Selective Excitotoxic Degeneration of Adult Pig Retinal Ganglion Cells In Vitro

Xianmin Luo, Valérie Heidinger, Serge Picaud, George Lambrou, Henri Dreyfus, José Sabel, and David Hicks

PURPOSE. Excitotoxicity is proposed to play a prominent role in retinal ganglion cell (RGC) death ensuing from diseases such as glaucoma and ischemia, but cell culture studies have used tissue from newborn rodents, yielding conflicting data that implicate either N-methyl D-aspartate (NMDA) or non-NMDA glutamate (Glu) receptor-mediated pathways. Excitotoxic RGC death was examined in vitro in this study, using adult pigs, a large-animal model for human retina.

METHODS. Adult pig retina (and for comparative purposes young and adult rat retina) were dissociated and maintained in monolayer culture. Medium was supplemented with Glu or pharmacologic agonists or antagonists, and surviving RGCs and other retinal neurons were quantified using specific immunolabeling methods. Electrophysiological responses to externally applied Glu of RGCs in culture were recorded using whole-cell patch–clamp techniques.

RESULTS. Application of Glu led to selective, dose-dependent losses in large RGCs (maximal 37% decrease at 1 mM; median effective dose [ED50], ~80 μM) and neurite damage in surviving RGCs. Application of Glu agonists and Glu receptor sub-class antagonists showed that large RGC death was mediated through both NMDA and non-NMDA receptor pathways. Small RGCs, amacrine cells, and all other retinal neurons were resistant to Glu-induced death. By comparison, rat retinal cultures displayed heightened RGC vulnerability to Glu, mediated exclusively by non-NMDA receptor-mediated pathways. Amacrine cells were unaffected by NMDA but were very sensitive to kainate application (≥90% loss). Other retinal neurons were unaffected by any treatment.

CONCLUSIONS. The molecular pathways underlying excitotoxic RGC death in vitro (non-NMDA or NMDA-prefering Glu receptors) vary among species and developmental stages. The selective elimination of adult pig large RGCs by NMDA receptor-mediated pathways more closely resembles human and animal glaucoma in vivo than other published culture models, providing a simplified experimental system for investigating the pharmacologic and toxicologic bases of glaucoma-like neuronal death. (Invest Ophthalmol Vis Sci. 2001;42:1096–1106)

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R etinal ganglion cells (RGCs) relay visual information from the neural retina to the processing centers in the brain. They are vulnerable to several retinal diseases, foremost of which are retinal ischemia, diabetic retinopathy, and glaucoma. The latter affects some 67 million people worldwide, and although the fundamental causes underlying glaucoma are still unknown, they are suggested to include genetic factors, pressure-induced damage, ischemia, and trophic factor deprivation. Most reports indicate that in human and experimental glaucoma, specifically the RGCs and no other neuronal types die.3–9 One key component is proposed to be excitotoxicity stemming from malfunctions in the regulation of extracellular levels of glutamate (Glu).10,11 Patients with glaucoma12 and animal models of glaucoma13,14 show elevated vitreal levels of Glu, and prolonged injection of Glu induces RGC death in rats.15 Ionotropic Glu receptors (GluR) of the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA) subtypes are all expressed in the neural retina, principally in the inner layers. These GluRs are formed of different combinations of subunits, GluR1 through −716,17 and NMDA receptors (R)-1 and R-2,18–21 which confer the pharmacologic characteristics of individual receptors.

Modeling of RGC pathologic responses has been performed extensively in vivo and in vitro. Whereas injections in vivo of excitatory amino acids (EAAs; Glu22,23 and KA+ NMDA24–27) show that the most affected structures are the inner nuclear layer (INL) and especially the ganglion cell layer (GCL), treatments with EAAs in vitro typically lead to much more widespread cell loss.28–30 Owing to the facility of experimental manipulation, adapted monolayer culture models of RGC toxicology would be of considerable use for studies of glaucoma and ischemia. We have investigated the responses by RGCs and other neurons to excitotoxicity, by using cultures prepared from fully adult pig retinas, and we report that compared with cultures prepared from newborn and adult rat retina, the pattern of EAA-induced RGC death observed in pig retina most resembles that observed in human and experimental glaucoma in vivo.

