Corneal Nerve Alterations in Diabetes Mellitus

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• The morphologic status of corneal innervation was studied in rats with streptozocin-induced diabetes. Animals were killed at 1, 4, 16, and 36 weeks. Corneal innervation was studied by light and electron microscopy using nonspecific cholinesterase reaction, gold chloride impregnation, and plastic-embedded sections. Increased irregularity in the periodicity of nerve fiber beading was observed in diabetic corneas with gold impregnation. Ultrastructural evidence of irregularities in the basal lamina of Schwann cells was demonstrated in 16- and 36-week-old diabetic animals, along with occasional axonal degeneration. These alterations constitute a constellation of early pathologic manifestations in the innervation of diabetic cornea. To our knowledge, this study represents the first demonstration of neural changes in diabetic corneas as well as nerve fiber changes in an avascular tissue in diabetes.

(*Arch Ophthalmol* 1984;102:1380-1384)

A number of ocular complications have been reported secondary to diabetes mellitus, many of which can lead to irreversible blindness. Although retinal complications and cataract formation have been recognized and studied extensively, the awareness of corneal complications has occurred in recent years.[1,2] Persistent epithelial defects,[3-5] decreased corneal sensitivity,[6-10] neurotrophic corneal ulceration,[11] and Descemet's folds[12] constitute a gamut of diabetic corneal complications. The exact pathogenesis of these alterations, however, is not clear.

Alterations in the basement membrane of corneal epithelium may be responsible for some of the previously described clinical manifestations.[13-15] This phenomenon, however, cannot explain decreases in corneal sensitivity and neurotrophic ulceration. These two alterations may be the result of a generalized peripheral neuropathy characteristic of diabetes mellitus.

We addressed this question by studying the status of corneal innervation in rats made diabetic by injection of streptozocin.

MATERIALS AND METHODS

Thirty-day-old Long-Evans hooded rats weighing approximately 110 to 130 g were used as experimental animals. They were divided into two groups of diabetic and control rats, with 21 animals in each group. Five animals were sacrificed at each of four different time points: 1, 4, 16, and 36 weeks following the induction of diabetes.

Induction of Diabetes

Rats were made diabetic by injection of streptozocin (65 mg/kg of body weight in 0.9% acidified saline [pH, 4.5]) into the tail vein after 16 hours of fasting. Control animals were injected with the vehicle alone. The animals were regularly tested for fasting blood glucose level and the presence of urine sugar and ketone with reagent strips (Dextrostix and Keto-Diastix) after 12 hours of fasting. The body weight and the daily urinary output of the animals were recorded. All animals were maintained on the same standard rodent diet, and were given water ad libitum.

Histologic Techniques

Gold chloride impregnation, nonspecific cholinesterase reaction, semithin plastic-embedded sections, and transmission electron microscopy were used to evaluate the status of corneal innervation. With the use of pentobarbital sodium anesthesia, the eyes were enucleated at each of the time points. The right cornea of each animal was always used for semithin sections and electron microscopy. The left cornea was reserved for gold impregnation and nonspecific cholinesterase reaction.

Gold Impregnation.—The left cornea with a thin scleral rim was immediately isolated from the eye and dissected into two halves in 0.1M cacodylate buffer. Samples were placed into citric acid–phosphate buffer (pH, 2.5) at 20 to 22 °C for 15 to 20 minutes. They then were transferred into 1% gold chloride for 15 minutes and immersed into acidulated distilled water (6 drops of acetic acid in 50 mL of distilled water for eight to 12 hours). The endothelium, along with a few stromal lamellae from the deeper parts of the cornea, was then gently dissected and removed in 70% ethyl alcohol. Additional dehydration and a clearing in toluene were then performed. The corneas were flat-mounted in a synthetic mounting medium (Malinol). After mounting, the samples were immediately observed, and the nerve fibers in the central cornea were photographed under the light microscope. One hundred nerve-beading intervals of ten nerves per each sample (ten nerve-beading spaces per one nerve) were measured in prints magnified ×950.

