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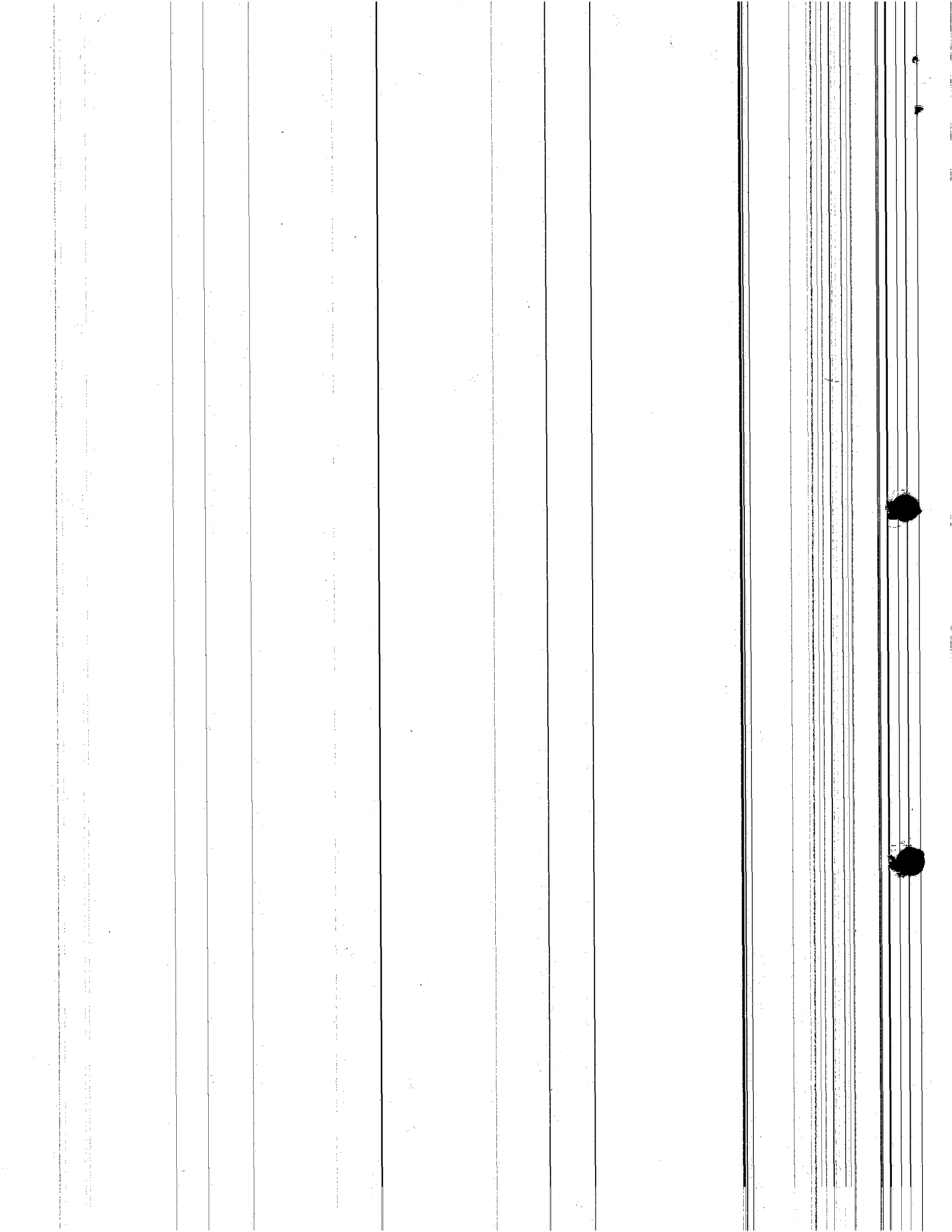
Ref.: Expediente No. 02.089.153
Solicitud de Patente a nombre de Novartis AG y Université Louis Pasteur

Yo, JIMENA ESCOBAR URIBE, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 94-76, Of. 305, obrando como apoderada de las sociedades Novartis AG, domiciliada en Basilea, Suiza y Université Louis Pasteur, domiciliada en Strasbourg, Francia, solicitantes de la patente de la referencia, en respuesta a su oficio No. 11194 notificado por fijación en lista el día 5 de octubre de 2006, me permito acompañar los siguientes documentos solicitados por ese despacho:

- CARWILE MARY E ET AL: "Rod outer segment maintenance is enhanced in the presence of bFGF, CNTF and GDNF." Database accession no. PREV199800399007 XP002135774 & EXPERIMENTAL EYE RESEARCH JUNE, 1998, vol. 66, no. 6, juin 1998 (1998-06), pages 791-805
- J. SAHEL ET AL.: "Glial cel line-derived neurotrophic factor (GDNF) induces histological and functional neuroprotection of rod photoreceptors in the retinal degeneration (rd) mouse." INVESTIGATIVE OPHTHALMOLOGY & VISUL SCIENCE, vol. 40, no. 4, 15 mars 1999 (1999-03-15), page S966 XP000891599 ST. LOUIS, US
- M. FRASSON ET AL.: "Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse." INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE, vol. 40, no. 11, octobre 1999 (1999-10), pages 2724-2734, XP000891598 ST. LOUIS, US

Espero con esto haber dado cabal cumplimiento a lo solicitado por ese despacho y de conformidad con lo indicado en los artículos 3°, inciso 3, 35 inciso 2 y 59 inciso 2 del CCA, al analizar este memorial, atentamente solicito se traten todas las cuestiones planteadas con motivo del mismo.

Señor Superintendente,



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Rod Outer Segment Maintenance Is Enhanced in the Presence of bFGF, CNTF and GDNF

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We employed a morphological assay of outer segment collapse to determine if growth factors or other supplements directly affect dissociated rod photoreceptors in vitro. The morphological changes in outer segments were correlated with the light responsiveness of rods. Time-lapse video microscopy was used to observe the collapse of rod outer segments from isolated single cells and small clumps of cells. A consistent pattern of outer segment collapse into the inner segment was observed, yielding a convenient assay of the effects of neurotrophic factors on photoreceptor functional maintenance. The functional state of rods, defined as light-responsiveness, was measured with suction electrode recordings and matched with the various stages of outer segment collapse. Ciliary neurotrophic factor (CNTF) and glial cell-line-derived neurotrophic factor (GDNF) at a high concentration, yielded statistically significant improvements in rat outer segment survival times. Basic fibroblast growth factor (bFGF), which rescues photoreceptors in several rodent models of retinal degeneration, produced a significant increase in survival time in the presence of the cofactor heparin. In 4 out of 10 cases using human tisue, bFGF also yielded a significant increase in survival times. When brain-derived neurotrophic factor (BDNF) was applied to rat rods, outer segment survival times did not change. Outer segments collapsed more quickly when either pigment epithelial cell derived factor (PEDF) or sugar *N*-acetyl D-galactosamine (NAD-gal) were present. Our results show that rod photoreceptors can respond to bFGF, GDNF and CNTF in vitro and provide evidence for a direct effect of these neurotrophic factors on rods. The rapid collapse of isolated photoreceptors in this model provides a convenient means for testing various neurotrophic agents and the induced cellular responses.

Key words: rat; human; retina; photoreceptor; rod; growth factor; bFGF; CNTF; GDNF; BDNF; PEDF.

1. Introduction

Several pathological conditions result in loss of vision due to degeneration of photoreceptors, including retinitis pigmentosa and macular degeneration. The etiology of the photoreceptor degeneration in these cases is not known, and as yet there is no known effective treatment. Several growth factors and neurotrophic factors have been shown to have survival-promoting activity in retinal tissues. Brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) are protective against pressure-induced retinal ischemia, and many growth factors and neurotrophins have shown some ability to prevent light-induced photoreceptor degeneration in rodents (LaVail et al., 1992; Unoki and LaVail, 1994). Treatment with bFGF also reduces rod loss due to laser injury (Schuschereba et al., 1994) as well as age-related or scrapie-induced photoreceptor cell loss in rodents (Fraser et al., 1997; Lin et al., 1997). CNTF has been shown to slow photoreceptor degeneration in mouse models such as *rd* (retinal degeneration) and rhodopsin mutant mice (LaVail et al., 1996). The retinal effects of growth factors in these in vivo models are seen after injection of the factor either intravitreally or subretinally into an intact eye. Commonly, the prevention of insult-induced outer nuclear layer (ONL) thinning in the test eye is used as an outcome measure. In these systems the effect of the growth factors could be directly on the photoreceptors or mediated indirectly through the actions of the retinal pigment epithelium (RPE), Müller cells or other retinal neurons. Glial cell-line-derived neurotrophic factor (GDNF) is a newly isolated factor which was originally characterized for its ability to cause a dose-dependent increase in the survival of dopaminergic neurons (Lin et al., 1993). Subsequently, GDNF has been found to be expressed in numerous areas including the retina and to have effects upon peripheral sensory and motor neurons (Trupp et al., 1995; Norsat et al., 1996). *N*-acetyl D-galactosamine (NAD-gal) is one of several sugars that has been shown to be permissive of outer segment formation in vitro in the absence of an RPE cell layer in Xenopus (Stiemke and Hollyfield, 1994). Pigment epithelial cell derived factor (PEDF) is a retinal factor that increases viability of cerebeller granule cells in vitro (Taniwaki et al., 1995, 1997).

Mature vertebrate photoreceptors have been maintained in culture for extended periods of time and

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continue to express opsin; however, these cells lack outer segments and compartmentalization of transduction proteins (Politi and Adler, 1986; Hicks and Courtois, 1992; Mandel, MacLeish and Townes-Anderson, 1993; Hicks et al., 1994). We have developed a model to observe the collapse of individual rod photoreceptor outer segments and have related these morphological changes to light responsiveness of the cells. We report here the effects of bFGF, BDNF, GDNF, NAD-gal, CNTF and PEDF on the maintenance of the outer segments of isolated single photoreceptors and show the usefulness of this model in quantifying the survival-promoting activity of neurotrophic factors. Preliminary reports of this work have been presented in abstract form (Carwile, Dooner and Kraft, 1995; Culbert et al., 1996).

2. Materials and Methods

Acquisition and Preparation of Tissue

Human donor eyes were acquired from the Alabama Eye Bank or from exenterations due to craniofacial tumors. Acceptance criteria were that the globe had to be available within 3 hr of death (Table I) and that the donor must not have been on a respirator for more than 3 days prior to enucleation. Specific times of acquisition for each case are given in Table I. No ocular complications were reported, but two donors were identified as septic. After removal of the sclera, RPE, and vitreous, the isolated retinas were cut in pieces approximately 8 × 5 mm and stored at 4°C for 3–9 days in Leibovitz's L-15 medium (Gibco, Grand Island, NY, U.S.A.) containing gentamicin (0·1 mg ml^{-1} or 10 μg ml^{-1}) (Gibco) and an additional 10 mM glucose. The storage medium was changed every 2 days. Human retinal samples were generally taken from peripheral retina outside the central 9 mm but at least 5 mm from the ora serrata. Donor tissues are typically exposed to significant levels of light during enucleation and corneal harvesting procedures. Because of this light exposure, it was necessary to regenerate visual pigment in human tissues used for electrophysiology. To regenerate visual pigment, tissue was stored in L-15 medium which included 11-*cis*-retinal (about 30 μg ml^{-1} in 0·5% ethanol). Photoreceptor dissociations and experiments with these tissues were performed under infrared and dim red light.

Hybrid Fisher 344-Brown Norway rats and Long-Evans hooded rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN, U.S.A.). When possible, litter mates were used in consecutive experiments testing the same compound. Rats were broadly categorized into two age groups younger, from 1–10 months of age and older, from 18–25 months of age. Rats were killed in a CO_2 chamber, and both eyes were immediately enucleated. The eyes were then sectioned through the pars plana. The neurosensory retina was peeled from the RPE, cut at the optic nerve and removed. Each retina was halved, and the hemiretinae were stored at 4°C in Leibovitz's L-15 medium containing 0·1 mg ml^{-1} gentamicin (Gibco, Grand Island, NY, U.S.A.). Generally four experiments were carried out per rat; interleaved trials of test and control media consisted of a paired control and test on the day of killing and a second pair of experiments on the day after killing. In some instances all experiments were performed on the day of killing.

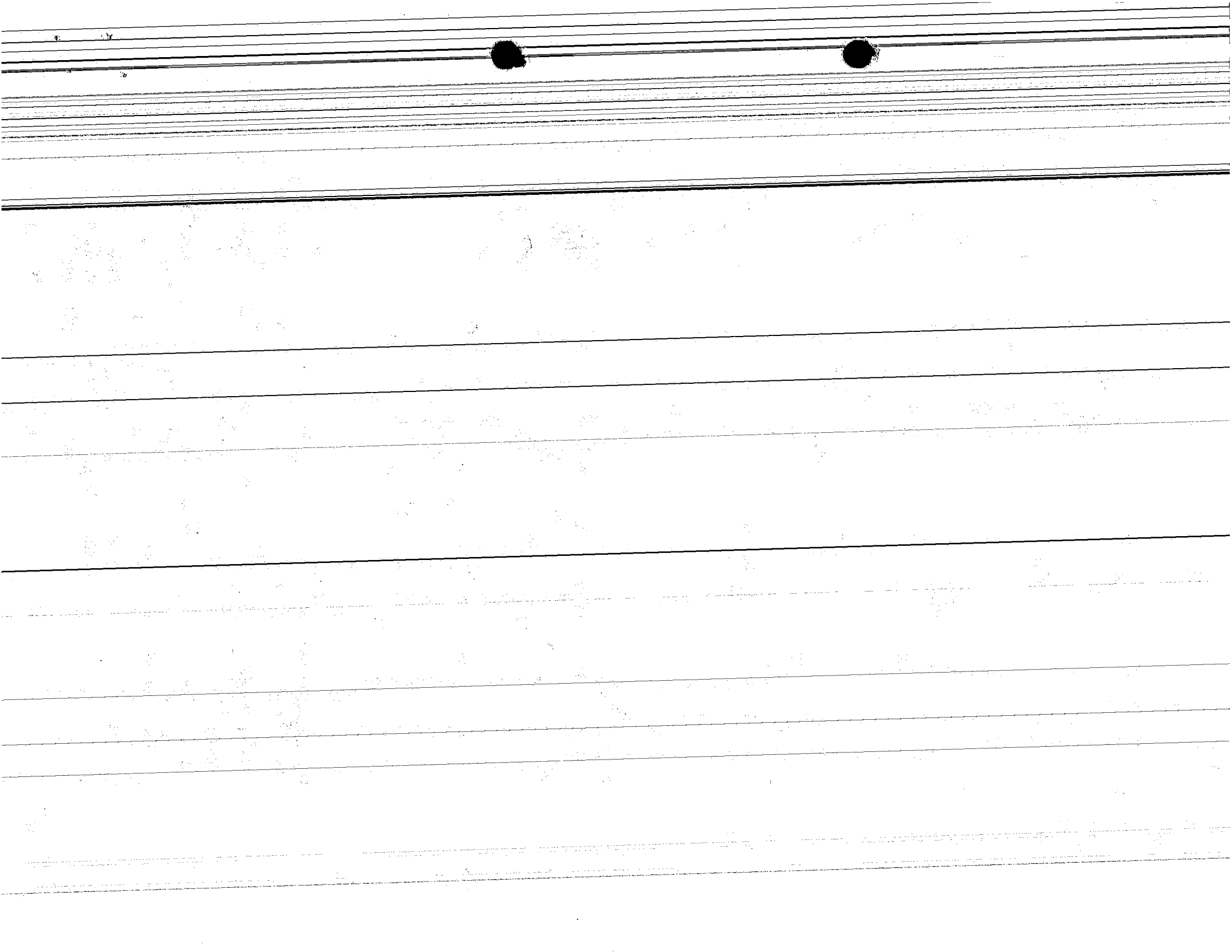
At the beginning of each experiment, a piece of retina was finely chopped in HEPES medium with a razor blade. The pieces were incubated in 0·5 ml DNase I (0·1 mg ml^{-1}, pH 7·4) (Sigma No. D-5025, St. Louis, MO, U.S.A.) for 15 minutes at 23°C. The temperature was elevated to 34·5°C for rat retina and 37°C for human retina by perfusion of the chamber

TABLE I

Tissue sources, sex, age and delay times

Eye	Sex, age	Enucleation delay (hr)[a]	Total delay (hr)[b]	Cause of death/enucleation
1	M88	1:27	3:04	post operative hemorrhage
2	M75	1:35	3:35	cardiopulmonary arrest
3	M73	0:56	1:41	myocardial infarction; sepsis
4	M75	2:01	3:41	myocardial infarction
5	F50	0:00	0:35	squamous cell carcinoma
6/7	M16	2:26	3:49	lymphoma
8	F79	1:59	2:24	chronic obstructive pulmonary disease
9	M78	1:40	3:02	multiple myeloma
10	M87	1:29	2:02	cardiogenic shock
11	F39	1:14	1:52	pulmonary hypertension; sepsis
12[c]	F79	1:10	2:12	lung cancer
13[c]	M69	1:30	2:35	myocardial infarction

[a] The first delay represents the time from cessation of retinal circulation to enucleation.
[b] The second delay includes the time before retinal isolation and storage at 4°C.
[c] Eyes used for electrophysiology.



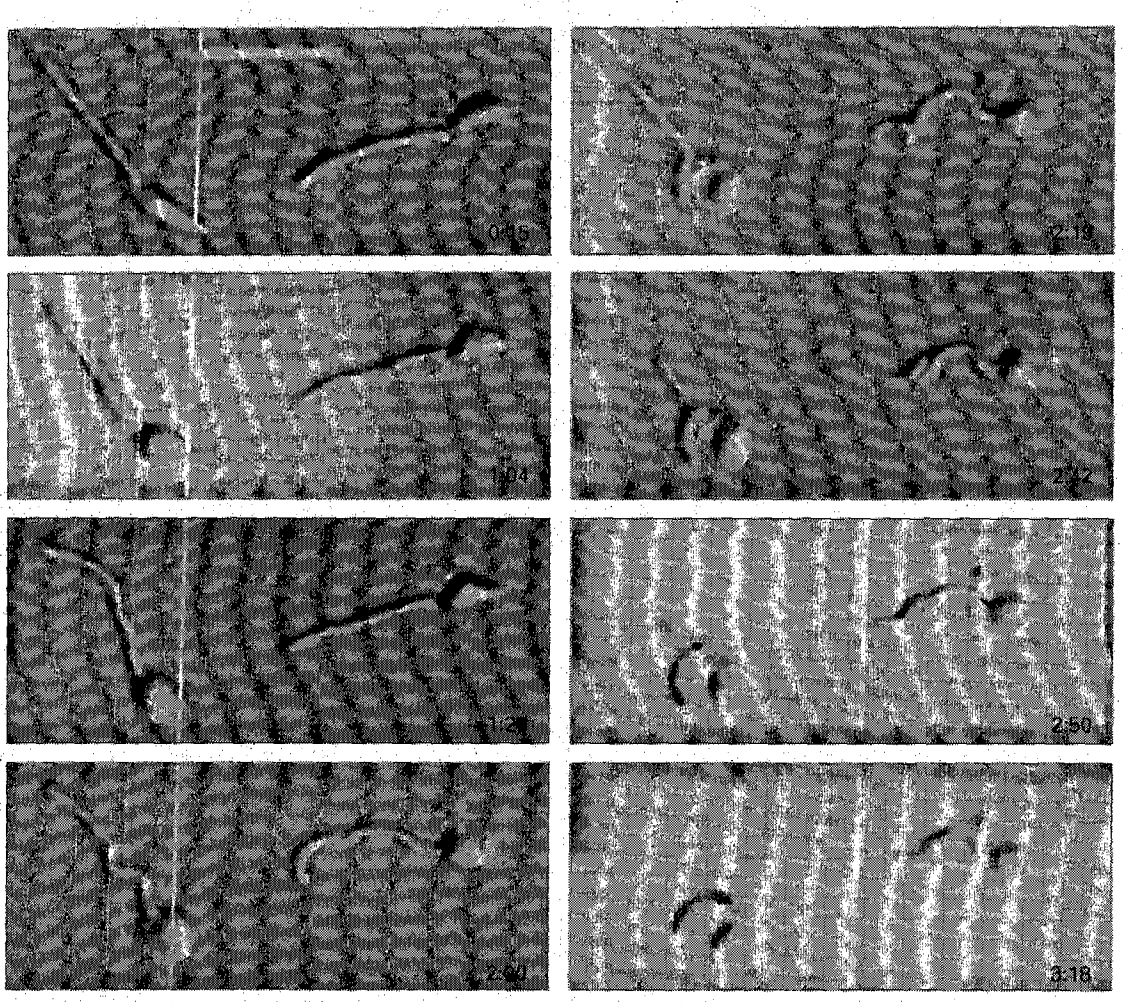


FIG. 1. Video images of human RIS/ROS taken during the course of a 4-hr experiment showing collapse of the outer segments at 37°C. Initial length of both outer segments was 24 μm. Time elapsed from the start of the experiment is shown in the lower right of each panel. Scale bar = 20 μm.

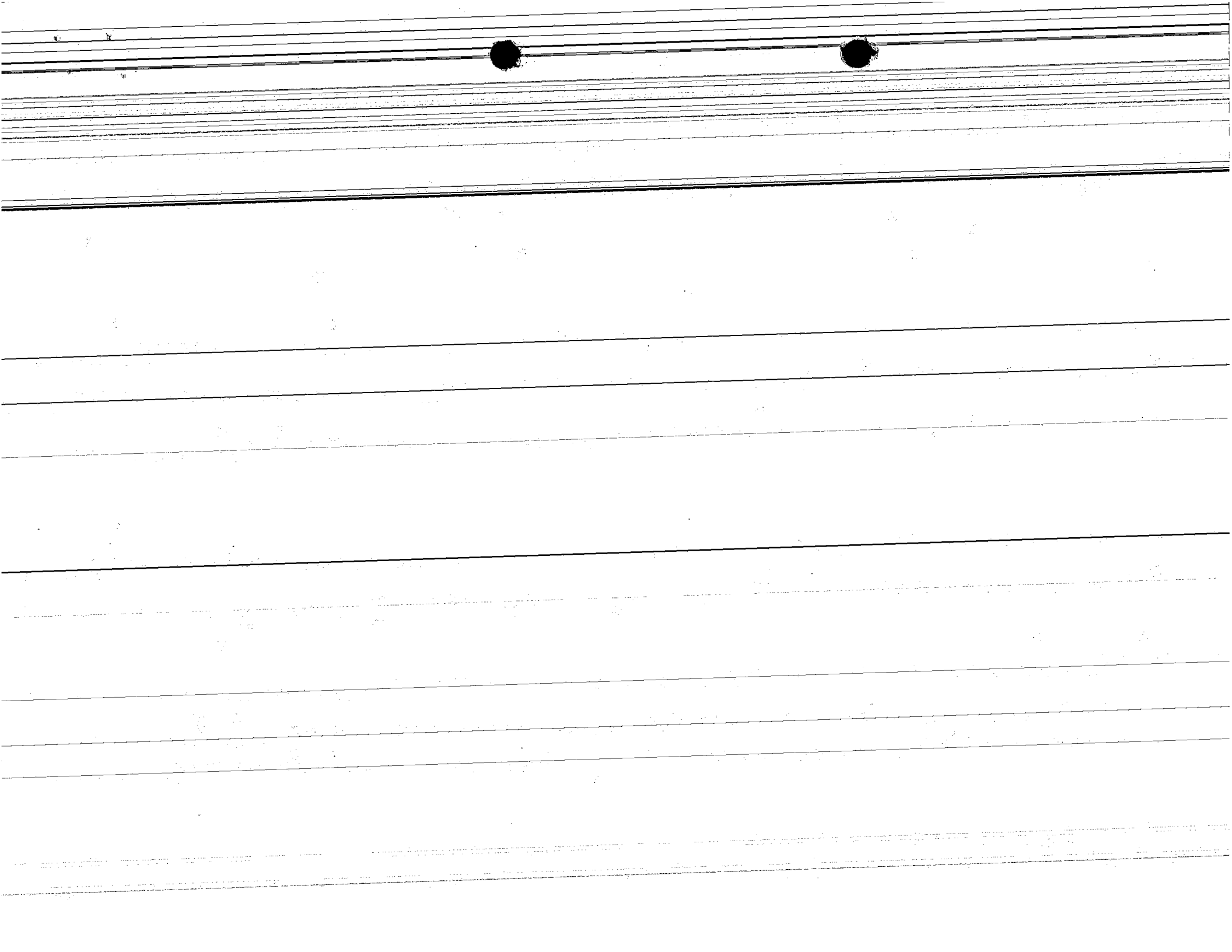
with heated solutions driven by a syringe pump (Razel, Inc., Stamford, CT, U.S.A.). The experimental chamber was a Teflon-coated brass ring with a round glass coverslip as the bottom. Carbon dioxide and O_2 were streamed over the solution in a ratio 5:95% to maintain a pH of 7·5. The experimental chamber was perfused at 7·7 ml hr^{-1}, yielding one volume exchange every 20 minutes. Syringes, delivery lines and the coverslip were changed for each solution to ensure no lingering effects of prior test media. For electrical recordings of light responses, all procedures were identical except that rats were dark-adapted for 1 hr prior to death, and experiments were performed under infrared and dim red light. Experiments were designed and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the tenets of the Declaration of Helsinki.

Dissociation trials performed in the presence of proteases greatly compromised rod outer segment survival. After a 10-minute exposure to papain solution (Makino et al., 1990), human rods changed almost immediately, beginning with a swelling and lengthening of outer segments. Pronase (1 mg ml^{-1}) treatment of the rat retina consisted of a single 12-minute incubation of the chopped retina after Lolley et al. (1986). Pronase treatments reduced survival times of rats rods by up to 60%. Because of the adverse effects of proteases, we discontinued these treatments, and all data presented below comes from cells isolated by mechanical chopping and DNase treatment.

Trypan blue solution (Sigma) was superfused onto isolated cells to test viability in four human retina experiments. In the first experiment the dye was administered at the start of the experiment (0·4% for 5 minutes) to test viability of single cells shortly after mechanical dissociation. The dye was applied at or near the end of the remaining experiments (0·05% for 10–30 minutes) to test viability of photoreceptors with completely collapsed outer segments. The experimental chamber was quickly rinsed with HEPES buffer to observe the uptake of dye.

Light Stimuli and Electrical Recordings

Photocurrents were recorded with suction electrodes (Baylor, Lamb and Yau, 1979) using an Axopatch 1-D amplifier (Axon Instruments, Foster



City, CA, U.S.A.) with data acquisition hardware and LabVIEW software (National Instruments, Austin, TX, U.S.A.). The averaging and analysis routines written using Igor (Wavemetrics, Lake Oswego, OR, U.S.A.) were kindly provided by Dr David Schneeweis (San Francisco, CA, U.S.A.). The optical bench focused a 425-μm-diameter spot of light at the specimen plane of a Zeiss (Hanover, MD, U.S.A.) Axioscope microscope. Neutral density filters (Reynard, San Clemente, CA, U.S.A.) were used to control stimulus intensity (Kraft, Schneeweis and Schnapf, 1993). Electrodes were slightly oversized to avoid any physical damage to outer segments; however, because of the large pipette tip diameters, electrode seals were poor, and current collection was relatively inefficient. Consistent recordings were obtained by using the same electrode with a known seal resistance to record from a given series of cells with differing morphologies. The solution in the recording pipette was HEPES solution, described below; it did not contain bovine serum albumin (BSA) or any neurotrophic factors.

Solutions

The control solution was Locke's solution with BSA (1 mg ml^{-1}). BSA was included in the media to block non-specific binding and ensure growth factor delivery. The Locke's solution contained the following in mM: NaCl (120), $NaHCO_3$ (20), KCl (3·6), $MgCl_2$ (2·4), $CaCl_2$ (1·2), HEPES (3·0), EDTA (0·02), and glucose (10), supplemented with vitamins and amino acids (Gibco, BME). The HEPES medium contained in mM NaCl (140), KCl (3·6), $MgCl_2$ (2·4), $CaCl_2$ (1·2), HEPES (3·0), and glucose (10). Crystalline BSA (Sigma, A7511) was used for about 1/2 of the assays. After a test of the crystalline vs. Fraction V BSA (Sigma No. PB1600–100) showed no difference in outer segment survival times, Fraction V BSA was used in the remaining experiments. The test media contained one of the following: BDNF (25 ng ml^{-1}), GDNF (0·1 or 1 μg ml^{-1}), CNTF (10 ng ml^{-1}), PEDF (100 ng ml^{-1}), bFGF (25 ng ml^{-1}) or bFGF with heparin (40 ng ml^{-1}) (Sigma, No. H-3393). Heparin was added to the bFGF medium to facilitate binding of bFGF to high-affinity receptors (Klagsburn and Baird, 1991). Human recombinant bFGF was purchased from Quality Controlled Biochemicals (Hopkinton, MA, U.S.A.). Human recombinant BDNF was a gift of Regeneron Pharmaceuticals Inc. (Tarrytown, NY, U.S.A.), human recombinant GDNF was a gift from Amgen, Inc. (Thousand Oaks, CA, U.S.A.), and human PEDF was a gift of Dr Joyce Tombran-Tink (NEI). Rat recombinant CNTF was purchased from RBI (Natick, MA, U.S.A.). The BSA concentration was 1 mg ml^{-1} except when a higher concentration (5 mg ml^{-1}) was used for the GDNF experiments based on the advice of Dr Jean-Claude Louis (Amgen, Inc.).

In addition to growth factors, the sugar NAD-gal, was tested (0·5 mM, Sigma, A-2795). In the first experiment the retina was stored overnight in L-15 as described earlier. In the second experiment the retina was stored overnight in L-15 with NAD-gal. A mixture of several growth factors (MIX) was tested; MIX contained the following growth factor/cofactor concentrations (ng/ml): BDNF (25), GDNF (100), CNTF (10), PEDF (100), bFGF (10) and heparin (40). The MIX medium also contained the sugar, NAD-gal (0·5 mM), and taurine (6 μM). Taurine was not tested independently.

