since these may alter greatly the glutamate activity we have attempted to modulate in this study.[5,59,60]

In summary, our results support glutamate modulation as a viable neuroprotective strategy with applications to glaucoma. We show for the first time successful group II mGluR modulation of RGC death. Furthermore, our investigation suggests that blockade of the NR2B subunit of the NMDA receptor alone may not be sufficient to achieve maximal neuroprotection in glutamate-mediated RGC apoptosis. We suggest that multiagent neuroprotective regimens be further investigated in glaucoma, as our study demonstrates that combination NMDA/mGluR therapy at doses derived to limit neurotoxic effects of MK801, appears to be effective, although the complex nature of the glutamatergic systems involved in RGC apoptosis was also clearly underlined. We demonstrate that strategies for minimizing secondary RGC degeneration effects may be most useful in glaucoma. Finally, our results support the use of our SSP-induced RGC apoptosis model as a useful tool in the assessment of neuroprotective strategies with strong data achievable within a relatively short time, from which optimal regimens can be identified and easily applied to OHT glaucoma models.

References

1. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci*. 1995;36:774–786.
2. Guo L, Moss SE, Alexander RA, Ali RR, Fitzke FW, Cordeiro MF. Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure (IOP) and IOP-induced effects on extracellular matrix. *Invest Ophthalmol Vis Sci*. 2005;46:175–182.
3. Cordeiro MF, Guo L, Luong V, et al. Real time imaging of single nerve cell apoptosis in retinal neurodegeneration. *Proc Natl Acad Sci USA*. 2004;101:13352–13356.
4. Osborne NN, Ugarte M, Chao M, et al. Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv Ophthalmol*. 1999;43:S102–S128.
5. Martin KR, Levkovitch-Verbin H, Valenta D, Baumrind L, Pease ME, Quigley HA. Retinal glutamate transporter changes in experimental glaucoma and after optic nerve transection in the rat. *Invest Ophthalmol Vis Sci*. 2002;43:2236–2243.
6. Vorwerk CK, Gorla MS, Dreyer EB. An experimental basis for implicating excitotoxicity in glaucomatous optic neuropathy. *Surv Ophthalmol*. 1999;43:S142–S150.
7. Hollmann M, Heinemann S. Cloned glutamate receptors. *Annu Rev Neurosci*. 1994;17:31–108.
8. Yang XL. Characterization of receptors for glutamate and GABA in retinal neurons. *Prog Neurobiol*. 2004;73:127–150.

