Loss of Neurons in Magnocellular and Parvocellular Layers of the Lateral Geniculate Nucleus in Glaucoma

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Objective: To determine whether there is loss of lateral geniculate nucleus relay neurons, which convey visual information to the visual cortex, in experimental glaucoma in monkeys.

Methods: Four cynomolgus monkeys with experimentally induced glaucoma in the right eye (referred to as the glaucoma group) and 5 control monkeys were studied. In both groups, the same conditions of fixation, tissue processing, staining, and measurement were used. In each monkey, the left lateral geniculate nucleus target neurons in magnocellular layer 1 and parvocellular layers 4 and 6, connected to the right glaucomatous eye, were studied. Immunocytochemistry with antibody to parvalbumin was used to specifically label relay neurons connecting to the visual cortex. The number of parvalbumin-immunoreactive neurons was estimated using an unbiased 3-dimensional counting method. The t test was used to compare the experimental and control groups.

Results: The mean (±SD) number of neurons in magnocellular layer 1 was significantly decreased in the glaucoma group compared with the control group (20 692 ± 9567 vs 37 687 ± 8017; P = .02). The mean (±SD) number of neurons in parvocellular layers 4 and 6 was significantly decreased in the glaucoma group compared with the control group (100 141 ± 44 906 vs 174 090 ± 39 136; P = .03). Data are given as the mean ± SD.

Conclusion: Significant loss of lateral geniculate nucleus relay neurons terminating in the primary visual cortex occurs in the magnocellular and parvocellular layers in an experimental monkey model of glaucoma.

Clinical Relevance: Knowledge of the fate of neurons in the central visual system may lead to a better understanding of the nature and progression of visual loss in glaucomatous optic neuropathy.


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SUBJECTS AND METHODS

SUBJECTS

All studies were performed following the guidelines of the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. Four young adult cynomolgus monkeys (Macaca fascicularis) with experimental glaucoma were studied. The survival time after laser treatment was 14 months.

In monkeys with experimental glaucoma, intraocular pressure (IOP) measurements were performed with a pneumotonometer (Digilab, Norwell, Mass) under light sedation (intramuscular injection of ketamine hydrochloride, 5 mg/kg) and topical anesthesia (5% proparacaine hydrochloride). The IOP was measured in each eye of each monkey 3 times before laser treatment and 2 times during a 14-day period after laser treatment. The interval between IOP measurements ranged from 5 to 39 days (mean, 26 days). The mean, median, minimum, and maximum IOPs of control and glaucomatous eyes have been previously described (rows 1, 4, 5, and 6, Tables 1 and 2). In control monkeys, the baseline IOP was within 12 to 19 mm Hg.

Based on previously reported optic nerve fiber counts of the monkeys with experimental glaucoma (rows 1, 4, 5 and 6, Tables 3 and 4), and using previously described morphometric techniques, the brains of 4 monkeys in which optic nerve fiber loss were previously described (rows 1, 4, 5, and 6, Tables 1 and 2). We examined the brains of 4 monkeys in which optic nerve fiber loss when compared with the fellow eye was 17%, 29%, 61%, and 100% (Table 1 and Table 2).

TISSUE PROCESSING

Under deep general anesthesia, animals were perfused through the heart with 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (pH 7.4), 0.1 mol/L. Control animals were anesthetized with an intramuscular injection of ketamine hydrochloride, 10 mg/kg, followed by an intramuscular injection of pentobarbital sodium, 35 mg/kg, at the University of Wisconsin, Madison. The animals with experimental glaucoma were anesthetized with an intramuscular injection of ketamine hydrochloride, 10 mg/kg, and acepromazine maleate, 0.5 mg/kg, followed by an intravenous injection of ketamine hydrochloride, 100 mg. After removal from the heart, the brains were fixed by immersion in 4% paraformaldehyde in phosphate buffer (pH 7.4), 0.1 mol/L, for at least 48 hours. The left LGNs were blocked in the coronal plane and cryoprotected by immersion in 10% glycerin and 2% dimethyl sulfoxide in phosphate buffer, 0.1 mol/L, for 2 days and in 20% glycerin and 2% dimethyl sulfoxide in phosphate buffer, 0.1 mol/L, for 5 days. The blocks were frozen in isopentane cooled by a mixture of 100% alcohol and dry ice. Coronal sections (50 μm) of the entire LGN were cut serially on a sliding microtome. Every seventh section was mounted onto a glass slide and stained with cresyl violet. Care was taken to use the same tissue processing procedures for all monkey brains. The remaining sections were stored in 25% glycerin and 30% ethylene glycol in sodium phosphate, 0.05 mol/L, and stored at −20°C.

