Assessment of glutamate loss from the ganglion cell layer of young DBA/2J mice with glaucoma

Heather C. Low, DVM; Juliet R. Gionfriddo, DVM, MS; James E. Madl, DVM, PhD

Objective—To determine whether glutamate contents are decreased in the ganglion cell layer (GCL) of retinas of DBA/2J mice with glaucoma, compared with unaffected control mice.

Sample Population—20 eyes from DBA/2J mice (9-week-old mice [n = 8] and 4- to 6-month-old [4] mice) and 17 eyes from control CD-1 (7) and C57BL/10 (10) mice of similar age.

Procedure—After euthanasia, the eyes were rapidly dissected and fixed. Serial 0.5-μm sections were prepared from eyecups and stained with toluidine blue (to identify damaged cells) or immunogold (to localize glutamate). Microscopic images were captured digitally for comparison; immunostaining densities were assessed via special software.

Results—In the GCL of control mice, few cells appeared damaged; large amounts of glutamate were detected in 83 ± 8.3% of cells. In DBA/2J mice > 9 weeks of age, damaged neurons were observed in retinal sections; the level of glutamate immunoreactivity was high in a few cells near areas of damage (13 ± 3.2%) and in many cells in less-damaged regions of the same sections (82 ± 4.2%). Many neurons with low amounts of glutamate in damaged regions did not appear damaged histologically.

Conclusions and Clinical Relevance—In retinas of young DBA/2J mice, damaged and undamaged GCL cells had decreased levels of immunostaining for glutamate, compared with less-damaged adjacent regions or retinas from control mice. The loss of neuronal glutamate in damaged retinal regions suggests that glutamate is contributing to early retinal damage prior to changes in intraocular pressure. (Am J Vet Res 2006;67:302–309)

Glaucoma is characterized by a progressive loss of retinal ganglion cells that is often associated with increased intraocular pressure and decreased retinal blood flow. Several pathologic mechanisms may contribute to glaucomatous damage of retinal ganglion cells, including high extracellular concentrations of glutamate, ischemia, and lack of growth factors. As laboratory animals, inbred DBA mice have been used in thousands of studies of immune responses, drug metabolism, epilepsy, and other processes.

Sample Population—20 eyes from DBA/2J mice (9-week-old mice [n = 8] and 4- to 6-month-old [4] mice) and 17 eyes from control CD-1 (7) and C57BL/10 (10) mice of similar age.

Objective—To determine whether glutamate contents are decreased in the ganglion cell layer (GCL) of retinas of DBA/2J mice with glaucoma, compared with unaffected control mice.

Sample Population—20 eyes from DBA/2J mice (9-week-old mice [n = 8] and 4- to 6-month-old [4] mice) and 17 eyes from control CD-1 (7) and C57BL/10 (10) mice of similar age.

Procedure—After euthanasia, the eyes were rapidly dissected and fixed. Serial 0.5-μm sections were prepared from eyecups and stained with toluidine blue (to identify damaged cells) or immunogold (to localize glutamate). Microscopic images were captured digitally for comparison; immunostaining densities were assessed via special software.

Results—In the GCL of control mice, few cells appeared damaged; large amounts of glutamate were detected in 83 ± 8.3% of cells. In DBA/2J mice > 9 weeks of age, damaged neurons were observed in retinal sections; the level of glutamate immunoreactivity was high in a few cells near areas of damage (13 ± 3.2%) and in many cells in less-damaged regions of the same sections (82 ± 4.2%). Many neurons with low amounts of glutamate in damaged regions did not appear damaged histologically.

