The Number and Diameter Distribution of Axons in the Monkey Optic Nerve

Ralph M. Sanchez, Gregory R. Dunkelberger, and Harry A. Quigley

Using an automated image analysis system, cross-sections from optic nerves of 17 normal cynomolgus monkeys were examined. The number of nerve fibers, their density, and the distribution of their diameters for whole nerves and for various regions of the nerve cross-section were estimated. The mean total number of fibers in the optic nerve was 1.2 million. The mean diameter of axons was 0.8 μm. The method of tissue fixation substantially affected the measurements. Histograms of fiber diameter suggested a trimodal distribution of fiber size with peaks at 0.5, 0.8, and 1.5 μm. The relative proportions of these fiber peaks differed significantly in different regions of the nerve. The highest proportion of large fibers was in the superior nerve periphery. The highest concentration of smallest fibers and the highest density of all fibers were located centrally in the infero-temporal quadrant. The observation that higher fiber density and smaller mean fiber diameter are skewed toward the inferior pole appears to coincide with the inferior position of the fovea with respect to the optic nerve head. This finding has importance for interpretation of pathologic changes in the optic disc. Invest Ophthalmol Vis Sci 27:1342-1350, 1986
Fig. 1. A, Cross-section of monkey optic nerve, taken 2 mm posterior to the globe, post-fixed in osmium tetroxide and stained with paraphenylenediamine (×75). B, Nerve shown at left is divided into the 16 segments used in this study. Eight zones are denoted A through H, and each of these is divided into a central and a peripheral half, so that subdivisions are called Cc or Hp as shown.

we first infused saline to clear red blood cells, then ¼ strength fixative, then full strength fixative into the aorta toward the heart and brain, having first opened the right ventricle. The eyes were enucleated and the optic nerves were marked with one razor slit superiorly and two slits nasally to determine the orientation after sectioning.

In seven further monkeys, eyes were fixed by immersion in Karnovsky’s fixative immediately following enucleation. In each monkey, only one nerve was obtained for analysis. All procedures used in this study were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

In 15 monkeys, a 1 mm section of optic nerve was taken 1–3 mm posterior to the sclera. In two monkeys, sections were only taken 5 mm behind the globe. Each specimen was postfixed in 2% osmium tetroxide in cacodylate buffer, dehydrated in alcohol, and embedded in epoxy resin. Thick sections (1 μm) were taken perpendicular to the major axis, stained with p-phenylenediamine, and mounted on glass slides.

Each nerve cross section was photographed (30×) and a 100× enlargement printed on 16 × 20 inch paper. The nerve was divided into 16 labeled segments of roughly equal area (Fig. 1), as described in a previous study. Each nerve was divided into 16 segments, excluding the regions containing central retinal vessels. The area of the nerve bundles and of the connective tissue were measured for each of the 16 segments using a planimeter. The central retinal vessels and their surrounding connective tissue were eliminated from all measurements.

In order to estimate the density of nerve fibers, we photographed each nerve using oil immersion, phase contrast (1000×). In most cases, four photographs were taken per segment at randomly selected positions within nerve bundles. We used Kodak (Rochester, NY) 35 mm SO-115 tech pan film at ASA 100 and developed for four minutes in undiluted Kodak D-11 developer to maximize contrast. Each photograph was printed at a magnification of 3400× and placed in a digital image analyzer. A black-and-white TV camera was used to convert the photograph into digital data. In 2500 μm² areas at a time. Any axon touching the border of the limiting window was excluded. An observer prevented any objects that were not axons from being counted.

Each axon was counted and its minimum diameter was measured using the following algorithm. The center of gravity was first determined and the radii along 32 axes from the center of gravity to the innermost ring of myelin were measured. The minimum diameter was taken as twice the shortest radius. The diameters given here do not, therefore, include the myelin sheath. This algorithm was selected to solve the problem of measuring diameter as accurately as possible when it is recognized that a substantial minority of axons are sectioned to some degree obliquely to their long axis instead of precisely perpendicular to it. No correction for shrinkage in fixation or processing was made.