MATERIALS AND METHODS

Tissue Collection and Cell Culture

All animal experimentation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Newborn (3–7 postnatal days) rat pups were decapitated and the eyes removed into warm DMEM/—CO2. Porcine eyes were obtained from a local abattoir. Glutaraldehyde was removed from the animals within minutes of death and were transported to the laboratory in cold DMEM/—CO2 on crushed ice (average delay before dissection, 1–2 hours). Retinal cultures were prepared as we have described previously.31,32 Retinas were isolated into fresh DMEM/—CO2 by circumfer-
vential section of the cornea and removal of the anterior chamber. Major blood vessels were excised, and retinas were then chopped into small fragments, washed in Ringer’s solution without Ca²⁺ or Mg²⁺, supplemented with 0.1 mM EDTA, and incubated in 0.5 ml 0.2% activated papain (Sigma-Aldrich, Saint Quentin Fallavier, France) in the same buffer for 20 minutes at 37°C. The tissue was dissociated by repeated gentle trituration and seeded in DMEM/HamsF12 (Gibco), supplemented with 5% fetal calf serum (FCS; Gibco) and penicillin-streptomycin (10 IU/ml), into 24-well tissue culture plates containing coverslips previously coated with poly-L-lysine (2 μg/cm² for 2 hours) followed by laminin (1 μg/cm² overnight; both from Sigma-Aldrich). Microscopic inspection using trypan blue exclusion showed that viability was more than 95%, 84%, and 90% for newborn rat, adult rat, and adult pig retinas, respectively, at the time of seeding. Seeding was performed at an initial density of 2.5 or 5 × 10⁶ viable cells/cm², and cells were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

**In Vitro Excitatory Amino Acid and Drug Treatments**

Cultures were treated by addition of different EAs (Glu, KA, and NMDA, all diluted in DMEM and added to a final concentration of 20–1000 μM, 50 μl/well; all from Sigma–Aldrich) at the time of seeding (defined as 0 days in vitro). Initial experiments showed that addition of NMDA alone in DMEM had no effect on retinal neuronal survival. To fully investigate NMDA’s effects, we performed one or both of the following: Either NMDA was coapplied with KA (KA + NMDA), the latter serving to depolarize neurons and permit NMDAR activation through removing the voltage-sensitive Mg block, or addition of NMDA:KA was performed in DMEM with reduced Mg²⁺ (0.1–0.3 mM) and elevated Ca²⁺ (10 mM) concentrations. In some wells, 6-cyano-7-nitroquinolinoxide-2′,3′-dione (CNQX; 25–50 μM) or (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,β)-cyclohepten-5,10-imine maleate (MK-801; 10 μM; Tocris Cookson, Bristol, UK), specific antagonists of AMPA/KAR and NMDAR, respectively, were added at the same time as EAAs to the culture medium. To examine whether length of time in culture alters cell responses, some cultures were treated after 3 or 5 days. Treatments were performed for 48 hours, after which cells were gently washed twice, replenished with culture medium, and allowed to grow for a total of 7 days (i.e., cultures treated at 0 days were maintained a further 5 days, and those treated at 5 days were fixed immediately after treatment).

**Immunocytochemical Characterization of Retinal Neurons**

After a total of 7 days in vitro, cells were washed twice in phosphate buffered saline (PBS; pH 7.3) and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were rinsed twice in PBS, permeabilized with 0.1% Triton X-100 for 5 minutes, and incubated in PBS containing 0.1% bovine serum albumin, 0.1% Tween 20, and 0.1% NaCl (buffer A) for 15 minutes. Different primary antibodies (all diluted in buffer A and used at a final concentration of 10 μg/ml) for 2 hours, washed six times with PBS, and exposed for 1 hour to goat anti-mouse IgG/Bodipy FL (10 μg/ml; for monoclonal primary antibodies) or goat anti-rabbit IgG/Texas red (10 μg/ml; for polyclonal primary antibodies; both from Molecular Probes, Portland, OR). Nuclei of cultured cells were stained with 4,6-diamidino-2-phenylindole (DAPI), incubated together with fluorescent secondary antibodies. Finally, coverslips and sections were washed thoroughly, mounted, and viewed under a confocal microscope (Optiphot 2; Nikon, Tokyo, Japan), equipped with Nomarski differential interference optics and epifluorescence illumination.