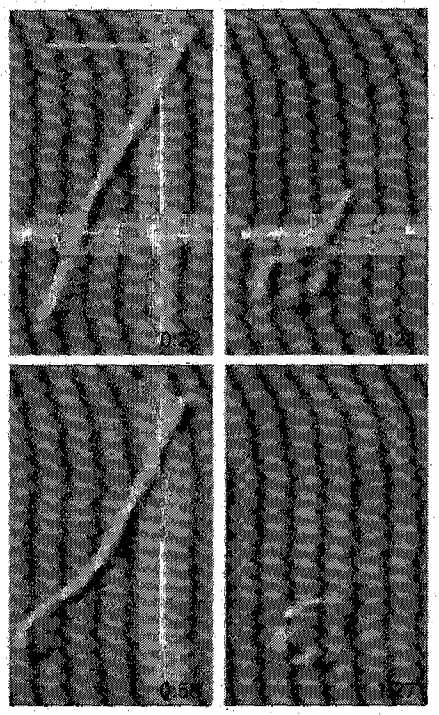
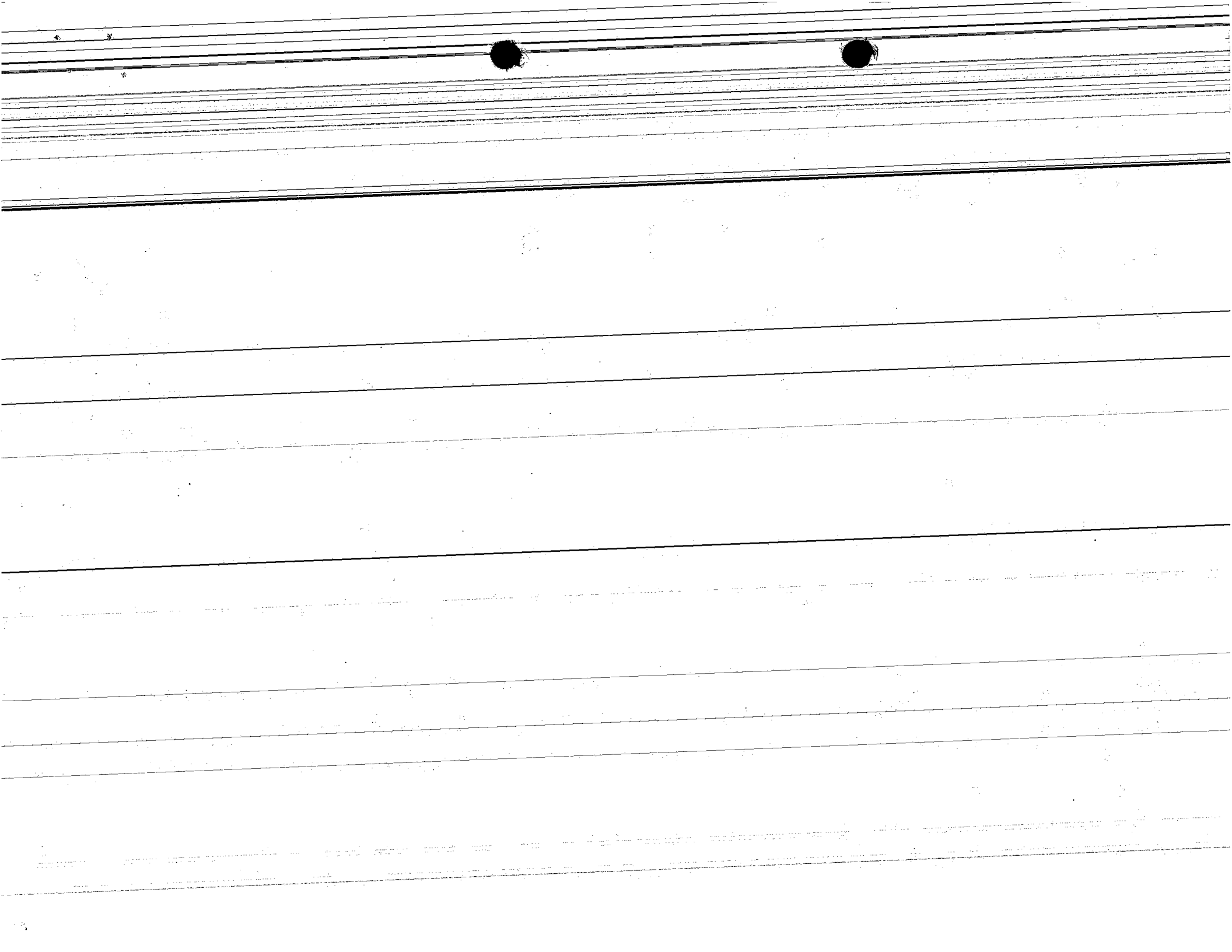


FIG. 2. Video images of a single rat RIS/ROS taken during the course of a 2-hr experiment showing collapse of the outer segment at 34·5°C. Initial outer segment length was 24 μm. Scale bar = 10 μm.

Video and Statistical Analysis

Cells were examined under 40X and 63X water immersion objectives with differential interference contrast optics and white light. Fine chopping of the retina produced small clumps of photoreceptors, single rod inner and outer segments lacking nuclei and synaptic endings (RIS/ROS), and many isolated outer segments. Rods chosen for observation had long (human mean = 21 μm; rat mean = 22 μm), straight outer segments that were clearly visible to the point of attachment with the inner segments (Figs 1 and 2, upper left panels).

The starting time of the experiments (t = 0) was defined as the time that DNase was rinsed away and warm perfusion medium began to fill the chamber. Images of the outersegments were digitized at 20-minute intervals in human experiments and 7-minute intervals in rat experiments (Data Translation Quick Capture or Scion LG-3 frame grabber, NIH *Image* software) until all or almost all the outer segments



under observation had completely collapsed. T_{50} values, defined as the time required for an outer segment to reach 50% of its starting length, were averaged and compared statistically. For data on human tissue, results from interleaved trials of control and test conditions collected over several days were pooled and compared using the Student *t* test. For the rat data, tissues were obtained from a more controlled and uniform source, and the results for all experiments were pooled and analysed using analysis of variance with Dunnett's post test (SAS software version 6.12). Rats were separated into two age groups for statistical analysis: young rats from 1 to 10 months-old, and old rats between 18 and 25 months-old.

3. Results

Time-lapse Observations of Outer Segments

Isolated RIS/ROS (rod inner and outer segments) have the highly organized appearance of photoreceptors in the intact eye (Fig. 1, t = 15 min). The mechanical dissociation technique yielded small clusters of 5–30 photoreceptor cells and many single RIS/ROS. Single bipolar and multipolar neurons that are commonly seen with enzymatic digests were not observed in this preparation. Red blood cells released from the retinal vessels during the chopping procedure were seen frequently and maintained their shape throughout the experiments. Figure 1 illustrates a typical sequence of changes in two human rods within a few hours after isolation at body temperature. Initilly, there were long, straight outer segments with spindle-shaped inner segments. This shape remained remarkably unchanged over the first hour of this experiment. The first visible changes were a rounding of the inner segment and a slight bending of the outer segment (Fig. 1, t = 1:25). Over the first 2 hr of the experiment, the outer segment length was constant even though bends and folds had developed. The inner segment became granular and continued to enlarge, and often its organelles became visible, suggesting that it may have been flattening against the coverslip (Fig. 1, t = 2:00). The outer segment then quickly collapsed into the inner segment. In short, the first third of the experiment showed little or no changes, the middle third included inner segment enlargement and outer segment bending, and in the final third, the outer segment degenerated or collapsed into the inner segment. To quantify the time course of these changes, we plotted the outer segment length vs. time and found the time required for the outer segment to be reduced to 50% of its starting length (T_{50}).

Viewing several areas of the experimental chamber and following up to 20 rods, we quantitatively described the results of an experiment as the mean T_{50} for the collection of cells observed. The T_{50} was determined for an average of 15 cells per human experiment and 14 cells per rat experiment. Most of

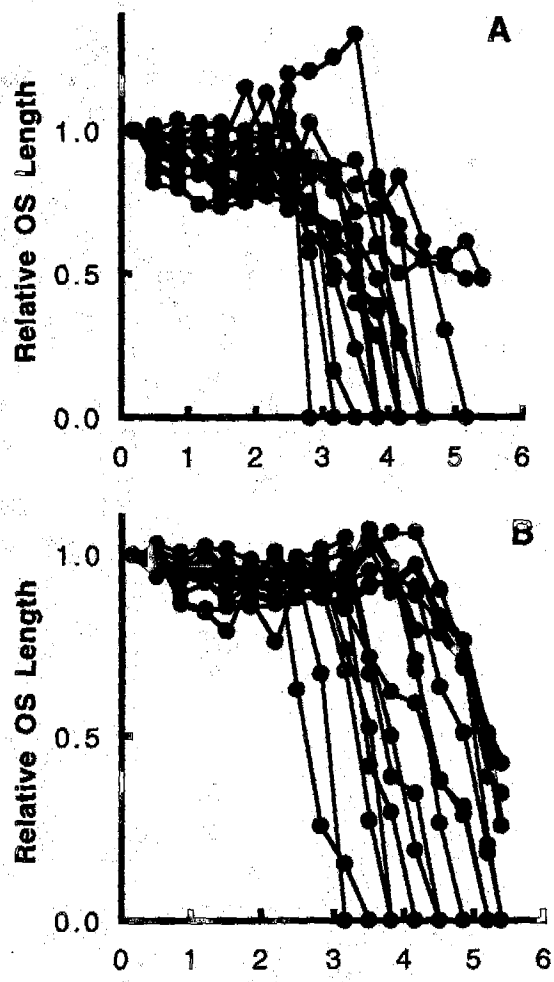


FIG. 3. Measurements of outer segment lengths from human experiments. Lengths have been normalized to starting lengths. (A) BSA experiment (1 mg ml^{-1}), T_{50} = 3·73 ± 0·75 (n = 17). (B) bFGF experiment (25 ng ml^{-1}), T_{50} = 4·11 ± 0·85 (n = 15).

the outer segments degenerated beyond 50% and eventually collapsed fully. The outer segments of roughly 5% of observable cells were excluded from consideration due to atypical changes. The cells appeared very granular and were essentially disintegrating on the coverslip, but the outer segment length was static because the distal portions of it remained attached to the glass. The degeneration rate of an outer segment was not related to its starting length; an analysis of T_{50} vs. starting length of 799 rat rods under the control conditions (BSA) resulted in a slope of −0·02. The mean starting length of the rat outer segments was 21·6 ± 2·8 μm (mean ± S.D.).

Features of rat rod outer segment collapse are depicted in Fig. 2. Rat rod inner segments were smaller and thinner than human inner segments. Often, the cilium connecting the outer and inner segments was visible. Fewer rat rods survived the isolation procedure, and outer segment collapse was much faster in preparations of rat tissue than in human tissue. Therefore, the temperature was lowered to 34·5°C for the rat experiments to slow morphological changes for accurate recording of outer segment length. On

TABLE II

Mean T_{50} values for single cells vs. clumped cells

	Singles	Groups
Eye 1, Locke's Soln*	0·96±0·14 (5)	1·73±0·11 (20)
Eye 2, Locke's Soln	1·67±0·14 (7)	1·77±0·13 (13)
Eye 1, BSA	2·00±0·50 (4)	2·13±0·10 (27)
Eye 2, BSA	2·83±0·17 (6)	2·63±0·14 (17)
Eye 1, bFGF	2·72±0·12 (7)	2·69±0·17 (25)
Eye 2, bFGF	2·67±0·20 (13)	2·70±0·12 (14)

Results are mean T_{50} values (hr)±S.E.M. for the number of cells in parentheses. *$P < 0·002$, statistically significant difference.

TABLE III

Effect of bFGF on human outer segment maintenance

	BSA	bFGF
Eye 1	2·11±0·10 (31)	2·69±0·13 (32) (†)
Eye 2	2·68±0·11 (23)	2·68±0·11 (27) (n.s.)
Eye 3	5·00±0·30 (16)	6·99±0·49 (18) (‡)
Eye 4	4·74±0·42 (12)	8·00±0·47 (9) (*)
Eye 5	9·44±0·62 (9)	7·49±0·73 (7) (n.s.)
Eye 6/7[a]	4·20±0·12 (65)	4·31±0·09 (87) (n.s.)
Eye 8	4·89±0·13 (64)	5·20±0·13 (47) (n.s.)
Eye 9	4·65±0·21 (41)	4·96±0·26 (43) (n.s.)
Eye 10	6·77±0·21 (55)	5·86±0·19 (43) (†)
Eye 11	4·78±0·18 (39)	5·83±0·32 (37) (†)

Results are mean T_{50} values (hr)±S.E.M. for the number of cells indicated in parentheses.

P values indicate statistically significant differences. *$P < 0·001$, †$P < 0·01$ and ‡$P < 0·05$.

[a] Eyes 6 and 7 came from the same donor and results were pooled.

average the rat outer segments survived only one-third to one-quarter as long as human outer segments despite the lower temperatures. Other small morphological differences were noted in the collapse of rat outer segments. Changes in the inner segment were not as apparent or early as in human rods. The outer segments often would straighten out again just before the inner segment rounded. Immediately thereafter, the outer segment rapidly collapsed. During the rapid phase of outer segment collapse (10–20 seconds), we readily observed the incorporation of 5–10 μm-lengths of outer segment into the inner segment as it occurred.

Figure 3 shows the normalized outer segment lengths for one BSA and one bFGF experiment from the same human eye. The pattern of maintained length and rapid collapse of the outer segment depicted by Figs 1, 2 and 3 occurred repeatedly in all experiments. Although the total experiment duration varied across tissues (Table III), the outer segment length vs. time curves had strikingly consistent shapes. The longest experiment on human tissue lasted 12 hr, and the mean survival time for outer segments in that experiment was 9·4 hr (Table III).

In this assay, both single RIS/ROS and outer

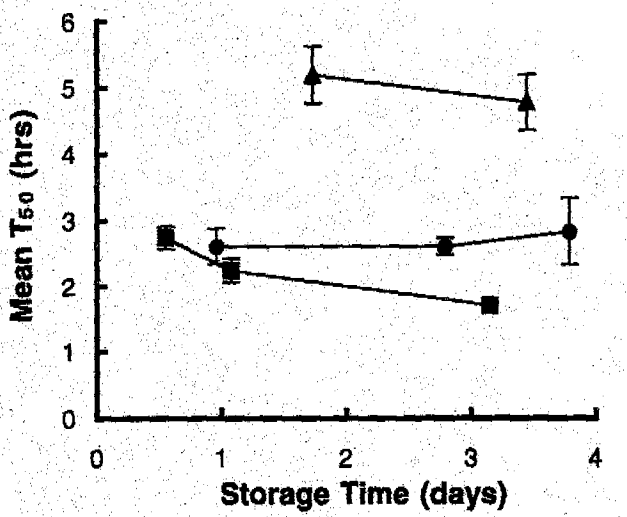
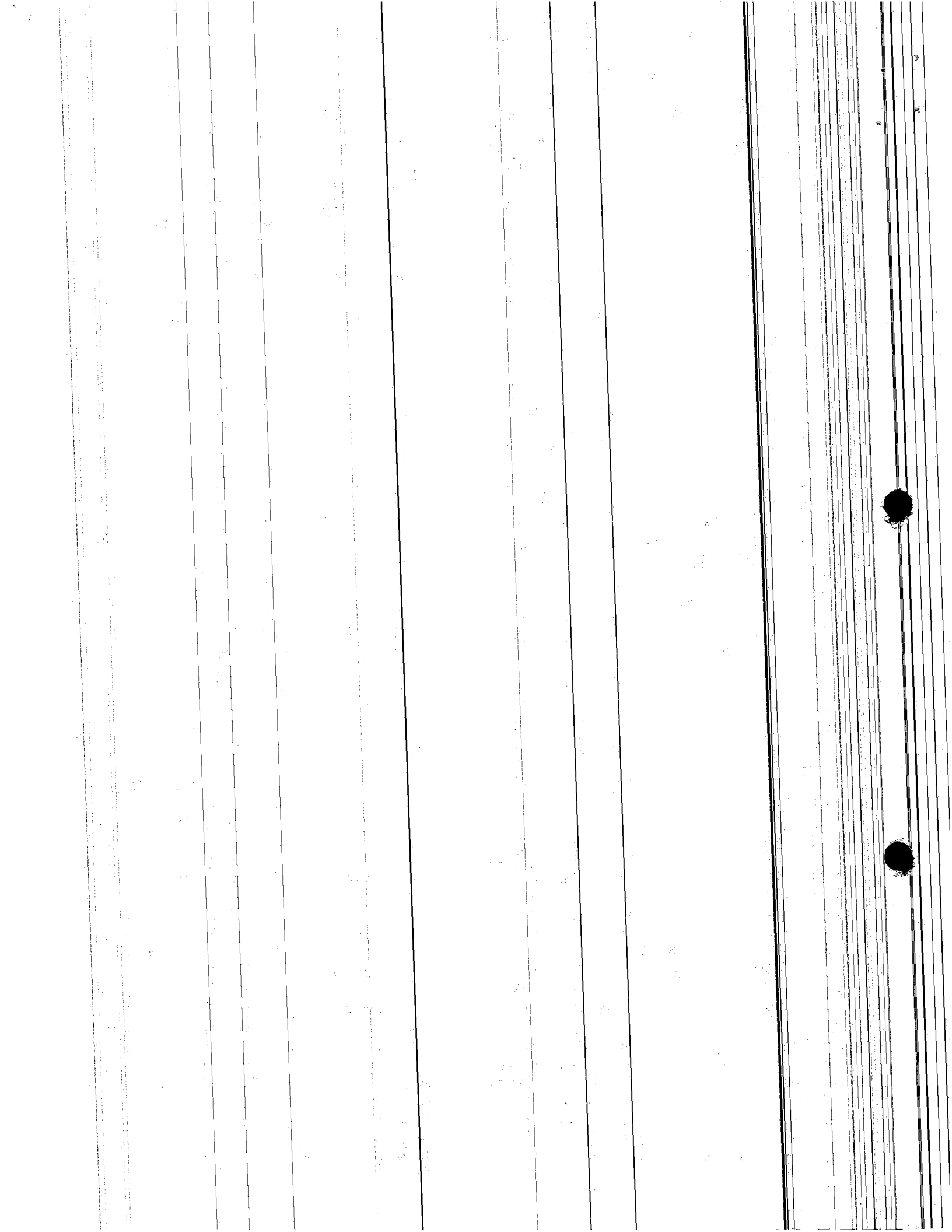


FIG. 4. Survival time of human rods isolated from the same eye stored for several days. Before experiments, tissue was stored in Leibovitz's L-15 at 4°C. Experiments were performed at 37°C. Mean T_{50} values for repeated experiments with BSA-containing medium are shown for tissue from three different donors (represented by different symbols).

segments on small pieces of retina were measured. To ensure that the presence of other cell types in the small groups did not significantly alter our results, an analysis of six human experiments included a comparison of the mean T_{50} values for single cells and those in clumps. A normal approximation to the Wilcoxon Rank Sum test, which allows the comparison of two populations with small and dissimilar sample sizes, yielded no difference in mean T_{50} between singles and groups in BSA or bFGF (Table II). In one out of two comparisons of single RIS/ROS vs. clusters in Locke's solution (no additives), there was a significantly greater survival time for cells in clusters ($P < 0·002$). It is possible that in our isolation procedure rods in clusters undergo less mechanical damage than single cells, but overall the effect is minimal.

Tissue Longevity and Viability

Isolated human retinal tissue was stored at 4°C for up to 9 days in Leibovitz's L-15 medium exchanged every two days. Repeated experiments several days apart using the same medium and tissue from the same eye produced very consistent results. Figure 4 shows stable T_{50} values obtained in repeated experiments over a 4-day period for tissues from three eyes using medium containing BSA. The outer segment survival times (mean T_{50}) varied considerably between cases, which is not surprising given the unknown and highly variable physiologic state of donor tissue received (Table I). However, the consistency of repeated experiments for a given tissue shows that the outer segment collapse assay is reliable, allowing collapse rates under test and control conditions to be compared statistically.



As expected, freshly dissociated RIS/ROS, exposed to trypan blue just after DNase treatment, excluded the dye. In addition, cells with straight, bent, or broken outer segments and even inner segments with no outer segments excluded dye applied at the end of experiments (up to 12 hr later), indicative of continued cell viability.

Relationship of Rod Outer Segment Morphology and Light Responses.

The goal of these experiments was to enhance the survival time of functional isolated photoreceptors, where function is defined in terms of light responsiveness. We have tested whether bFGF can prolong the structural integrity of rod outer segments and have related that integrity to rod function. To relate morphological observations with the functional state of the cells, we made suction electrode recordings from outer segments with various degrees of bends and lengths.

Figure 5 shows video frame images of three different RIS/ROS and their corresponding light responses. A cell with a long, straight outer segment [Fig. 5(A)] responded to saturating and sub-saturating 500-nm light with faster rising phase and recovery kinetics [Fig. 5(B)] than another cell, with a slightly bent outer segment [Fig. 5(C), (D)]. Any morphological disturbance of the outer segment beyond a slight bending was indicative of an unresponsive cell [Fig. 5(E) and 5(F)]. These results have been observed repeatedly in recordings from this and other human tissues. To relate the physiologic responses of a human rod whose outer segment is collapsing inside the recording pipette, we have made continuous recording of human rods for 4–7 hr. The amplitudes of light responses over the course of 7 hr were reduced as the outer segment partially collapsed inside the electrode [Fig. 6(A)]. Outer segment blebbing and a gradual 30% decline in outer segment length (▲) were accompanied by a substantial decline in flash sensitivity S_f, picoamperes of current generated per incident photon (○), and an increase in the time-to-peak of the dim flash response (dashed line) [Fig. 6(B)]. A second experiment of this type on the following day yielded similar outer segment collapse and decrease in light responsiveness. The flash sensitivity and intensity required for half-maximal response ($I_{1/2}$) in these experiments were comparable to published values (Kraft et al., 1993).

The loss of light responses from rat (2-month-old) rods preceded morphological changes in the outer segments. Rods with long, straight outer segments responded to brief flashes of 500-nm light. However, 35 minutes after DNase dissociation was the latest time at which a light response could be recorded. Seven out of 12 rods were light-responsive within the first 35 minutes (up to 4 pA, saturating). Out of 22 rods none were light-responsive after 35 minutes.

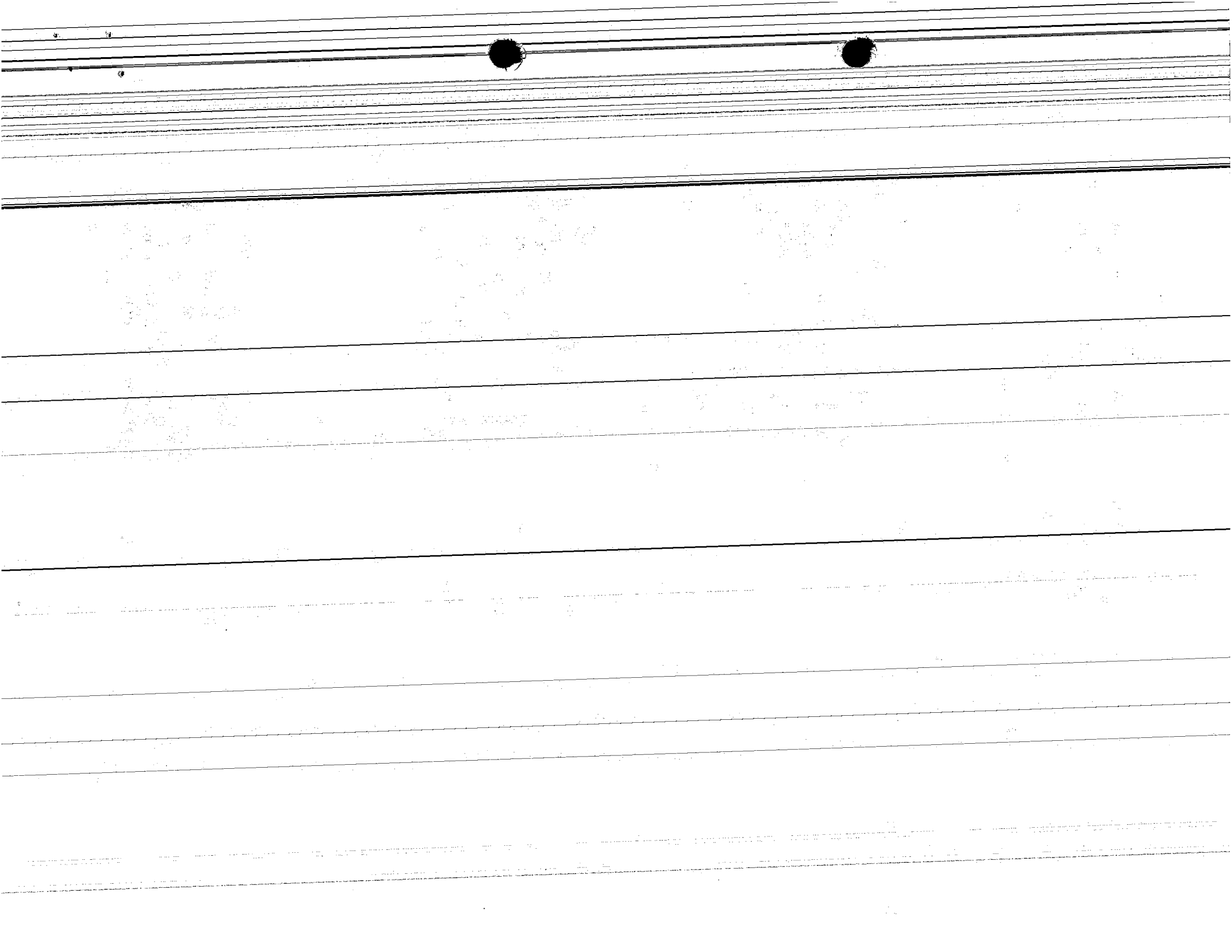
Effect of bFGF on Human Rods

The addition of a non-specific protein (BSA) to the medium enhanced the survival time of the outer-segments in seven out of eight experiments performed on human tissues from five donor eyes (mean improvement 46%), and in four out of four experiments on rat tissues (mean improvement 59%). The presence of extracellular protein had a marked effect in stabilizing the outer segment structure; thus, all remaining experiments were done with solutions containing BSA in order to concentrate on the effects of bFGF.

Experiments on four eyes from different donors showed a statistically significant bFGF effect (Table III and Fig. 7, ●). The straight line with a slope of one represents zero effect. Survival times were increased by $28 \pm 6\%$, $40 \pm 10\%$, $68 \pm 9\%$, and $22 \pm 7\%$ in Eyes 1 ($P < 0.01$), 3 ($P < 0.05$), 4 ($P < 0.001$) and 11 ($P < 0.01$), respectively. Two eyes came from donors with sepsis; in both of these cases bFGF produced a significant improvement in outer segment survival times. Experiments with bFGF on tissue from five cases yielded no effect, and in one eye the presence bFGF reduced OS survival times by $13 \pm 7\%$ (Fig. 7, ▲). In one additional experiment on tissue from eye 8, the cofactor heparin was included. In this case the combination of bFGF and heparin produced a statistically significant increase in outer segment survival time whereas bFGF alone had not, $T_{50} = 5.54 \pm 0.33$ ($n = 16$) ($P < 0.02$). The enhancement of an bFGF effect by heparin was also seen in the results from rat retina. The addition of heparin alone had no effect on outer segment survival times.