Nonspecific Acetylcholinesterase Reaction.—The remainder of the left cornea was immediately transferred to a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M sodium cacodylate. The cornea with a thin scleral rim was then dissected into quadrants. After 60 minutes in fixative, each sample was washed with 0.44M sucrose and refrigerated overnight. Samples were incubated at room temperature for 24 hours in Karnovsky-Root medium[16] (a modification of the Koelle-Friedenwald formula[17]), without inhibitor for demonstration of nonspecific acetylcholinesterase activity. These samples were dehydrated with ethyl alcohol, cleaned in toluene, and flat-mounted in synthetic mounting medium. They were observed by light microscopy, and the parenchymal nerve density of the periphery was quantified with the use of an ocular reticle as described elsewhere.[18] The data were statistically analyzed by an unpaired Student's t test.

Plastic Embedding of Tissue for Light and Electron Microscopy.—After surgical removal, the eyes were immediately immersed in a mixture of 2% paraformal-

Accepted for publication Feb 17, 1984.

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dehyde and 2% glutaraldehyde in 0.1M sodium cacodylate with 0.01% calcium chloride. The eyes were slit under fixative and allowed to fix for approximately 48 hours at 4 °C. Specimens were rinsed in 0.1M sodium cacodylate in 5% dextrose, postfixed in 2% osmium tetroxide for two hours, stained enbloc in aqueous 2% uranyl acetate, dehydrated with an alcohol series, and embedded in epoxy (Poly-Bed 812) resin. Sections for optical microscopy were cut at central, midcentral, and limbal regions and stained with Stevenel's blue.[19,20] With 1-μm thick sections as a guide, pyramidal mesas were trimmed in the plastic blocks. Ultrathin sections containing the desired area of the cornea were cut with a diamond knife; these sections were stained with lead citrate and studied under the electron microscope.

RESULTS

The diabetic status of each animal was assessed regularly with the outlined clinical laboratory tests. The resulting morphologic changes are described in chronologic sequence.

Week 1

No substantial morphologic differences between the diabetic and control animals could be detected.

Week 4

Beginning four weeks after the induction of diabetes and at all subsequent time points, all diabetic rats demonstrated a significant difference from the controls in all the observed clinical variables (Table 1).

No significant differences were noticed at this four-week point between the diabetic and control groups with any of the histologic techniques used for qualitative or quantitative analysis of corneal innervation.

Week 16

Ultrastructural analysis demonstrated changes in the corneal nerves. Irregularities in the basal lamina of Schwann cells appeared in the form of thickening and thinning more frequently in diabetic rats than in controls (Fig 1 and Table 2). In spite of these changes in Schwann cells, the myelinated part of corneal nerves and intraepithelial nerves did not demonstrate any significant alterations.

Gold impregnation revealed marked irregularity in the periodicity of nerve fiber beading in the diabetic animals as compared with periodicity in controls (Figs 2 through 4) ($P < .01$ by Student's t test).