Conclusions and Clinical Relevance—In retinas of young DBA/2J mice, damaged and undamaged GCL cells had decreased levels of immunostaining for glutamate, compared with less-damaged adjacent regions or retinas from control mice. The loss of neuronal glutamate in damaged retinal regions suggests that glutamate is contributing to early retinal damage prior to changes in intraocular pressure. (Am J Vet Res 2006;67:302–309)

Glaucoma is characterized by visual field defects and may be the result of nonuniform loss of ganglion cells and their axons. We hypothesized that such regional damage may be associated with selective release and redistribution of glutamate in the retinal regions with the most severe damage. Recently, our group reported focal ischemialike changes in the distribution of glutamate in retinas of dogs with primary glaucoma, compared with findings in dogs with unaffected eyes. These changes included loss of glutamate from neuronal cell bodies and dendrites and an accumulation of glutamate in Müller cells. Changes in glutamate concentration were greatest in damaged regions of the retina, which was consistent with increased glutamate release and excitotoxicosis within these damaged regions. However, primary glaucoma in dogs results in loss of cells from all neuroretinal layers within days of clinical onset. In our previous study, a substantial depletion of ganglion cells had already occurred in the retinas of dogs with primary glaucoma by the time the disease was diagnosed and samples were obtained. This prevented us from determining whether glutamate was lost from increased intraocular pressure has been identified. These changes suggest that these mice may be useful as a model for pigment-related glaucoma that may develop in several breeds of dog and perhaps for primary glaucoma in dogs that is often classified as angle-closure or narrow-angle glaucoma.

Taken together, the results of several types of studies support the concept that high extracellular concentration of glutamate is toxic to retinal neurons, increases in glutamate concentration in the vitreous body have been reported in primates with primary open-angle glaucoma, dogs with primary glaucoma, quail with hereditary angle-closure glaucoma, and AKXD-28/Ty mice with hereditary pigmentary glaucoma. The NMDA antagonist memantine has been shown to decrease ganglion cell death in DBA/2J mice and primates, indicating that activation of glutamate receptors contributes to cell death. Factors that may lead to high extracellular concentration of glutamate associated with some types of glaucoma have also been reported, including decreased blood flow (which may increase the release of glutamate) and decreased expression of glutamate transporters (which may lead to decreased uptake of glutamate into cells).

Glaucoma is characterized by visual field defects and may be the result of nonuniform loss of ganglion cells and their axons. We hypothesized that such regional damage may be associated with selective release and redistribution of glutamate in the retinal regions with the most severe damage. Recently, our group reported focal ischemialike changes in the distribution of glutamate in retinas of dogs with primary glaucoma, compared with findings in dogs with unaffected eyes. These changes included loss of glutamate from neuronal cell bodies and dendrites and an accumulation of glutamate in Müller cells. Changes in glutamate concentration were greatest in damaged regions of the retina, which was consistent with increased glutamate release and excitotoxicosis within these damaged regions. However, primary glaucoma in dogs results in loss of cells from all neuroretinal layers within days of clinical onset. In our previous study, a substantial depletion of ganglion cells had already occurred in the retinas of dogs with primary glaucoma by the time the disease was diagnosed and samples were obtained. This prevented us from determining whether glutamate was lost from increased intraocular pressure has been identified. These changes suggest that these mice may be useful as a model for pigment-related glaucoma that may develop in several breeds of dog and perhaps for primary glaucoma in dogs that is often classified as angle-closure or narrow-angle glaucoma.

Taken together, the results of several types of studies support the concept that high extracellular concentration of glutamate is toxic to retinal neurons, increases in glutamate concentration in the vitreous body have been reported in primates with primary open-angle glaucoma, dogs with primary glaucoma, quail with hereditary angle-closure glaucoma, and AKXD-28/Ty mice with hereditary pigmentary glaucoma. The NMDA antagonist memantine has been shown to decrease ganglion cell death in DBA/2J mice and primates, indicating that activation of glutamate receptors contributes to cell death. Factors that may lead to high extracellular concentration of glutamate associated with some types of glaucoma have also been reported, including decreased blood flow (which may increase the release of glutamate) and decreased expression of glutamate transporters (which may lead to decreased uptake of glutamate into cells).

Glaucoma is characterized by visual field defects and may be the result of nonuniform loss of ganglion cells and their axons. We hypothesized that such regional damage may be associated with selective release and redistribution of glutamate in the retinal regions with the most severe damage. Recently, our group reported focal ischemialike changes in the distribution of glutamate in retinas of dogs with primary glaucoma, compared with findings in dogs with unaffected eyes. These changes included loss of glutamate from neuronal cell bodies and dendrites and an accumulation of glutamate in Müller cells. Changes in glutamate concentration were greatest in damaged regions of the retina, which was consistent with increased glutamate release and excitotoxicosis within these damaged regions. However, primary glaucoma in dogs results in loss of cells from all neuroretinal layers within days of clinical onset. In our previous study, a substantial depletion of ganglion cells had already occurred in the retinas of dogs with primary glaucoma by the time the disease was diagnosed and samples were obtained. This prevented us from determining whether glutamate was lost from increased intraocular pressure has been identified. These changes suggest that these mice may be useful as a model for pigment-related glaucoma that may develop in several breeds of dog and perhaps for primary glaucoma in dogs that is often classified as angle-closure or narrow-angle glaucoma.