The diameter measurements were sorted into 40 bins of 0.1 μm increments from 0–4 μm. Photographs from the same segment of a nerve were analyzed together, and the absolute number of axons counted in each photograph were cumulatively added to the bins. After all photographs for a segment had been analyzed, the total count in each bin for each segment of a nerve was printed as raw data.

More than 30,000 axons were counted per nerve, representing a sampling of 3% of the estimated total
Table 2. Repeat counts in one nerve
distributions.

| Diameter | 0.849 | 0.855 (-1%) | 0.863 (+2%) |

In 17 eyes, the estimated mean number of fibers per nerve was 1,067,926 (standard error = 71,447). Table 1 shows the neural area, density (fibers per mm² within nerve bundles), and total number of fibers for each of the 17 eyes measured. The mean diameter of optic nerve fibers for the 17 eyes was 0.80 ± 0.09 μm (standard deviation). Mean fiber diameter among the 17 nerves ranged from a low of 0.68 to a high of 1.00 μm (Table 1).

In order to estimate possible effects of fixation and/or proximity of the cross section to the eye, we grouped the data by fixation method and location. Between 10 eyes fixed by perfusion and 7 eyes fixed by immersion, no statistically significant differences were found in fiber number, neural area, or diameter (P > 0.05). The immersion-fixed group did have 10% lower density, 14% lower neural area, and, hence, 20% lower mean fiber count compared to those fixed by perfusion. In addition, nerves fixed by immersion had smaller mean fiber diameters than those in the perfusion group, though again this difference was not significant (P > 0.05; Table 1).

Next, we excluded the data for two nerves taken 5 mm behind the globe from the group fixed by immersion. The resulting fiber count of 769,016 for the remaining five immersion-fixed eyes was significantly lower (P < .01) than the 1,169,227 found in the perfusion group. There did appear to be a variation in total fiber count and possibly in other variables attributable to fixation method. To avoid the problem of confounding variables, analysis of fiber distribution by segment was limited to the ten nerves that were perfusion fixed and taken 1–3 mm behind the eye.

We also wished to estimate the reproducibility of the method of estimation used. To do this, we carried out the major steps of the method on the same nerve three separate times. The measurement of the area of nerve bundles on the planimeter has been previously shown to have less than a 1% variation, so this was not repeated. But repeat photomicrographs were taken in 64 different, randomly selected areas for the three counts, and these were separately measured by the image analysis system. The second and third estimates of nerve fiber number differed from the first one by 1% and 5% (Table 2). Fiber diameter estimates were even more reproducible, differing by less than 1.6% for the three analyses.

The mean fiber diameter had a consistent pattern of regional difference around the 16 segments of the nerve. The smallest mean diameter was in the central infero-temporal quadrant (labelled Cc), and the largest mean fiber diameter was the superior nasal periphery (segment Hp) (Fig. 1).

Mean fiber diameters for the perfusion fixed optic nerves were averaged by segment, and a grey scale representation of the results are shown in Figure 2. The

Results

In 17 eyes, the estimated mean number of fibers per nerve was 1,067,926 (standard error = 71,447). Table

<table>
<thead>
<tr>
<th>Count 1</th>
<th>Count 2</th>
<th>Count 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>289,563</td>
<td>292,350 (+1%)</td>
</tr>
<tr>
<td>Fiber number</td>
<td>1,425,487</td>
<td>1,439,210 (+1%)</td>
</tr>
<tr>
<td>Diameter</td>
<td>0.849</td>
<td>0.855 (-1%)</td>
</tr>
</tbody>
</table>
nerve appeared to have a smaller mean fiber diameter temporally compared to nasally, inferiorly compared to superiorly, and centrally compared to peripherally.