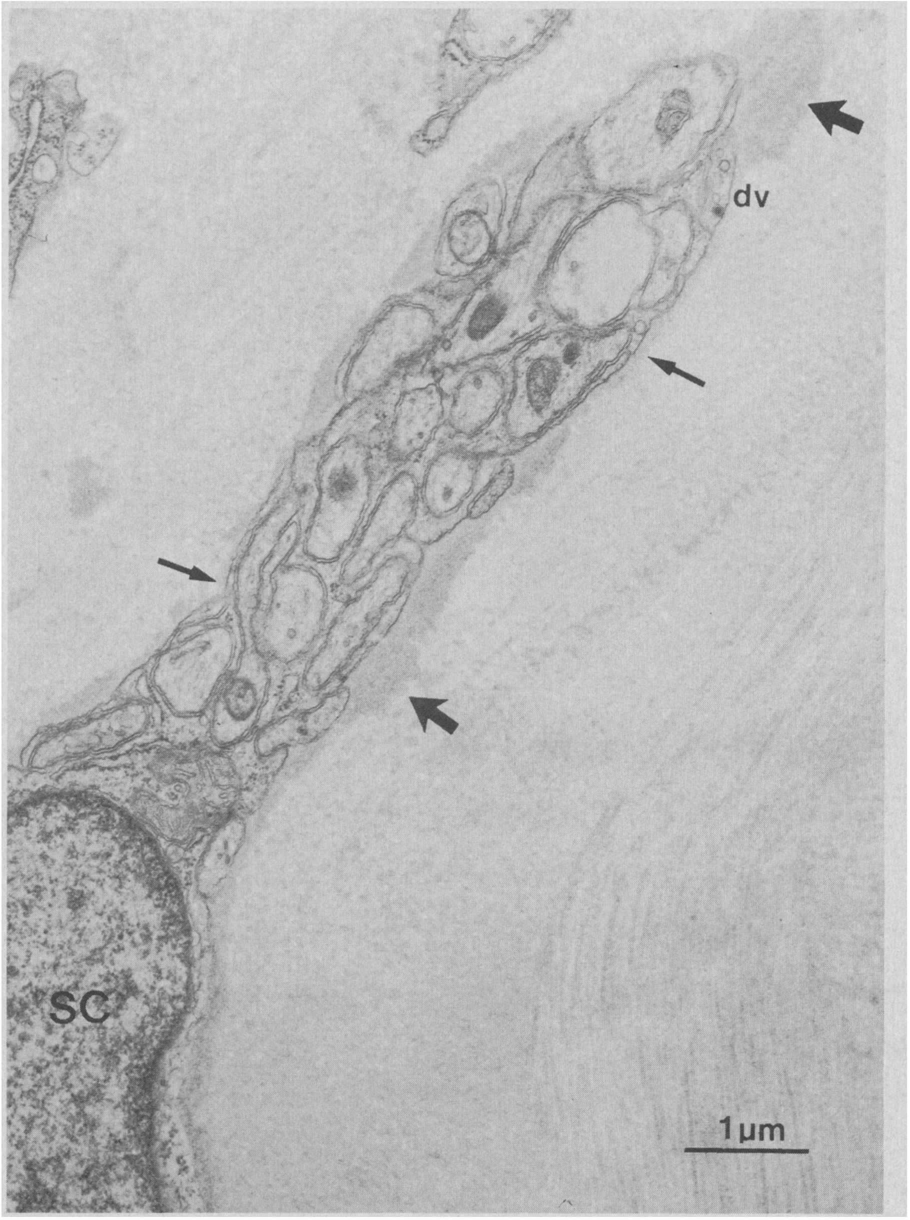
Week 36

The difference in the nerve-beading pattern between the two groups became much more pronounced (Figs

Table 1.—Indicators of Diabetic Status

Group and Time, wk	No. of Rats	Fasting Blood Glucose, mg/dL	Body Wt, g	Urine Output, g/Day	Urine Glucose, mg/dL	Urine Ketones, mg/dL
1						
Control	6	45-90	157.3 ± 7.6	...	100 or negative	Negative
Diabetic	6	>250	134.2 ± 25.2	...	≥2,000	80-160
4						
Control	5	45-90	290.8 ± 14.6	13.3 ± 3.7	100 or negative	Negative
Diabetic	5	>250	181.6 ± 14.7	>100	≥2,000	80-160
16						
Control	5	45-90	495.2 ± 46.8	17.6 ± 4.8	100 or negative	Negative
Diabetic	5	>250	291.7 ± 26.9	>100	≥2,000	80-160
36						
Control	6	45-90	546.6 ± 51.8	25.8 ± 3.3	100 or negative	Negative
Diabetic	6	>250	300.6 ± 43.0	>100	≥2,000	80-160

Fig 1.—Cornea in 16-week-old diabetic rat demonstrating irregularities in basal lamina of Schwann cells in form of thickening (thick arrows) and thinning (thin arrows). SC indicates Schwann cell; dv, dense core vesicle (original magnification ×17,640).