9. 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.
10. Lam TT, Siew E, Chu R, Tso MO. Ameliorative effect of MK-801 on retinal ischemia. *J Ocul Pharmacol Ther*. 1997;13:129–137.
11. Calzada JI, Jones BE, Netland PA, Johnson DA. Glutamate-induced excitotoxicity in retina: neuroprotection with receptor antagonist, dextromethorphan, but not with calcium channel blockers. *Neurochem Res*. 2002;27:79–88.
12. Osborne NN, Cazevieille C, Wood JP, et al. Flupirtine, a nonopioid centrally acting analgesic, acts as an NMDA antagonist. *Gen Pharmacol*. 1998;30:255–263.
13. Pang IH, Wexler EM, Nawy S, DeSantis L, Kapin MA. Protection by eliprodil against excitotoxicity in cultured rat retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 1999;40:1170–1176.
14. Fletcher EL, Hack I, Brandstatter JH, Wassle H. Synaptic localization of NMDA receptor subunits in the rat retina. *J Comp Neurol*. 2000;420:98–112.
15. Kwon YH, Rickman DW, Baruah S, et al. Vitreous and retinal amino acid concentrations in experimental central retinal artery occlusion in the primate. *Eye*. 2005;19:455–463.
16. Lotery AJ. Glutamate excitotoxicity in glaucoma: truth or fiction? *Eye*. 2005;19:369–370.
17. Salt TE, Cordeiro MF. Glutamate excitotoxicity in glaucoma: throwing the baby out with the bathwater? (Letter). *Eye*. 2005;10: 10.
18. Levin LA. Retinal ganglion cells and neuroprotection for glaucoma. *Surv Ophthalmol*. 2003;48:S21–S24.
19. Kaushik S, Pandav SS, Ram J. Neuroprotection in glaucoma. *J Postgrad Med*. 2003;49:90–95.
20. Schori H, Kipnis J, Yoles E, et al. Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. *Proc Natl Acad Sci USA*. 2001;98:3398–403.
21. Levkovitch-Verbin H, Quigley HA, Martin KR, Zack DJ, Pease ME, Valenta DF. A model to study differences between primary and secondary degeneration of retinal ganglion cells in rats by partial optic nerve transection. *Invest Ophthalmol Vis Sci*. 2003;44: 3388–3393.
22. Levkovitch-Verbin H, Quigley HA, Kerrigan-Baumrind LA, D'Anna SA, Kerrigan D, Pease ME. Optic nerve transection in monkeys may result in secondary degeneration of retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2001;42:975–982.
23. Osborne NN, Chidlow G, Layton CJ, Wood JP, Casson RJ, Melena J. Optic nerve and neuroprotection strategies. *Eye*. 2004;18:1075–1084.
24. Chaudhary P, Ahmed F, Sharma SC. MK801: a neuroprotectant in rat hypertensive eyes. *Brain Res*. 1998;792:154–158.
25. WoldeMussie E, Yoles E, Schwartz M, Ruiz G, Wheeler LA. Neuroprotective effect of memantine in different retinal injury models in rats. *J Glaucoma*. 2002;11:474–480.
26. Hare WA, WoldeMussie E, Weinreb RN, et al. Efficacy and safety of memantine treatment for reduction of changes associated with experimental glaucoma in monkey, II: Structural measures. *Invest Ophthalmol Vis Sci*. 2004;45:2640–2651.
27. Hare WA, WoldeMussie E, Lai RK, et al. Efficacy and safety of memantine treatment for reduction of changes associated with experimental glaucoma in monkey, I: Functional measures. *Invest Ophthalmol Vis Sci*. 2004;45:2625–2639.
28. Pin JP, Duvoisin R. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology*. 1995;34:1–26.
29. Nicoletti F, Bruno V, Copani A, Casabona G, Knopfel T. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci*. 1996;19:267–271.
30. Shen W, Slaughter MM. Metabotropic and ionotropic glutamate receptors regulate calcium channel currents in salamander retinal ganglion cells. *J Physiol*. 1998;510:815–828.
31. Hartveit E, Brandstatter JH, Enz R, Wassle H. Expression of the mRNA of seven metabotropic glutamate receptors (mGluR1-7) in the rat retina: an in situ hybridization study on tissue sections and isolated cells. *Eur J Neurosci*. 1995;7:1472–1483.
32. Tehrani A, Wheeler-Schilling TH, Guenther E. Coexpression patterns of mGLuR mRNAs in rat retinal ganglion cells: a single-cell RT-PCR study. *Invest Ophthalmol Vis Sci*. 2000;41:314–319.
33. Robbins J, Reynolds AM, Treseder S, Davies R. Enhancement of low-voltage-activated calcium currents by group II metabotropic glutamate receptors in rat retinal ganglion cells. *Mol Cell Neurosci*. 2003;23:341–350.
34. Higgs MH, Lukasiewicz PD. Activation of group II metabotropic glutamate receptors inhibits glutamate release from salamander retinal photoreceptors. *Vis Neurosci*. 2002;19:275–281.
35. Dyka FM, May CA, Enz R. Metabotropic glutamate receptors are differentially regulated under elevated intraocular pressure. *J Neurochem*. 2004;90:190–202.
36. Kermer P, Klocker N, Bahr M. Modulation of metabotropic glutamate receptors fails to prevent the loss of adult rat retinal ganglion cells following axotomy or N-methyl-D-aspartate lesion in vivo. *Neurosci Lett*. 2001;315:117–120.
37. Guo L, Tsatourin V, Luong V, et al. En-face optical coherence tomography (OCT): a new method to analyze structural changes of the optic nerve head in rat glaucoma. *Br J Ophthalmol*. 2005;89: 1210–1216.
38. Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC. A rat model of chronic pressure-induced optic nerve damage. *Exp Eye Res*. 1997;64:85–96.
39. Lipton SA. Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. *J Alzheimers Dis*. 2004;6:S61–S74.
40. Farlow MR. NMDA receptor antagonists: a new therapeutic approach for Alzheimer's disease. *Geriatrics*. 2004;59:22–27.
41. Ullian EM, Barkis WB, Chen S, Diamond JS, Barres BA. Invulnerability of retinal ganglion cells to NMDA excitotoxicity. *Mol Cell Neurosci*. 2004;26:544–557.
42. Lipton SA. Retinal ganglion cells, glaucoma and neuroprotection. *Prog Brain Res*. 2001;131:712–718.
43. Sun Q, Ooi VE, Chan SO. N-methyl-D-aspartate-induced excitotoxicity in adult rat retina is antagonized by single systemic injection of MK-801. *Exp Brain Res*. 2001;138:37–45.
44. Lipton SA. Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx*. 2004;1:101–110.
45. Williams AJ, Dave JR, Lu XM, Ling G, Tortella FC. Selective NR2B NMDA receptor antagonists are protective against staurosporine-induced apoptosis. *Eur J Pharmacol*. 2002;452:135–136.
46. Monn JA, Valli MJ, Massey SM, et al. Design, synthesis, and pharmacological characterization of (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740): a potent, selective, and orally active group 2 metabotropic glutamate receptor agonist possessing anticonvulsant and anxiolytic properties. *J Med Chem*. 1997; 40:528–537.
47. Kingston AE, O'Neill MJ, Lam A, Bales KR, Monn JA, Schoepp DD. Neuroprotection by metabotropic glutamate receptor glutamate receptor agonists: LY354740, LY379268 and LY389795. *Eur J Pharmacol*. 1999;377:155–165.
48. Bond A, O'Neill MJ, Hicks CA, Monn JA, Lodge D. Neuroprotective effects of a systemically active group II metabotropic glutamate receptor agonist LY354740 in a gerbil model of global ischaemia. *Neuroreport*. 1998;9:1191–1193.
49. Dreyer EB, Pan ZH, Storm S, Lipton SA. Greater sensitivity of larger retinal ganglion cells to NMDA-mediated cell death. *Neuroreport*. 1994;5:629–631.
50. Suyama K, Saito K, Chen G, Pan BS, Manji HK, Potter WZ. Alterations in cyclic AMP generation and G protein subunits following transient ischemia in gerbil hippocampus. *J Cereb Blood Flow Metab*. 1995;15:877–885.
51. Allen JW, Ivanova SA, Fan L, Espey MG, Basile AS, Faden AI. Group II metabotropic glutamate receptor activation attenuates traumatic neuronal injury and improves neurological recovery after traumatic brain injury. *J Pharmacol Exp Ther*. 1999;290:112–120.