Mapping of fiber density (fibers per mm²) by segment revealed a similar pattern (Fig. 3) to that of fiber size. The highest density of fibers within nerve bundles was found in the central segments of the infero-temporal quadrant (Dc), coinciding with the segment where fibers have the smallest diameter (Cc and Dc). Lowest fiber density was in the superior periphery (Hp) where mean fiber diameter was largest.

A histogram showing fiber density vs fiber diameter for the average of 17 eyes shows peak densities occurred at 0.55, 0.85, and 1.55 μm (Fig. 4). We wished to know whether the apparent trimodality of the data was a chance occurrence, or whether it indicated a true grouping of three different fiber diameter types. First,
we tested the data to see if it fit a normal distribution, one way to determine if the shape simulated unimodality. The distribution was clearly not normally distributed (Kolmogorov-Smirnov method, \( P < 0.01 \)), suggesting a heterogeneous population. Further analysis was carried out to determine if the data seemed to consist of three separate groups, each a gaussian distribution. This was not the case, using a method described by Bhattacharya. Fibers of 0.55 and 0.85 \( \mu m \) diameter occur in roughly equal number (40% and 50% respectively) with the largest fibers (1.55 \( \mu m \)) comprising about 8% of the total. Histograms for nerves grouped by fixation method showed the same three peaks but in slightly different ratios. Nerves fixed by immersion showed a significantly \(( P < .05 )\) lower density of 0.85 \( \mu m \) fibers. The density of 0.55 and 1.55 \( \mu m \) fibers were not significantly different \(( P > .05 )\) between the two groups.

The three diameters, 0.55, 0.85, and 1.55 \( \mu m \), were observed as distinct peaks in each segment of every nerve. While fibers of every size were observed throughout the nerve, the density ratio for these three fiber sizes changed substantially from region to region. The most dramatic differences were observed between the superior periphery (Hp) and the central segment of the infero-temporal quadrant (Cc). For example, segment Cc has twice the density of 0.55 \( \mu m \) fibers but only half the density of 1.55 \( \mu m \) fibers compared to
segment Hp (Fig. 5). Each of the three major peaks in segment Hp differs significantly ($P < .05$) from its corresponding peak in segment Cc.

To demonstrate more clearly the regional differences in fiber size distribution, we mapped fiber density for each of the three peak fiber sizes (Fig. 6, Table 3). The density of the smallest axons (0.55 μm) was highest centrally in the infero-temporal half of the nerve (segments Be, Cc, and Dc) and lowest in the superior periphery. Density of 0.85 μm fibers was highest in the central nerve in segments Ac and Dc (Fig. 6). Again, this group’s lowest density was in the superior periphery. The largest axons (1.55 μm) form a complementary pattern to the smallest fiber group, with the highest density in the superior periphery (segment Hp) and the lowest density in the central temporal quadrant (segments Bc and Cc).

### Discussion

Our estimate of the number of axons in the monkey optic nerve is not surprising when compared with previous studies. Other studies estimated 1.2 and 1.8 million optic nerve fibers in the Macaque monkey. None of these reports examined more than two monkey nerves, and, in three reports, the data was limited to one adult monkey eye. Our finding that the 10 perfusion-fixed nerves had a mean of 1.2 million fibers places the number of ganglion cells in Macaca fascicularis at the low end of the previously reported range, assuming one axon per ganglion cell. The number of fibers in the monkey nerve is quite similar to the available estimates in human eyes. Tissue fixation had a significant influence on fiber number estimates. Our immersion-fixation group contained some nerves in which there was a delay of 5 min after enucleation before the optic nerve was placed in fixative. This group also contained one animal that died during anesthesia. While its eyes were removed promptly, these and the other immersion-fixed nerves represented the extremes, usually the low end of our fiber number estimates. For this reason, we limited most of our data presentation to the perfusion-fixed group of ten eyes.