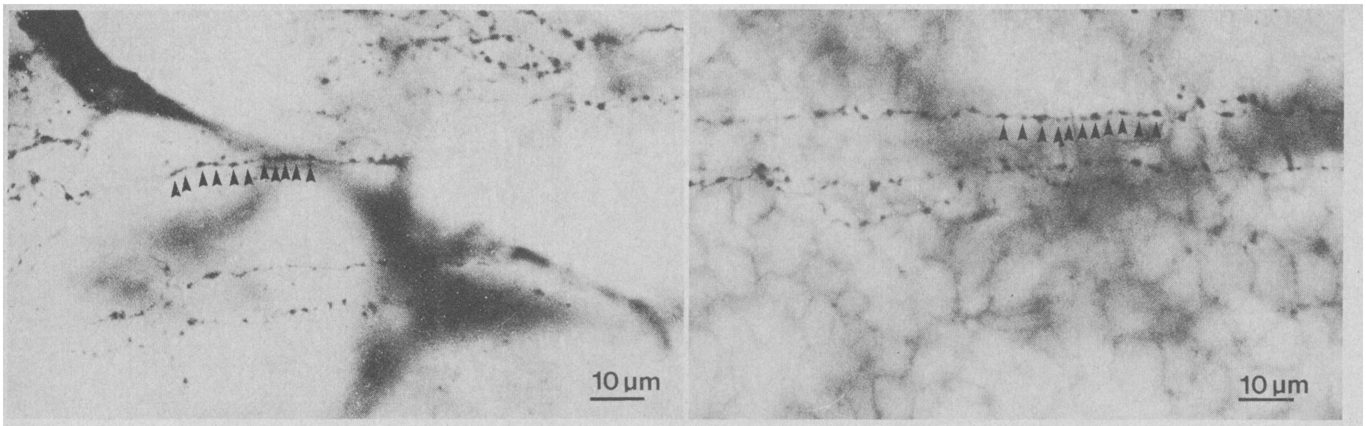


Fig 2.—Nerve beading by gold chloride impregnation (arrowheads) in control rats at 16 (left) and 36 weeks (right) (×950).

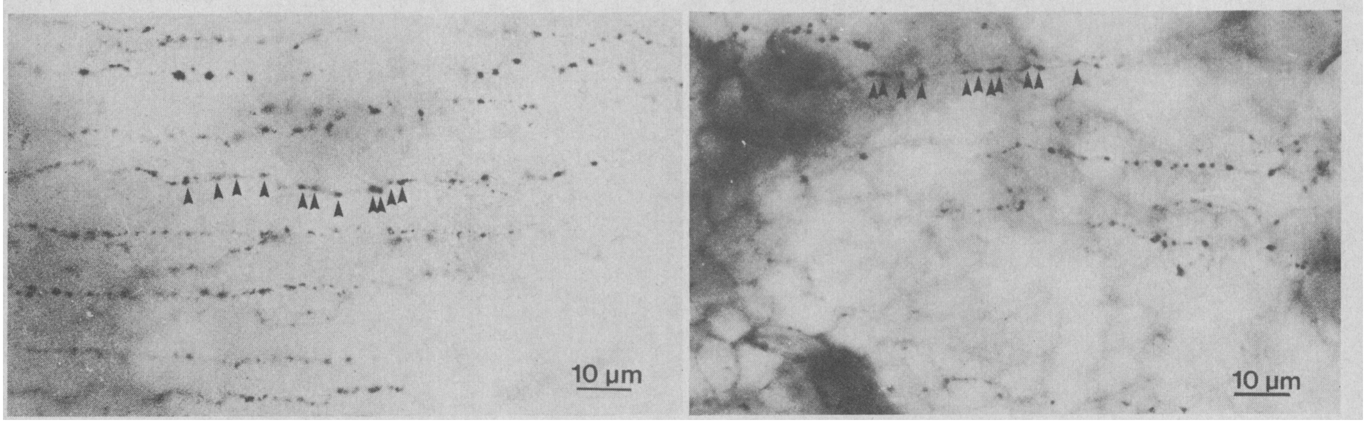


Fig 3.—Nerve beading by gold impregnation (arrowheads) in diabetic rats at 16 (left) and 36 weeks (right) for comparison with beading in Fig 2. Note interbeading spaces of diabetic rats are irregular (×950).

Table 2.—Number of Schwann Cells Showing Irregularity of Basal Lamina

	No. (%) Irregular*	
Time, wk	Diabetic Rats	Control Rats
4	2/37 (5.4)	2/40 (5.0)
16	6/69 (8.7)	2/42 (4.8)
36	12/55 (21.8)	4/40 (10.0)

*Number of cells with irregular basal lamina over total number of observed Schwann cells.