Taken together, the results of several types of studies support the concept that high extracellular concentration of glutamate is toxic to retinal neurons, increases in glutamate concentration in the vitreous body have been reported in primates with primary open-angle glaucoma, dogs with primary glaucoma, quail with hereditary angle-closure glaucoma, and AKXD-28/Ty mice with hereditary pigmentary glaucoma. The NMDA antagonist memantine has been shown to decrease ganglion cell death in DBA/2J mice and primates, indicating that activation of glutamate receptors contributes to cell death. Factors that may lead to high extracellular concentration of glutamate associated with some types of glaucoma have also been reported, including decreased blood flow (which may increase the release of glutamate) and decreased expression of glutamate transporters (which may lead to decreased uptake of glutamate into cells).

Glaucoma is characterized by visual field defects and may be the result of nonuniform loss of ganglion cells and their axons. We hypothesized that such regional damage may be associated with selective release and redistribution of glutamate in the retinal regions with the most severe damage. Recently, our group reported focal ischemialike changes in the distribution of glutamate in retinas of dogs with primary glaucoma, compared with findings in dogs with unaffected eyes. These changes included loss of glutamate from neuronal cell bodies and dendrites and an accumulation of glutamate in Müller cells. Changes in glutamate concentration were greatest in damaged regions of the retina, which was consistent with increased glutamate release and excitotoxicosis within these damaged regions. However, primary glaucoma in dogs results in loss of cells from all neuroretinal layers within days of clinical onset. In our previous study, a substantial depletion of ganglion cells had already occurred in the retinas of dogs with primary glaucoma by the time the disease was diagnosed and samples were obtained. This prevented us from determining whether glutamate was lost from increased intraocular pressure has been identified. These changes suggest that these mice may be useful as a model for pigment-related glaucoma that may develop in several breeds of dog and perhaps for primary glaucoma in dogs that is often classified as angle-closure or narrow-angle glaucoma.
damaged ganglion cells during the early stages of the disease. The acute mechanisms of pathogenesis of glaucoma in dogs may not resemble those seen in other species or types of glaucoma that may take years to reach their end stage. Therefore, we undertook studies of early ganglion cell death in DBA/2J mice. DBA/2J mice have 2 genetic defects\textsuperscript{10,11} that affect melanosomal proteins. These mutations cause the mice to develop anterior segment defects by 6 months of age.\textsuperscript{20,21} These changes, including pigment dispersion and anterior and posterior synechia, lead to angle closure and significantly increased intraocular pressure in most mice by 6 to 9 months of age.\textsuperscript{20,21} The loss of ganglion cells at 9 months of age in DBA/2J mice may be decreased by the glutamate antagonist memantine.\textsuperscript{18} However, in a recent study,\textsuperscript{20} it was determined that some ganglion cells of DBA/2J mice may die as early as 3 months of age through an apoptotic mechanism, before increases in intraocular pressure are detected. The purpose of the study reported here was to determine whether glutamate loss occurs from the ganglion cell layer of young DBA/2J mice with glaucoma. The intent was to examine whether the ischemialike losses of neuronal glutamate detected in damaged retinas of dogs with primary glaucoma also occur in DBA/2J mice (which are used as a laboratory animal model of pigmentary glaucoma).

Materials and Methods

All mice used in the study were treated in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the Colorado State University Animal Care and Use Committee. The DBA/2J mice,\textsuperscript{7} control CD-1 (ICR) nonpigmented mice,\textsuperscript{7} and control C57BL/6j (pigmented) mice\textsuperscript{7} used in the study were housed at the laboratory animal facility of Colorado State University; they were maintained on an alternating cycle of 12 hours of light and 12 hours of dark and fed a standard mouse chow.