There are other substantial differences in methodology between our study and others. Potts et al. attempted to count every fiber in two nerves by placing in register photographs encompassing whole nerve cross-sections. While not subject to the potential errors in our random sampling method, their method may be more likely to count the same fiber twice, leading to an overestimate.

Even within the perfusion group, however, there was some variation from one animal to the other in nerve fiber count. This exceeded the variation in repeat counts of the same nerve three times. There is, therefore, a substantial difference in the number of ganglion cell fibers from one monkey to another. One report has suggested that there is a decrease by 100% in the number of optic nerve fibers in the monkey eye from the fetus to the term infant. This elimination of fibers probably is part of a natural process of selection in the establishment of synaptic connections in higher centers. Whether there is a continued loss of optic nerve fibers with age in adulthood is as yet unclear. One examination of this question in human eyes found no statistically significant decline in fiber number with age when the best-fixed nerves were evaluated. We found no relation between age and optic nerve fiber number in five human adults. This factor is not a likely explanation of the variation in our material, since our monkeys were all young adults. The sex of the animal likewise played no significant role.

Our previous estimation of the optic nerve fiber number in five normal, adult human eyes had only half the variation of the monkeys measured here (judged by the standard deviation). It will be important to expand our data in human eyes to elucidate this point. This is relevant to the clinical evaluation of optic disks. The size of the disc cup is used as a parameter to judge neural loss in glaucoma management. It has been assumed that most eyes have the same amount of neural tissue in the disc, and that variations in cup size derive from variations in optic disc canal diameter. Recent clinical studies have attempted to measure the neuroretinal rim area of human optic discs. If the

### Table 3. Fiber density of three peak fiber diameter groups by segment

<table>
<thead>
<tr>
<th>Segment</th>
<th>0.5 μm group</th>
<th>0.8 μm group</th>
<th>1.5 μm group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ap</td>
<td>15,800</td>
<td>24,316</td>
<td>6,221</td>
</tr>
<tr>
<td>Ac</td>
<td>25,168</td>
<td>34,779</td>
<td>3,958</td>
</tr>
<tr>
<td>Bp</td>
<td>24,979</td>
<td>33,863</td>
<td>3,400</td>
</tr>
<tr>
<td>Bc</td>
<td>33,063</td>
<td>33,758</td>
<td>3,411</td>
</tr>
<tr>
<td>Cp</td>
<td>24,263</td>
<td>33,021</td>
<td>3,895</td>
</tr>
<tr>
<td>Cc</td>
<td>32,551</td>
<td>32,086</td>
<td>3,016</td>
</tr>
<tr>
<td>Dp</td>
<td>21,688</td>
<td>31,588</td>
<td>4,350</td>
</tr>
<tr>
<td>Dc</td>
<td>37,966</td>
<td>37,349</td>
<td>3,611</td>
</tr>
<tr>
<td>Ep</td>
<td>22,292</td>
<td>28,573</td>
<td>5,859</td>
</tr>
<tr>
<td>Ec</td>
<td>29,968</td>
<td>33,937</td>
<td>4,095</td>
</tr>
<tr>
<td>Fp</td>
<td>25,685</td>
<td>28,277</td>
<td>6,059</td>
</tr>
<tr>
<td>Fc</td>
<td>29,874</td>
<td>31,453</td>
<td>4,453</td>
</tr>
<tr>
<td>Gp</td>
<td>18,926</td>
<td>27,537</td>
<td>5,832</td>
</tr>
<tr>
<td>Gc</td>
<td>24,346</td>
<td>25,211</td>
<td>5,384</td>
</tr>
<tr>
<td>Hp</td>
<td>14,347</td>
<td>20,863</td>
<td>7,453</td>
</tr>
<tr>
<td>Hc</td>
<td>21,212</td>
<td>26,141</td>
<td>5,529</td>
</tr>
<tr>
<td>Mean</td>
<td>25,133</td>
<td>30,172</td>
<td>4,845</td>
</tr>
<tr>
<td>S.D.</td>
<td>6,364</td>
<td>4,500</td>
<td>1,230</td>
</tr>
<tr>
<td>Relative % total</td>
<td>41.8%</td>
<td>50.2%</td>
<td>8.0%</td>
</tr>
</tbody>
</table>
number of nerve fibers varies only slightly, the neuroretinal rim area would vary little among normal eyes. In addition, it would be easier to distinguish early glaucoma damage from normal if the normal variation is small. However, in 33 normal human eyes, the clinical method suggested that the variation in rim area is similar to the variation seen in our nerve counts. The size of the standard deviation in normal rim area was 13% of the mean region area, while the standard deviation of nerve counts was 19% of the mean nerve count. Thus, both methods suggest a substantial variation among primate and human eyes in the amount of neural tissue.