**Assessment of Cellular Injury**

Neuronal cell injury was assessed as described previously, by counting of immunolabeled cells exhibiting continuous plasma membranes, with no signs of vacuolation, and well-developed neuritic processes. Cells showing perikaryal swelling, nuclear pyknosis, or fragmentation as visualized by DAPI staining were not included in the counts. Data are expressed as total cell number per coverslip (for adult pig retina, average total number NF-immunopositive RGCs was 220 large and 500 small RGCs; for adult rat retina, average total 300 RGCs; for newborn rat retina, average total 800 RGCs; for newborn pig retina, average total 500 RGCs/control coverslip).

**Electrophysiological Recording of Large RGCs**

We were able to record from large RGCs in adult pig retinal cultures, because these cells were easily recognizable under high-power inverse-phase contrast optics by their size and neurite morphology, even in the absence of antibody labeling. Cultured RGCs were recorded with the whole-cell patch–clamp technique, according to a procedure previously described. For these experiments a minimum of 20 randomly chosen optical fields per coverslip were counted under ×20 objectives. Each experiment was repeated separately a minimum of three times with triplicate treatments per experiment. Statistical analysis was performed by computer (StatWorks Data Student’s t Statistic software; Cricket Software, Malvern, PA), using the parametric method for unpaired series for one variable.

**RESULTS**

**Immunocytochemical Identification of Retinal Neurons In Vitro**

**Adult Pig Retina.** In adult pig retinal cultures, NF-immunopositive cells could be categorized into three groups: (1) large RGCs, which exhibited large circular somata (30–45 μm in diameter), with the nucleus displaced to one side and several long, smooth processes sometimes running great distances. NF immunolabeling was intense and continuous, coiled tightly within the cell body, and present throughout the length of the neurites (Figs. 1A, 1B); (2) small RGCs, typically mono- or bipolar, with circular or ovoid cell bodies less than 30 μm in diameter. NF immunolabeling was of moderate intensity and discontinuous along neurites (Fig. 1C); and (3) horizontal cells, distinguishable from RGCs by their distinctive morphology (~30 μm in diameter, stellate cell bodies with multiple long and thick processes) and PGP9.5 immunonegativity (Fig. 1D). These cells were excluded from measures of toxic effects on
RGCs. Control coverslips typically contained approximately 200 large and 600 small RGCs, although the amount ranged from 100 to 400 large and 400 to 1000 small RGCs, depending on individual experiments.

In adult pig retinal cultures PGP9.5-immunopositive cells represented approximately 10% of total neurons and were of various sizes and shapes, bipolar or multipolar, with cell bodies 15 to 40 μm in diameter (Fig. 1E). RGCs were strongly PGP9.5 immunopositive, and PGP9.5-immunopositive, NF-immunonegative cells represented a major subpopulation (~50%) of ACs. Immunocytochemically identified horizontal cells accounted for less than 0.5%, BCs for approximately 20%, and PRs for more than 50% total neurons (data not shown). Therefore, the proportions of distinct cell types in vitro approximately matched those observed in vivo.

**Rat Retina.** NF immunoreactivity was essentially identical in both young and adult rat retinal cultures, revealing scattered neurons (soma diameter, 25 ± 5 μm) with multiple long neurites (Figs. 1F, 1G, respectively). We could not distinguish clear subpopulations of RGCs based on soma size or neurite morphology, and quantitative data are given for total RGCs. As was true of pig retinal cell cultures, horizontal cells were excluded from cell counts. Neonatal rat cultures contained an average of 1100 NF-immunoreactive RGCs per coverslip, whereas adult rat cultures contained approximately 450 such cells. In preliminary observations of fixed sections we detected only faint PGP9.5 expression in newborn rat retina and weak PGP9.5 immunoreactivity in young rat cultures. Intense PGP9.5 expression was seen in multipolar neurons (soma diameter 15–40 μm) in adult rat.
retinal cultures (Fig. 1H). Double labeling showed these immunopositive neurons to be RGCs and ACs, as in pig cell cultures.