Twenty eyes from DBA/2J mice 9 weeks (n = 8) and 4 (4), 6 (4), and 12 months of age (4) and 17 eyes from control CD-1 (7) and C57BL/6j (10) mice of similar age were used. Both eyes of each mouse were utilized. Mice were anesthetized with halothane in a closed chamber and euthanatized by opening the thoracic cavity to air as a simple and effective means of inducing death. Eyes were rapidly dissected from the head of each mouse, and retinas were rapidly fixed with 4% paraformaldehyde and 0.3% glutaraldehyde in 100mM phosphate buffer. Corneas were first punctured with a pointed scalpel blade, and the anterior segment was flushed with fixative by use of a 27-gauge needle.\textsuperscript{12} After 5 minutes, the eyes were hemisectioned along the ora serrata and the cornea, lens, and vitreous body were removed.\textsuperscript{18} Eyecups were quartered and immersion fixed for approximately 16 hours; quarters were then processed and embedded in epoxy resin, as previously described.\textsuperscript{31} Serial 0.5-µm sections were obtained from each eyecup quarter. Sections were stained with toluidine blue (for detection of morphologic signs of damage) or with an immunogold solution (to determine the distribution of glutamate and GFAP). The silver-intensified immunogold technique used was essentially the method that Perlman et al\textsuperscript{32} used to localize amino acids in ischemic retinas obtained from rats. Sections were etched in sodium ethoxide diluted 1:5 in ethanol for 20 minutes and rinsed in ethanol and then in water. Sections were blocked with 5% goat serum in PBS solution (pH, 7.4; 0.05M phosphate) for 15 minutes and rinsed in PBS solution. Sections were then incubated in rabbit primary antiseria against glutamate that was diluted 1:4,000 or in GFAP\textsuperscript{33} that was diluted 1:100 in PBS solution. Sections were rinsed in PBS solution and incubated for 4 hours at 23°C in ultrasmall gold-labeled secondary antibodies\textsuperscript{7} that were diluted 1:40 in PBS solution containing 1% bovine serum albumin. After rinsing in PBS solution for 45 minutes followed by final brief rinses in distilled water, silver intensification was performed for 6 minutes by use of freshly prepared solution (8.4mM silver nitrate, 0.05mM hydroquinone, and 0.13M citrate buffer [pH, 4.85]). In absorption control specimens (in which the antibodies were preincubated with glutamate conjugated to bovine serum albumin), there was almost no staining of sections (data not shown).

Image capture and analysis—Only retinal regions free from the trauma of dissection and quartering were used for analysis of glutamate distribution. Retinal regions that were distorted because of folding or were near the edges of scissor cuts were associated with a decrease of staining for glutamate in all layers of the retina, compared with retinal regions without these artifacts; regions that had homogeneous loss of staining for glutamate were excluded from the study.

Digital images (130 X 100 µm) were captured by use of a microscope\textsuperscript{4} with special software.\textsuperscript{5} For quantification of the staining density, images were analyzed with image-analysis software\textsuperscript{4} by use of methods similar to those previously described.\textsuperscript{33}
Immunostaining densities were measured after inversion accomplished by use of the software’s inversion function. For measurements of immunostaining density, the background staining density of regions of blank plastic was subtracted from retinal staining levels.

To determine the proportion of cells of the ganglion cell layer with high amounts of glutamate, the number of cells with a high density of immunostaining for glutamate in a region was compared with the total number of cells with visible nuclei that were at least 4 µm in diameter in that region of the ganglion cell layer. Cell numbers were determined on a neighboring section stained with toluidine blue. A cell was considered to have a high density of staining for glutamate if more than half of its profile had a staining density > 90 (gray scale), as determined by use of the software’s threshold function.

To determine whether glutamate immunostaining was reproducible, repeat measurements of the same region were performed. Six 50 × 100-µm regions of the INL of retinas from DBA/2J mice (from 6 quarters from 3 eyes from 3 mice) were classified by an observer as having homogenously high-, medium-, and low-density staining for glutamate. Immunostaining of serial sections of these regions was repeated the next day. The density of glutamate staining (gray scale) of each region was compared with the density of staining obtained the following day.