IMMUNOCYTOCHEMISTRY

The primary antibody was a monoclonal antibody against parvalbumin (clones PA-235; Sigma-Aldrich Corp, St Louis, Mo). Farvalbumin, a calcium-binding protein, labels relay neurons in the LGN layers that project axons to the visual cortex. Sections were washed with Tris-buffered saline, 0.1 mol/L, and incubated with 0.2% octylphenoxypolyethoxyethanol (Triton-X; Sigma-Aldrich Corp) in Tris-buffered saline, 0.1 mol/L, for 15 minutes, followed by 3% normal goat serum for 1 hour. Sections were incubated in 1:1000 diluted antibody in phosphate-buffered saline with 3% normal goat serum overnight at 4°C. After thorough washing in repeated changes of phosphate-buffered saline and Tris-buffered saline, they were reacted with secondary immunoglobulins using avidin-biotin-peroxidase. A supersensitive detection system kit (Biogenex, San Ramon, Calif) and peroxidase were used to localize the antigen by incubation in 0.02% 3,3-diaminobenzidine and hydrogen peroxide. Sections were mounted onto slides coated with silane-based reagent (Vectabound; Vector, Burlingame, Calif), dehydrated, cleared, and cover-slipped. A negative control was obtained by omitting the primary antibody.

COUNTING METHODS

Morphometry was performed using bright-field microscopy with a color video camera (JVC, Yokohama, Japan), video and computer monitors, and a computer. Stereological procedures that provide unbiased estimates of cell numbers were used. Neurozoom software (Human Brain Project, La Jolla, Calif) enabled digital superposition of the sampling grids on the tissue. Neuronal density and layer surface area measurements were performed on immunostained and cresyl

LIGHT MICROSCOPY

At low power, the overall appearance of LGNs with glaucoma (Figure 1, right) appeared atrophic compared with the control LGNs (Figure 1, left). Immunoreactivity in layers 4 and 6 was less in glaucomatous (Figure 1, right) compared with control (Figure 1, left) LGNs. Immunoreactivity in layers 1, 2, 3, and 5 showed no apparent difference between glaucomatous and control LGNs (Figure 1, left and right).

At high power, immunoreactivity was seen in the cell body and in neuronal processes. In glaucomatous LGNs, the cell bodies of neurons appeared smaller and fewer in layers 4 and 6 (Figure 2, bottom). In addition, fewer neuronal processes were present between neurons compared with the control (Figure 2, top). In layer 1, although no difference in neuronal density was apparent, the size of the cell bodies appeared to be reduced.
stained sections, respectively. The 6 layers of the LGN were easily identified on stained sections. The layers were identified as layer 1 to layer 6 from ventral to dorsal. Ventral layers 1 and 2 are magnocellular layers, while the remaining dorsal layers 3 through 6 are parvocellular layers. Layers 1, 4, and 6 of the left LGN are connected to the glaucomatous right eye, while layers 2, 3, and 5 are connected to the nonglaucomatous left eye. To determine whether neurons are lost in magnocellular and/or parvocellular LGN layers connected to a glaucomatous eye, neurons in the left LGN layers 1, 4, and 6 were counted, and the counts were compared with those from the left LGN layers 1, 4, and 6 in control monkeys. Retinal ganglion cells of the right nasal hemiretina and fovea project to the left LGN layers 1, 4, and 6, and compose approximately 50% of the right eye retinal ganglion cells. The difference in nerve fiber loss between the nasal and temporal quadrants of the right optic nerves was not statistically significant (P > .05) for the monkeys examined in this study; therefore, changes observed in left LGN layers 1, 4, and 6 are most representative for changes observed in target LGN neurons. Left LGN layers 1, 4, and 6 of monkeys with a normal visual system were used as controls rather than right LGN layers 1, 4, and 6 of the laser-treated monkeys, since significant decrease in cell size has been observed in the underdeprived layers in some monocular experimental conditions. In addition, our preliminary results show that the neuronal number in the left LGN layers connected to the control fellow eye in laser-treated monkeys appears to be reduced compared with the neuronal number in the left LGN in control monkeys (unpublished data, 1999). Analysis was performed for magnocellular layer 1 and parvocellular layers 4 and 6, the latter sharing the same morphologic and functional properties.

LAYER VOLUME

Measurements of the surface area for each layer were made on equally spaced sections (interval, 350 µm) containing all 6 layers of the LGN, starting with a section selected randomly. Areas between the LGN layers were excluded. Lateral geniculate nucleus layers at low power (×2.5 objective) and a point counting grid generated by the software were visualized on the computer monitor. Using the mouse, the operator marked the points located on a layer. Each point corresponded to an area of 0.01 mm² for the grid used for layer 1, and 0.0225 mm² for the grid used for layers 4 and 6. Layer surface area was calculated for each section by the software by multiplying the number of points on the layer with the area corresponding to a grid point. Layer surface area for each section was multiplied by the interval (350 µm) between sections and summed by the software to calculate layer volume.