Growth Factor Effects on Survival of Rat Rod Outer Segments

As with the human tissue the outer segment collapse of rat rods followed a regular pattern. There was typically a plateau phase during which the cells underwent very little change; the plateau phase was followed by a rounding of the cell body and then a rapid collapse of the outer segment. Effective compounds prolonged the phase in which the full outer segment length was maintained, but did little to reduce the speed the outer segment collapsed after the process had started. In order to more easily summarize numerous experiments on rat tissues all data were pooled. Table IV and Figs 8 and 9 summarize the ANOVA analysis of 151 experiments on 44 rats containing a total of 2,100 observations of the outer segment collapse and measurement of T_{50}. The rats were grouped into two age categories, old (18–25 month-old) and young (1–10 months-old). Figure 8(A) shows results of adding bFGF with and without the cofactor heparin in young rats. The addition of 25 ng ml^{-1} bFGF caused a significant decrease in O.S. survival time, while the addition of heparin alone was



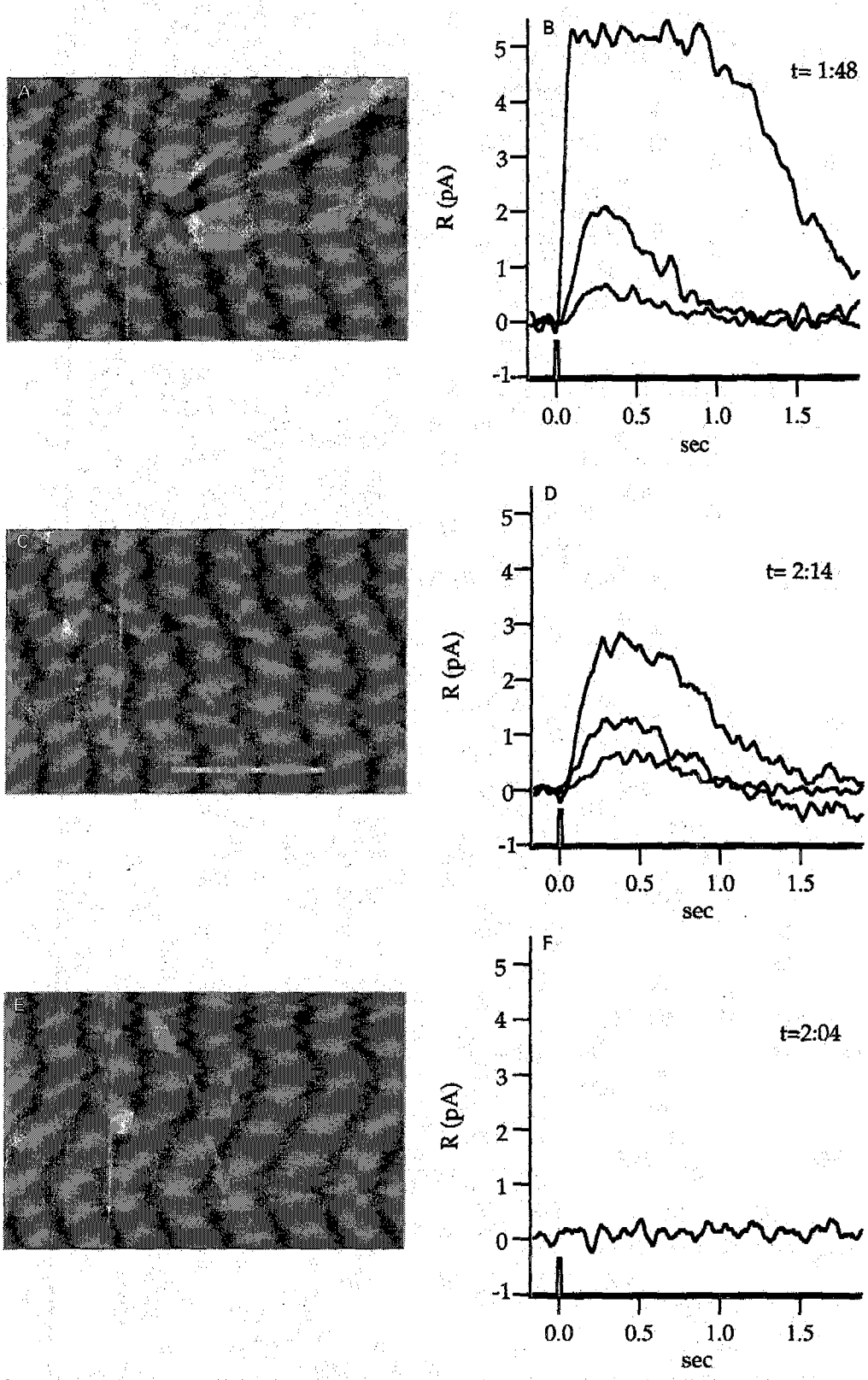
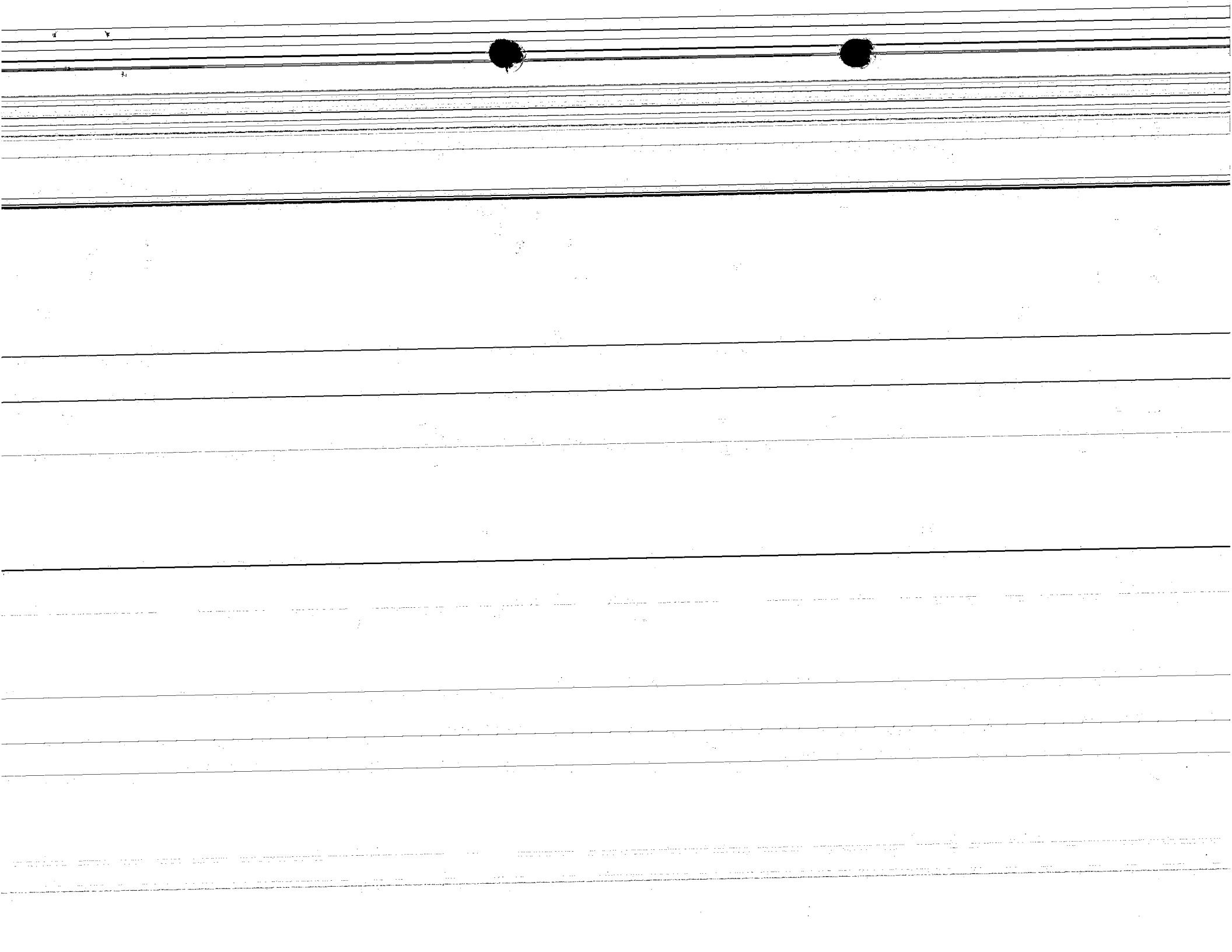


FIG. 5. Flash responses from human rods compared with their structural integrity at the time of recording. A stimulus marker for a 20-ms flash at 500 nm is given below the current traces. Current responses were the averages of 5–20 individual traces. T = 35°C, DC to 50 Hz. The times in the upper right of each graph are times post-DNase. (A) Freshly isolated rod with a straight outer segment which was pulled into a suction electrode for recording. (B) Three responses of the rod in A to brief flashes of light. Intensities were 1200 photons μm^{-2} ($n = 5$), 43 photons μm^{-2} ($n = 10$) and 10 photons μm^{-2} ($n = 15$). (C) A rod with a single bend in the outer segment. (D) Three responses of the rod in C to brief flashes of light. Intensities were 85 photons μm^{-2} ($n = 10$), 43 photons μm^{-2} ($n = 10$), and 10 photons μm^{-2} ($n = 20$). (E) Single RIS/ROS with several bends along the outer segment. (F) Averaged current traces show no response from the cell in E for a light intensity of 1200 photons μm^{-2} ($n = 10$).



not significant. The combination of heparin and bFGF caused a significant increase in O.S. survival time when compared with control ($P < 0{\cdot}05$) and heparin solutions ($P < 0{\cdot}0001$). In older rats the addition of 25 ng ml^{-1} bFGF also caused a significant decrease in O.S. survival time [Fig. 8(B)]. Supplementing the medium with either heparin alone or heparin plus bFGF did not have a significant effect in older rats.

While much attention has been given to bFGF as a potential therapeutic agent for retinal degeneration and we have shown it can have a marked effect in our assay, a number of other growth factors and cytokines have been shown to affect photoreceptor survival in rodent models of retinal degeneration. Figure 9 and Table IV summarize results from experiments with five other factors. Asterisks indicate significant differences from the control solution (T_{50} of 1·44 hr shown by dashed line). Neither BDNF (25 ng ml^{-1}) nor GDNF (100 ng ml^{-1}) significantly changed outer segment survival times. A higher concentration of GDNF (1 μg ml^{-1}) and CNTF (10 ng ml^{-1}) resulted in a significant increase in outer segment survival time. When either PEDF (100 ng ml^{-1}) or the sugar NAD-gal were present, the outer segment survival times significantly decreased.

A mixture of several growth factors (MIX) was also tested (see methods for components of MIX). The T_{50} declined 15% in the presence of multiple growth factors. The mean T_{50} in BSA was $1{\cdot}38 \pm 0{\cdot}05$ hr ($n = 65$), and the mean T_{50} in MIX medium was $1{\cdot}18 \pm 0{\cdot}02$ hr ($n = 67$) ($P < 0{\cdot}001$).

4. Discussion

The condition of the outer segment is a crude indicator of the functional status of the rod photoreceptor in the sense that rods cannot signal the presence or absence of light without an organized outer segment. The loss of light sensitivity coincident with loss of the rod outer segment in our assay has been demonstrated. It has been suggested that rounding of the inner segment corresponds to fusion of the inner and outer segments (Townes-Anderson, 1995); this event would be a logical precursor to the incorporation of the outer segment into the inner segment seen so typically in our assays.

We have tested a variety of factors which have, in other systems, proven effective in maintaining photoreceptors or other retinal neurons. An attractive advantage of this model is that it quickly measures the direct effect of the growth factors on photoreceptors. A disadvantage is that factors requiring longer times to affect outer segment survival, e.g. by alterations in gene expression, would require a modified assay. However, it is now becoming clear that a number of different growth factors do have a rapid effect on cell function (Scharfman, 1997; Suen et al., 1997; Taniwaki et al., 1997). Thus, the results we observed with incubation periods of a few hours are not surprising.

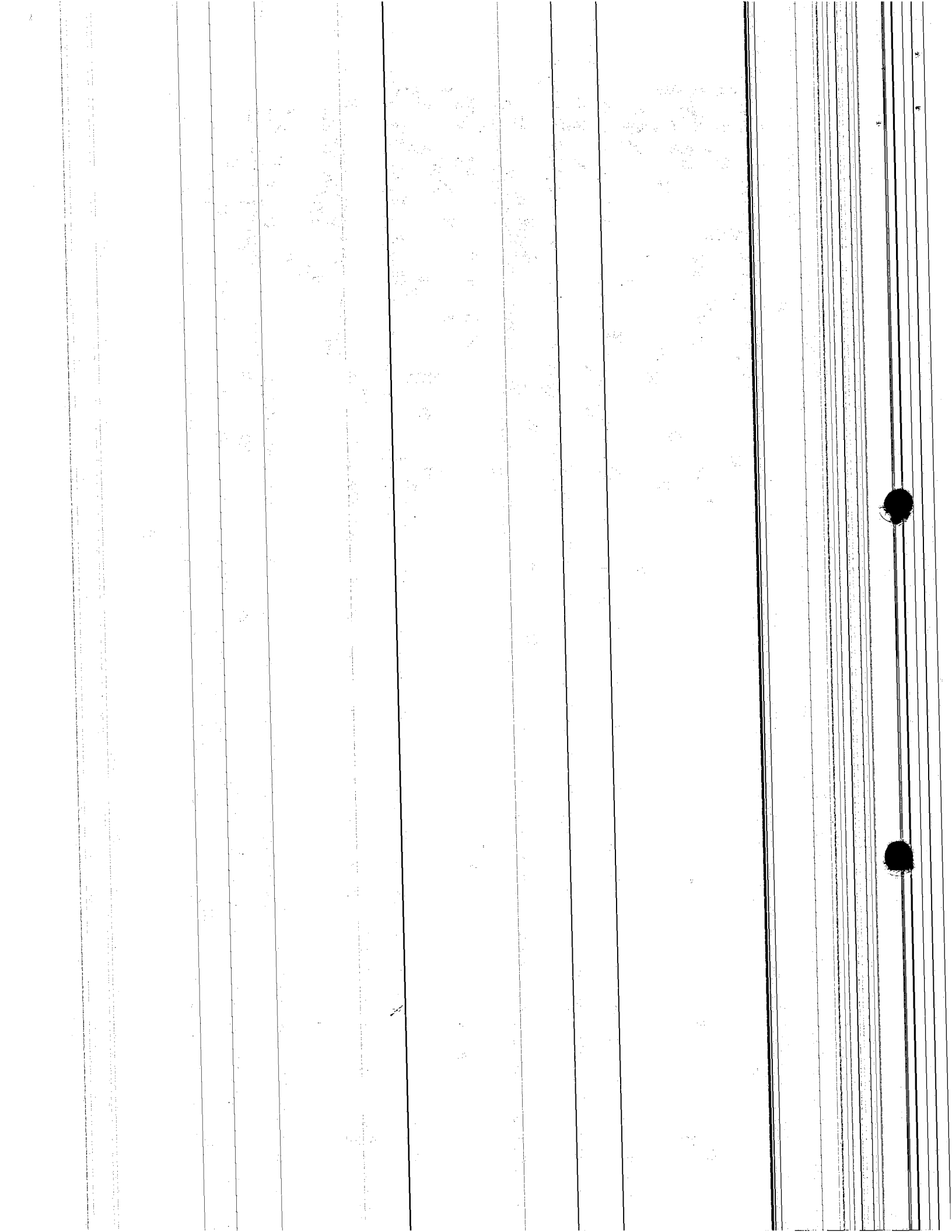
CNTF and GDNF both enhanced outer segment survival of rodent rods. The effect with GDNF appears to be dose-dependent; however, both doses of GDNF used were considerably higher than those previously shown to be effective in neural tissue (Lin et al., 1993). It is possible that a specific receptor for GDNF on the rod cell has a very low affinity, but it is also conceivable that the high concentrations used allowed nonspecific binding to receptors, thus stimulating a non-physiologic effect of GDNF. In our assay the use of all-*trans* retinol as a cofactor for GDNF was without effect.

The concentration of BDNF used was similar to those previously reported as effective (Johnson et al., 1986), yet we observed no effect on the photoreceptors themselves. The BDNF receptor, trkB, has been localized immunohistochemically in the rat retina to the ganglion cell layer, but this technique failed to detect the receptor in the photoreceptor or RPE layers (Perez and Caminos, 1995; Rickman and Brecha, 1995; Rickman and Rickman, 1996). Intravitreal injection of BDNF does not prevent photoreceptor degenerations in cat eyes with detached retinas (Fisher et al., 1995). In their model BDNF does prevent the reduction of outer segment length, suggesting the existence of some type of receptor in the outer retina. Our results argue against a direct effect of BDNF on photoreceptors.

CNTF has been shown in previous studies to prevent photoreceptor degeneration in vivo. (LaVail et al., 1992; 1996; Cayouette and Gravel, 1997) and is upregulated in response to mechanical injury (Wen et al., 1995). The CNTF-receptor alpha subunit has been localized, by in situ hybridization, to ganglion and horizontal cells, but not photoreceptors in postnatal day 6 and adult rat retina (Kirsch et al., 1997). In our studies CNTF produced a small but significant increase in rod outer segment survival suggesting a direct effect on photoreceptors. Differentiation of photoreceptors, measured by expression of opsin, is dramatically reduced by CNTF in cultured neonatal rat cells, suggesting that CNTF receptors are present on these cells (Ezzeddine et al., 1997).

Initially bFGF was studied for its mitogenic roles, controlling entry into the cell cycle, and mediating differentiation (Gospodarowicz, 1985; Gospodarowicz et al., 1987). Fetal human retinal cells have been shown to survive in culture for up to 300 days; in these cultures bFGF stimulates a six-fold proliferation over baseline (Kelley, Turner and Reh, 1995). Differentiation of photoreceptors, measured by expression of opsin, is increased by bFGF in cultured neonatal rat cells (Hicks et al., 1992). Recent attention has been directed toward the dynamic response of bFGF in the diseased or physically stressed retina (Gao and Hollyfield, 1996).

A partial functional rescue by bFGF in retinas from light-damaged rats has been demonstrated with ERG



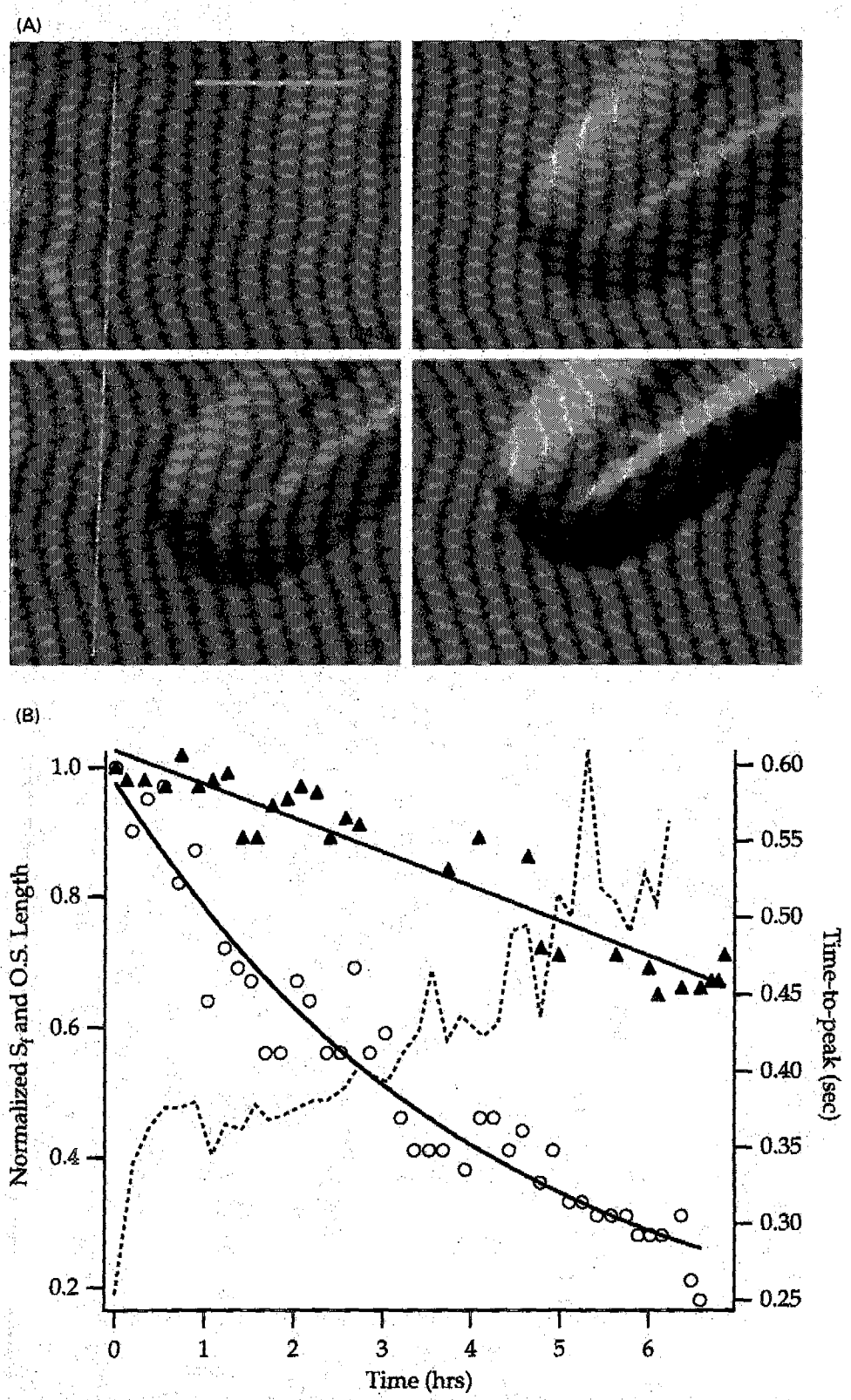


FIG. 6. (A) Changes in the appearance of a human rod outer segment monitored while light responses were recorded with a suction electrode (total experimental duration = 7 hr); scale bar = 20 μm. (B) Outer segment collapse (▲), flash sensitivities (S_f) (○), and times-to-peak (——) from the dark-adapted rod in A over several hours. Flash sensitivities at 500 nm and 31 photons μm^{-2} declined drastically as the outer segment collapsed within the pipette. An initial jump in the time-to-peak was followed by a steady rise in time-to-peak (over 6 hr). Responses were still recordable at 7 hr post-DNase. T = 35°C.

recordings (Masuda et al., 1995). Our study also suggests that functional rescue of rods by bFGF in vitro is possible, an effect which appears to be independent of other retinal cell types. It is the first to describe bFGF's ability to maintain single rod outer segments, structures that are crucial to the generation of light

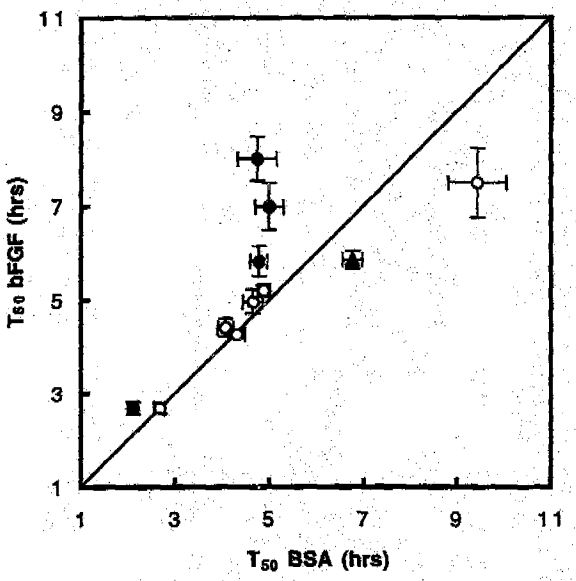


FIG. 7. Survival times of human rod outer segments in BSA plotted against survival times of tissue from the same patient in bFGF (±S.E.M.). Survival times were those required for outer segments to degenerate to 50% of their initial lengths. The mean number of outer segments measured per experiment was 32. Filled symbols indicate statistically significant differences in favor of bFGF (circles) ($P < 0.001$, $P < 0.01$, $P < 0.05$) or BSA (triangle) ($P < 0.01$). The straight line with a slope of one represents zero effect.

responses. The relationship between changes in morphology and light responses was evident in human tissue where flash sensitivity and response kinetics decreased substantially as the outer segment shortened. This relationship was not documented with the more fragile rat rods as light responsiveness was lost before notable morphological changes occurred. Nevertheless, during the early critical period in which recordings could be made, rat RIS/ROS with long,

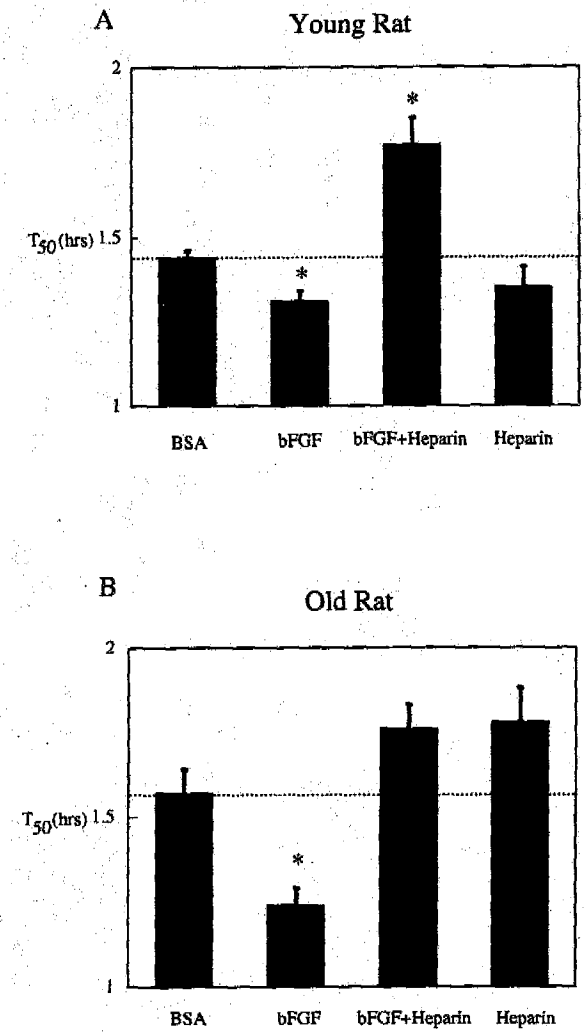


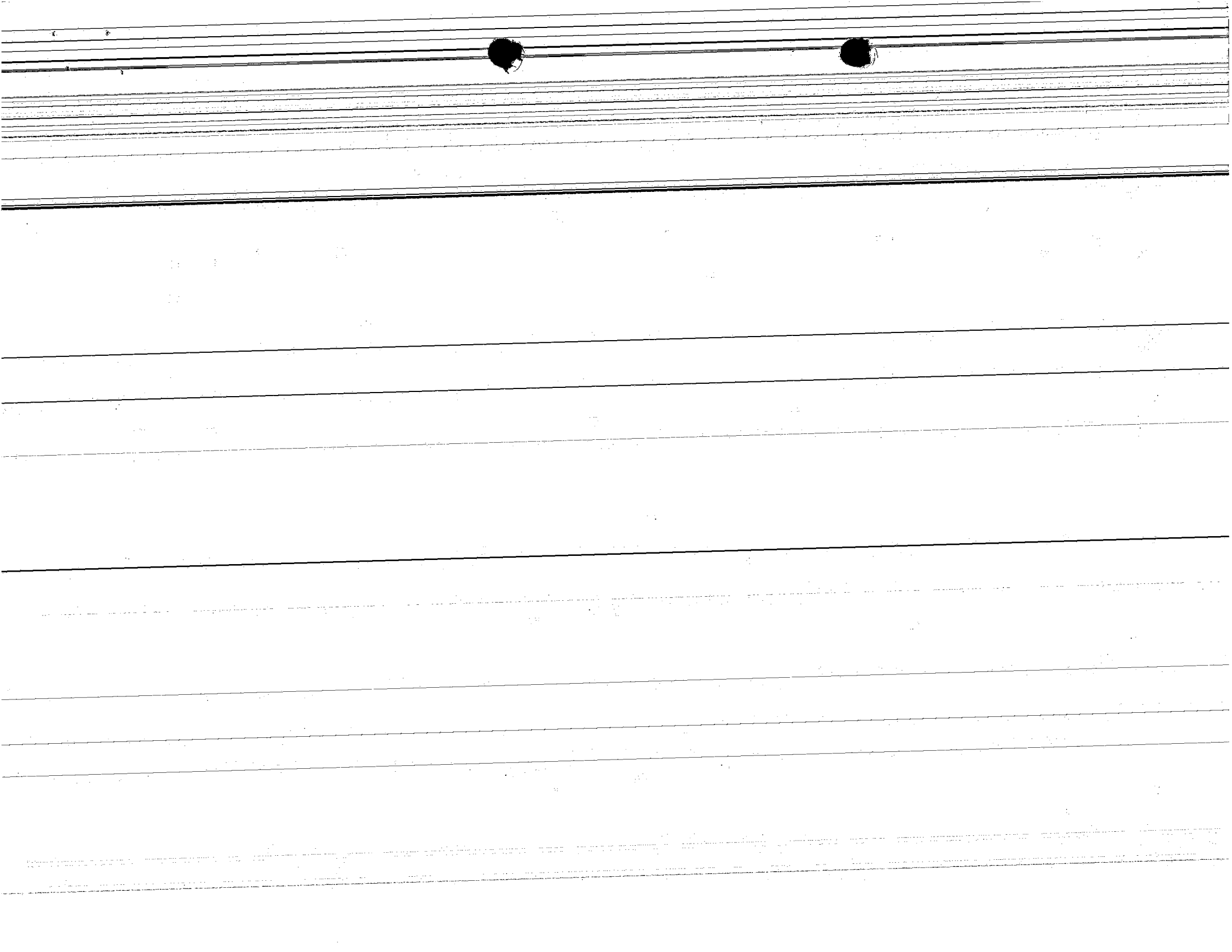
FIG. 8. bFGF effects on the outer segment survival times of rods isolated from (A) young rat retina and (B) old rat retinas. Each data set was compared to the control condition (BSA) indicated by the dashed line. Bar heights indicate the mean and standard error of the mean T_{50} in hours for each condition. An asterisk indicates statistical significance ($P < 0.05$).

TABLE IV

Rat outer segment maintenance

Solutions	Young rats mean T_{50}	Old rats mean T_{50}
BSA	1·44±0·02 (799) {34}	1·57±0·07 (74) {4}
bFGF	1·31±0·03 (155) {6}*	1·24±0·05 (73) {4}*
bFGF+Heparin	1·77±0·08 (118) {5}*	1·76±0·07 (56) {2}
Heparin	1·35±0·06 (110) {5}	1·78±0·10 (41) {2}
BDNF	1·48±0·03 (225) {9}	
CNTF	1·68±0·04 (78) {3}*	
GDNF (0·1 μg ml^{-1})	1·39±0·03 (186) {8}	
GDNF (1·0 μg ml^{-1})	2·65±0·10 (46) {2}*	
PEDF	1·01±0·04 (61) {2}*	
Sugar (NAD-gal)	0·90±0·06 (36) {2}*	
Locke's Solution	0·90±0·03 (42) {2}*	

Results are mean T_{50} values (hr)±S.E.M. for the number of cells indicated in parentheses. The number of animals is given in brackets. An asterisk indicates a statistically significant difference ($P < 0.05$) was found when compared to the control solution (BSA). Young rats are 1–10 months-old, old rats are 18–25 months-old.



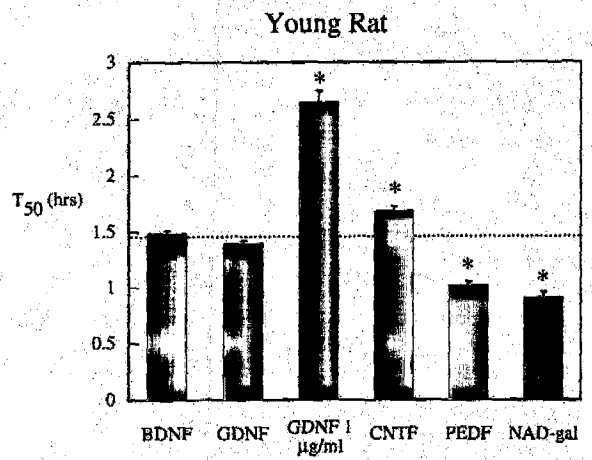


FIG. 9. Results from tests of six additional factors on rat rods from young animals. Bar heights indicate the mean and standard error of the mean T_{50} in hours for each condition compared to the control solution (BSA, indicated by dashed line). BDNF and GDNF (0·1 μg ml^{-1}) were not different from the control condition. All other factors resulted in T_{50}'s which were significantly different from that measure under control conditions ($P < 0·05$).

straight outer segments generated responses as robust as previously described (Robinson et al., 1993).

Some variability of whether a given tissue responded to bFGF in vitro or the extent to which it responded was noted. The effective level of bFGF delivered to low and high affinity receptors would be one possible explanation. Some cell types require heparin as a co-factor to observe mitogenic effects of bFGF (Klagsbrun and Baird, 1991). In the retina, more photoreceptors are rescued in the presence of bFGF with heparin than with bFGF alone (LaVail et al., 1992). Heparin combined with bFGF in our system improved the bFGF response in the only human retina tested and had a dramatic effect in improving O.S. survival time in cells from young rat retinas. The mRNA for bFGF has been found in human photoreceptor inner segments (Ohsato et al., 1997). Recently, Li, Chang and Milam (1997) have described a gradient of bFGF immunoreactivity in human rods; from 0·5% of rods in the macula increasing to as much as 88% of rods in the far periphery. Thus the variability in our results may have represented an inadvertent sampling bias. The nature of and amount of extracellular matrix surrounding the isolated rods in our system was unknown and could have been another possible source of variability. In addition, the disease state of the human tissue was generally unknown and may be related to the response variability.