Statistical analysis—Data were analyzed to detect significant differences by use of t tests. Statistical software was used for the calculations. Variability was expressed as the SEM unless otherwise specified. A value of P < 0.05 was considered significant.

Results

Assessment of the ganglion cell layer of control and DBA/2J mice—Morphologic signs of damage were observed microscopically in cells of the ganglion cell layers of eyes obtained from DBA/2J mice. In 9-week-old DBA/2J mice, the retinal sections stained with toluidine blue contained cells with darkly stained, irregularly shaped nuclei and dark cytoplasm that was frequently foamy in appearance (Figure 1). In control ICR (age, 9 weeks) and C57BL/6J (age, 8 weeks) mice, similar darkly stained cells were observed rarely (less than or equal to one quarter of each retina). In sections of 27 eyecup quarters from four 9-week-old DBA/2J mice, each contained at least 1 damaged cell and 8 regions with multiple damaged cells in < 100 µm of retinal length that were identified as damaged and used for additional studies. In control ICR mice, no regions with multiple damaged cells were found in 9 eyecup quarters from 6 eyes of three 9-week-old mice or 7 eyecup quarters from 2 eyes of a 4-month-old mouse. In control C57BL/6J mice, 1 of 11 eyecup quarters from 4 eyes of two 8-week-old mice had a region with 3 damaged cells. Other signs of retinal damage observed in 9-week-old DBA/2J mice included lightly stained, swollen structures in the nerve fiber layer that were consistent with degenerating axons. Definitive identifi-
cation of these structures would require electron microscopic examination in future studies.

Six- and 12-month-old DBA/2J mice also had ganglion cell layer cells with similar signs of damage, but such cells were detected less frequently than they were in retinal specimens from 9-week-old mice (Figure 2). Compared with findings in sections from eyecups obtained from the younger mice, a greater number of swollen cells and processes appeared to be present in the ganglion cell layer and INL of retinas of the older DBA/2J mice, but these differences were not quantified. By 6 months of age and continuing through 12 months of age, immunostaining for GFAP was increased in DBA/2J mice, compared with that detected in both C57/BL6J and ICR control mice, which suggested that reactive glia may be present in retinas of those DBA/2J mice.

Assessment of glutamate immunostaining density in ganglion cell layer regions of control and DBA/2J mice—The ganglion cell layer of retinas obtained from control ICR mice contained a high proportion of cells with dense immunogold staining for glutamate (Figure 3). The mean ± SEM staining density of Müller cell bodies of the INL was low (57 ± 6.6 [gray scale]) in sections from 6 eyecup quarters from four 9-week-old ICR mice. The staining density of most cells of the ganglion cell layer in sections from 6 eyecup quarters from 4 young DBA/2J mice ranged from 100 to 180 (gray scale), which was consistent with the different types of ganglion cells and displaced amacrine cells observed in the ganglion cell layer of these control mice. In 83 ± 8.3% of the cells of the ganglion cell layer in regions quantified. However, in some regions of retinas from 9-week-old control ICR mice and 9-
week-old DBA/2J mice, glutamate immunoreactivity was decreased throughout all layers of the retina, compared with undamaged regions. Often, these retinal regions with low glutamate immunoreactivity were near obvious folds or cut edges of the specimen, which led us to speculate that these areas of uniform loss of glutamate immunoreactivity were artifacts resulting from dissection. To exclude these regions of presumptive artifact from the study, all regions of retinas from control and DBA/2J mice that were used for data collection had cells with high levels of glutamate immunoreactivity in the INL (Table 1). The control ICR mice are nonpigmented mice that are not likely to develop problems with pigment dispersion, such as those associated with DBA/2J mice. In addition, densities of immunogold staining for glutamate were measured in a pigmented strain of mice (C57/BL6J) that is commonly used as a control group. Control C57/BL6J mice also had a high proportion of cells with dense immunogold staining for glutamate (94 ± 2.4%) in 8 regions of 8 quarters of 4 eyes from 2 mice.