EAA Neurotoxicity of Retina In Vitro

**Adult Pig Retina. Total Neurons.** Cultures were exposed to 1 mM Glu, and total neuronal numbers were measured at 7 days. Glu did not significantly affect total neuronal survival (Fig. 2A). This was corroborated on two immunologically identified neuronal subpopulations, PRs and BCs, neither of which exhibited changes in numbers after Glu addition (Fig. 2A). To observe influences of KA and NMDA on global neuronal survival, cultures were exposed to 1 mM KA alone or KA+NMDA and examined after 7 days. Results showed that both treatments led to similar, significant reductions (~35%, average) in the number of total neurons. BCs and PRs were unaffected by addition of KA or NMDA (Fig. 2B).

**Large RGCs.** After 7 days in vitro, examination of cultures exposed to increasing concentrations of Glu (20–1000 μM) revealed that the number of large RGC decreased in a dose-dependent manner compared with the number in untreated control cultures. Small reductions (8% total RGCs, 19% maximal loss) were already visible at 20 μM and differed significantly from control cultures at 50 μM (P < 0.05), with a median effective dose (ED₅₀) of approximately 80 μM (19% total RGCs) and a maximal effective dose of 1000 μM (37% reduction, P < 0.001; Fig. 3A). Application of Glu at 5 and 10 mM did not decrease survival further relative to 1 mM (data not shown). The neurotoxic effects of Glu (1 mM) could be significantly blocked by simultaneous addition of MK-801 (10 μM), whereas treatment with 50 μM CNQX had no effect. Addition of 10 μM MK-801 or 50 μM CNQX alone to these cultures did not influence RGC survival in comparison with untreated control cultures (Fig. 3B). We found that KA alone had dose-dependent neurotoxic effects on large RGCs, maximal at 1 mM (18% cell loss, statistically significant). This loss was completely prevented by inclusion of CNQX but not by MK-801 (Fig. 4A). NMDA alone did not lead to any loss of RGCs, whereas KA+NMDA (1 mM each) led to a significantly greater decrease...
low-Mg/high-Ca medium and showed dose-dependent toxic effects in all cases. KA toxicity was maximal at 1 mM with more than 40% loss of cells. NMDA was maximally toxic at 0.5 mM (25% loss), and KA+NMDA (NMDA, 1 mM; KA, 0.1–1 mM) led to significantly greater cell loss than did KA alone (maximum of 60% decrease; Fig. 4B).

We also found the morphology of surviving large RGCs was adversely affected in cultures exposed to 1 mM Glu. Compared with morphologic features in untreated cultures (Fig. 5A), neurite length and degree of branching in the remaining large RGCs were severely reduced (Fig. 5B). Inclusion of MK-801 partially restored the normal aspect of neurites in residual RGCs treated with 1 mM Glu (Fig. 5C), whereas addition of CNQX did not rescue normal neurite outgrowth in Glu-treated RGCs (Fig. 5D). Application of MK-801 or CNQX alone did not modify neurite morphology in comparison with control cultures. Addition of KA alone had no visible effect on surviving RGC neurites (Fig. 5E; although when applied in low-Mg/high-Ca medium KA also induced neurite damage in surviving RGCs; data not shown), whereas KA+NMDA resulted in neurite damage (Fig. 5F).

**Small RGCs and ACs.** Maximal concentrations of Glu (1 mM) showed small but significant effects on the number of small RGCs (86.7% ± 11.3% surviving cells relative to control cultures, \( P < 0.05 \)), but had no significant effect on the number of ACs (95.2% ± 6.5% surviving cells relative to control cultures). As shown in Table 1, small RGCs and ACs were very strongly affected by KA treatment, with an approximate 90% loss after addition of KA alone or in combination with NMDA. To study GluR pathways underlying KA toxicity in small RGCs and ACs, specific antagonists were added to cultures. Toxic effects of KA+NMDA on small RGCs were partially blocked by CNQX but not by MK-801. ACs were significantly protected from KA toxicity by CNQX.

**Adult Rat Retina.** Because most in vitro studies on RGC excitotoxicity have been performed on rats, we wanted to compare this species with pig. Furthermore, we decided to conduct experiments with tissue from both newborn and adult rats, to examine possible age differences. Treatment with Glu (1 mM) led to marked reductions in total RGC survival compared with untreated control cultures, a mean 37% loss (\( P < 0.001 \); Fig. 6A). Glu did not significantly affect the number of surviving ACs, or any other cell type. To investigate which GluR mediated the neurotoxicity of Glu on young rat retina in vitro, we placed adult rat retinal cultures in low-Mg2+/high-Ca2+ medium and showed dose-dependent toxic effects in all cases. KA toxicity was maximal at 1 mM with more than 40% loss of cells. NMDA was maximally toxic at 0.5 mM (25% loss), and KA+NMDA (NMDA, 1 mM; KA, 0.1–1 mM) led to significantly greater cell loss than did KA alone (maximum of 60% decrease; Fig. 4B).