The ability of bFGF to protect diseased or damaged neurons in the retina has been clearly demonstrated in rodent models, but the mechanism is not understood (LaVail et al., 1992; Faktorovich et al., 1992). A wide variety of possibilities has been described. Binding of iodinated bFGF has been described in preparations of rod outer segments and disk membranes in bovine retina (Mascarelli, Raulais and Courtois, 1989) as well as bFGF-binding to photoreceptor inner segments and pigment epithelium of neonatal mouse retina (Fayein, Courtois and Jeanny, 1990). Potential sites of bFGF action have also been identified with anti-rat bFGF antibody in the outer segment layer of mouse retina and Müller and RPE cells of the rat (Gao and Hollyfield, 1992). The high-affinity receptors for bFGF and aFGF, *flg* and *bek*, were immunolocalized to photoreceptors, the plexiform layers, and ganglion cell layer in the rat retina (Blanquet and Jonet, 1996). The hereditary defect in the RCS rat is in the RPE cells, and localization of bFGF receptors on the RPE (Sternfield et al., 1989; Rakoczy et al., 1993) leads to some question as to the site(s) of action for bFGF in the retinas of these animals. Because of the low cell density, continuous fluid exchange, and the relatively short duration of the assay, the survival-promoting action of bFGF in our preparation is almost certainly a direct effect. Our comparison of single isolated RIS/ROS vs. rods in small clusters of cells supports this view. We cannot distinguish outer versus inner segment location of the bFGF receptor(s), but the pre-synaptic terminal and cell nucleus were not present on the isolated RIS/ROS and, therefore, can be ruled out. The intracellular events activated by bFGF in the outer retina are unknown. Although bFGF commonly alters gene expression, in some cell types, ion channels are affected by bFGF within a few minutes. Examples include a subclass of K^+ channels and a Na^+/H^+ exchanger, both in BALB/c 3T3 fibroblasts (Halperin and Lobb, 1987; Panet, Amir and Atlan, 1986). In photoreceptors light responses are slowed within a few minutes by intracellular application of bFGF (Schmidt et al., 1995). However, effects of extracellularly applied bFGF on photoreceptors have not been reported. Because altered gene expression is not always required for bFGF effects, isolated RIS/ROS are sufficient for a first examination of bFGF's influences on photoreceptors' ionic currents or specifically on photocurrents. In addition, bFGF-mediated regulation of intracellular calcium, which may or may not involve altered gene expression, has been reported in culture studies with hippocampal and cortical neurons (Mattson and Cheng, 1993; Mattson et al., 1993), and such a process might also occur in photoreceptors.

The photoreceptor response to bFGF in developing retinas, adult retinas, and stressed retinas is often quite different and is likely to be regulated (Faktorovich et al., 1990; Gao and Hollyfield, 1996). Development of the normal mouse retina proceeds without high levels of bFGF, but large quantities of bFGF and bFGF mRNA are found in the developing *rd* mouse (Gao and Hollyfield, 1995). Dureau, Hick and Putterman (1994) found no survival-promoting effect of bFGF on cultured sheets of adult rat photoreceptors. However, exogenously applied bFGF has been shown to rescue rat photoreceptors in vivo when they are diseased or

injured by constant light (LaVail et al., 1992; Faktorovich et al., 1990; 1992). Photoreceptor bFGF mRNA increases after mechanical injury in albino rats (Wen et al., 1995), degenerating *rds* mouse, and light-stressed albino rat retinas (Gao and Hollyfield, 1996). In addition, studies of the response to brain injuries also have shown focal elevation in the levels of bFGF and its mRNA (Logan et al., 1992). A recent report showed an increase of bFGF binding in cultured human RPE cells in response to low levels of oxygen (Khaliq et al., 1996). We acquired two donor eyes from patients with septicemia, a known risk factor for endogenous bacterial endophthalmitis which includes vitritis and chorioretinitis (Pflugfelder and Flynn, 1992). Our positive finding of an bFGF effect with these tissues support the recent evidence for bFGF's protective ability as a dynamic response of the retina under stress.

Isolating photoreceptors from the supportive environment of the RPE and Müller cells as well as from other photoreceptors is a drastic change, poorly substituted for by the simple media devised thus far. Yet through this model we can learn which identified factors directly affect photoreceptors as well as test less defined supplements, like conditioned medium from other cell culture systems or partially purified protein preparations. Moreover, it may provide a model for the investigation of photoreceptors' stress-dependent growth factor responsiveness and intracellular mechanisms.

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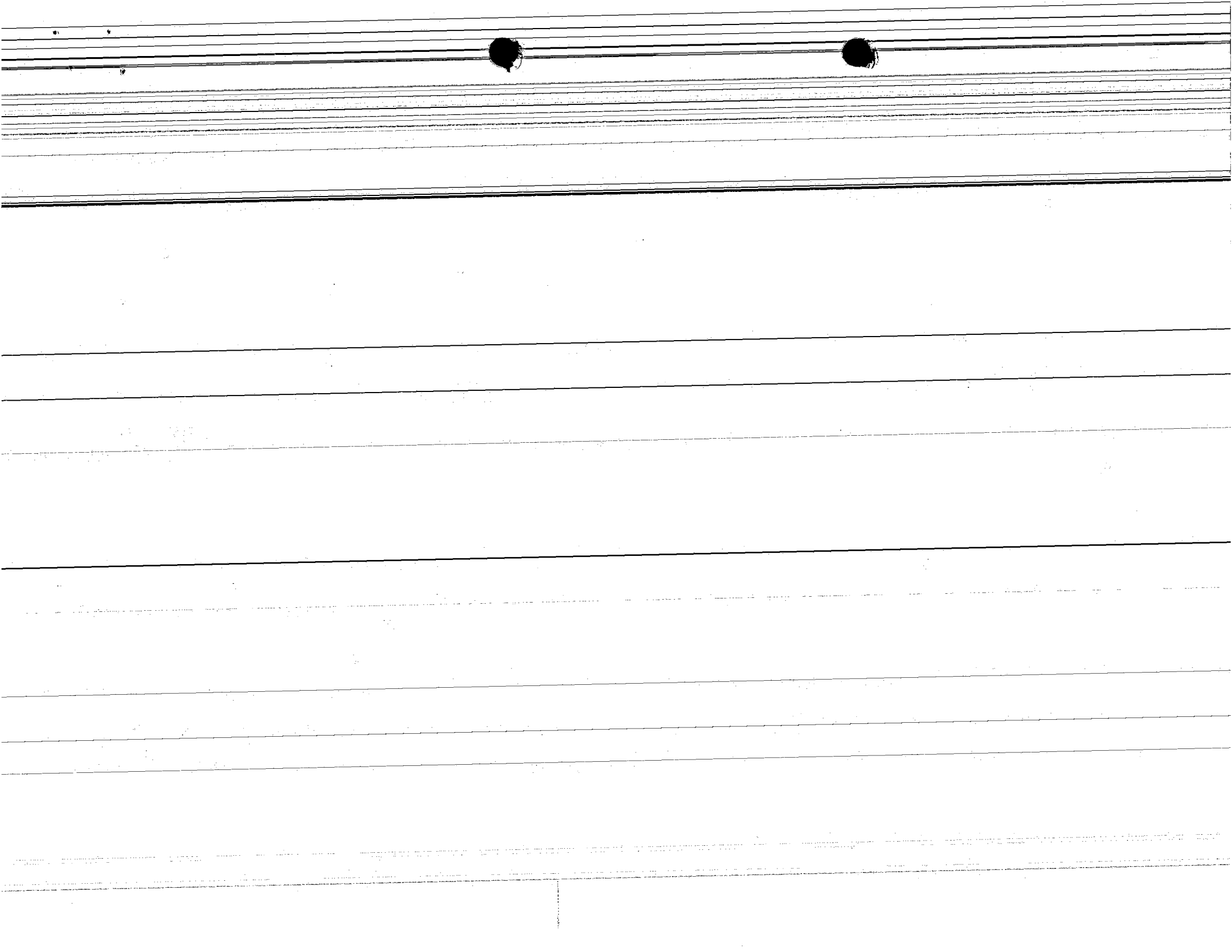

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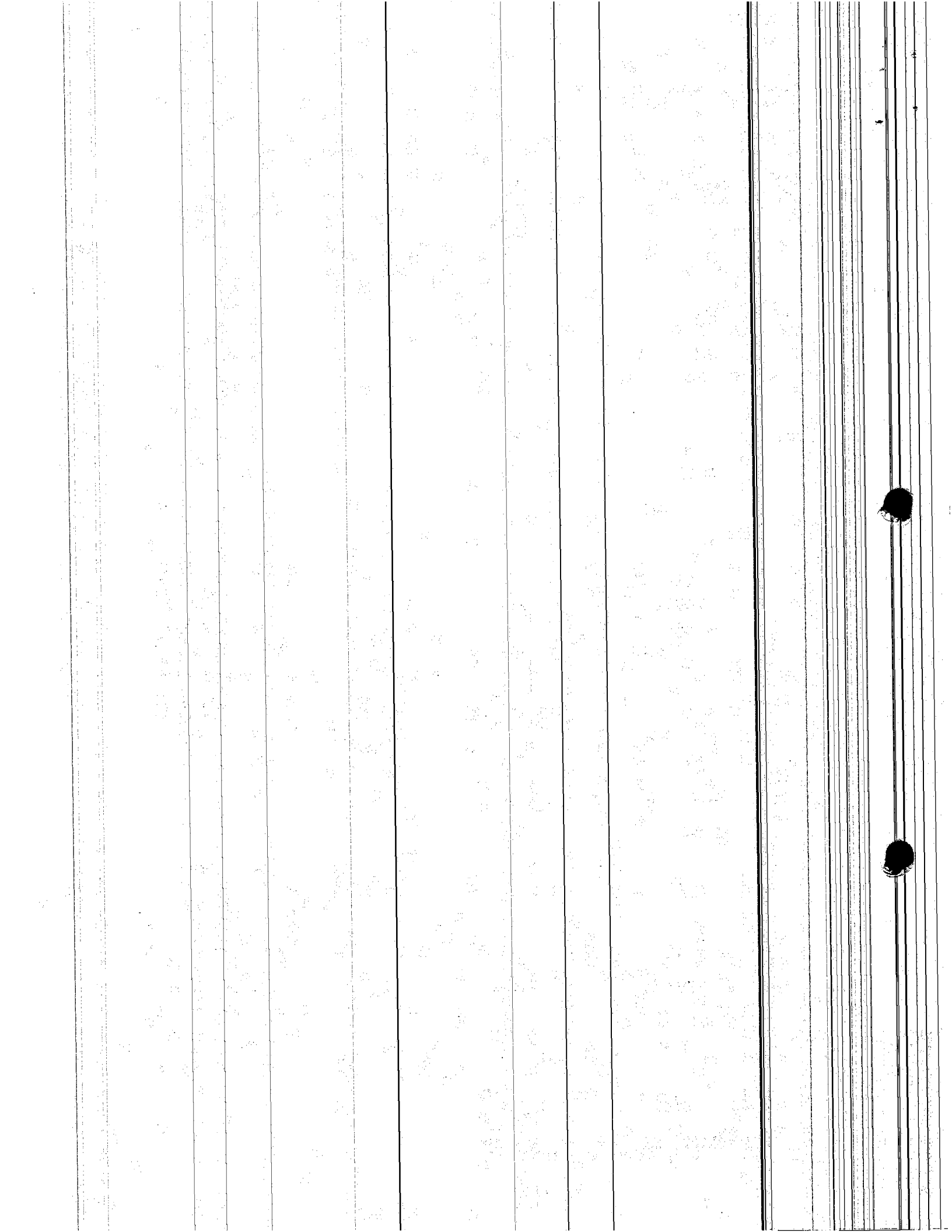

Room 305
Friday 8:30 — 10:15 AM

Retinal Cell Biology, Anatomy & Pathology
Photoreceptor Apoptosis: Neuroprotection

MODERATORS: David Hicks
Gail M Seigel

PGM#	TIME	AUTHORS
5091	8:30	Sahel, Frasson, Picaud, Leveillard, Dreyfus, Hicks
5092	8:45	Doly, Luyckx, Gressier, Cluzel, Martinez, Luyckx
5093	9:00	Ohira, Kria, Gong, Kodama
5094	9:15	Seigel, Paxhia
5095	9:30	Droy-Lefaix, Ranchon, Doly
5096	9:45	Wang, Wiegand, Peterson
5097	10:00	Jablonski, Ervin, Wohabrebbi

NOTE: Potential conflicts of interest of presenters and authors are noted at the end of the Abstract either by "None" or with codes (see code definitions under the section "Abstract Content/Commercial Relationships/Disclosure Codes" in the front matter).

5091—8:30

Glial Cell Line-Derived Neurotrophic Factor (GDNF) Induces Histological and Functional Neuroprotection of Rod Photoreceptors in the Retinal Degeneration (rd) Mouse J. Sahel, M. Frasson, S. Picaud, T. Leveillard, H. Dreyfus, D. Hicks. Hopital Civil/Univ. Louis Pasteur, Strasbourg, France.

Purpose. To examine the efficacy of GDNF in reducing photoreceptor loss in inherited retinal degeneration. Methods. GDNF (n=10) or PBS only (n=10) were injected into the subretinal space of homozygous *rd* mice at 13 and 17 days of age. At 22 days, mice were examined for visual function by electroretinography (ERG). At 25 days the mice were killed and their retinas processed for histological and immunohistochemical analyses. Rod photoreceptors were labelled with rod opsin antibody, and their numbers assessed in both wholemounted and transversely sectioned retinas by unbiased stereological techniques. Endogenous GDNF mRNA levels were measured in *rd* mice by semi-quantitative RT-PCR. Results. At 22 days ERG were still detectable in 4/10 mice injected with GDNF, while no ERG signal could be observed in sham- or non-operated controls. Counting of immunolabelled rod photoreceptors in whole flatmounted retinas revealed highly significantly greater numbers for GDNF (89000 mean entire retina) vs PBS (61200) or non-operated controls (41900, $p<0.002$ and 0.001 respectively). PBS-injected retinas also contained more rods than uninjected eyes ($p<0.001$). These differences were confirmed by examination of transverse frozen sections of treated and control retinas. GDNF (and to a lesser extent PBS) treatment also increased Muller glial expression of glial fibrillar acidic protein compared to controls. Semi-quantification of endogenous GDNF mRNA revealed no changes in expression levels during the course of photoreceptor loss in *rd* mice. Conclusions. GDNF exerts neuroprotective effects on rod photoreceptors in *rd* mice, and to the best of our knowledge this constitutes the first report of functional rescue in an animal model of Retinitis Pigmentosa in which the gene defect lies within the photoreceptors themselves. GDNF hence represents a potentially important candidate for the treatment of inherited retinal degeneration.
CR: None Support: British RP Soc., Fond. De l'Avenir, A&M Suchert Found., ULP, Retina France, FNAHV, ADRET, EFG, IES, Essilor Intl.

5092—8:45

EFFECTS OF CYANINOSIDE CHLORIDE AND DIETHYLAMINE CHROMOCARBE ON PHOTO-OXIDATIVE STRESS IN THE RAT RETINA . M. Doly[1], M. Luyckx[2], B. Gressier[2], J. Cluzel[2], V. Martinez[1], J. Luyckx[3]. Laboratoire de Biophysique Sensorielle, Clermont-Ferrand[1], Laboratoire de Pharmacologie et Pharmacie Clinique, Lille[2], Transphyto, Clermont-Ferrand, France[3].

Purpose: Since the model of light-induced photoreceptor degeneration has been suggested to be partially dependent of the production of free radicals, we have investigated the effect of two compounds on the respiratory burst response and their in vivo capacity to protect the retina after light exposure. Methods: Antioxidant effect of diethylamine chromocarbe and cyaninoside chloride has been investigated on the hydrogen peroxide and hypochlorous acid generation by PMA (16nM) stimulated human PMNs (1h ; 37°C). Electroretinograms were recorded on albinos rat that received or not the treatment by IP injection (50mg/kg) for 14 days before and following the light exposure. Light sensitivity curves were analysed at the following time points : prior to light exposure; 1 day and 14 days after exposure. Animals were illuminated 24h with 1700lux white fluorescent light. At the end, eyes were processed for light microscopy. Results: For diethylamine chromocarbe, H_2O_2 production was decreased in a dose dependant manner with an IC50 value of 180mg/l, whereas the IC50 value for cyaninoside chloride on HOCl production was 6.4mg/l. Photo-oxidative stress to light results in significant reduction of ONL layer and decline of the b-wave amplitude. In cyaninoside chloride treated group, both morphometric and electrophysiologic data show a better preservation of the retina. By contrast, there were no electrophysiologic significant changes in the diethylamine chromocarbe group compared to exposed animals, although retinal protection was obvious histologically. Conclusions: We have demonstrated antioxidant effects for both substances. Scavenging activity of cyaninoside results in strong protective effects on neuronal cells, whereas prevention of retinal integrity from photo-oxidative stress by diethylamine chromocarbe was good but incomplete.
CR: None

5093—9:00

ENHANCED EXPRESSION OF MANGANESE SUPEROXIDE DISMUTASE (Mn SOD) BY EXPOSURE OF THE RETINA TO LIGHT ((A. Ohira[1], L. Kria[2], H.Q. Gong[2], T. Kodama[1]) Department of Ophthalmology, Shimane Medical University[1]; Nagasaki University School of Medicine[2], Japan.

Purpose. Manganese superoxide dismutase (Mn SOD) is a naturally occurring scavenger of reactive oxygen intermediates. To determine if Mn SOD expression is enhanced as a defensive mechanism against oxidative challenges, such as intense light exposure.

Methods. Experimental rats were exposed to cyclic light (80 lux) for 2 weeks, intense light (1,800 lux) for 24 hours, and then again exposed to cyclic light till rats were sacrificed. Control rats were exposed to cyclic light only. Experimental and control eyes were evaluated at 3 hrs, 1, 3, 7, and 14 days after exposure and protein expression was examined immunohistochemically using rabbit antisera against rat Mn SOD. Morphology was evaluated by light and electron microscopy.

Results. Both retinal pigment epithelial (RPE) cells and photoreceptor inner segments in the normal retina were labeled for Mn SOD. Mn SOD labeling also were found in the RPE cells on days 3, 7, and 14 and in the photoreceptor inner segments on day 14 after light challenge in experimental animals. Electron microscopy revealed that inner segment mitochondria and the RPE were substantially damaged at 3 hrs and 1 day in intense light exposed rats, but not at 14 day. Mn SOD labeling was again apparent on 14 days after light exposure.

Conclusion. These results suggest that induction of Mn SOD in the photoreceptor inner segments and RPE might protect against retinal light damage.

CR: None

5094—9:15

Insulin-Like Growth Factor 1 Protects Neuroretinal Cells From Hypoxia-Induced Cell Death G. M. Seigel, A. Paxhia. University of Rochester.

Purpose: Visual loss secondary to retinal ischemia/hypoxia can play a significant role in diabetic retinopathy and other retinal vascular disease processes. We sought to determine the susceptibility of retinal cells to hypoxic insult, as well as attempt to provide neuroprotection from hypoxia-induced cell death with the use of Insulin-Like Growth Factor 1 (IGF-1), a cytokine with known neuroprotective properties. Methods: Our immortalized, non-tumorigenic R28 rat retinal cell line, as well as Y79 human retinoblastoma cells were exposed to hypoxic conditions (95% Nitrogen/5% CO2) for up to 8 hours. Cell viability was measured by trypan blue exclusion assay. Apoptosis was assessed by TUNEL-in situ immunoreactivity, coupled with ssDNA analysis, a definitive assay for apoptosis (Frankfort, et al. Exp. Cell Res. 226: 387-397, 1996). Fas/FasL and IGF-1 immunoreactivity was measured by quantitative immunocytochemistry. Results: R28 cells responded to hypoxic conditions by a 30% decline in trypan-blue viability and an increase in the number of TUNEL+, ssDNA+ cells over the course of 8 hours. After 4 hours of hypoxia, R28 cell immunoreactivity for Fas increased by 40-fold and FasL by 60-fold; in addition, the number of IGF-1 immunoreactive R28 cells decreased by 50%. Replacement with 50 ng/ml IGF-1 (added 18 hours prior to hypoxia treatment) allowed R28 cells to remain 90% viable for up to 8 hours under hypoxic conditions. In contrast, Y79 retinoblastoma cells were resistant to hypoxic insult, with greater than 90% viability after 4 hours of hypoxia and 24 hours after a return to normoxia. Conclusions: IGF-1 appears to provide significant neuroprotection to hypoxia-sensitive retinal cells, and offers promise as a potential therapeutic agent for ischemic retinal disease.
CR: None Support: NEI grant EY10676

5095—9:30

ROLE OF OXYGENATED FREE RADICALS OF LIGHT-INDUCED RETINAL APOPTOSIS M. Droy-Lefaix[1], I. Ranchon[2], M. Doly[2]. IPSEN Laboratory, Paris, France[1], University of Auvergne, Laboratory of Sensorial Biophysic, Clermont-Ferrand, France[2].

Purpose: We have established that light-induced morphological and functional retinal injuries are partially prevented by free radical scavengers such as dimethylthiourea (DMTU) or Ginkgo biloba extract (EGb 761). The aim of the present work is i) to demonstrate neuronal apoptosis associated with light-induced retinal degeneration ii) to evaluate the involvement of oxygen free radicals in triggering apoptosis and iii) to investigate Bax and Bcl-2 proteins. Methods: Retinal degeneration is induced by exposing Wistar rats to 24 h of 1700 lux light. Treatments consist in DMTU (Sigma ; 500 mg/kg ; i.p.) or EGb 761 (IPSEN ; 100 mg/kg/day ; per os). One day after the light exposure, apoptotic cells were detected using ApopTag® Plus kit (Becton-Dickinson). Bax and Bcl-2 proteins were detected by use of specific polyclonal antibodies. Results: We established that light exposure induces a significant increase in apoptotic cell staining at the level of photoreceptor layer, while after treatment with DMTU or EGb 761 a significant decrease in apoptotic photoreceptors is observed. Bcl-2 and Bax were detected in exposed retinas and localized in the outer nuclear layer. Treatments with DMTU or EGb 761 are able to totally inhibit such proteins expression. Conclusions: This preventive effect of radical scavengers suggests a major role of oxygenated free radicals in light-induced retinal apoptosis.
CR: None

Glial Cell Line–Derived Neurotrophic Factor Induces Histologic and Functional Protection of Rod Photoreceptors in the *rd/rd* Mouse

Maria Frasson, Serge Picaud, Thierry Léveillard, Manuel Simonutti, Saddek Mohand-Said, Henri Dreyfus, David Hicks, and José Sahel

PURPOSE. To evaluate the neuroprotective potential of glial cell line–derived neurotrophic factor (GDNF) in the retinal degeneration (*rd/rd*) mouse model of human retinitis pigmentosa.

METHODS. Subretinal injections of GDNF were made into *rd/rd* mice at 13 and 17 days of age and electroretinograms (ERGs) recorded at 22 days. Control mice received saline vehicle injections or underwent no procedure. At 23 days of age, retinas from treated and control mice were fixed and processed for wholemount immunohistochemistry using an anti-rod opsin antibody, and rod numbers were estimated using an unbiased stereological systematic random approach. Subsequent to counting, immunolabeled retinas were re-embedded and sectioned in a transverse plane and the numbers of rods recalculated.

RESULTS. Although ERGs could not be recorded from sham-operation or nonsurgical *rd/rd* mice at 22 days of age, detectable responses (both a- and b-waves) were observed in 4 of 10 GDNF-treated mice. Stereological assessment of immunolabeled rods at 23 days showed that control *rd/rd* retinas contained 41,880 ± 3,890 (mean ± SEM; $n = 6$), phosphate-buffered saline (PBS)–injected retinas contained 61,165 ± 4,932 ($n = 10$; $P < 0.001$ versus control retinas) and GDNF-injected retinas contained 89,232 ± 8,033 ($n = 10$; $P < 0.001$ versus control retinas, $P < 0.002$ versus PBS). This increase in rod numbers after GDNF treatment was confirmed by cell counts obtained from frozen sections.

CONCLUSIONS. GDNF exerts both histologic and functional neuroprotective effects on rod photoreceptors in the *rd/rd* mouse. Thus rescue was demonstrated in an animal model of inherited retinal degeneration in which the gene defect was located within the rods themselves, similar to most forms of human retinitis pigmentosa. GDNF represents a candidate neurotrophic factor for palliating some forms of hereditary human blindness. (*Invest Ophthalmol Vis Sci.* 1999;40: 2724–2734)

Retinitis pigmentosa (RP) is the term given to a group of inherited retinal dystrophies resulting in progressive photoreceptor loss and eventual extreme visual handicap. There is no known treatment for this condition, which can be caused by a large number of mutations in a variety of genes that code for proteins involved in phototransduction (rhodopsin, cyclic guanosine monophosphate [cGMP]–dependent phosphodiesterase)[1,2] or in structural roles (peripherin/*rds*).[3] Several strategies are under examination for limiting or preventing the photoreceptor loss associated with this disease: gene therapy,[4] pharmacologic treatment (growth factor application[5,6] or vitamin supplementation[7]), and retinal transplantation.[8,9] Among these approaches, the use of growth factors has been advocated since pioneering work by the group of Faktorovich et al.[10] showed that intraocular injections of basic fibroblast growth factor (FGF-2) delayed photoreceptor degeneration in an animal model of RP, the Royal College of Surgeons rat. FGF-2's effects on photoreceptor survival were subsequently demonstrated by the same group in rat models of light-induced photoreceptor degeneration.[5,11] In addition, in vitro studies have demonstrated the differentiative effects of FGF-2 on newborn rat photoreceptors.[12] Yet, photoreceptor degeneration in the RCS rat as well as after constant light damage does not correlate to any currently known form of human retinal degeneration.

The retinal degeneration (*rd/rd*) mouse is a model of human RP that has been investigated for more than 70 years because photoreceptor cell degeneration follows a similar evolution to human RP. Most rod cells undergo apoptosis over the first 3 weeks,[13] followed by death of cone photoreceptors.[14] Furthermore, rod cell death has been found to result from a mutation in the β subunit of cGMP-dependent phosphodiester-

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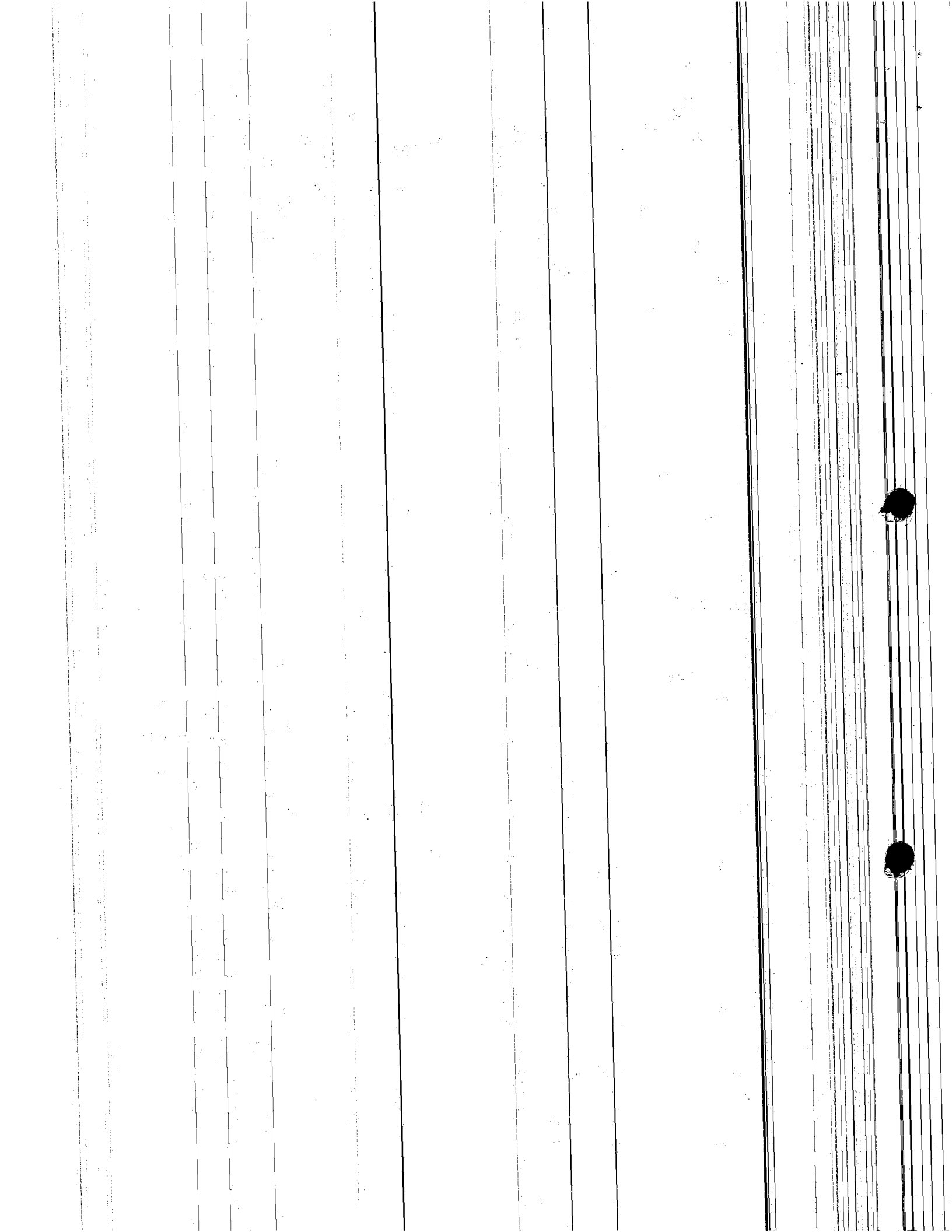


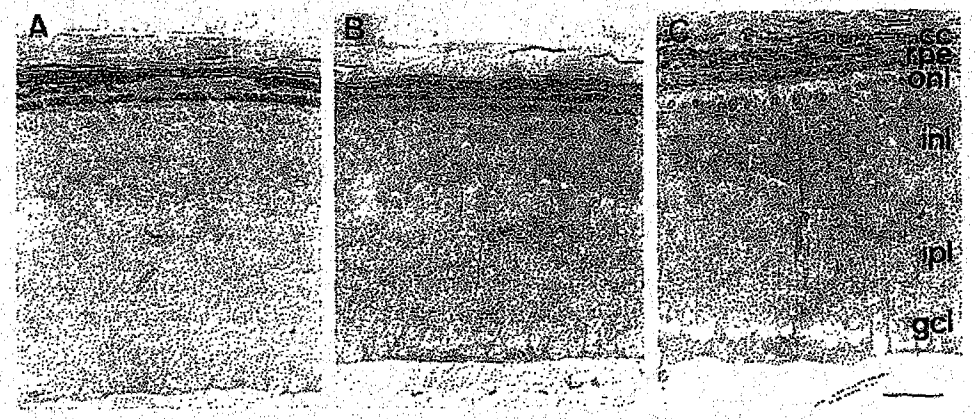


FIGURE 3. Histologic sections of whole eyes from 23-day *rd/rd* mice injected with (A) GDNF or (B) PBS or (C) noninjected. Semithin epoxy sections were stained with toluidine blue. There was no detectable new blood vessel formation within the injected eyes or visible gliosis or macrophage invasion into the subretinal or vitreal spaces. All micrographs were taken in proximity to the optic nerve head and exposed for the same times. cc, choriocapillaris; rpe, retinal pigmented epithelium; onl, outer nuclear layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Scale bar, 20 μm.

formed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

In Vivo Application of GDNF in the *rd/rd* Mouse

GDNF was injected into C3H mice at 13 and 17 days of age (with the day of birth defined as day 0). Mice (n = 10) were anesthetized with an intraperitoneal injection (16.7 μl/g body weight) of etomidate (0.5 mg/ml) and midazolan (0.5 mg/ml). The pupils were dilated with 0.5% tropicamide, and the cornea was anesthetized with 0.5% topical proparacaine. GDNF (1 μl; recombinant human GDNF; Promega, Madison, WI) was injected at a dose of 330 ng/μl in 0.1 M sterile PBS with a Hamilton syringe and a 30-gauge needle 1 mm from the limbus into the subretinal space. The contralateral eye was injected with the same volume of PBS solution only. It was necessary to perform bilateral ocular surgery to distinguish between the rescue effect due to GDNF per se and that due to experimental manipulation. In rare cases, partial backflow was observed. After injection, fundoscopy was performed to confirm the induction of a local retinal detachment. This was observed in every case at the time of injection, and we observed that the detachment created by the initial injection was resorbed by the time the second was administered. Processing of fixed retinas after recording (description follows) indicated that retinas were correctly attached at the end of the experimental period.