To minimize the effect of staining variation, the glutamate distribution in damaged and nearby less-damaged retinal regions of DBA/2J mice was examined in the same sections. In 9-week-old DBA/2J mice, undamaged regions of the ganglion cell layer that had a large proportion of cells with high glutamate staining density were identified (Figure 3). The percentage of cells in these regions with high densities of glutamate immunostaining (82 ± 4.2%) was not significantly (P > 0.6) different from the percentage of ganglion cell layer cells with high densities of glutamate immunostaining in 9-week-old control ICR mice (83 ± 8.3%). However, in 8 regions (from 8 quarters of 6 eyes) that had a high frequency of damaged cells in DBA/2J mice, the proportion of ganglion cell layer cells with high densities of glutamate immunostaining was significantly (P < 0.01) decreased to 13 ± 3.2%. Almost all of the cells with morphologic signs of damage had low amounts of glutamate, and many of the neighboring cells that had no obvious signs of damage also had low densities of immunostaining for glutamate (Figure 4).

Table 1—Minimal and maximal densities (± SEM) of immunogold staining of glutamate in cells of the INL and ganglion cell layer in 6 damaged and 6 neighboring undamaged regions of the retinas from 6 eyes of 3 DBA/2J mice. Cells with the highest and lowest levels of glutamate immunoreactivity were selected for measurement in each region.

<table>
<thead>
<tr>
<th>Location</th>
<th>Cell classification</th>
<th>Damaged retinal regions</th>
<th>Undamaged retinal regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>INL</td>
<td>Heavily stained</td>
<td>129 ± 6.0</td>
<td>128 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>Lightly stained</td>
<td>40 ± 5.1</td>
<td>32 ± 3.0</td>
</tr>
<tr>
<td>Ganglion cell layer</td>
<td>Heavily stained</td>
<td>119 ± 6.6*</td>
<td>144 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>Lightly stained</td>
<td>20 ± 3.7†</td>
<td>56 ± 6.5</td>
</tr>
</tbody>
</table>

*Staining density (gray scale) of cells after background staining (assessed by use of blank plastic) was subtracted. Values were determined from a minimum of 17 cells in 6 sections, except for lightly stained ganglion cell layer cells in undamaged areas for which only 6 cells with low-density staining were measured.†Value significantly (P < 0.01) different from that of similarly stained cells in undamaged retinal regions.
obtained the following day. The mean staining density was highly correlated ($R = 0.91$), indicating that staining densities were similar when repeated. Secondly, measurements of maximal and minimal staining densities of INL cells in the damaged and undamaged retinal regions were performed. No significant difference in the staining density of heavily labeled cells of the INL in damaged and less-damaged regions was detected, indicating that all immunostaining reagents had access to the INL of damaged retinal regions in sections (Table 1). Similarly, no significant difference in the staining density of the minimally labeled INL cells in the damaged and less-damaged regions was detected. However, despite the similarities of maximal and minimal staining densities of INL cells in damaged and less-damaged regions, the percentages of cells with high- and low-density staining often appeared quite different, but further studies would be required to quantify those differences in the INL. In contrast to findings in the INL, the most heavily stained cells and the least heavily stained cells in damaged regions of the ganglion cell layer had significantly lower staining densities than the corresponding cells in undamaged regions of the ganglion cell layer, which was consistent with glutamate depletion in many cell types in the damaged regions.

To determine whether selective loss of cells that contained high concentrations of glutamate could account for glutamate depletion in damaged regions of the ganglion cell layer, the number of cells per millimeter of retina was counted in the damaged regions and in nearby apparently undamaged regions of the retina in 9-week-old DBA/2J mice. In damaged regions, there were $129 \pm 6$ ganglion cell layer cells/mm of retina, and in undamaged regions, there were $129 \pm 6$ ganglion cell layer cells/mm of retina. This small difference in the number of cells between damaged and undamaged retinal regions was neither significant ($P > 0.4$) nor sufficient to account for the greatly decreased number of cells with high glutamate immunoreactivity in damaged regions of retinas in 9-week-old DBA/2J mice. In DBA/2J mice that were 12 months of age, there were $47 \pm 3.4$ ganglion cell layer cells/mm of retina in regions with low glutamate immunoreactivity; this value was significantly ($P < 0.01$) less than the value in 9-week-old DBA/2J mice. This decrease in overall cell number in the older mice suggests that large numbers of cells died after 9 weeks of age.