52. Han Y, Wu SM. NMDA-evoked [Ca2+]i increase in salamander retinal ganglion cells: modulation by PKA and adrenergic receptors. *Vis Neurosci*. 2002;19:249–256.
53. Berrino L, Oliva P, Rossi F, Palazzo E, Nobili B, Maione S. Interaction between metabotropic and NMDA glutamate receptors in the periaqueductal grey pain modulatory system. *Naunyn Schmiedebergs Arch Pharmacol*. 2001;364:437–443.
54. Bruno V, Battaglia G, Copani A, et al. Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. *J Cereb Blood Flow Metab*. 2001;21:1013–1033.
55. Olney JW, Labruyere J, Price MT. Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science*. 1989;244:1360–1362.
56. Fix AS, Horn JW, Wightman KA, et al. Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK(+)801 (dizocilpine maleate): a light and electron microscopic evaluation of the rat retrosplenial cortex. *Exp Neurol*. 1993;123:204–215.
57. Hargreaves EL, Cain DP. Hyperactivity, hyper-reactivity, and sensorimotor deficits induced by low doses of the N-methyl-D-aspartate non-competitive channel blocker MK801. *Behav Brain Res*. 1992;47:23–33.
58. Lipton SA. Prospects for clinically tolerated NMDA antagonists: open-channel blockers and alternative redox states of nitric oxide. *Trends Neurosci*. 1993;16:527–532.
59. Mawrin C, Pap T, Pallas M, Dietzmann K, Behrens-Baumann W, Vorwerk CK. Changes of retinal glutamate transporter GLT-1 mRNA levels following optic nerve damage. *Mol Vis*. 2003;9:10–13.
60. Vorwerk CK, Naskar R, Schuettauf F, et al. Depression of retinal glutamate transporter function leads to elevated intravitreal glutamate levels and ganglion cell death. *Invest Ophthalmol Vis Sci*. 2000;41:3615–3621.