Recording ERGs in the *rd* Mouse

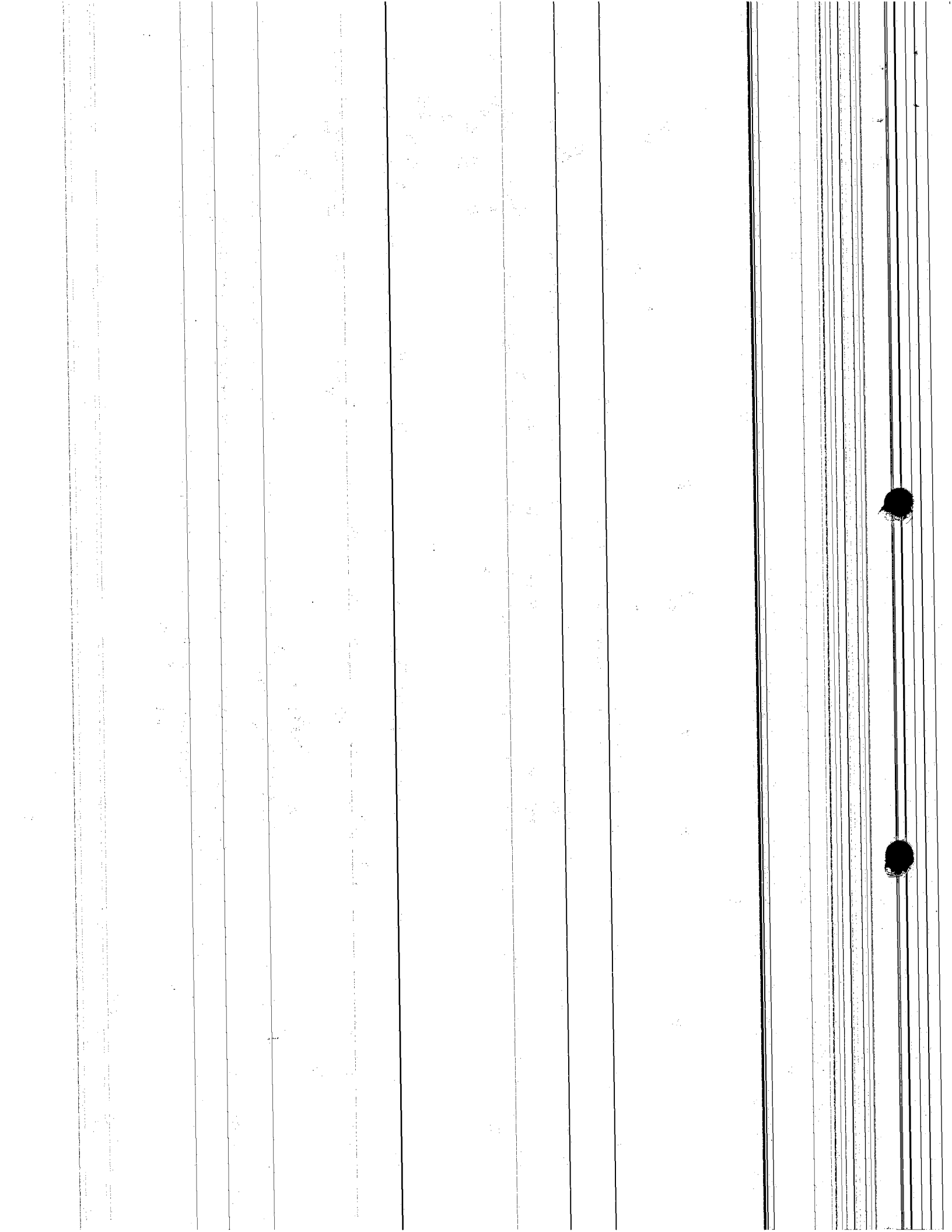
ERGs were picked up by a cotton wick connected to an Ag:AgCl electrode with saline solution directed to the apex of cornea by a micromanipulator. A stainless steel reference electrode was placed subcutaneously on top of the head. Responses were amplified at a gain of 10,000 and filtered with the low 0.1-Hz and high 1000-Hz cutoff filters from an amplifier (Universal; Gould, Ballainvilliers, France). Responses were digitized using a data acquisition labmaster board (Scientific Solutions, Solon, OH), mounted on an IBM-compatible personal computer. Experimental data were acquired and analyzed using the Patchit and Tack software packages (Scientific Solutions).[25] After a period of 24 hours, dark-adapted mice were prepared under dim red illumination. They were anesthetized as above and placed in a Faraday cage, the head resting in a U-shaped holder. The upper and lower lids were retracted to hold open and proptose the eye. The light stimulus was obtained from a 150-watt quartz-xenon lamp bulb (Müller Instruments, Moosinning, Germany). The light beam was focused to infinity through a heat-absorbing filter onto a hole in the Faraday cage. The illumination intensity of the light was 2.9 log cd/m^2 as measured with a luxmeter at the level of the eye. A computer-controlled shutter was used to deliver flashes of 300-msec duration. For GDNF-injected mice, recordings from both eyes were obtained successively, and the investigator was not aware of which eye had received the GDNF injection.

Histology of Injected Eyes

Mice were killed by anesthetic overdose at 23 days. Whole eyes from GDNF- and PBS-injected and noninjected *rd/rd* eyes (n = 2 for each group), were removed from mice at 23 days, fixed in 2.5% glutaraldehyde, and embedded in epoxy resin. Eyes were sectioned along a perpendicular plane close to the optic nerve head, and semithin sections were processed for histologic examination after toluidine blue counterstaining.

Immunohistochemical Labeling of Retinas

Mice that underwent surgery (n = 10 for both GDNF-injected and sham-operation mice) were killed by anesthetic overdose at 23 days. Nonsurgical eyes were also sampled from a further six 23-day-old *rd/rd* mice. Eyecups were removed and fixed in 4% paraformaldehyde for 2 hours. Retinas were dissected from the posterior eyecup, washed three times in PBS, permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes, and incubated in blocking buffer (PBS containing 0.1% bovine serum albumin, 0.1% Tween 20, and 0.1% sodium azide ([buffer A]) for 15 minutes. They were then incubated with rod photoreceptor-specific monoclonal antibody rho-4D2[26] (10 μg/ml in buffer A for 1 hour), washed and incubated in goat anti-mouse IgG-Texas red (10 μg/ml in buffer A for 1 hour; Molecular Probes, Portland, OR). Retinas were washed and flatmounted in PBS-glycerol (50:50 vol/vol), with the photoreceptor layer facing up, and examined under a photomicro-



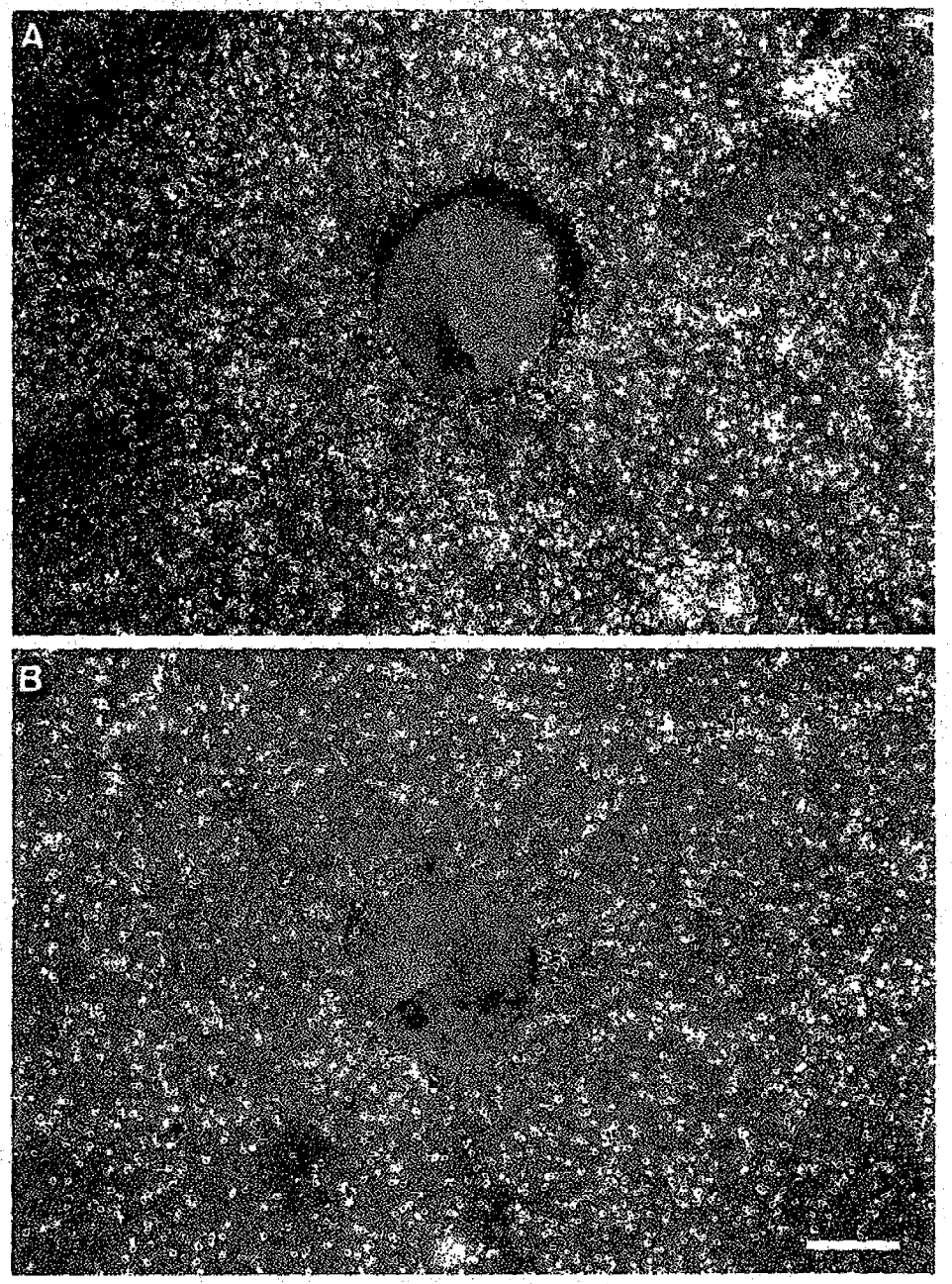


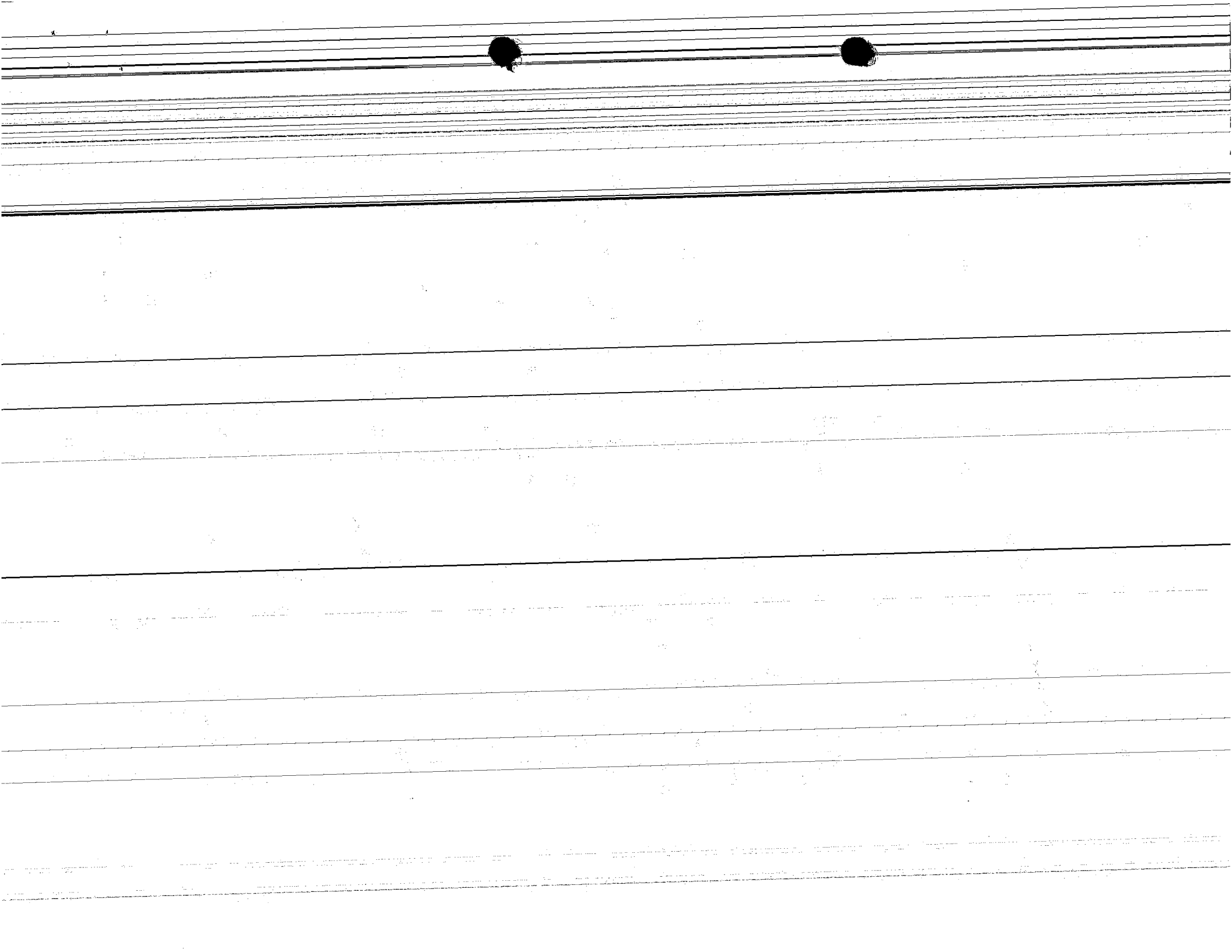
FIGURE 4. Low-power view of GDNF-injected (A) and PBS-injected (B) wholemounted *rd/rd* retinas immunolabeled with rho-4D2. The greater numbers of surviving rod photoreceptors after GDNF treatment are apparent. This example represents the mouse showing the greatest difference between the GDNF-treated eye and the PBS-injected contralateral eye. Scale bar, 100 μm.

scope (Optiphot 2; Nikon, Melville, NY) equipped with differential interference and fluorescence optics.

After stereological counting of rod numbers (see below), five wholemounted immunolabeled retinas from each experimental group were processed further by re-embedding and cryostat sectioning in a transverse plane. Frozen sections were collected from near the central retina, mounted on microscope slides, and examined for rho-4D2 staining. After labeled rods were counted (see later description), sections were incubated with anti-glial fibrillar acidic protein (GFAP) polyclonal antibody (Dako, Trappes, France), diluted 1:400 in buffer A for 2 hours. Sections were washed and incubated in goat anti-rabbit IgG-Bodipy FL (Molecular Probes) and the nuclear dye 4′-6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich, Saint Quentin Fallavier, France), both 10 μg/ml in buffer A for 1 hour. Slides were washed thoroughly and examined as above, with different filter sets allowing visualization of only rho-4D2–immunolabeled rods (single-band-pass, filter XF42), only anti-GFAP-immunolabeled retinal glia (single-band-pass, filter XF22), simultaneous rho-4D2 and anti-GFAP staining (double-band-pass, filter XF53) and DAPI-labeled nuclei (single-band-pass, filter XF03; all fluorescence filters from Omega Optical, division of Molecular Probes). For photography, picture series were taken with similar exposure times (HP5 film; Ilford, Basildon, UK) and printed using identical times.

Cell Counts

For quantification of rods in flatmounted retinas we used a stereological approach permitting unbiased sampling.[27] Immunolabeled cells were observed by microscope (Optiphot 2; Nikon) under epifluorescence illumination with a plan 40/070 differencial interference contrast (DIC) (160/0.17)



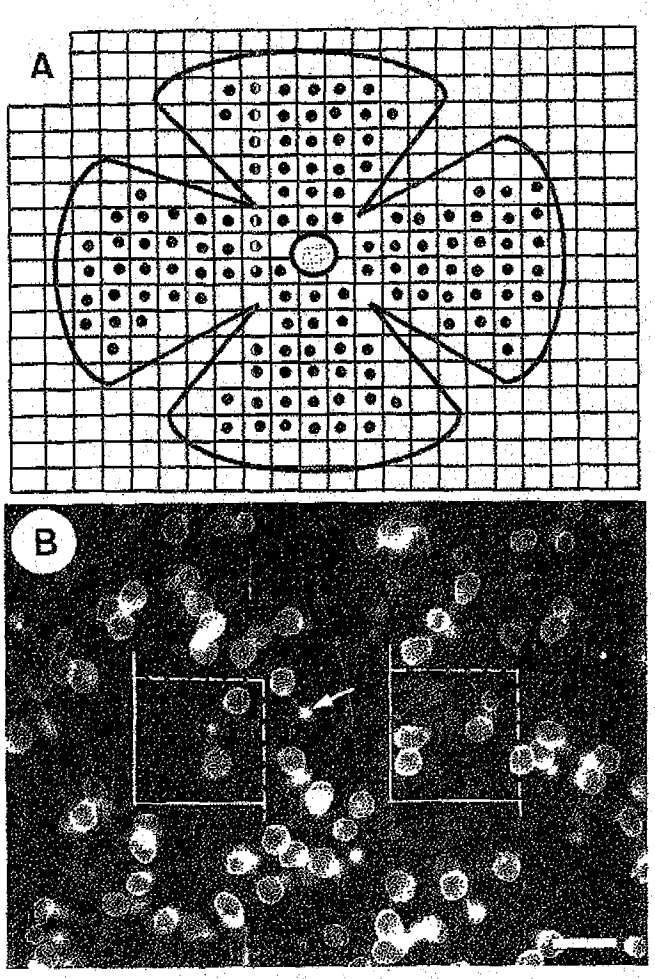


FIGURE 5. Illustration of the systematic random sampling approach used (A) and high-power view of 23-day-old *rd/rd* retina showing aspect of immunolabeled rod cells and superimposed counting frame (B). The antibody stains the entire cell body and vestigial outer segment. Scale bar (B). 10 μm.

objective. One hundred twenty fields of 8000 μm^2 were selected throughout the entire retinal surface (approximately 13 mm^2) using a stage encoder and a systematic random procedure.[28] Fields were viewed with a Sony Trinitron color graphic display camera and digitized software (Automator for Windows; Biocom, Lyon, France). In each of the 120 fields, cells were counted in two unbiased counting frames of 900 μm^2 generated by the software. The total number of rod cells in the entire retina was then estimated by normalizing these numbers to the entire retinal surface area, which was measured using an image analysis system (Optiscan; Macintosh LC II Ci; Apple Computer, Cupertino, CA). For counting of rods in transverse sections, a single section from each of five retinas for each treatment, passing through the optic nerve head and traversing the entire retinal width, was mounted on a microscope slide and stained with DAPI (10 μg/ml in PBS for 10 minutes). Viewing sections with the two appropriate filter sets allowed visualization of rho-4D2–labeled rods and total DAPI-labeled nuclei. Images were captured on the computer as described along the entire length of each section.

Reverse Transcription–Coupled Polymerase Chain Reaction Analysis of GDNF mRNA

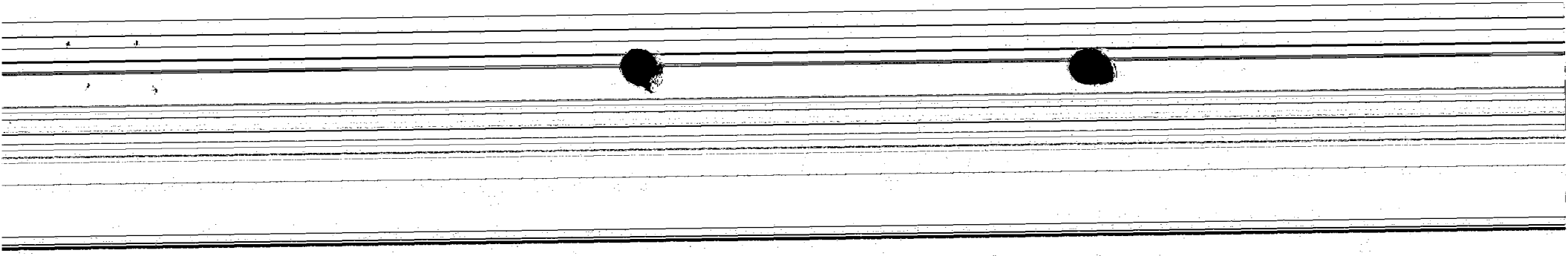
Total RNA was isolated with an RNA-DNA extraction kit (Qiagen; Valencia, CA) with standard protocol. RNA samples (1 μg) were primed with random hexamer and incubated 2 hours at 37°C with Moloney murine leukemia virus reverse transcriptase. cDNA (1:20; 2 μl) was amplified for 35 cycles with specific pairs of primers. Primer sequences 5′ to 3′were: rhodopsin, AAGCCGATGAGCAACTTCC, TCATC TCCCAGTGGATTCTT; GDNF, ACCAGATAAACAAGCGGCAG, TCAGATACATCCA CACCGTTTAG; and glucose-6-phosphate dehydrogenase (MG6PDH), GCAGTCACCAAGAACATTCAAG, CCC AAATTCATCAA AATAGCCC. Amplified products were visualized on agarose gels stained with ethidium bromide and digitized with a camera. Quantitative measurement of band intensity was made using commercial software (Phoretix International, Newcastle-upon-Tyne, UK). To quantify the amplification products, the exponential phase of the reaction was experimentally determined using different cycling numbers for each primer pair. Under these conditions the amount of the amplification product reflects the initial concentration of transcripts.[29]

RESULTS

ERGs

Figure 1 illustrates the comparative evolution of ERGs from wild-type (C57) and *rd/rd* mice. In wild-type mice, the amplitude of a- and b-waves steadily increased from postnatal days 12 to 24. In *rd/rd* mice, the ERG was similar to the wild-type at postnatal day 12, but thereafter a- and b-waves steadily decreased from postnatal day 15 onward, becoming unrecordable by 22 days (Fig. 1). The light intensity for triggering a signal was 0.3 log cd/m^2, which corresponds approximately to the threshold for evoking cone responses.

To determine whether GDNF could improve visual functions in *rd/rd* mice, subretinal injections of this trophic factor were administered at 13 and 17 days, together with PBS vehicle injections into the contralateral eye to serve as control eyes. ERGs were recorded in animals at 22 postnatal days when ERG signals had disappeared completely in noninjected *rd/rd* mice. None of the eyes that received PBS exhibited a detectable ERG (n = 10). In contrast, ERGs were measurable in 4 of 10 GDNF-injected eyes (Fig. 2A, numbers 3, 4, 7, and 9). It should be noted that two of the six mice in which no signal was detected (Fig. 2A, numbers 1 and 5) showed prominent cataracts in both eyes, and they were not considered in the functional scores. A typical ERG recorded from a GDNF-injected eye is shown in Figure 2B. The average amplitudes of a- and b-waves (±SEM) for the four GDNF-injected mice showing a response were 8.5 ± 2.8 μV and 15.5 ± 2.3 μV, respectively. These represent responses that would normally be obtained from 18.5-day-old *rd/rd* mice, or 22% of the maximal response observed in *rd/rd* mice (Fig. 1B). These responses had implicit times (a-wave 120 ± 14 msec; b-wave 242 ± 43 msec; n = 4) similar to those observed in 15- to 16-day-old control *rd* animals (a-wave 144 ± 10 msec; b-wave 274 ± 23 msec; n = 6) but longer than those of control C57 mice at 24 days (a-wave 31 ± 3 msec; b-wave 110 ± 12 msec; n = 4). These data indicate that GDNF treatment prolonged visual function by approxi-



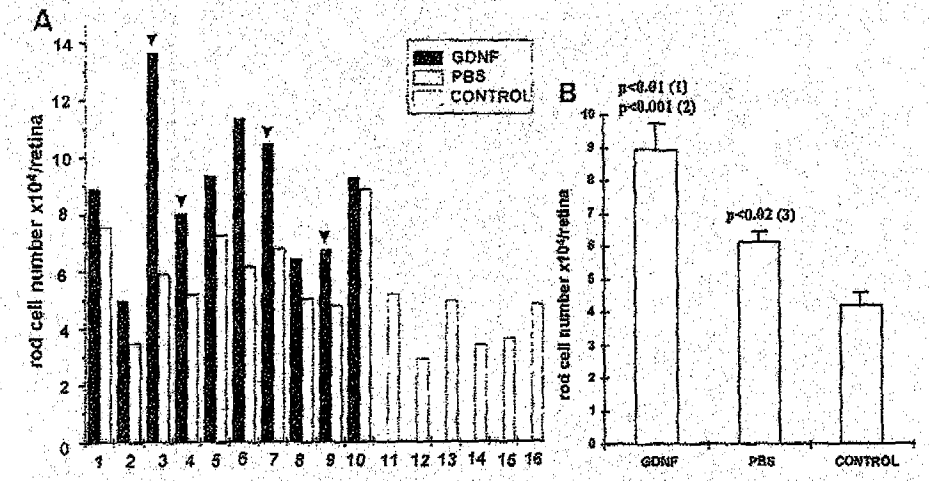


FIGURE 6. Immunolabeled rod cell counts in GDNF-, PBS- and noninjected 23-day *rd/rd* mice. (A) Total surviving rod numbers for each mouse (*x*-axis) injected with GDNF in one eye (*black bars*) and PBS vehicle in the other eye (*dark gray bars*). The consistent beneficial effect of growth factor treatment compared with vehicle-injected eyes can be observed. Those mice in which ERGs were successfully recorded were numbers 3, 4, 7, and 9 (*arrowheads*). Total surviving rod numbers for nonsurgical *rd/rd* retinas from an additional five mice are shown by bars 11 to 15 (*light gray*). It can be seen that surgery itself led to variation in rod numbers compared with nonsurgical eyes and that PBS injection produced a smaller but statistically significant increment in rod numbers. (B) Average (mean ± SEM) rod cell numbers calculated from (A). The GDNF-induced increase in rod numbers compared with PBS-treated and untreated control eyes is visible. Probabilities indicated above GDNF are comparisons with PBS treatment (1: $P < 0.01$) and nonsurgical control eyes (2: $P < 0.001$) and above PBS is comparison with nonsurgical control eyes (3: $P < 0.02$).