**Discussion**

Glaucoma is characterized by a nonhomogeneous loss of retinal ganglion cells that results in visual field defects. In DBA/2J mice with glaucoma, neuronal death is decreased by the NMDA antagonist, memantine, which suggests that extracellular glutamate may contribute to retinal damage. We hypothesized that selective release of glutamate in damaged retinal regions may mediate cell death. In rats, glutamate release as a result of ischemia and other metabolic insults, such as hypoxia and hypoglycemia, has been detected immunohistochemically and involves a characteristic loss of glutamate from neuronal cell bodies and dendrites. Retinal damage in dogs with primary glaucoma is greater in regions where glutamate immunostaining is decreased in neurons than in regions with normal glutamate immunostaining, suggesting that there is increased glutamate release in these regions. In the dogs of those studies, it was not possible to perform time-course assessments of neuronal damage and glutamate loss. Primary glaucoma in dogs is frequently classified as narrow-angle or angle-closure glaucoma. In the present study, we examined the relationship between neuronal damage and glutamate loss in young DBA/2J mice, a strain of mouse that is used to investigate angle-closure glaucoma.

Damaged neurons were observed in the ganglion cell layer of DBA/2J mice at 9 weeks of age and older. Via light microscopy, these damaged cells appeared to have undergone an apoptotic type of cell death similar to that identified via electron microscopy by Schuettauf et al in DBA/2J mice at 3 months of age. In our study, the pathologic changes of neurons in 9-week-old DBA/2J mice included homogeneous dark staining of nuclei, irregular nuclear shapes, and dark cytoplasm interspersed with vacuoles. Swollen cell bodies and swelling of the inner plexiform layer (which is characteristic of complete ischemia in rat retinas) were not observed in the DBA/2J mice at 9 weeks of age, but they were detected in older mice (at an age at which necrotic types of cell death predominate in retinas of DBA/2J mice). The DBA/2J mice that were 12 months old also had increased immunostaining for GFAP, a marker for reactive glia. Increased amounts of GFAP in Müller cells are consistent with increased numbers of intermediate filaments that have been identified in Müller cells of 6- to 8-month-old DBA/2J mice. Increased amounts of GFAP have also been reported in...
In some regions of the retinas obtained from DBA/2J mice, glutamate immunoreactivity was reduced, compared with undamaged regions or control mice. To our knowledge, there are no data to suggest that there are large regional differences in the percentage of cells in the ganglion cell layer that contain high amounts of glutamate in clinically normal vertebrate retinas. In the retinal sections examined in the present study, 2 types of regional variation were detected. The first type of regional variation was a uniform loss of glutamate immunoreactivity from all layers of the retina in the control ICR and C57BL/6J mice and in the DBA/2J mice. This loss of glutamate immunoreactivity seemed to be associated with retinal defects induced by dissection and was considered an artifact. Retinal regions with uniform glutamate depletion were therefore excluded from the assessments of staining density. The second type of regional variation in glutamate immunoreactivity was detected in damaged regions of the ganglion cell layer of DBA/2J mice; this pathologic loss of glutamate immunoreactivity was distinguished from artifact because glutamate immunoreactivity in the associated INL and other layers was unaffected.

Glutamate immunoreactivity was decreased in damaged regions of ganglion cell layer in the retinas of DBA/2J mice. In some morphologically undamaged retinal regions from 9-week-old DBA/2J mice, glutamate immunoreactivity was high in most cells of the ganglion cell layer and the distribution of glutamate was similar to that detected in clinically normal rodents and other species. Most cells of the ganglion cell layer in many undamaged regions had high densities of immunostaining for glutamate. However, in regions with clusters of damaged cells, far fewer ganglion cell layer cells with high density of immunostaining for glutamate were found. Compared with control mice, immunostaining for glutamate was decreased in neuronal cell bodies and also in structures that might have been degenerating axons in the nerve fiber layer. Essentially all of the damaged cells and a large proportion of the nearby apparently undamaged cells had low densities of immunostaining for glutamate.