Because also in pig cultures NMDA toxicity was less apparent in normal medium, we placed adult rat retinal cultures in low-Mg2+/high-Ca2+ medium. Even under these conditions NMDA had no effect on the number of RGCs. Similar to pig under these conditions, KA toxicity was enhanced (>70% loss; data not shown).

**Neonatal Rat Retina.** Glu, KA, and KA+NMDA (each 1 mM) were added to neonatal retinal cultures at the time of seeding. Each treatment significantly adversely affected the survival of total RGCs: losses of 47%, 78%, and 83% for Glu, KA, and KA+NMDA, respectively (Fig. 6B). To explore which GluR mediated the neurotoxicity of Glu on young rat retina in vitro, CNQX (50 \( \mu \)M) and MK-801 (10 \( \mu \)M) were added simulta-

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**Figure 4.** In adult pig retina in vitro, exogenous KA and NMDA also induced excitotoxicity in large RGCs that was prevented by ligand-specific antagonists and exacerbated in Mg2+/Ca2+-depleted culture conditions. (A) In cultures containing normal Mg2+/Ca2+ levels (0.8 mM), treatment with KA (1 mM) led to significant decreases in RGCs compared with control cultures, and inclusion of CNQX completely blocked toxicity, whereas MK-801 had no effect. **P < 0.01** above KA and ***P < 0.001** above KA/NMDA: significantly different from untreated control cultures; \( P < 0.05 \) above KA+NMDA/MK-801 and **P < 0.01** above KA/CNQX: comparison with corresponding treatments without antagonist. (B) When similar treatments were performed in low-Mg2+/high-Ca2+ medium, KA, NMDA, and KA+NMDA led to greater cell losses than identical treatments in normal medium. Values are normalized with respect to control cultures in low-Mg2+/high-Ca2+ medium, which contained 10% to 15% less large RGCs compared with control cultures in normal medium. x-axis: concentrations of KA and NMDA (in millimolar) added to cultures. \( P < 0.05 \), **P < 0.01, and ***P < 0.001** above KA (0.1–1 mM) and NMDA (0.1–1 mM): significantly different from control cultures. \( P < 0.05 \) above KA (0.1–1 mM)/NMDA (1 mM): significantly different from KA (0.1–1 mM) alone.

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**Table 1.** Toxic properties of KA and NMDA in retinal cultures from adult and neonatal rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total RGCs Survival (%)</th>
<th>Small RGCs Survival (%)</th>
<th>ACs Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KA (1 mM)</td>
<td>37.5</td>
<td>27.3</td>
<td>95.2</td>
</tr>
<tr>
<td>KA+NMDA (1 mM)</td>
<td>8.5</td>
<td>9.2</td>
<td>95.2</td>
</tr>
<tr>
<td>MK-801 (10 ( \mu )M)</td>
<td>21.3</td>
<td>20.4</td>
<td>95.2</td>
</tr>
<tr>
<td>MK-801 (10 ( \mu )M)+CNQX (50 ( \mu )M)</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

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**Figure 5.** Effects of KA and NMDA on RGC neurite morphology in vitro. (A) Phase-contrast images of RGC neurites in control cultures. (B) Effects of KA and NMDA on neurite morphology in adult rat retina. KA (1 mM) was added to cultures, and neurite length and degree of branching were severely reduced (Fig. 5B). Inclusion of MK-801 partially restored the normal aspect of neurites in residual RGCs treated with 1 mM Glu (Fig. 5C), whereas addition of CNQX did not rescue normal neurite outgrowth in Glu-treated RGCs (Fig. 5D). Application of MK-801 or CNQX alone did not modify neurite morphology in comparison with control cultures. Addition of KA alone had no visible effect on surviving RGC neurites (Fig. 5E; although when applied in low-Mg/high-Ca medium KA also induced neurite damage in surviving RGCs; data not shown), whereas KA+NMDA resulted in neurite damage (Fig. 5F).
neously to Glu (1 mM). CNQX greatly improved RGC survival, whereas MK-801 had no effect (Fig. 6B). As expected, inclusion of CNQX prevented much of the loss induced by KA, whereas MK-801 was without effect (data not shown). Use of low-Mg/high-Ca medium for young rat cultures led to extensive neuronal, and especially RGC, death, even in the absence of added EAAs. It was not possible to examine the effects of EAAs under these conditions.