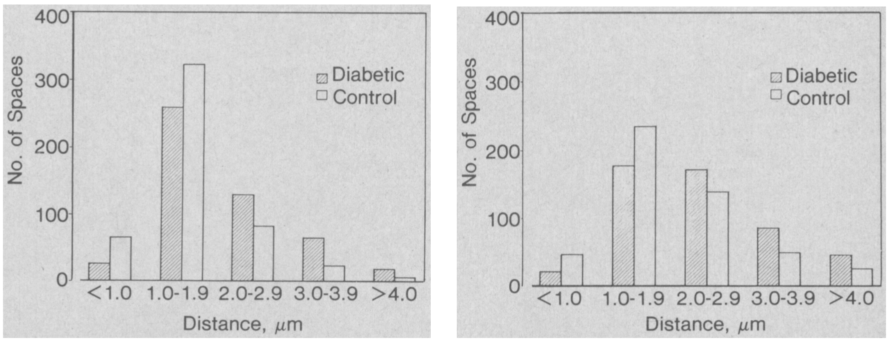


Fig 4.—Histograms of periodicity of nerve beading in diabetic and control groups. Differences of distribution between groups were observed at 16 weeks (left). Differences were more pronounced at 36 weeks (right).

3 and 4) ($P<.01$ by Student's t test). In addition, evidence of the irregularities in the thickness of the basal lamina of Schwann cells became much more frequent as compared with the controls (Fig 5 and Table 2). A notable electron microscopic feature at this time was the occasional presence of axonal degeneration in the diabetic rats but not in the controls (Fig 5). Even at this stage, changes were conspicuously absent in myelinated nerves, in intraepithelial nerves, and in the peripheral stromal nerve density using nonspecific acetylcholinesterase reaction ($P>.05$). With the use of 1-μm thick-plastic-embedded sections, no demonstrable difference was found between the two groups on light microscopy.

COMMENT

Corneal complications of diabetes mellitus are poorly understood clinical phenomena.[1] Decreased corneal sensitivity,[6-11] noted in these patients, may be an underlying factor for some of these problems.[21] It is not clear, however, if this change in sensitivity is a reflection of altered corneal innervation. A number of reports[22-25] have alluded to the changes in other peripheral nerves of the body in diabetics, whereas others have demonstrated duplication and thickening of the basal lamina surrounding the