Mechanisms other than release of glutamate may contribute to the neuronal glutamate loss detected in damaged regions of retinas obtained from DBA/2J mice. The decreased amounts of intracellular glutamate suggested by the low-density staining for glutamate in damaged retinal regions may also be the result of altered uptake or metabolism of glutamate. In rats, the expression of glutamate transporters may be altered in association with glaucoma, perhaps contributing to changes in intracellular glutamate concentrations. Altered glutamate metabolism is also suggested by increased amounts of glutamine (a precursor of glutamate) that have been detected in Müller cells in monkeys with glaucoma. It has been suggested that these changes in glutamine may reflect an increased glutamate release during development of glaucoma.

Of interest is the relationship of glutamate redistribution to the pathogenesis of glaucoma. Compared with undamaged regions of DBA/2J retinas, glutamate immunoreactivity in neurons of the ganglion cell layer was decreased in damaged regions of retinas that were 9 weeks old (the earliest age at which retinal damage was assessed). The fact that the number of cells in these regions of decreased glutamate immunoreactivity in young mice was the same as the number of cells in the undamaged retinal areas suggests that glutamate depletion develops early in the disease before substantial cell losses occur. Similar losses of glutamate from neurons occur during ischemia and other insults. These results are consistent with the hypothesis that glutamate is released from damaged and from neighboring, morphologically undamaged neurons of the ganglion cell layer of the retina during development of glaucoma. This may lead to further glutamate receptor-mediated damage in these retinal regions and thus progression of the disease.

In addition to providing an indication of pathologic glutamate release, decreased intracellular glutamate concentration may also contribute to neuronal damage or dysfunction. Glutamate is not only a major excitatory transmitter but also is a direct precursor for the synthesis of glutathione and γ-aminobutyric acid. Depletion of intracellular glutamate may alter synaptic transmission through decreased loading of glutamate or its metabolite, γ-aminobutyric acid, into synaptic vesicles. Glutamate depletion may also decrease the synthesis of the antioxidant glutathione in the retina, perhaps resulting in increased oxidative stress in these regions. Oxidative stress has been reported to contribute to retinal damage in some types of glaucoma.

Our data confirm the finding that morphologically evident cell damage develops in DBA/2J mice before intraocular pressure increases at 6 months of age. Furthermore, results of the immunohistochemical evaluation of retinal specimens from DBA/2J mice with glaucoma indicated that neuronal glutamate concentration may be decreased selectively in damaged retinal regions. This loss of glutamate from neurons is similar to that identified in damaged retinal regions in dogs with primary glaucoma. However, in DBA/2J mice, the glutamate loss occurs early in the disease, before intraocular pressure is increased. It remains unknown whether similar loss of glutamate and damage to neurons precede the clinical onset of disease in dogs with primary glaucoma. Depletion of neuronal glutamate in damaged regions is consistent with pathologic release of glutamate in these regions and supports the concept that memantine and other glutamate antagonists may be effective in preventing ganglion cell death in this animal model of angle-closure glaucoma.

a. Jackson Laboratories, Bar Harbor, Me.
b. Charles River, Wilmington, Mass.
c. Rabbit anti-glutamate, Sigma Chemical Co, St Louis, Mo.
d. Glial fibrillary acidic protein, DakoCytomation, Carpinteria, Calif.
f. Zeis Axioplan 2, Carl Zeiss Microimaging Inc, Thornwood, NY.
g. Axiovision, version 3.1, Carl Zeiss Microimaging Inc, Thornwood, NY.
h. ImageJ, version 1.28, National Institutes of Health, Bethesda, Md.
References


16. Lucas DR, Newhouse JP. The toxic effects of sodium L-gluta-


17. Anderson MG, Smith RS, Savinova OV, et al. Genetic modi-


24. Naskar R, Verwerk CK, Dreyer EB. Concurrent downregu-


30. Dyka FM, May CA, Enz R. Metabotropic glutamate recep-


tivities during forebrain ischemia: a semiquantitative electron micro-


42. Carter-Dawson L, Shen F, Harwerth RS, et al. Glutamate immuno- 


43. Reichelt W, Stabel-Burow J, Pannicke T, et al. The glu-

tamine level in the retina of rats with primary angle-closure glauco-