The Influence of Treatment’s Timing on EAA Neurotoxicity. We observed a strong dependency of EAA-induced RGC death on timing of treatments in the various experimental models. In both adult rat and pig cultures, whereas Glu addition at 0 days invariably produced toxicity, delay of treatment until after 3 or 5 days resulted in a complete absence of effect (Figs. 7A, 7B, 7C). Adult rat RGCs and pig large RGCs also become refractory to KA and KA+NM2A within 3 days (Figs. 7A, 7C), whereas pig small RGCs still exhibited marked KA toxicity even after 5 days (Fig. 7B). In contrast, neonatal rat retinal cultures exposed to Glu, KA, or KA+NM2A (each 1 mM) after 3 and 5 days in vitro exhibited increased RGC neurotoxicity compared with toxicity at 0 days: 47% at 0 days to more than 90% at 5 days with Glu, 78% at 0 days to more than 99% at 5 days with KA, and 83% at 0 days to more than 99% at 5 days with KA+NM2A (Fig. 7D). KA toxicity in ACs persisted throughout the culture period, ranging from approximately 90% loss at the 0-day treatment to 50% loss at the 3- and 5-day treatments.

Comparative EAA Toxicity among Culture Models. We compared maximal Glu neurotoxicity on total RGCs between neonatal rat, adult rat, and adult pig retinas (pooled data from large and small RGCs) in vitro. A hierarchy of vulnerability was observed in the order neonatal rat RGCs > adult rat RGCs >
TABLE 1. Deleterious Effects of KA and KA/NMDA Treatment on Adult Pig Small RGC and AC Survival In Vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Small RGCs</th>
<th>ACs</th>
</tr>
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<tbody>
<tr>
<td>KA*</td>
<td>11.9 ± 4.9</td>
<td>11.7 ± 5.7</td>
</tr>
<tr>
<td>KA/CNQX†</td>
<td>49.1 ± 4.8</td>
<td>61.5 ± 16.0</td>
</tr>
<tr>
<td>KA/MK801</td>
<td>6.9 ± 3.0</td>
<td>ND</td>
</tr>
<tr>
<td>KA/CNQX/MK801†</td>
<td>59.6 ± 9.1</td>
<td>ND</td>
</tr>
<tr>
<td>KA/NMDA*</td>
<td>10.8 ± 4.5</td>
<td>ND</td>
</tr>
<tr>
<td>KA/NMDA/CNQX†</td>
<td>24.9 ± 5.4</td>
<td>ND</td>
</tr>
<tr>
<td>KA/NMDA/MK801†</td>
<td>10.5 ± 4.2</td>
<td>ND</td>
</tr>
<tr>
<td>KA/NMDA/CNQX/MK801†</td>
<td>27.5 ± 8.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

Addition of either KA alone or in combination with NMDA led to large losses in both small RGCs and ACs, expressed as percentage of control (100%). Addition of CNQX partially prevented cell losses from KA and KA/NMDA, whereas MK-801 had no effect. ND, not determined.

* P < 0.001 relative to untreated control cultures.
† P < 0.001 relative to EAA alone.

adult pig RGCs. Neonatal rat RGCs were by far the most vulnerable (>90% dead with treatment at 5 days). Adult rat RGCs showed 40% loss, and adult pig RGCs showed only 17% loss overall (the latter two with treatment at 0 days). Similar comparisons for KA also showed neonatal RGCs to be the most vulnerable (>99% loss when treated at 5 days) compared with adult rat and pig (60% and 70% loss, respectively, when treated at 0 days). We also compared the extent of neuroprotection afforded by NMDA or non-NMDAR blockers. In all cases KA-induced toxicity was greatly reduced by CNQX, but not MK-801, as expected. Whereas rat RGCs were significantly protected from Glu-induced toxicity only by CNQX, showing no NMDA sensitivity at all, adult pig RGCs were significantly protected from Glu-induced toxicity by both CNQX and MK-801.