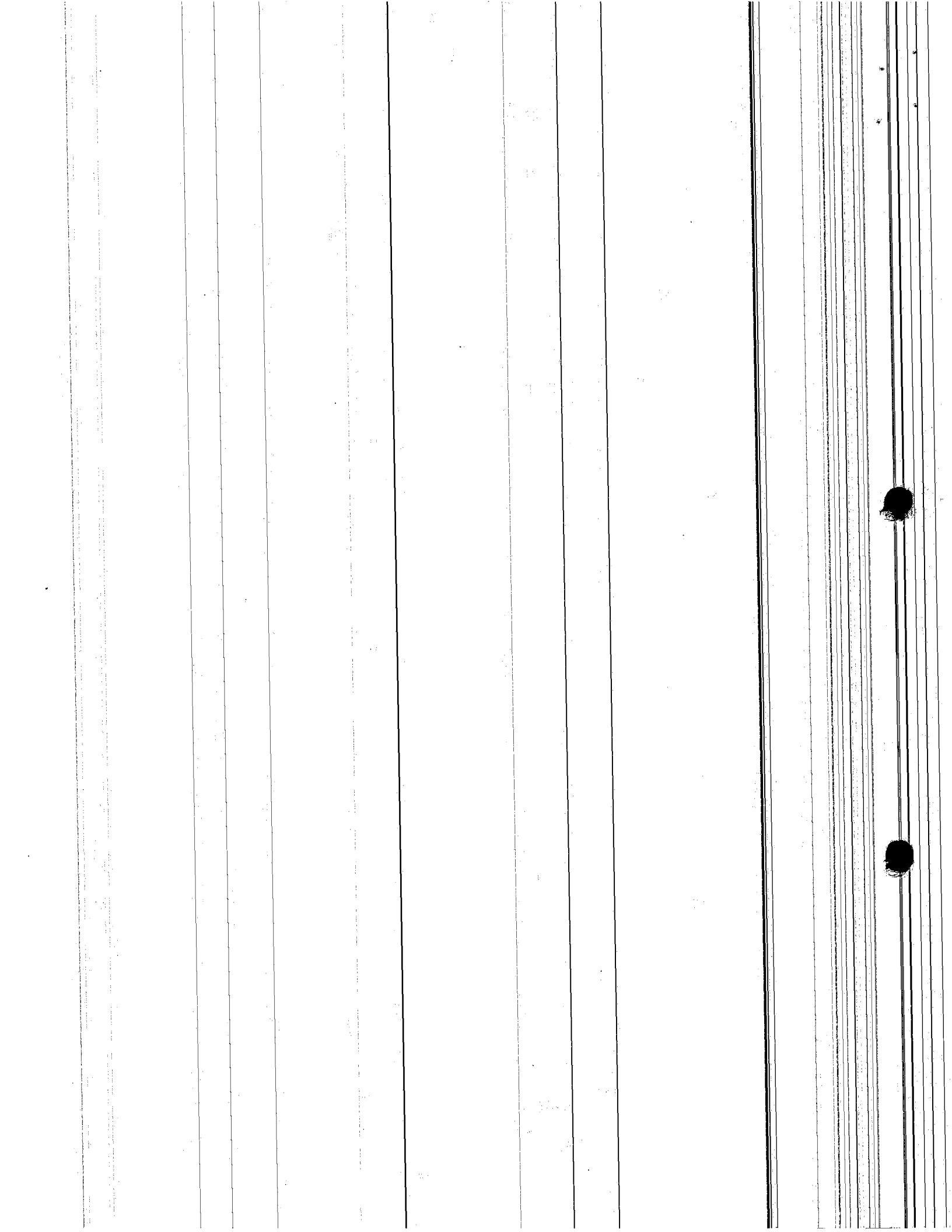
tely 30% relative to the period of massive photoreceptor ith in this strain.

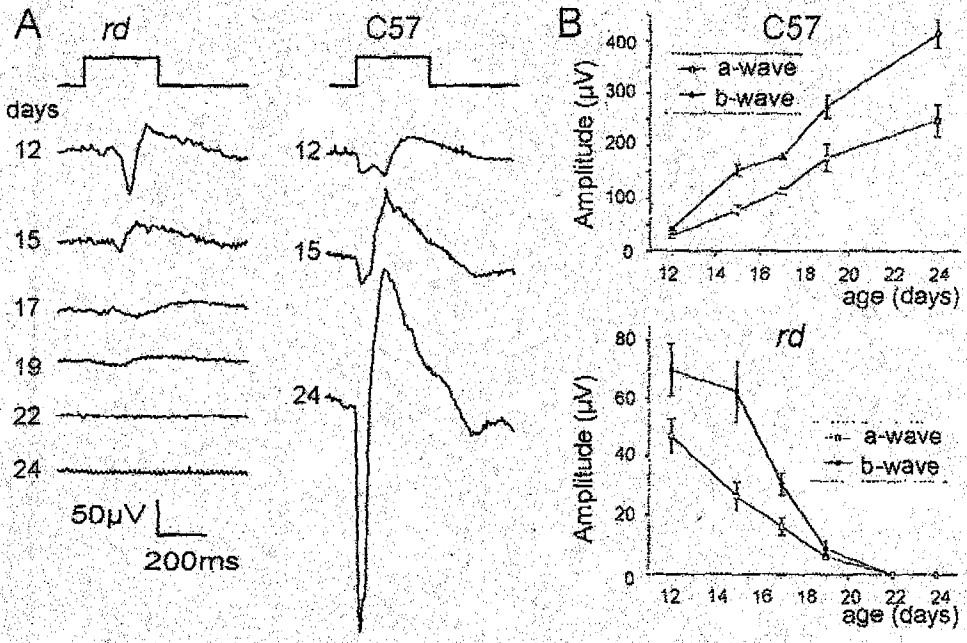
stologic, Immunohistochemical, and rphometric Analyses

er ERGs were recorded, the animals that received GDNF l PBS injections ($n = 10$) and a second group of control e of the same age ($n = 6$) were killed and the eyes fixed dissected. Epon-embedded histologic sections of surgi- or control eyes showed that injection of GDNF or PBS did lead to complications such as neovascularization, retinal ring, or macrophage invasion by 6 days after the final ction (Fig. 3).

Figure 4 shows a low-power view of GDNF-injected . 4A) and PBS-injected (Fig. 4B) wholemounted *rd/rd* nas immunolabeled with rho-4D2. The overall greater ibe surviving rod photoreceptors after GDNF treat- t are apparent. Higher power photographs showed that antibody stained the entire rod photoreceptor cell, in- ing the cell body and vestigial outer segment (Fig. 5). stereological counting frame and the principle of the om systematic sampling procedure used are also shown gure 5. Rod cell counts for individual animals are shown gure 6A (paired GDNF-treated and PBS-injected *rd/rd* [1–10] and nonsurgical *rd/rd* mice[11–16]). The variability ween individual mice in opsin-immunolabeled rod num- is evident, but in each case GDNF treatment led to er cell counts. Correspondence between the degree of e and functionality was suggested by the fact that three four mice in which ERGs were detected (Fig. 6A, mice and 7) showed the highest relative differences in rod numbers between GDNF- and PBS-injected animals: 70% for the ERG-positive group ($n = 4$) and 34% for the ERG-negative group ($n = 6$). However, the differences between these two groups were not statistically significant. The average (±SEM) rod cell number found in GDNF-injected eyes (89,232 ± 8,033; $n = 10$) showed a 46% increase compared with the number in PBS-injected eyes (61,165 ± 3,259; $n = 10$) and a 113% increase compared with control noninjected eyes (41,880 ± 3,890; $n = 6$; Fig. 6B). PBS injection also led to an increase (36% more) in rod numbers compared with the number in untreated eyes. These increases were all statistically significant: GDNF versus PBS, $P < 0.01$; GDNF versus control, $P < 0.001$; PBS versus control, $P < 0.02$. These data expressed relative to total rod numbers found in normal adult C57 mice (estimated by us as 4.13×10^6; data not shown) yield 2.2%, 1.5%, and 1.0% for GDNF, PBS, and noninjected *rd/rd* retinas, respectively.

To compare these data more easily with previously published results on rod loss in the *rd/rd* mouse, we sectioned five wholemounted retinas from each experimental group and re-examined rod cell labeling. Figure 7 shows representative areas from each experimental group and clearly shows immunolabeled rods scattered among immunonegative cells within the vestigial outer nuclear layer (ONL). Noninjected control *rd/rd* retinas had only sparsely scattered rods, appearing as a single discontinuous layer along the scleral border (Figs. 7A, 7B). PBS-injected retinas had more immunolabeled rods, appearing as a single-to-double discontinuous layer (Figs. 7C, 7D). GDNF-injected retinas often contained two to three rows of immunolabeled rods, although areas of rod opsin-immunonegative cells within the ONL were still apparent (Figs. 7E, 7F). Sections





GURE 1. Evolution of *rd/rd* and ild-type mice ERG on light stimula- n. (A) ERG recordings at different stnatal ages for *rd/rd* and wild- pe (C57) mice. Postnatal days are licated on the *left* of the ERG re- rdings. (B) Evolution of a- and b- ve amplitude from 12 to 24 post- tal days. Note the continuous crease toward complete disap- arance of the a- and b-waves in *rd* mice from postnatal days 15 to in contrast to the regular increase served in wild-type mice. The plot each age represents data from 5 imals, except for the latest time int for the *rd/rd* mouse (24 days), ich represents 10 animals.

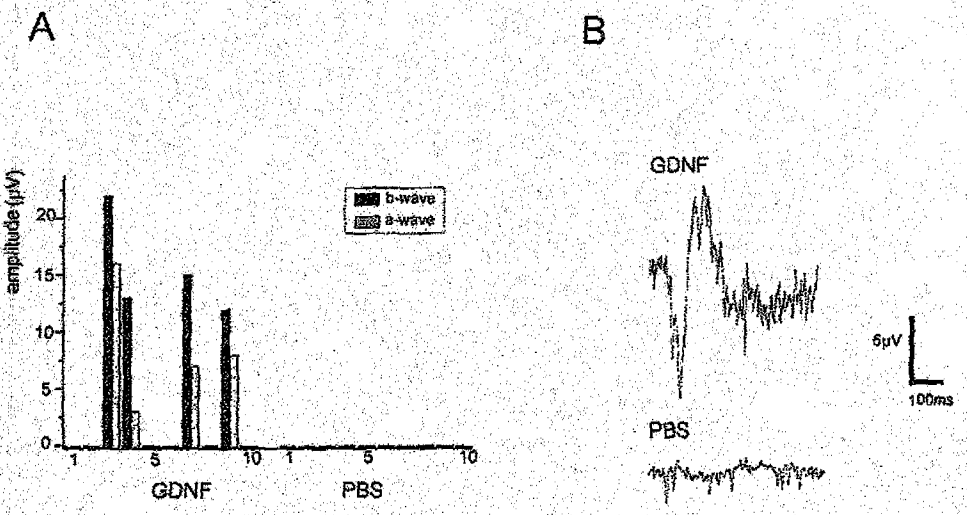
[15] r to some human families affected by the disease.[16] cent ong a large variety of neurotrophic factors in- ted into *rd/rd* eyes, only ciliary neurotrophic factor (CNTF) duced histologic photoreceptor cell loss.[6]

Glial cell line–derived neurotrophic factor (GDNF), which longs to the transforming growth factor β superfamily, was st described as a stimulant of survival of dopaminergic neu- ns in vitro.[17] Subsequently, its protective effects were dem- strated in in vivo models of Parkinson disease[18–20] or on veloping motoneurons.[21] Because GDNF is synthesized in retina,[22] has been found to stimulate survival of newborn ce photoreceptors in vitro,[23] and recently was shown to lay photoreceptor outer segment collapse in vitro,[24] we nducted electrophysiological, histologic, immunohisto- emical, morphometric, and molecular biologic studies to see ether it could stimulate photoreceptor survival in the *rd/rd* ouse after intraocular injections in vivo. We showed that h treatment leads to functional improvement, as indicated the presence of recordable electroretinograms (ERGs) in some GDNF-injected retinas. Increased numbers of immunolabeled rods were detected using two methods of counting, and retinal glial cell physiology was also affected. In contrast, we could exclude the involvement of decreased levels of endogenous GDNF in rod photoreceptor cell death in the *rd/rd* mouse.

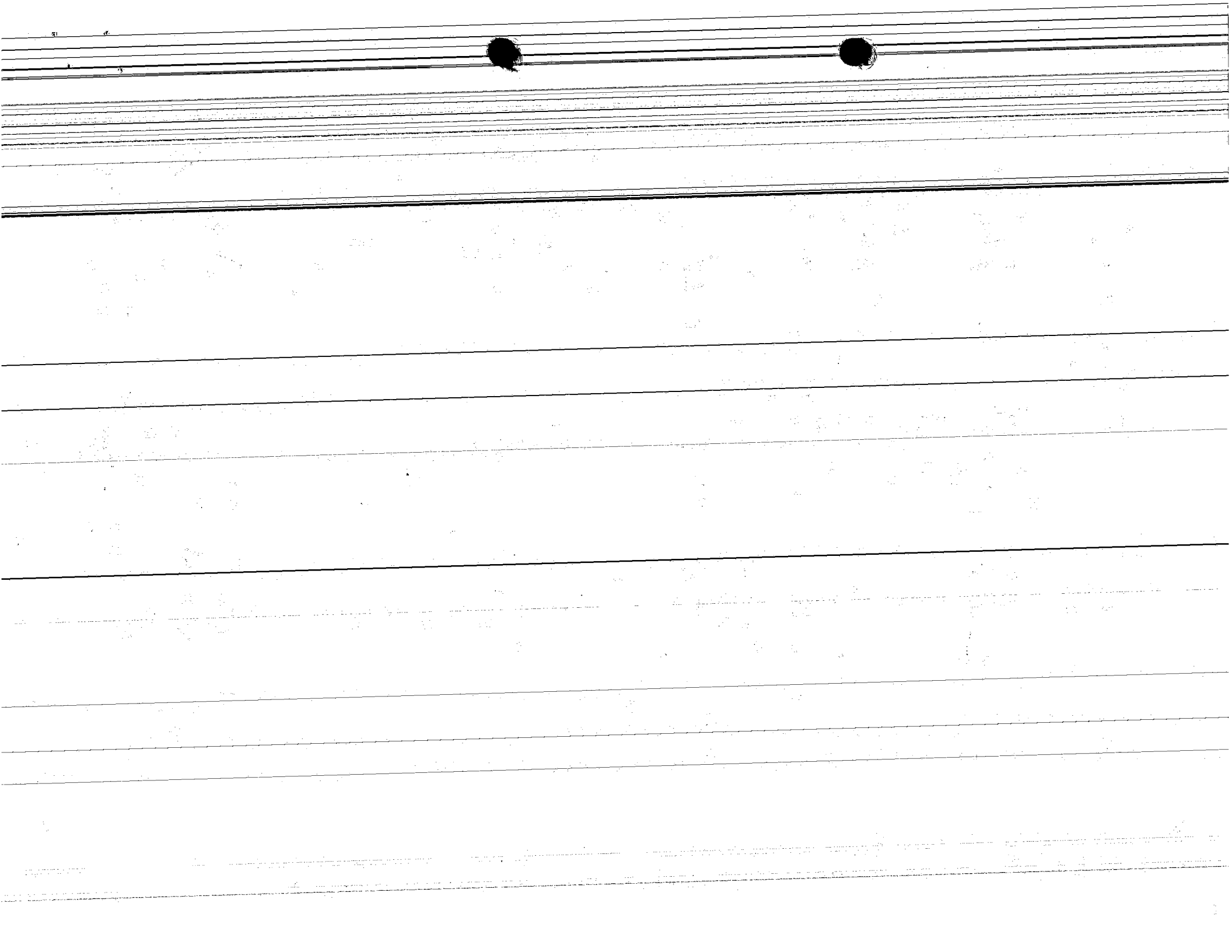
Materials and Methods

Animals

C3H/He/J mice homozygous for the *retinal degeneration* (*rd*) gene (*rd/rd* mice) and C57/BL6/J control mice aged 12, 15, 17, 19, and 24 days were obtained from single breeding colonies maintained exclusively for our laboratory by Charles River Animal Suppliers (St. Aubin-les-Elbeuf, France), maintained in clear plastic cages, and subjected to standard light:dark cycles of 12 hours All procedures con-



ure 2. Preservation of record- ERG in GDNF- compared with injected 22-day *rd/rd* mouse. (A) ogram showing amplitudes of G and b-waves from individual mals. (B) ERGs recorded from NF- and PBS-injected eyc. The re- dings were made from mouse 7 in and represent average traces three separate recordings.



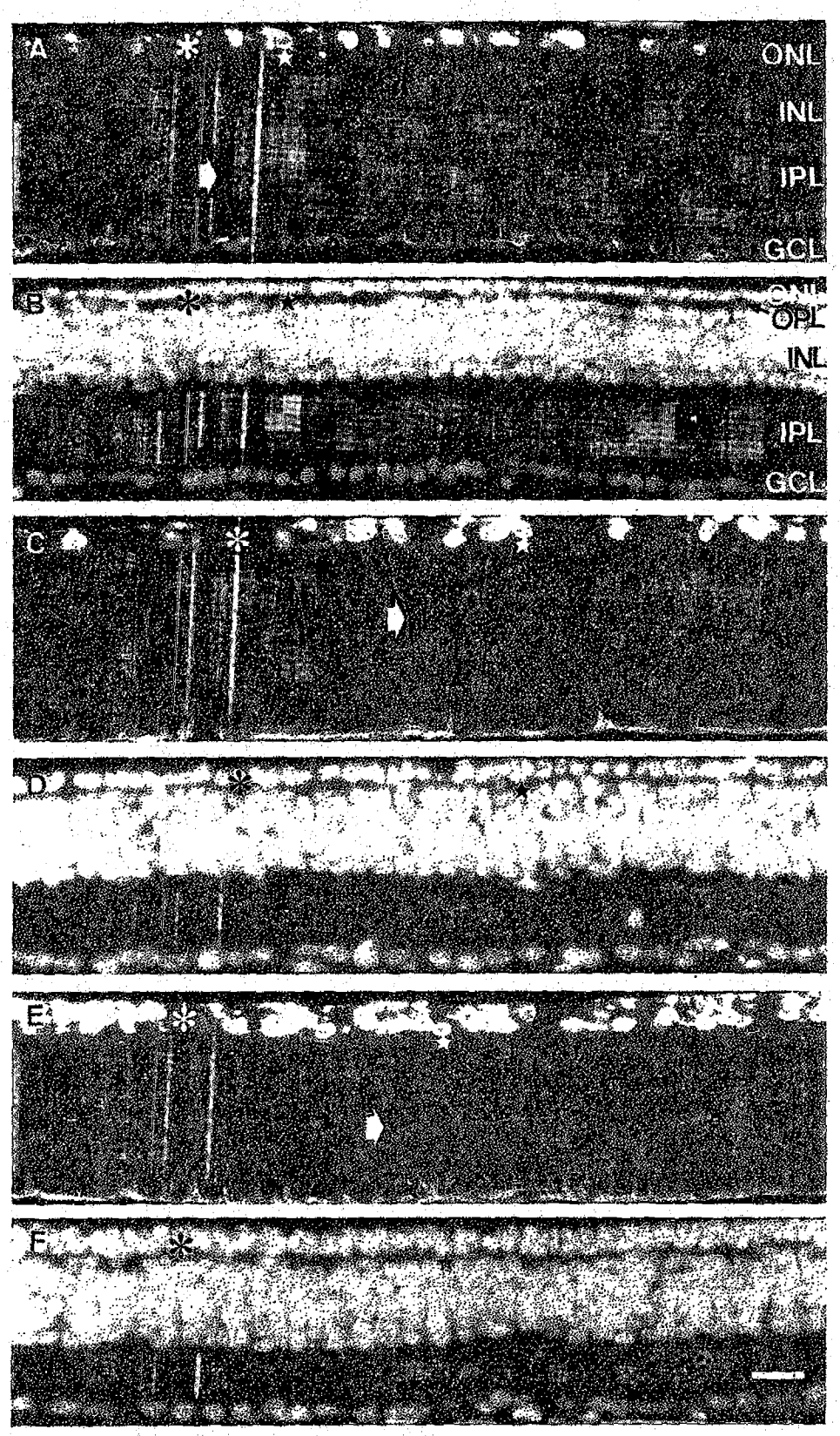
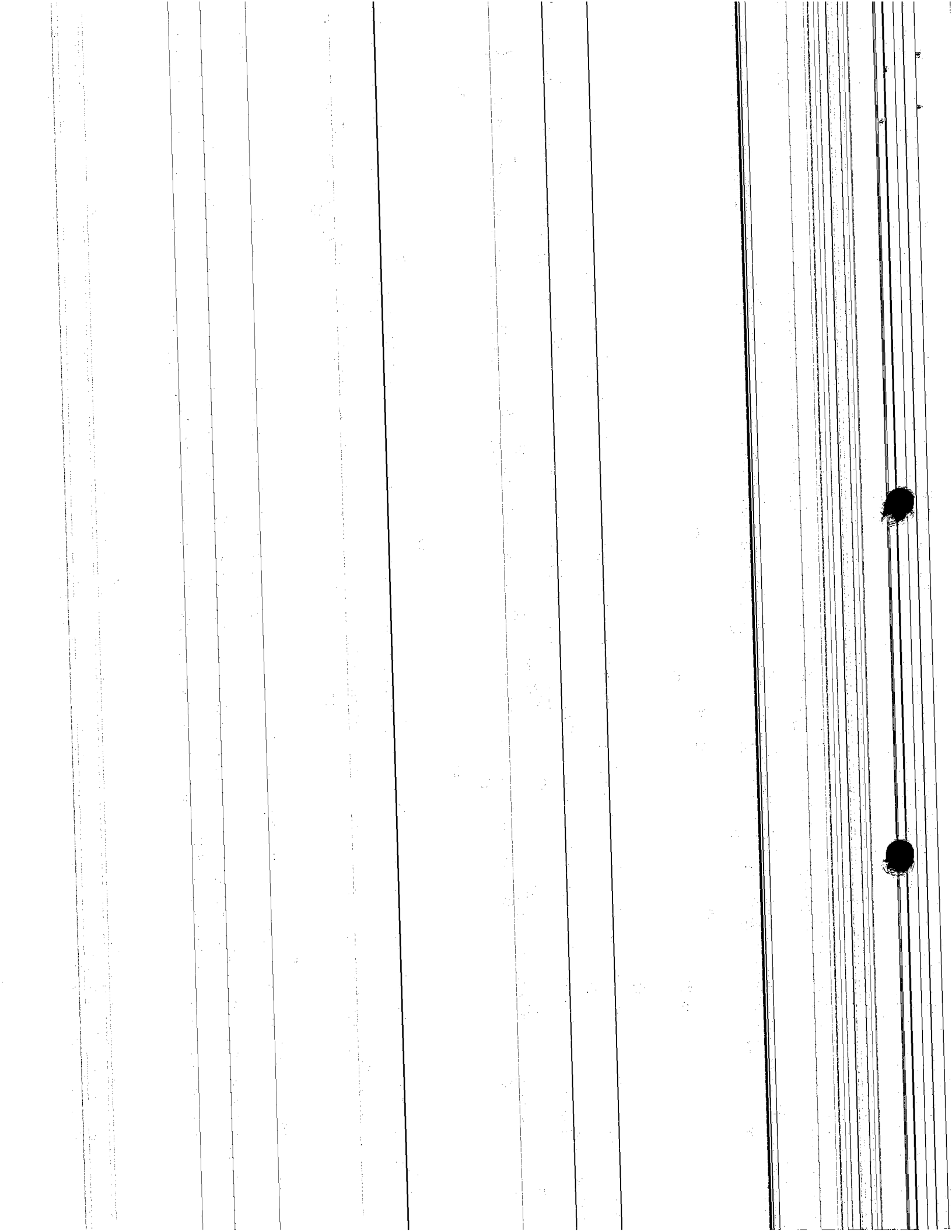


FIGURE 7. Transverse sections taken from wholemounts of 23-day *rd/rd* retinas prelabeled with rho-4D2 and relabeled with anti-GFAP. (A) and (B) are the same section from noninjected, (C) and (D) from PBS-injected, and (E) and (F) from GDNF-injected retinas. (A, C, E) Simultaneous visualization of rod opsin and GFAP immunoreactivity; (B, D, F) DAPI labeling of all nuclei. The rho-4D2–immunopositive rods are visible as brightly labeled, small, round cells lying along the vestigial ONL (*stars* in A, C, E, with the corresponding cell indicated by *stars* in B, D, F). Rho-4D2–immunonegative cells can be seen as unlabeled areas in the ONL (*asterisks* in A, C, and E, with corresponding cells indicated by *asterisks* in B, D, and F). The different number of immunolabeled rods after no injection ($n = 16$), PBS alone ($n = 22$), or GDNF treatment ($n = \sim 40$) can be clearly seen (A, C, E, respectively). Also, the increasing intensity of radial Müller glial fibers labeled by anti-GFAP antibody can be seen in the order GDNF-injected > PBS-injected > noninjected (E, C, and A, respectively, *arrows*), whereas astrocyte staining by the same antibody remains approximately constant along the vitreal border. All micrographs were taken in proximity to the optic nerve head and exposed for the same times. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 23 μm.

were subsequently immunolabeled with anti-GFAP antibody and the two labels visualized simultaneously with a double-band-pass fluorescence filter. GFAP immunoreactivity was visible as bright labeling of astrocytes within the nerve fiber layer and faint transverse fibrous labeling of retinal Müller glia. In noninjected retinas, GFAP immunoreactivity was mostly confined to the astrocytes, with only rare GFAP-immunopositive fibers (Fig. 7A). PBS-injected retinas showed increased numbers of such fibers (Fig. 7C), and GDNF-injected retinas contained large numbers (Fig. 7E). Quantitative analysis of rod



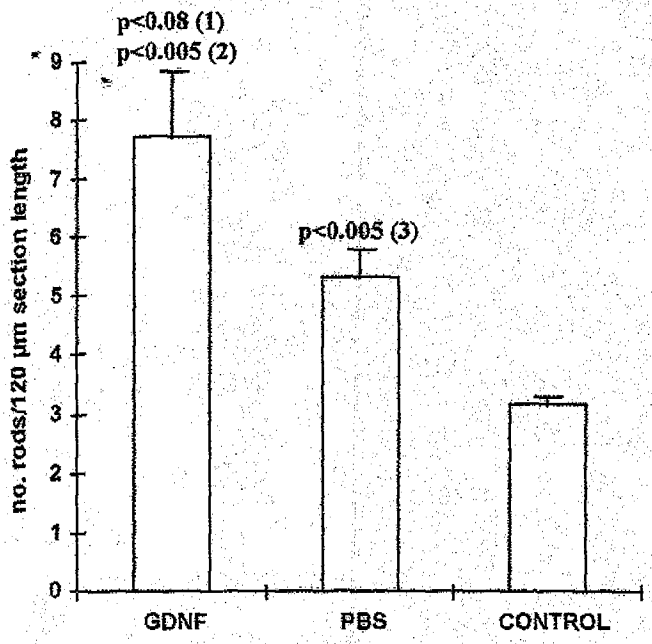


FIGURE 8. Rod numbers determined from transverse sections of GDNF-, PBS-, and noninjected *rd/rd* retinas. Average numbers (mean ± SEM) of immunolabeled rods were calculated from the entire width of sing[illegible]ction passing through the optic nerve head from retinas of ach experimental group (n = 5). Rod numbers were not statistically ifferent between GDNF- and PBS-treated retinas (1: $P < 0.08$). Rod umbers were significantly higher in GDNF- compared with noninjected retinas (2: $P < 0.005$) and in PBS- compared with noninjected etinas (3: $P < 0.005$).

umbers from transverse sections revealed an average (±SEM) f 7.70 ± 1.10 rods/120 µm (n = 5) for GDNF-injected, 5.30 ± .48 rods/120 µm (n = 5) for PBS-injected, and 3.14 ± 0.13 ods/120 µm (n = 5) for noninjected retinas (Fig. 8). The ifference in rod numbers between GDNF-treated, PBS-treated, nd nonsurgical retinas was not significant between GDNF and BS ($P < 0.08$), but was significant between GDNF and nonargical control eyes ($P < 0.005$) and between PBS-injected nd nonsurgical control eyes ($P < 0.005$).

xpression of GDNF mRNA in *rd/rd* Mice

o study the possible involvement of GDNF as an endogenous ophic factor for rods, we analyzed the expression of endogenous GDNF mRNA in mouse retina (Fig. 9A). Lane 1 shows hat GDNF mRNA was expressed in higher steady state mounts in retina compared with the remainder of the eye atio 6:4). Twelve-day-old *rd/rd* mice expressed rod-specific odopsin mRNA at levels comparable to 35-day-old C57 mice ig. 9B, lanes 1, 10, and 11). Large decreases in rhodopsin RNA expression were observed in *rd/rd* mice between days 2 and 120 (lanes 1 through 9). During the whole period, levels f GDNF mRNA (lanes 12 through 20) and those of the housekeeping enzyme G6PDH (lanes 23 through 31) remained unhanged and were similar to their respective expression intenties in C57 mouse eye at 35 days (lanes 21, 22, 32, and 33).