The mean diameter of monkey fibers estimated here was 0.8 μm. Our measurement did not include the myelin sheath. While Potts reported measuring the size of fibers, it was given as the area within the myelin. We found that no cross-section of the nerve cuts every axon precisely perpendicular to its long axis. Since some axons are cut obliquely, their area is not an accurate measure of diameter. Ogden and Miller measured the diameter of fibers in one nerve, including the myelin sheath (different from our method), but taking the "smallest overall diameter" (probably similar to our system). Despite these variations in method, our data and that of these two groups did not differ dramatically in mean fiber diameter. Potts indicated that most fibers were included in two bins, 0.5 and 0.75 μm. Ogden and Miller show a curve with "peak frequency" of 1.2 μm. The mean optic fiber diameter in a number of species is not greatly different from our figure.2

It is in the histogram of fiber diameters that our study offers new information. In previous studies, the distribution appeared unimodal. However, the data was placed in bins 0.2 μm or more wide in these reports. The tendency toward three peaks in our data would have been obscured using their method. The separation between these three apparent groups is not striking, yet the distribution is proven distinct from a unimodal Gaussian. However, the unwary reader should not assume that we have demonstrated the existence of three fiber groups that are cleanly separated biostatistically. There may, in fact, be no true separation among the fiber sizes measured. Yet, every eye showed the same tendency toward trimodality. In addition, the position and relative heights of the three groups is similar to fiber diameter groups in one cat optic nerve. Williams and Chalupa also found two modes in cat optic nerve fibers, with a smaller third group at a larger diameter. As in our monkey nerves, the two main groups of fibers each occupy about 45% of the total, while 8% of the total consists of the group of large diameter fibers. This distribution fits with the expected sizes and frequencies of the three classes of retinal ganglion cells in the cat eye. When these groups are described by cell size and dendritic pattern, they are designated gamma, beta, and alpha from smallest to largest. The three groups have been shown to correspond in general with three known physiologic groupings, W-, X-, and Y-cells, respectively.

Gamma cells (W-cells) have the smallest cell body and axon diameters, hence the slowest axonal conduction velocity. They exhibit a diversity of receptive field properties. They comprise a substantial minority of ganglion cells in the cat. This group may correspond to our fibers at 0.55 μm diameter. Beta (X-cell) soma and axon diameter is larger than gamma cells, but still near the mean somal size for all ganglion cells. Physiologically, they give sustained responses to stimuli, summing input linearly, and are differently reactive to colored targets. They seem most likely to be represented by our 0.85 μm diameter group. Alpha cells (Y-cells) are the largest in cell body and axon and the fastest in conduction. Their receptive field centers are large, their responses more transient, and they sum input non-linearly, all features of the group called Y-cells. The small group at 1.55 μm diameter in our monkey data may correspond to these cells.