Glutamate Responses of Large RGCs in Adult Pig Retina In Vitro

To partially characterize the current responses of large RGCs to Glu application, representative cells were recorded with the whole-cell patch–clamp technique after 4 to 5 days in vitro. Recorded cells were identified as large RGCs by their responses to voltage steps (Fig. 8A). When RGCs were held at a potential of ~70 mV in the absence of external Mg^2+, puff application of Glu (1 mM) generated an inward current. This response was mostly blocked (64% ± 12%, n = 3) by bath application of CNQX (100 μM) and suppressed by 91% ± 4% (n = 3) by application of CNQX + APV (100 μM; Fig. 8B). Full Glu-evoked responses were recordable after washout of inhibitors. Similar measurements from cultured RGCs obtained from postnatal rats produced comparable results (data not shown).

DISCUSSION

The present study reports the use of a novel adult large-animal culture model to demonstrate that increased Glu concentrations can reproduce, at least on a superficial level, the gradual toxic lesions produced by reduced blood flow, and/or trophic factor deprivation. In remodeling of the optic nerve head and component astrocytes is thought to affect RGC axons detrimentally, resulting ultimately in cell death. Problems in immune regulation have also been cited as possible causes. Glaucoma selectively involves RGC death and preferentially affects large RGCs. Increased vitreal Glu concentrations
correlate with the glaucomatous state\textsuperscript{12,14} and if excitotoxicity is causally implicated, it is expected that experimental manipulation of Glu exposure should mimic such features.

Intraocular injections of Glu or NMDA lead to selective cell loss in the GCL\textsuperscript{15-26} although lesions in the INL\textsuperscript{27} and even in the PR layer\textsuperscript{46} have also been reported. Previous studies on elevation of EAA levels in vitro either report more generalized neuronal death\textsuperscript{28-30} or do not address the issue of selectivity.\textsuperscript{47-49} In pig retinas, large RGC excitotoxicity was meditated by both non-NMDAR and NMDAR, whereas rats showed an entirely non-NMDAR-mediated RGC death, especially prevalent in neonatal retinas, which are commonly used in this field. In contrast, KA was only moderately toxic for pig large RGCs but was highly toxic for small RGCs and ACs, as previously published regarding ACs.\textsuperscript{50}

There are several advantages to using adult instead of embryonic or young postnatal models in vitro to conduct pathologic studies. One of the most important in studying diseases of the adult central nervous system (CNS) is the maturation state of different cell types. Developmental changes in GluR expression levels and/or subunit composition are widely observed within the CNS,\textsuperscript{51} with, for example, changes in the relative levels of NR2A and NR2B\textsuperscript{52} and appearance of NR2C.\textsuperscript{53} The rank order of excitotoxic potency differs between immature and adult brain.\textsuperscript{54} KA has generally been shown to be more toxic for adult than for embryonic CNS tissue,\textsuperscript{55} whereas NMDA treatment is more deleterious for immature than for mature brain tissue,\textsuperscript{56} and neuroprotection through blocking NMDAR activation also changes with development.\textsuperscript{57} Within the RGCs, pharmacologic responses of immature cells differ from those of adult neurons,\textsuperscript{58-60} making extrapolation of data obtained from such models to adult human tissue difficult. It has recently been shown by single-cell polymerase chain reaction that juvenile rat RGCs exhibit a different profile of GluR mRNA expression than adult rat RGCs.\textsuperscript{61}

It is difficult to align precisely our data with those obtained from previous studies, owing to numerous differences in experimental design (species, age, culture conditions, identification of RGC loss, and length of treatment). Our data suggest that neonatal RGCs are more sensitive in general to experimental treatments than are adult RGCs, which parallels the more generalized cell loss observed in other studies.\textsuperscript{28-30} Such differences may be partially due to maturational changes in glial clearance of Glu,\textsuperscript{57} although this cannot explain the differences seen with KA. Previous in vitro studies using newborn rat RGCs have shown exactly opposite results. Otori et al.\textsuperscript{62} demonstrated that Glu-induced cell death of young rat RGCs is essentially mediated by AMPA/KAR, which we also observed in the present study. On the contrary, Pang et al.\textsuperscript{49} demonstrated an entirely NMDAR-mediated response to applied Glu in cultured neonatal rat RGCs. Neither of these studies apparently involved trials under conditions in which voltage-sensitive Mg blockade of NMDAR should have been lifted, and their results are hard to reconcile with the present data. In our studies, no NMDAR-mediated death was observed in young or adult rat RGCs in vitro, whereas NMDA clearly affects rat RGCs in vivo.\textsuperscript{26}