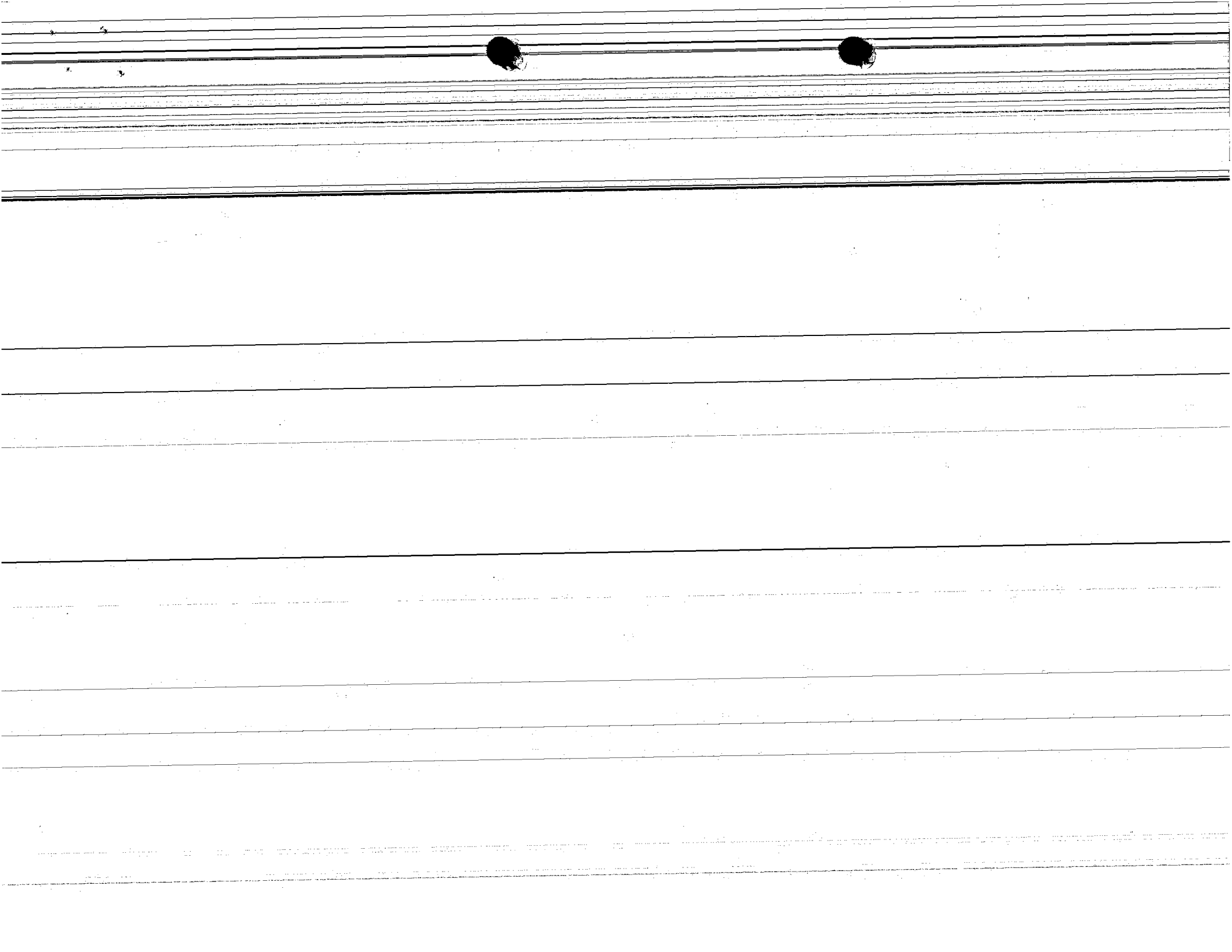
ISCUSSION

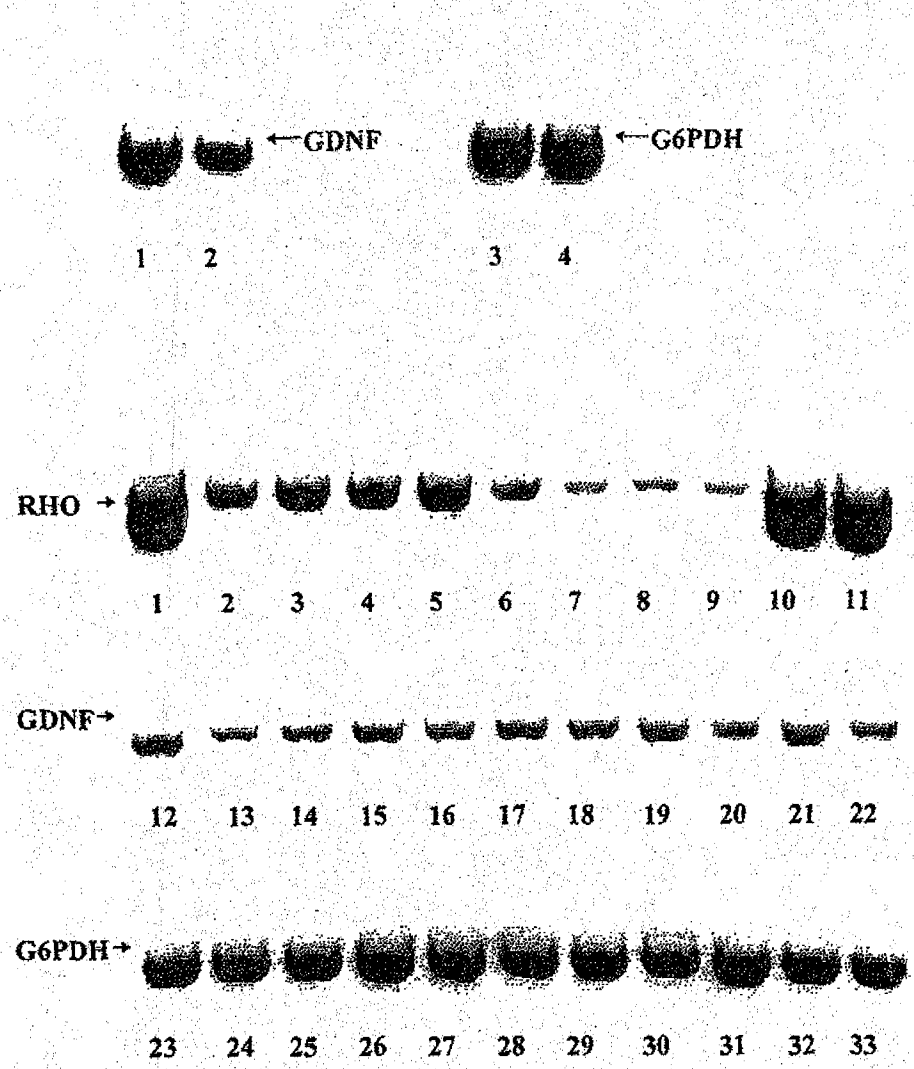
ur data show a stimulatory effect of subretinal injections of e neurotrophic factor GDNF on rod photoreceptor survival in the *rd/rd* mouse. This effect was demonstrated through immunohistochemical and morphometric analyses and through measurement of light-evoked responses. To the best of our knowledge this is the first report in which injection of a neurotrophic growth factor has been shown to promote improvement of retinal function in an animal model of retinal degeneration closely resembling human RP.

Faktorovich et al.[10] first showed the feasibility of using polypeptide growth factors to slow photoreceptor loss in inherited retinal degeneration. Using a rat model of RP they showed that intraocular injections of FGF-2 delayed rod photoreceptor loss. They also reported recently the effects of intraocular injection of multiple neurotrophic factors (CNTF, brain-derived neurotrophic factor, FGF-2, leukemia inhibitory factor, insulin-like growth factor II, neurotrophin-3 and -4, and nerve growth factor) on slowing rod photoreceptor loss in several mouse models of retinal degeneration including the *rd/rd* mouse.[6] These molecules were chosen because they are known to exhibit neuroprotective effects in other retinal models or elsewhere in the nervous system.[5,30,31] Of all these factors, only rat CNTF injected between postnatal days 7 and 10 had a protective effect 1 week later in *rd/rd* mice. In an independent study, CNTF delivered by adenoviral vectors showed histologic and functional protection of rod photoreceptors in another mutant, the retinal degeneration slow (*rds*) mouse.[32] Rescue effects of PBS injections alone have not been previously observed in mouse *rd* mutants, whereas in our study PBS injection alone induced a significant rescue effect at the histologic level, but did not lead to detectable functional improvement in any animal examined. Transient rescue effects of PBS injections have been previously reported in the rat[33] and are thought to result from local release of endogenous trophic factors as a result of tissue damage.

GDNF was one of the few neurotrophic factors not tested in the exhaustive recent trials by LaVail et al.[6] It was shown recently, when used at relatively high concentrations, to have protective effects against photoreceptor outer segment collapse in short-term in vitro assays.[34] It is impossible to know the final concentration of the subretinally injected GDNF used in the present study, although the dose administered lies in the range normally used for intraocular growth factor injections.[6,10] Although it is possible that GDNF may constitute one of the few identified neurotrophic factors influencing rod photoreceptor survival in inherited retinal degenerations in which the gene defect is localized to the rod cell, other criteria such as timing or ocular site of injection and approach were probably critical. Our injections were performed at relatively late times, after the initial onset of rod loss in this strain.[14] These ages were chosen to permit injection into the subretinal space, which was not routinely possible in younger animals. The stereological method of counting, originally developed for assessment of neuronal numbers in brain regions,[27,34] allows an accurate unbiased estimation of total cell numbers, reducing greatly the variability induced by regional differences (superior versus inferior hemisphere, site of injection). Nevertheless, increased rod numbers were also reliably scored in transverse retinal sections as routinely used by other investigators.[6,10]

GDNF may be a particularly interesting neuroprotective molecule, because unlike FGF-2 it is not reported to be angiogenic and thus should not lead to neovascular complications.[35] Histologic observations in the present study also did not record the presence of new retinal blood vessels or invading macro-



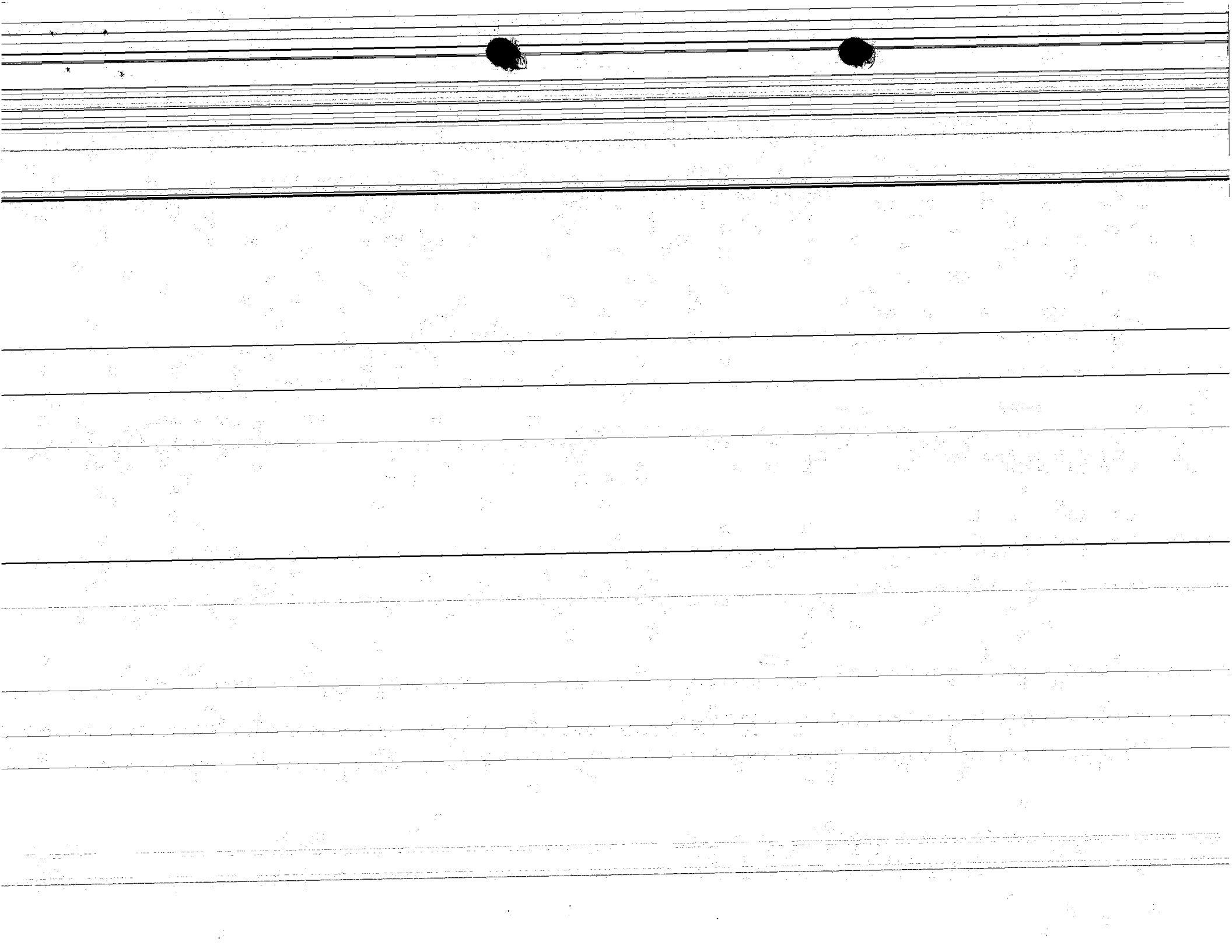


Age (days)	12	22	28	35	120	35
Strain	C3H	C3H	C3H	C3H	C3H	C57Bl6

FIGURE 9. GDNF mRNA expression was not altered during photoreceptor degeneration in the *rd/rd* mouse. GDNF mRNA expression was studied by semiquantitative RT-PCR. (A) GDNF expression was compared between retina and the remainder of the eye. Total RNA was isolated from *rd/rd* retina (*lanes 1, 3*) and the remainder of the eye (*lanes 2, 4*) and analyzed with primers specific for GDNF[1,2] and G6PDH.[3,4] (B) Total RNA isolated from whole eyes of 12- (*lanes 1, 12, 23*), 22- (*lanes 2, 3, 13, 14, 24, 25*), 28- (*lanes 4, 5, 15, 16, 26, 27*), 35- (*lanes 6, 7, 17, 18, 28, 29*) and 120- (*lanes 8, 9, 19, 20, 30, 31*) postnatal day *rd/rd* mice, and from 35-day postnatal normal mice (C57Bl6) (*lanes 10, 11, 21, 22, 32, 33*) was analyzed with primers specific for rhodopsin (RHO, *upper row*), GDNF (*middle row*), and the housekeeping gene glucose-6-phosphate dehydrogenase (G6PDH, *lower row*). The reduction in opsin mRNA levels during the course of rod degeneration and the uniform profile of steady state mRNA levels for GDNF and G6PDH can be seen.

phages. Although this distant member of the transforming growth factor-β superfamily was originally thought to be a specific survival factor for dopaminergic neurons,[17] GDNF is now known to stimulate a wide variety of autonomic, sensory, and motoneurons[21,36,37] and has an important role in kidney development.[38] Previous studies have indicated the potential value of GDNF as a neuroprotective agent for the retina through the demonstration that subnanomolar concentrations of GDNF increase cell survival in enriched photoreceptor cultures prepared from newborn mice and that rat photoreceptor cultures possess high-affinity GDNF receptors.[23] GDNF is expressed in the rat retina from embryonic day (E) 15 to E19, mostly in the innermost layer.[22] In the mouse embryo, it is expressed from E8.5 in the neuroectoderm surrounding the optic vesicle and later in the mesenchymal components of the developing eye.[39] Another member of the GDNF subfamily, neurturin and its receptor components GFRα2 and Ret, were also recently detected in normal and *rd* mouse retina throughout the life span.[40] In the present study, two histologic observations may be of particular importance. The first is that GDNF injection was effective at slowing rod loss even though treatment was begun after the initiation of rod degeneration in this strain.[41] Such findings may be of clinical significance, because they suggest growth factor treatment could be effective after the onset of photoreceptor death in RP patients. Enhanced photoreceptor survival after gene transfer of CNTF in *rds* mice,[32] gene transfer of the normal *PDE* gene in *rd* mice,[42] or targeted digestion of mutant opsin mRNA by adenovirally delivered ribozymes in transgenic retinal degeneration rats[43] have all been obtained when treatment began long before photoreceptor loss. The second is that intraocular injection of GDNF (and to a lesser extent PBS) led to upregulation of GFAP expression, indicating that, similar to FGF-2,[44] this growth factor may regulate phenotypic expression in retinal Müller glia and that its effects on rod survival may be mediated through an indirect pathway.[45] We are currently exploring this hypothesis.

The detection of an ERG in GDNF-injected eyes is an especially important finding in the present study, indicating that visual function was extended in treated *rd/rd* retina. Our



ata on ERG decrease in young *rd/rd* mice are in agreement ith several previous studies.[46–48] The persistence of record-ble ERG correlated in three of four cases, with the largest elative increases in rod cell numbers. However, because ERG ere absent in several *rd/rd* retinas containing at least as many ods as one animal in which signals were detected, recording as probably also influenced by anesthesia and cataract forma-on. Experimental constraints (dark adaptation) prevented onitoring of ocular status before recording, but overt cataract rmation was noted in both eyes of two animals that did not roduce a detectable ERG. In addition, under the experimental onditions used, rods and cones would both have been stim-lated and would have contributed to the signal. It should be ressed that ERG recordings and stereological assessment ere performed by a naive observer unaware of which eye had eceived GDNF.

We showed previously in the *rd/rd* mouse model the rotective effects of rod photoreceptor transplantation on host one cell survival and suggested that this effect was mediated y a diffusible factor released by rods.[49] Additional evidence or the existence of such diffusible signals produced by normal etina and influencing cone survival was obtained using cocul-ure models.[50] Although exogenous GDNF protects rod pho-orec[illegible]ors, it is unlikely to represent this endogenous signal or s[illegible]l reasons. GDNF is expressed in the *rd/rd* mouse etina even after complete loss of rods, suggesting that GDNF s not expressed in these cells (although it is possible that its xpression may be upregulated in inner retinal cells during hotoreceptor degeneration as has been shown to occur for GF-2 and CNTF during retinal injury[51]). Because the cone-urvival–promoting effect observed in our previous coculture tudies was dependent on the presence of rods,[50] GDNF is nlikely to constitute this diffusible molecule. Endogenous GDNF is not efficient in counteracting the apoptotic process in utant rods, and it may have functions other than photorecep-or cell survival in the mouse retina.

In conclusion, although the endogenous expression of GDNF was not sufficient to prevent photoreceptor loss in the *d/rd* mouse, intraocular injections were able to delay such osses significantly. Administration of GDNF using injections or ene delivery systems may be very useful to protect against hotoreceptor degeneration in humans.

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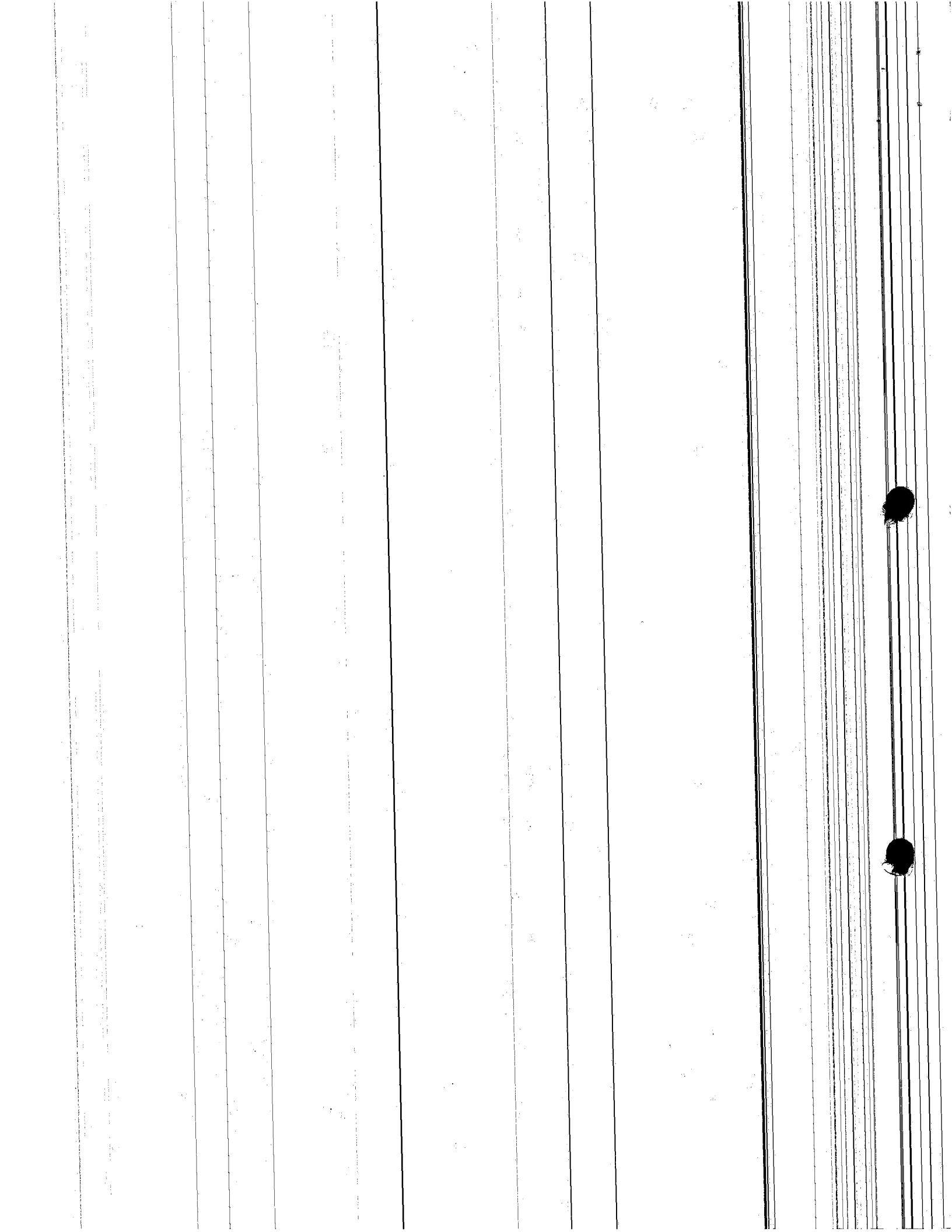

Glial Cell Line–Derived Neurotrophic Factor Induces Histologic and Functional Protection of Rod Photoreceptors in the *rd/rd* Mouse

Maria Frasson, Serge Picaud, Thierry Léveillard, Manuel Simonutti, Saddek Mohand-Said, Henri Dreyfus, David Hicks, and José Sahel

PURPOSE. To evaluate the neuroprotective potential of glial cell line–derived neurotrophic factor (GDNF) in the retinal degeneration (*rd/rd*) mouse model of human retinitis pigmentosa.

METHODS. Subretinal injections of GDNF were made into *rd/rd* mice at 13 and 17 days of age and electroretinograms (ERGs) recorded at 22 days. Control mice received saline vehicle injections or underwent no procedure. At 23 days of age, retinas from treated and control mice were fixed and processed for wholemount immunohistochemistry using an anti-rod opsin antibody, and rod numbers were estimated using an unbiased stereological systematic random approach. Subsequent to counting, immunolabeled retinas were re-embedded and sectioned in a transverse plane and the numbers of rods recalculated.

RESULTS. Although ERGs could not be recorded from sham-operation or nonsurgical *rd/rd* mice at 22 days of age, detectable responses (both a- and b-waves) were observed in 4 of 10 GDNF-treated mice. Stereological assessment of immunolabeled rods at 23 days showed that control *rd/rd* retinas contained 41,880 ± 3,890 (mean ± SEM; $n = 6$), phosphate-buffered saline (PBS)–injected retinas contained 61,165 ± 4,932 ($n = 10$; $P < 0.001$ versus control retinas) and GDNF-injected retinas contained 89,232 ± 8,033 ($n = 10$; $P < 0.001$ versus control retinas, $P < 0.002$ versus PBS). This increase in rod numbers after GDNF treatment was confirmed by cell counts obtained from frozen sections.

CONCLUSIONS. GDNF exerts both histologic and functional neuroprotective effects on rod photoreceptors in the *rd/rd* mouse. Thus rescue was demonstrated in an animal model of inherited retinal degeneration in which the gene defect was located within the rods themselves, similar to most forms of human retinitis pigmentosa. GDNF represents a candidate neurotrophic factor for palliating some forms of hereditary human blindness. (*Invest Ophthalmol Vis Sci.* 1999;40: 2724–2734)

Retinitis pigmentosa (RP) is the term given to a group of inherited retinal dystrophies resulting in progressive photoreceptor loss and eventual extreme visual handicap. There is no known treatment for this condition, which can be caused by a large number of mutations in a variety of genes that code for proteins involved in phototransduction (rhodopsin, cyclic guanosine monophosphate [cGMP]-dependent phosphodiesterase)[1,2] or in structural roles (peripherin/*rds*).[3] Several strategies are under examination for limiting or preventing the photoreceptor loss associated with this disease: gene therapy,[4] pharmacologic treatment (growth factor application[5,6] or vitamin supplementation[7]), and retinal transplantation.[8,9] Among these approaches, the use of growth factors has been advocated since pioneering work by the group of Faktorovich et al.[10] showed that intraocular injections of basic fibroblast growth factor (FGF-2) delayed photoreceptor degeneration in an animal model of RP, the Royal College of Surgeons rat. FGF-2's effects on photoreceptor survival were subsequently demonstrated by the same group in rat models of light-induced photoreceptor degeneration.[5,11] In addition, in vitro studies have demonstrated the differentiative effects of FGF-2 on newborn rat photoreceptors.[12] Yet, photoreceptor degeneration in the RCS rat as well as after constant light damage does not correlate to any currently known form of human retinal degeneration.

The retinal degeneration (*rd/rd*) mouse is a model of human RP that has been investigated for more than 70 years because photoreceptor cell degeneration follows a similar evolution to human RP. Most rod cells undergo apoptosis over the first 3 weeks,[13] followed by death of cone photoreceptors.[14] Furthermore, rod cell death has been found to result from a mutation in the β subunit of cGMP-dependent phosphodiester-

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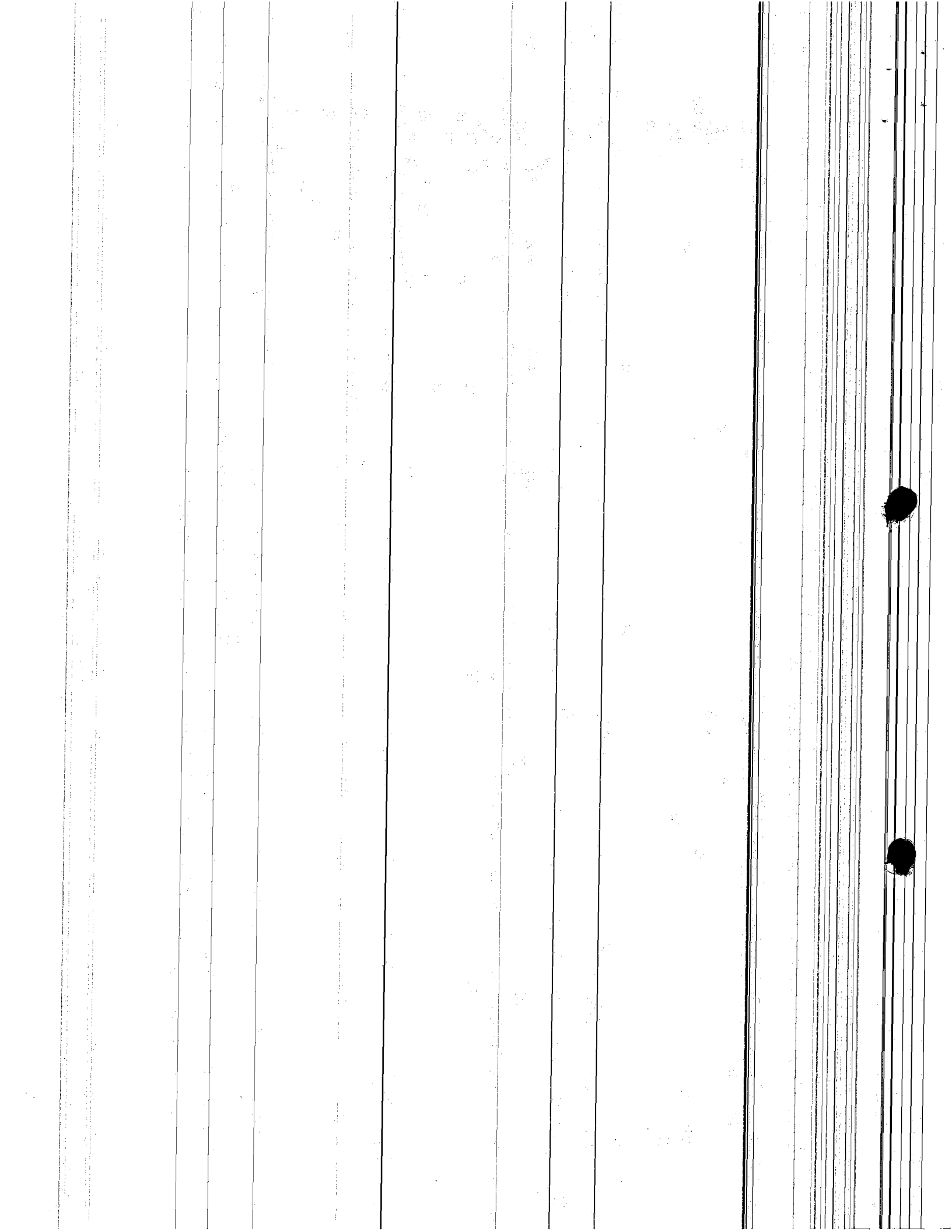


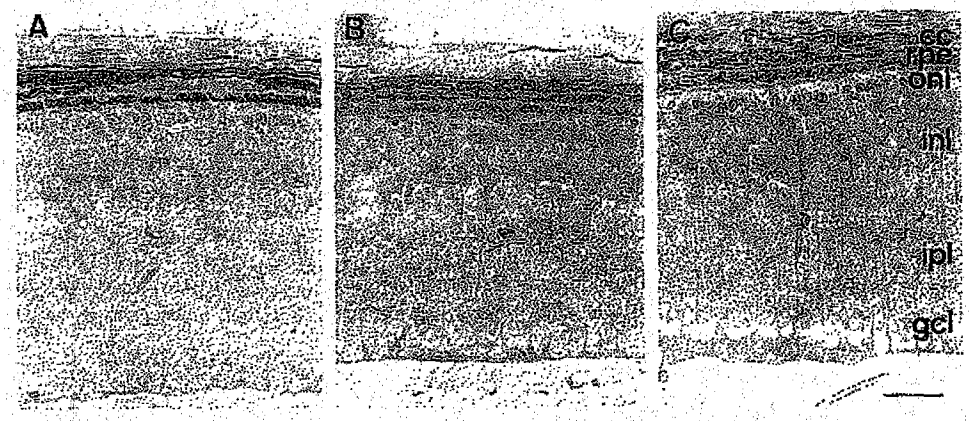


FIGURE 3. Histologic sections of whole eyes from 23-day *rd/rd* mice injected with (A) GDNF or (B) PBS or (C) noninjected. Semithin epoxy sections were stained with toluidine blue. There was no detectable new blood vessel formation within the injected eyes or visible gliosis or macrophage invasion into the subretinal or vitreal spaces. All micrographs were taken in proximity to the optic nerve head and exposed for the same times. cc, choriocapillaris; rpe, retinal pigmented epithelium; onl, outer nuclear layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Scale bar, 20 μm.

formed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

In Vivo Application of GDNF in the *rd/rd* Mouse

GDNF was injected into C3H mice at 13 and 17 days of age (with the day of birth defined as day 0). Mice (n = 10) were anesthetized with an intraperitoneal injection (16.7 μl/g body weight) of etomidate (0.5 mg/ml) and midazolan (0.5 mg/ml). The pupils were dilated with 0.5% tropicamide, and the cornea was anesthetized with 0.5% topical proparacaine. GDNF (1 μl; recombinant human GDNF; Promega, Madison, WI) was injected at a dose of 330 ng/μl in 0.1 M sterile PBS with a Hamilton syringe and a 30-gauge needle 1 mm from the limbus into the subretinal space. The contralateral eye was injected with the same volume of PBS solution only. It was necessary to perform bilateral ocular surgery to distinguish between the rescue effect due to GDNF per se and that due to experimental manipulation. In rare cases, partial backflow was observed. After injection, fundoscopy was performed to confirm the induction of a local retinal detachment. This was observed in every case at the time of injection, and we observed that the detachment created by the initial injection was resorbed by the time the second was administered. Processing of fixed retinas after recording (description follows) indicated that retinas were correctly attached at the end of the experimental period.