NEURON DENSITY

Neuron density measurements were made on 3 parvalbumin-immunostained sections taken from the anterior, middle, and posterior LGN with all 6 layers at high power using an oil immersion objective (×100, numerical aperture = 1.32), a bright-field microscope, and a color video camera. Immunostained neurons were visualized on the computer and video monitors. Cell counts were made at tissue locations determined by a random and systematic sampling procedure using a superimposed grid method. Only sample locations within the LGN layers were used for neuron density measurements. The size of the sampling grid was adjusted for each layer so that there were at least 80 samples for that layer through the nucleus. To measure neuronal density, the optical dissector method was used. A 3-dimensional optical dissector composed of x-, y-, and z-axes that were 50 × 50 × 10 µm, respectively, was placed within the tissue section with a guard area above and below the optical dissector. The neurons in focus intersecting the top surface of the dissector were not counted. Only the first encountered profile of the new cell bodies that came into focus within the optical dissector and intersecting its right, back, and bottom surfaces was counted. The excursion along the focusing axis (10 µm) and the thickness of the section were measured with a microcator (model MT12; Heidenhain, Traunreut, Germany) mounted on the microscope stage. Neuronal density per layer was estimated by calculating the average neuronal density for at least 80 optical dissectors. The average neuronal density was adjusted for tissue shrinkage occurring during the tissue processing.

NUMBER OF NEURONS

The number of neurons in each layer was calculated by multiplying the average density of neurons (neurons per cubic millimeter) by layer volume.

STATISTICAL ANALYSIS

The t test was used to compare layer volume, neuronal density, and number of neurons in the LGN of glaucomatous vs control monkeys. Data are given as mean ± SD unless otherwise indicated.

STEREOLOGICAL MORPHOMETRY

Morphometric studies were performed in left LGN magnocellular layer 1 and parvocellular layers 4 and 6 connected to the right optic nerve in monkeys with experimental glaucoma in the right eye (n = 4) and in control monkeys (n = 5).

MAGNOCYELLULAR LAYER 1

Table 1 summarizes the volume, neuronal density, and number of neurons for magnocellular layer 1 in the control and glaucoma groups.

Layer Volume

The volume of layer 1 ranged from 0.70 to 2.50 mm³ in the glaucoma group, and from 2.00 to 3.04 mm³ in the control group. The mean ± SD volume of layer 1 was significantly decreased in the glaucoma group compared with the control group (P = .04).

Neuronal Density

Neuronal density in layer 1 ranged from 11,848 to 17,041 neurons per cubic millimeter in the glaucoma group, and
from 13 339 to 15 466 neurons per cubic millimeter in the control group. The mean density of neurons in layer 1 did not differ significantly between the glaucoma and the control groups (P > .05).

Number of Neurons

The number of neurons in magnocellular layer 1 ranged from 11 929 to 33 339 in the glaucoma group, and from...
26,611 to 46,941 in the control group. The mean number of neurons in magnocellular layer 1 was significantly decreased in the glaucoma group compared with the control group ($P = .02$) \((\text{Figure 3, top})\). The number of neurons in magnocellular layer 1 in the glaucomatous monkeys showed a tendency to decrease with increasing optic nerve fiber loss \((\text{Figure 3, bottom})\).

**PARVOCELLULAR LAYERS 4 AND 6**

Table 2 summarizes the volume, neuronal density, and number of neurons for parvocellular layers 4 and 6 in the control and glaucoma groups.

**Layer Volume**

The combined volume of layers 4 and 6 ranged from 3.36 to 8.06 mm$^3$ in the glaucoma group, and from 7.45 to 12.84 mm$^3$ in the control group. The mean volume of layers 4 and 6 was significantly decreased in the glaucoma group compared with the control group \((P = .02)\).

**Neuronal Density**

Neuronal density in layers 4 and 6 ranged from 16,608 to 18,426 neurons per cubic millimeter in the glaucoma group, and from 15,701 to 18,857 neurons per cubic millimeter in the control group. The mean density of neurons in layers 4 and 6 did not differ significantly between the glaucoma and the control groups \((P > .05)\).

**Number of Neurons**

The number of neurons in parvocellular layers 4 and 6 ranged from 56,439 to 151,822 in the glaucoma group, and from 124,971 to 227,005 in the control group. The mean number of neurons in parvocellular layers 4 and 6 was significantly decreased in the glaucoma group compared with the control group \((P = .03)\) \((\text{Figure 4, top})\). The number of neurons in parvocellular layers 4 and 6 in the glaucomatous monkeys showed a tendency to decrease with increasing optic nerve fiber loss \((\text{Figure 4, bottom})\).