Obvious caveats that have to be mentioned in the interpretation of our data are the validity of the immunocytochemical-based method of detection used and the possible selection of distinct RGC subpopulations in the different models. Many previous studies have used backfilling techniques to selectively label RGCs, but such an approach was not feasible in enucleated pig eyes. We chose an immunocytochemical assay that
rat RGCs to subgroups, although overall numbers of RGCs in this group is not yet certain. We were unable to reliably assign markers such as anti-syntaxin antibodies. Finally, protein kinase C and gave more reproducible results than alternative AC markers as robust marker of all RGCs and of many ACs within pig retina in vivo and in vitro.31,32,34

reliable markers of BP and PR cells, respectively, in the pig retina in vivo and in vitro.31,32,34 permitted sampling of all neuronal populations for which specific antibodies are available. Anti-NF antibodies clearly identified RGCs and horizontal cells in our cultures, the latter being discarded from cell counts through their distinctive morphologies and absence of PGP9.5 immunoreactivity. Small RGCs were observed only in pig cultures and corresponded to a population of small RGCs observed in pig retina in vivo (Luo et al, manuscript in preparation). It should be cautioned that the pharmacologic properties of small RGCs were indistinguishable from those of ACs, and because NF expression has been reported in a minor subset of ACs,53 definitive identification of this group is not yet certain. We were unable to reliably assign rat RGCs to subgroups, although overall numbers of RGCs were approximately equal between pig and neonatal rat when adjusted for seeding density (~1000 per coverslip; viability of adult rat RGCs was less good, ~450 per coverslip). Furthermore, electrophysiological characteristics of large RGCs resembled those published for neonatal rat RGCs.59,63 PGP9.556 was a robust marker of all RGCs and of many ACs within pig retina and gave more reproducible results than alternative AC markers such as anti-syntaxin antibodies. Finally, protein kinase Ca and arrestin antibodies have been shown by us to constitute reliable markers of BP and PR cells, respectively, in the pig retina in vivo and in vitro.31,52,54

We observed that timing of treatment in culture was a very important parameter in RGC toxicity. The effects Glu and NMDA decreased rapidly with time in vitro in adult models and increased rapidly in the neonatal model. KA sensitivity, however, was less affected, irrespective of the model or cell type. As mentioned earlier, changes in Glu efficacy may be related to glial detoxification, which could be operative in adult retinal cultures.37 Alternatively, these observations may indicate changing behavior of NMDAR and AMPA/KAR within RGCs under in vitro conditions. NMDAR or downstream elements including post-synaptic density 95 may depend on mechanisms such as activity- or target-derived factors to maintain them, whereas AMPA/KAR are relatively stable (or in the case of neonatal retina can occur in the absence of extrinsic factors).

In conclusion, Glu-induced toxicity of adult pig large RGCs in vitro resembles some aspects of that reported in glaucoma in both humans43,45,62 and experimental animal models.5,14 Furthermore, the neurite damage observed in residual large RGCs resembles dendritic degeneration, one of the earliest morphological signs of glaucoma,65 and suggests that many surviving cells are adversely affected. These data thus support a role for Glu-induced excitotoxicity in glaucomatous RGC death, although factors such as ischemia or trophic factor deprivation may also be important. The most straightforward explanation is that imbalances in Glu metabolism are only lethal to large RGCs because, alone among retinal neurons, they possess sufficient NMDAR (or NMDAR of a particular subunit composition) to trigger cell death under such conditions. Although previous studies have failed to demonstrate GluR subunit differences correlating with neuronal susceptibility to experimental glaucoma in vivo,6 our data are most easily explained by such a hypothesis. This system therefore represents a convenient alternative to in vivo large animal models and should be of value in exploring the molecular mechanisms underlying adult RGC death and in facilitating the screening of potential neuroprotective agents.

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