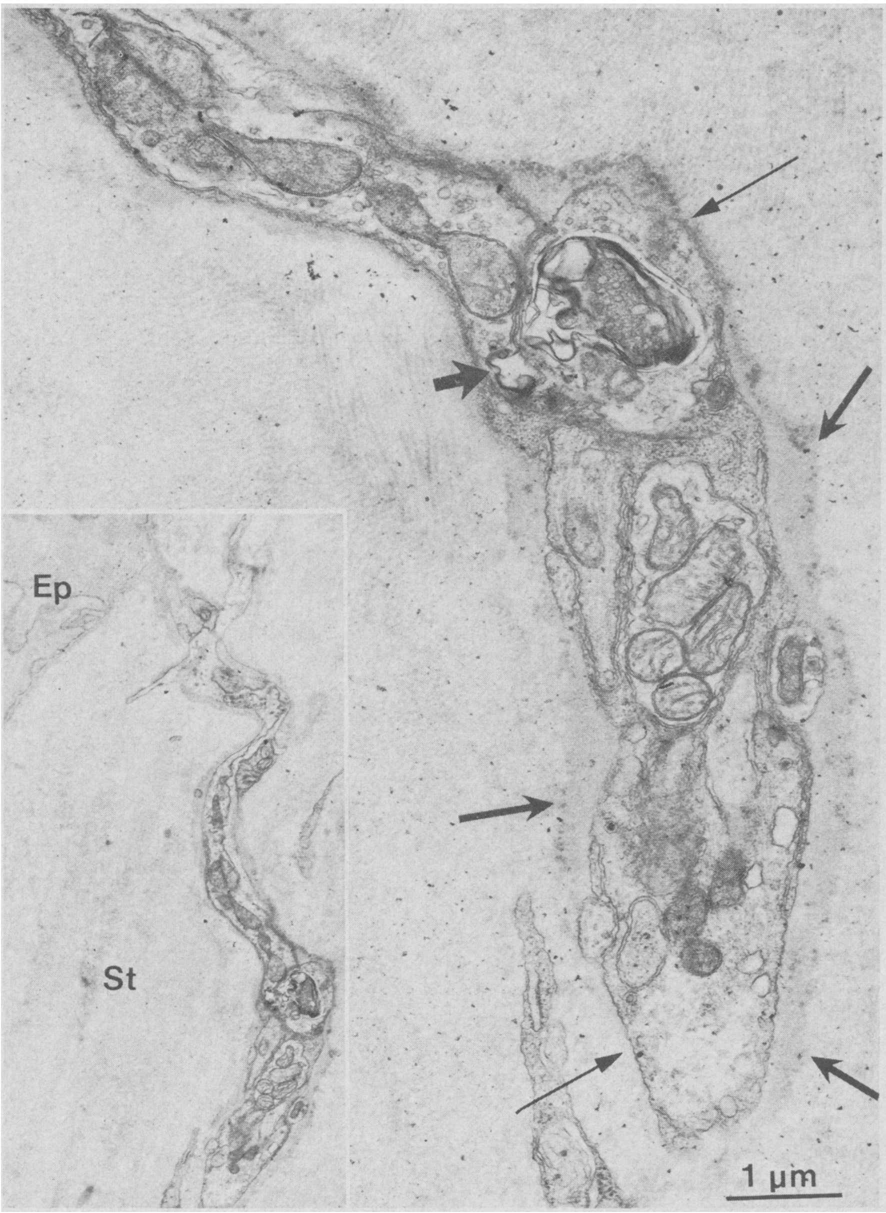


Fig 5.—Irregularities in basal lamina of Schwann cells in 36-week-old diabetic rat are demonstrated as thickening (thick arrows) and thinning (long thin arrows). Axonal degeneration (short arrow) is also seen (original magnification ×16,000). Inset, Note epithelium (Ep) and stroma (St) (original magnification ×4,060).

endoneural capillaries.[26-28]

In the present study, we observed that the basal lamina of Schwann cells of diabetic corneas had irregular patches of thickening and thinning. To our knowledge, this finding has not been previously reported. Rats began to demonstrate this phenomenon 16 weeks following induction of diabetes, with more marked and frequent changes occurring at 36 weeks, when compared with age-matched control animals. Although these changes are probably a manifestation of the aging process,[29] our study clearly demonstrated that such changes become much more pronounced and frequent in diabetics, suggesting that diabetes may accelerate these age-related alterations.

We observed occasional axonal degeneration in unmyelinated corneal nerves of the 36-week-old diabetic rats. This change could be the corneal component of distal diabetic polyneuropathy.[28,30-32] It is conceivable that in diabetes the metabolic support for the axon normally provided by Schwann cells may be impaired, which is a fact supported by alterations in basal lamina of Schwann cells in corneal nerves. At this time, however, it is not possible to rule out a concomitant effect on the axon or neuronal soma.

Segmental demyelination of the peripheral nerves has been reported as characteristic of diabetic peripheral neuropathy.[28,33,34] The myelinated portions of corneal nerves in the diabetic animals of the present study demonstrated no such change. Our results indicate that the early manifestations of corneal neuropathy in diabetes begin in nonmyelinated branches. Changes in the myelin, if they occur at all, may be a late complication.

Another significant observation in this study relates to the distribution of nerve beading. Diabetic rats had a marked irregularity in the periodicity of nerve beading. Since the exact functional correlates of nerve beading are not yet known,[35,36] the importance of these changes remains to be elucidated. However, these alterations could represent a morphologic counterpart of the changes in norepinephrine levels described by Felten et al[37] in rats with streptozocin-induced diabetes.

From the present study, it appears that alterations in the thickness of the basal lamina of Schwann cells, axonal degeneration, and irregular distribution of nerve beading constitute a constellation of early pathologic changes in corneal innervation of diabetics. These observations may provide a basis for some of the observed clinical phenomena. Since changes occur in nerve fibers innervating an avascular tissue, vascular involvement cannot be a necessary prerequisite for the development of diabetic neuropathy as is commonly believed.[22] In addition, our study highlights the deleterious effects of diabetes on nonmyelinated nerves, a phenomenon not previously observed to our knowledge in any other peripheral nerves.

In conclusion, the early manifestions of diabetes mellitus may indeed occur in the nonmyelinated nerves before manifesting in myelinated nerves. Our observations may provide a basis for the understanding of clinical corneal manifestations encountered in diabetic patients. The diabetic cornea provides an excellent model to study the effects of diabetes on peripheral nerve branches without additional effects of vascular abnormality or mechanical trauma that complicate the interpretation of results obtained by studying other peripheral nerves.

This study was supported in part by grant 5R01EY02632 from the National Institute (Dr del Cerro) and by funds from Coopervision, Inc (Dr Aquavella), and the Rochester Eye Bank (Dr Ishida).

Maria Mathe provided technical assistance.

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