These three groups have been studied extensively in cats, and monkey ganglion cells exhibit similar grouping, though the complexity in primate eyes is probably greater. Estimates of the relative proportions of the general groups seem similar to the cat. Our normal fiber diameter data therefore seem consonant with this present analysis of ganglion cell types. However, fiber diameter alone is only one of a number of means by which ganglion cells can be classified. Anatomic methods include cell body size and dendritic form, retinal...
location, and terminal connections. The physiological characteristics of various cell types are detailed above. In order to establish further whether the fiber diameter distribution in the monkey illustrates an important division into different cell groups, corroborative evidence will be needed.

When the fiber diameter data are analyzed by zone of the optic nerve, further support is evident for the existence of important cell groupings. The highest density of all types of fibers and the highest density of small fibers both occur in the central nerve inferiorly on the temporal side. This indicates that, at the level of the optic nerve 1–3 mm posterior to the eye, the distribution of fiber density and type is not symmetrical about the vertical or the horizontal axis. The clear explanation for this skew in the nerve fiber pattern is the fact that the fovea in the monkey and human eye is not centered on a horizontal line through the middle of the optic disk (Fig. 7). Rather the fovea is found closer to a line drawn to the inferior disk border than to a line through the disk center. If we assume that the same number of foveal and macular ganglion cells send axons to the disk from above and from below a horizontal line through the fovea, then it is clear that the density of fibers will be greater below the center of the disk and nerve than above it, at least near the globe. In other studies, differences in the distribution of fiber diameter around the nerve were found, but only in Potts’ study was orientation preserved precisely. In the two monkey and two human nerves they analysed, the fiber size was smallest and density greatest on the temporal side. It is not possible to state whether the tendency toward greater density below the horizontal was shown, although it is suggested in their diagrams.

The pattern of other fiber sizes follows this same skew toward the inferior temporal nerve. The axons found in the 0.85 μm bin have their highest density temporoinferiorly. The group of fibers in the 1.55 μm bin show the mirror image distribution, with their greatest density superonasally and decreasing density in a pattern that spreads downward and temporally about an axis from upper temporal to lower nasal. This skew may have implications for clinical observations of the optic disk. For example, in glaucoma, the loss of neural rim tissue from the disk is used as a parameter for degree of damage. It should be recognized that loss of inferior rim tissue is not equivalent to loss of the corresponding superior tissue. More fibers per square millimeter are likely to be present in the inferior rim, as they were in our retrobulbar nerve sections. Also, a higher percentage of small ganglion cells and a lower proportion of large ganglion cells are damaged by injury to the inferior rim as compared to superior rim. The functional significance of loss of tissue from areas above and below the mid-position of the disc almost surely differ.

A number of disorders cause loss of optic nerve fibers in characteristic patterns. Glaucoma, ischemic optic neuropathy, compressive optic neuropathy, and toxic amblyopia are conditions that we have investigated in detail for their selective effects on certain zones of the optic nerve. If we assume that the human and monkey nerves are similar, then the normal patterns of fiber diameter delineated here may be interpreted in the light of known findings in these disorders. Toxic amblyopia appears to affect the highest density, high proportion of small fiber zone in the temporal, central nerve. Ischemic optic neuropathy, on the other hand, spares this very area. Compressive lesions at the chiasm injure the fibers in the mid-nerve at that site; again, those that are found in the temporal, central nerve nearer to the eye. The pattern of glaucoma damage is diffuse, but with a selective preference for greater damage at the upper and lower poles of the nerve, in the shape of an hourglass. We have wondered whether this pattern derived solely from the weaker structural support tissue of the lamina cribrosa there, or from the presence in this hourglass-shaped area of certain fibers that might be more sensitive to glaucoma injury. None of the three fiber diameter types illustrated here are found at their greatest density in an hourglass shape as in the glaucoma damage pattern. While further details of the specific injury to ganglion cells by glaucoma are being investigated, this seems to suggest that the regional anatomy of the nerve head may play the most important role in producing the characteristic neural loss and field defects of glaucoma.

Key words: retina, ganglion cell, optic nerve, histology, axon, monkey, morphometry

Acknowledgments

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