**COMMENT**

Previous studies\(^ {22,27,28}\) in monkey LGN have used the Nissl stain, which labels all neurons, including relay neurons and interneurons. In the present study, parvalbumin was used to label only relay neurons connecting to the visual cortex in the magnocellular and parvocellular layers.\(^ {13,14}\) Neuron density measurements for the magnocellular and parvocellular layers were similar to measurements computed by Ahmad and Spear,\(^ {22}\) and comparable coefficients of error for neuron number \((9.5\%\) for layer 1 and 10\% for layers 4 and 6) were obtained. Since the stereological method used in this study has been shown to be unbiased by the size, orientation, or shape of the objects counted,\(^ {24,26}\) neuronal density measure-
ments are not biased by a possible reduction in size of neurons in magnocellular and parvocellular layers in glaucoma (qualitative observation and preliminary measurements).

Our study demonstrated neuronal loss in the magnocellular and parvocellular layers in experimental glaucoma. These results are in keeping with a descriptive study29 showing weaker immunoreactivity for synaptophysin and neurofilament in magnocellular and parvocellular layers, and with electrophysiologic studies30 reporting deficits in visually responsive cells in magnocellular and parvocellular layers. The differences in our findings might be explained by differences between human clinical and monkey experimental conditions, extent and/or duration of disease, and differences in method.

Histomorphometric studies32,33 in human clinical and monkey experimental glaucoma, including measurements of cell body size and of axon diameter of the surviving retinal ganglion cells, suggest preferential loss of large retinal ganglion cells. In addition, large retinal ganglion cells immunoreactive for neurofilament have been shown to be preferentially lost in experimental glaucoma.34 The researchers32,33 suggest that retinal ganglion cells projecting to the magnocellular layers (M) are preferentially lost in early glaucoma, based on the observation that M retinal ganglion cell bodies and axons are relatively larger compared with cell bodies and axons of retinal ganglion cells in normal retina projecting to the parvocellular layers (P). The identification of retinal ganglion cell types on size alone may not be reliable35 for several reasons: overlap in cell body size between M and P retinal ganglion cells occurs in normal monkey and human retina; S cells, a third class of retinal ganglion cells involved in the blue on-center system, are similar in size to M cells; and atrophic changes in glaucoma include a reduction in cell body size and axon diameter of retinal ganglion cells.36 In our study, the number of neurons in the magnocellular and parvocellular layers showed a tendency to decrease with increasing optic nerve fiber loss. Further studies of the LGN with a larger sample size and with optic nerve fiber loss ranging from 30% to 60% may provide evidence for preferential loss in these pathways.

Support for neuronal damage in magnocellular and parvocellular pathways in glaucoma comes from various functional tests. Motion-automated perimetry, high-frequency temporal flicker perimetry, and frequency-doubling perimetry reveal deficits in the magnocellular pathway in glaucoma.37-43 The parvocellular pathways are known to convey red-green color information.44,45 Color pattern–electroretinogram, sweep visual-evoked potentials, and psychophysical tests for red-green sensitivity show deficits in the parvocellular pathway in glaucoma.46-49 There is evidence to suggest that the blue-sensitive pathway is conveyed through a third information channel located in the interlaminar zones of the LGN,50,51 not examined in the present study. Short-wave automated perimetry testing blue-yellow sensitivity also reveals deficits in glaucoma.32-34

The significant loss of LGN relay neurons known to send their axons to the primary visual cortex13,14 suggests that in glaucoma, degenerative changes are also occurring in the primary visual cortex. The present study suggests that, in addition to retinal ganglion cell loss, the loss of target neurons in at least the LGN is part of the pathological process responsible for vision loss in glaucoma. Further studies to elucidate the mechanisms of neuronal death in the LGN are needed.

Accepted for publication October 6, 1999.
This study was supported by the Smith Barney Inc Research Fund of the Glaucoma Foundation, New York, NY (Dr Yucel); the Glaucoma Research Society of Canada, Toronto, Ontario (Dr Yucel); the Foundation for Eye Research, Rancho Santa Fe, Calif (Dr Yucel); grant EY02698 from the National Eye Institute, Bethesda, Md (Dr Kaufman); Research to Prevent Blindness Inc, New York, NY (Dr Kaufman); and the Joseph Drown Foundation, Los Angeles, Calif (Dr Weinreb).

We thank Terri Kozano, Bsc, and B’Ann Gabelt, MSc, for their excellent technical assistance at early stages of the study.

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