Recording ERGs in the *rd* Mouse

ERGs were picked up by a cotton wick connected to an Ag:AgCl electrode with saline solution directed to the apex of cornea by a micromanipulator. A stainless steel reference electrode was placed subcutaneously on top of the head. Responses were amplified at a gain of 10,000 and filtered with the low 0.1-Hz and high 1000-Hz cutoff filters from an amplifier (Universal; Gould, Ballainvilliers, France). Responses were digitized using a data acquisition labmaster board (Scientific Solutions, Solon, OH), mounted on an IBM-compatible personal computer. Experimental data were acquired and analyzed using the Patchit and Tack software packages (Scientific Solutions).[25] After a period of 24 hours, dark-adapted mice were prepared under dim red illumination. They were anesthetized as above and placed in a Faraday cage, the head resting in a U-shaped holder. The upper and lower lids were retracted to hold open and proptose the eye. The light stimulus was obtained from a 150-watt quartz-xenon lamp bulb (Müller Instruments, Moosinning, Germany). The light beam was focused to infinity through a heat-absorbing filter onto a hole in the Faraday cage. The illumination intensity of the light was 2.9 log cd/m^2 as measured with a luxmeter at the level of the eye. A computer-controlled shutter was used to deliver flashes of 300-msec duration. For GDNF-injected mice, recordings from both eyes were obtained successively, and the investigator was not aware of which eye had received the GDNF injection.

Histology of Injected Eyes

Mice were killed by anesthetic overdose at 23 days. Whole eyes from GDNF- and PBS-injected and noninjected *rd/rd* eyes (n = 2 for each group), were removed from mice at 23 days, fixed in 2.5% glutaraldehyde, and embedded in epoxy resin. Eyes were sectioned along a perpendicular plane close to the optic nerve head, and semithin sections were processed for histologic examination after toluidine blue counterstaining.

Immunohistochemical Labeling of Retinas

Mice that underwent surgery (n = 10 for both GDNF-injected and sham-operation mice) were killed by anesthetic overdose at 23 days. Nonsurgical eyes were also sampled from a further six 23-day-old *rd/rd* mice. Eyecups were removed and fixed in 4% paraformaldehyde for 2 hours. Retinas were dissected from the posterior eyecup, washed three times in PBS, permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes, and incubated in blocking buffer (PBS containing 0.1% bovine serum albumin, 0.1% Tween 20, and 0.1% sodium azide ([buffer A]) for 15 minutes. They were then incubated with rod photoreceptor-specific monoclonal antibody rho-4D2[26] (10 μg/ml in buffer A for 1 hour), washed and incubated in goat anti-mouse IgG-Texas red (10 μg/ml in buffer A for 1 hour; Molecular Probes, Portland, OR). Retinas were washed and flatmounted in PBS-glycerol (50:50 vol/vol), with the photoreceptor layer facing up, and examined under a photomicro-

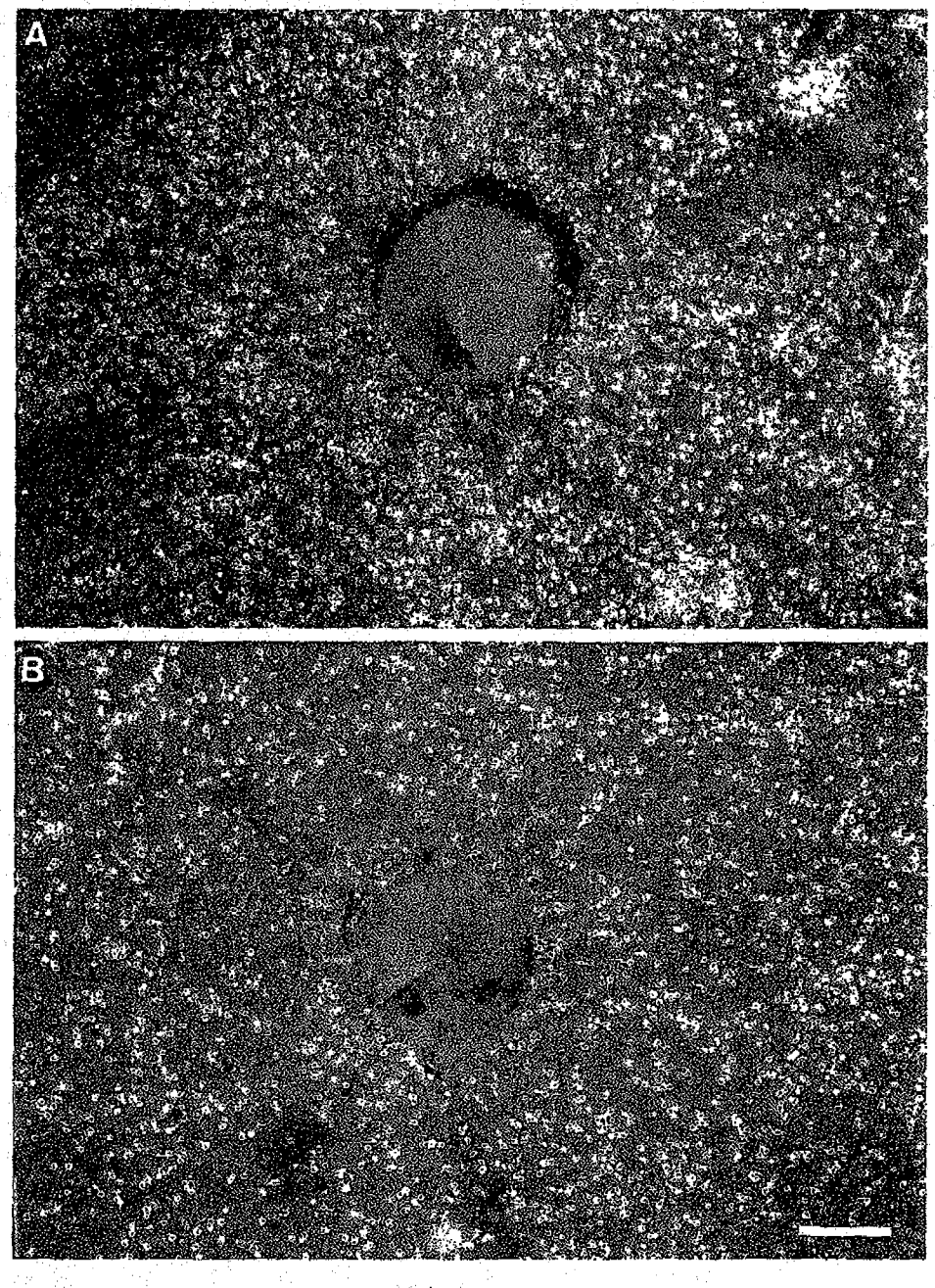


FIGURE 4. Low-power view of GDNF-injected (A) and PBS-injected (B) wholemounted *rd/rd* retinas immunolabeled with rho-4D2. The greater numbers of surviving rod photoreceptors after GDNF treatment are apparent. This example represents the mouse showing the greatest difference between the GDNF-treated eye and the PBS-injected contralateral eye. Scale bar, 100 μm.

scope (Optiphot 2; Nikon, Melville, NY) equipped with differential interference and fluorescence optics.

After stereological counting of rod numbers (see below), five wholemounted immunolabeled retinas from each experimental group were processed further by re-embedding and cryostat sectioning in a transverse plane. Frozen sections were collected from near the central retina, mounted on microscope slides, and examined for rho-4D2 staining. After labeled rods were counted (see later description), sections were incubated with anti-glial fibrillar acidic protein (GFAP) polyclonal antibody (Dako, Trappes, France), diluted 1:400 in buffer A for 2 hours. Sections were washed and incubated in goat anti-rabbit IgG-Bodipy FL (Molecular Probes) and the nuclear dye 4′-6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich, Saint Quentin Fallavier, France), both 10 μg/ml in buffer A for 1 hour. Slides were washed thoroughly and examined as above, with different filter sets allowing visualization of only rho-4D2–immunolabeled rods (single-band-pass, filter XF42), only anti-GFAP-immunolabeled retinal glia (single-band-pass, filter XF22), simultaneous rho-4D2 and anti-GFAP staining (double-band-pass, filter XF53) and DAPI-labeled nuclei (single-band-pass, filter XF03; all fluorescence filters from Omega Optical, division of Molecular Probes). For photography, picture series were taken with similar exposure times (HP5 film; Ilford, Basildon, UK) and printed using identical times.

Cell Counts

For quantification of rods in flatmounted retinas we used a stereological approach permitting unbiased sampling.[27] Immunolabeled cells were observed by microscope (Optiphot 2; Nikon) under epifluorescence illumination with a plan 40/070 differencial interference contrast (DIC) (160/0.17)

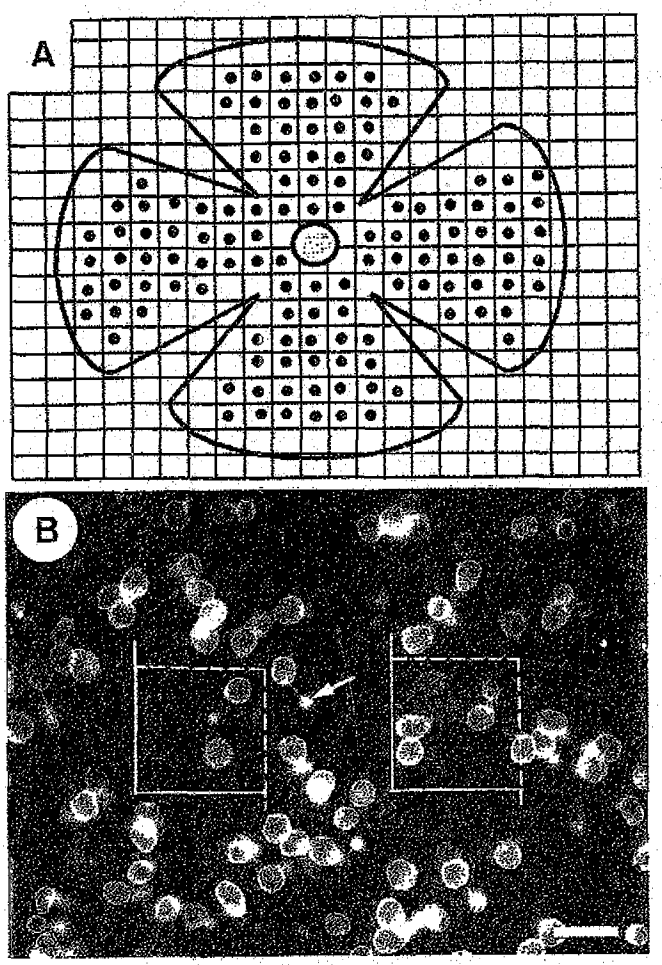


FIGURE 5. Illustration of the systematic random sampling approach used (A) and high-power view of 23-day-old *rd/rd* retina showing aspect of immunolabeled rod cells and superimposed counting frame (B). The antibody stains the entire cell body and vestigial outer segment. Scale bar (B), 10 μm.

objective. One hundred twenty fields of 8000 μm^2 were selected throughout the entire retinal surface (approximately 13 mm^2) using a stage encoder and a systematic random procedure.[28] Fields were viewed with a Sony Trinitron color graphic display camera and digitized software (Automator for Windows; Biocom, Lyon, France). In each of the 120 fields, cells were counted in two unbiased counting frames of 900 μm^2 generated by the software. The total number of rod cells in the entire retina was then estimated by normalizing these numbers to the entire retinal surface area, which was measured using an image analysis system (Optiscan; Macintosh LC II Ci; Apple Computer, Cupertino, CA). For counting of rods in transverse sections, a single section from each of five retinas for each treatment, passing through the optic nerve head and traversing the entire retinal width, was mounted on a microscope slide and stained with DAPI (10 μg/ml in PBS for 10 minutes). Viewing sections with the two appropriate filter sets allowed visualization of rho-4D2–labeled rods and total DAPI-labeled nuclei. Images were captured on the computer as described along the entire length of each section.

Reverse Transcription–Coupled Polymerase Chain Reaction Analysis of GDNF mRNA

Total RNA was isolated with an RNA-DNA extraction kit (Qiagen; Valencia, CA) with standard protocol. RNA samples (1 μg) were primed with random hexamer and incubated 2 hours at 37°C with Moloney murine leukemia virus reverse transcriptase. cDNA (1:20; 2 μl) was amplified for 35 cycles with specific pairs of primers. Primer sequences 5′ to 3′were: rhodopsin, AAGCCGATGAGCAACTTCC, TCATC TCCCAGTGGATTCTT; GDNF, ACCAGATAAACAAGCGGCAG, TCAGATACATCCA CACCGTTTAG; and glucose-6-phosphate dehydrogenase (MG6PDH), GCAGTCACCAAGAACATTCAAG, CCCAAATTCATCAA AATAGCCC. Amplified products were visualized on agarose gels stained with ethidium bromide and digitized with a camera. Quantitative measurement of band intensity was made using commercial software (Phoretix International, Newcastle-upon-Tyne, UK). To quantify the amplification products, the exponential phase of the reaction was experimentally determined using different cycling numbers for each primer pair. Under these conditions the amount of the amplification product reflects the initial concentration of transcripts.[29]

RESULTS

ERGs

Figure 1 illustrates the comparative evolution of ERGs from wild-type (C57) and *rd/rd* mice. In wild-type mice, the amplitude of a- and b-waves steadily increased from postnatal days 12 to 24. In *rd/rd* mice, the ERG was similar to the wild-type at postnatal day 12, but thereafter a- and b-waves steadily decreased from postnatal day 15 onward, becoming unrecordable by 22 days (Fig. 1). The light intensity for triggering a signal was 0.3 log cd/m^2, which corresponds approximately to the threshold for evoking cone responses.

To determine whether GDNF could improve visual functions in *rd/rd* mice, subretinal injections of this trophic factor were administered at 13 and 17 days, together with PBS vehicle injections into the contralateral eye to serve as control eyes. ERGs were recorded in animals at 22 postnatal days when ERG signals had disappeared completely in noninjected *rd/rd* mice. None of the eyes that received PBS exhibited a detectable ERG ($n = 10$). In contrast, ERGs were measurable in 4 of 10 GDNF-injected eyes (Fig. 2A, numbers 3, 4, 7, and 9). It should be noted that two of the six mice in which no signal was detected (Fig. 2A, numbers 1 and 5) showed prominent cataracts in both eyes, and they were not considered in the functional scores. A typical ERG recorded from a GDNF-injected eye is shown in Figure 2B. The average amplitudes of a- and b-waves (±SEM) for the four GDNF-injected mice showing a response were 8.5 ± 2.8 μV and 15.5 ± 2.3 μV, respectively. These represent responses that would normally be obtained from 18.5-day-old *rd/rd* mice, or 22% of the maximal response observed in *rd/rd* mice (Fig. 1B). These responses had implicit times (a-wave 120 ± 14 msec; b-wave 242 ± 43 msec; $n = 4$) similar to those observed in 15- to 16-day-old control *rd* animals (a-wave 144 ± 10 msec; b-wave 274 ± 23 msec; $n = 6$) but longer than those of control C57 mice at 24 days (a-wave 31 ± 3 msec; b-wave 110 ± 12 msec; $n = 4$). These data indicate that GDNF treatment prolonged visual function by approxi-

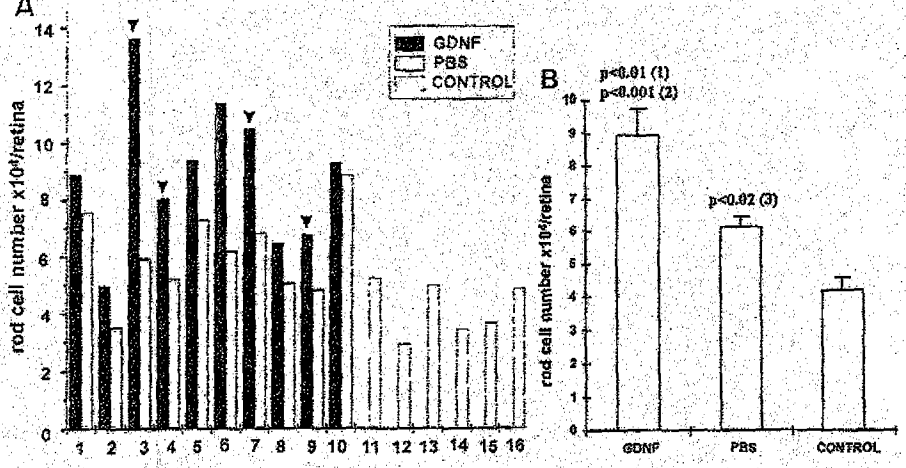


FIGURE 6. Immunolabeled rod cell counts in GDNF-, PBS- and noninjected 23-day *rd/rd* mice. (A) Total surviving rod numbers for each mouse (*x*-axis) injected with GDNF in one eye (*black bars*) and PBS vehicle in the other eye (*dark gray bars*). The consistent beneficial effect of growth factor treatment compared with vehicle-injected eyes can be observed. Those mice in which ERGs were successfully recorded were numbers 3, 4, 7, and 9 (*arrowheads*). Total surviving rod numbers for nonsurgical *rd/rd* retinas from an additional five mice are shown by bars 11 to 15 (*light gray*). It can be seen that surgery itself led to variation in rod numbers compared with nonsurgical eyes and that PBS injection produced a smaller but statistically significant increment in rod numbers. (B) Average (mean ± SEM) rod cell numbers calculated from (A). The GDNF-induced increase in rod numbers compared with PBS-treated and untreated control eyes is visible. Probabilities indicated above GDNF are comparisons with PBS treatment (1: $P < 0.01$) and nonsurgical control eyes (2: $P < 0.001$) and above PBS is comparison with nonsurgical control eyes (3: $P < 0.02$).

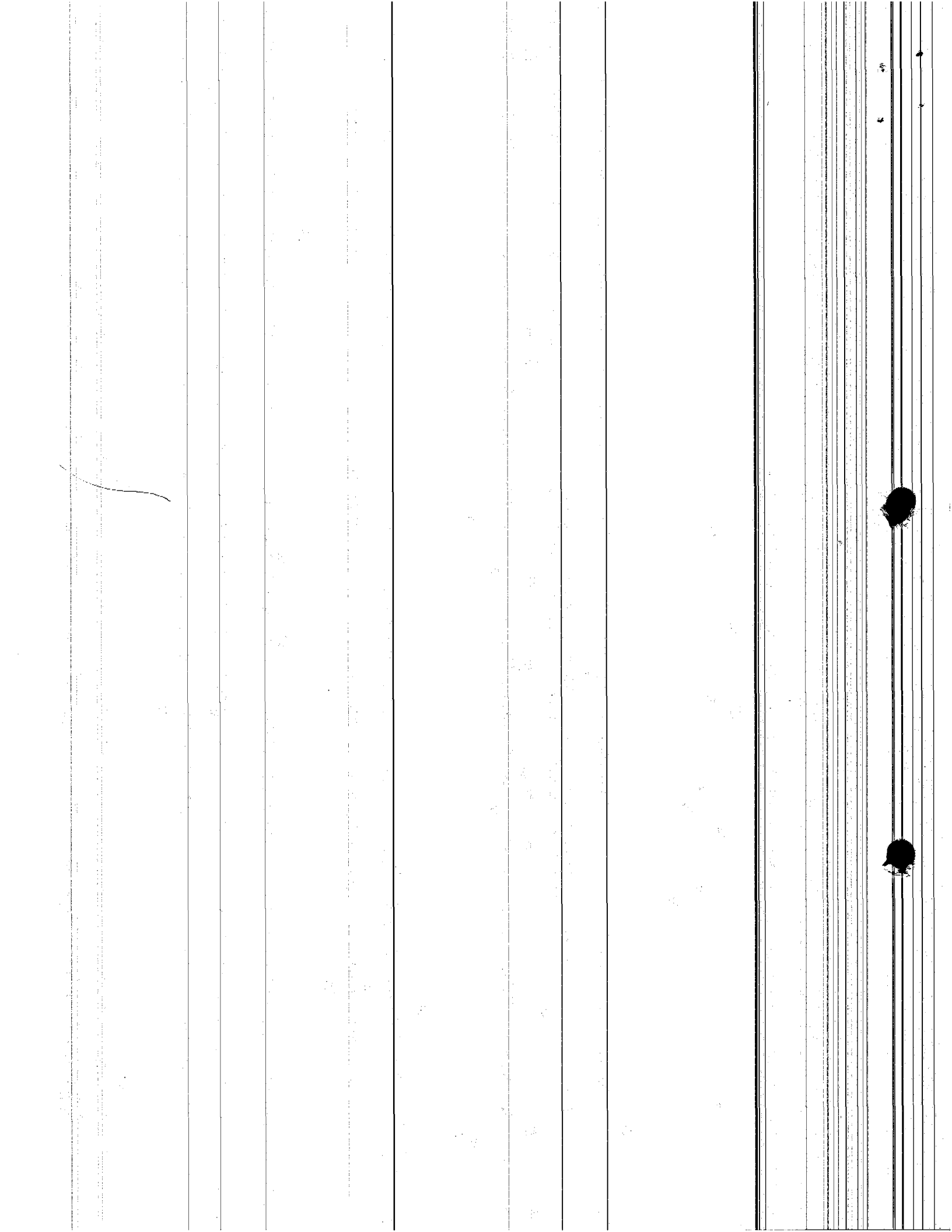
tely 30% relative to the period of massive photoreceptor ith in this strain.

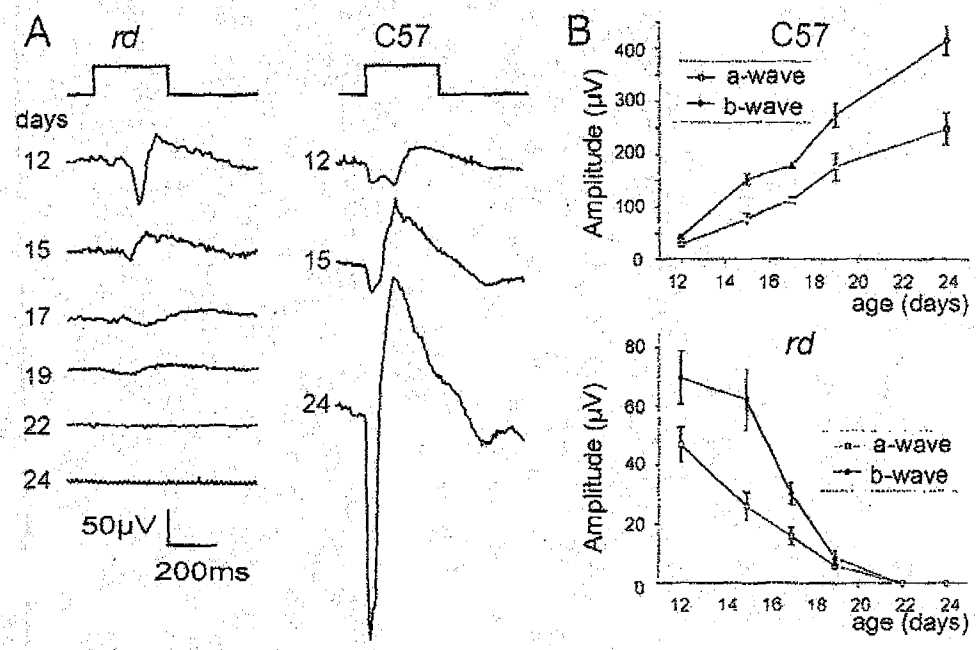
stologic, Immunohistochemical, and orphometric Analyses

er ERGs were recorded, the animals that received GDNF l PBS injections ($n = 10$) and a second group of control e of the same age ($n = 6$) were killed and the eyes fixed dissected. Epon-embedded histologic sections of surgi- or control eyes showed that injection of GDNF or PBS did lead to complications such as neovascularization, retinal ring, or macrophage invasion by 6 days after the final ction (Fig. 3).

Figure 4 shows a low-power view of GDNF-injected . 4A) and PBS-injected (Fig. 4B) wholemounted *rd/rd* nas immunolabeled with rho-4D2. The overall greater ber surviving rod photoreceptors after GDNF treat- t are apparent. Higher power photographs showed that antibody stained the entire rod photoreceptor cell, in- ing the cell body and vestigial outer segment (Fig. 5). stereological counting frame and the principle of the om systematic sampling procedure used are also shown gure 5. Rod cell counts for individual animals are shown gure 6A (paired GDNF-treated and PBS-injected *rd/rd* [1–10] and nonsurgical *rd/rd* mice[11–16]). The variability ween individual mice in opsin-immunolabeled rod num- is evident, but in each case GDNF treatment led to er cell counts. Correspondence between the degree of e and functionality was suggested by the fact that three four mice in which ERGs were detected (Fig. 6A, mice and 7) showed the highest relative differences in rod numbers between GDNF- and PBS-injected animals: 70% for the ERG-positive group ($n = 4$) and 34% for the ERG-negative group ($n = 6$). However, the differences between these two groups were not statistically significant. The average (±SEM) rod cell number found in GDNF-injected eyes (89,232 ± 8,033; $n = 10$) showed a 46% increase compared with the number in PBS-injected eyes (61,165 ± 3,259; $n = 10$) and a 113% increase compared with control noninjected eyes (41,880 ± 3,890; $n = 6$; Fig. 6B). PBS injection also led to an increase (36% more) in rod numbers compared with the number in untreated eyes. These increases were all statistically significant: GDNF versus PBS, $P < 0.01$; GDNF versus control, $P < 0.001$; PBS versus control, $P < 0.02$. These data expressed relative to total rod numbers found in normal adult C57 mice (estimated by us as 4.13×10^6; data not shown) yield 2.2%, 1.5%, and 1.0% for GDNF, PBS, and noninjected *rd/rd* retinas, respectively.

To compare these data more easily with previously published results on rod loss in the *rd/rd* mouse, we sectioned five wholemounted retinas from each experimental group and re-examined rod cell labeling. Figure 7 shows representative areas from each experimental group and clearly shows immunolabeled rods scattered among immunonegative cells within the vestigial outer nuclear layer (ONL). Noninjected control *rd/rd* retinas had only sparsely scattered rods, appearing as a single discontinuous layer along the scleral border (Figs. 7A, 7B). PBS-injected retinas had more immunolabeled rods, appearing as a single-to-double discontinuous layer (Figs. 7C, 7D). GDNF-injected retinas often contained two to three rows of immunolabeled rods, although areas of rod opsin–immunonegative cells within the ONL were still apparent (Figs. 7E, 7F). Sections





URE 1. Evolution of *rd/rd* and ld-type mice ERG on light stimula-n. (A) ERG recordings at different stnatal ages for *rd/rd* and wild-pe (C57) mice. Postnatal days are licated on the *left* of the ERG re-rdings. (B) Evolution of a- and b-ve amplitude from 12 to 24 post-tal days. Note the continuous crease toward complete disap-arance of the a- and b-waves in *rd* mice from postnatal days 15 to in contrast to the regular increase served in wild-type mice. The plot each age represents data from 5 imals, except for the latest time int for the *rd/rd* mouse (24 days), ich represents 10 animals.

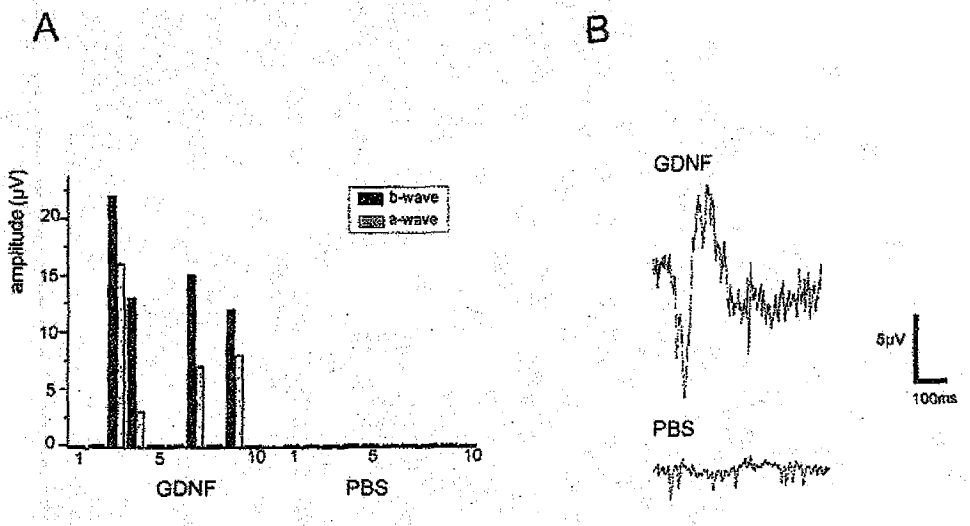
[15] lar to some human families affected by the disease.[16] cent among a large variety of neurotrophic factors in-cted into *rd/rd* eyes, only ciliary neurotrophic factor (CNTF) duced histologic photoreceptor cell loss.[6]

Glial cell line–derived neurotrophic factor (GDNF), which longs to the transforming growth factor β superfamily, was st described as a stimulant of survival of dopaminergic neu-ns in vitro.[17] Subsequently, its protective effects were dem-strated in in vivo models of Parkinson disease[18–20] or on veloping motoneurons.[21] Because GDNF is synthesized in e retina,[22] has been found to stimulate survival of newborn ne photoreceptors in vitro,[23] and recently was shown to lay photoreceptor outer segment collapse in vitro,[24] we nducted electrophysiological, histologic, immunohisto-emical, morphometric, and molecular biologic studies to see ether it could stimulate photoreceptor survival in the *rd/rd* ouse after intraocular injections in vivo. We showed that h treatment leads to functional improvement, as indicated the presence of recordable electroretinograms (ERGs) in some GDNF-injected retinas. Increased numbers of immunolabeled rods were detected using two methods of counting, and retinal glial cell physiology was also affected. In contrast, we could exclude the involvement of decreased levels of endogenous GDNF in rod photoreceptor cell death in the *rd/rd* mouse.

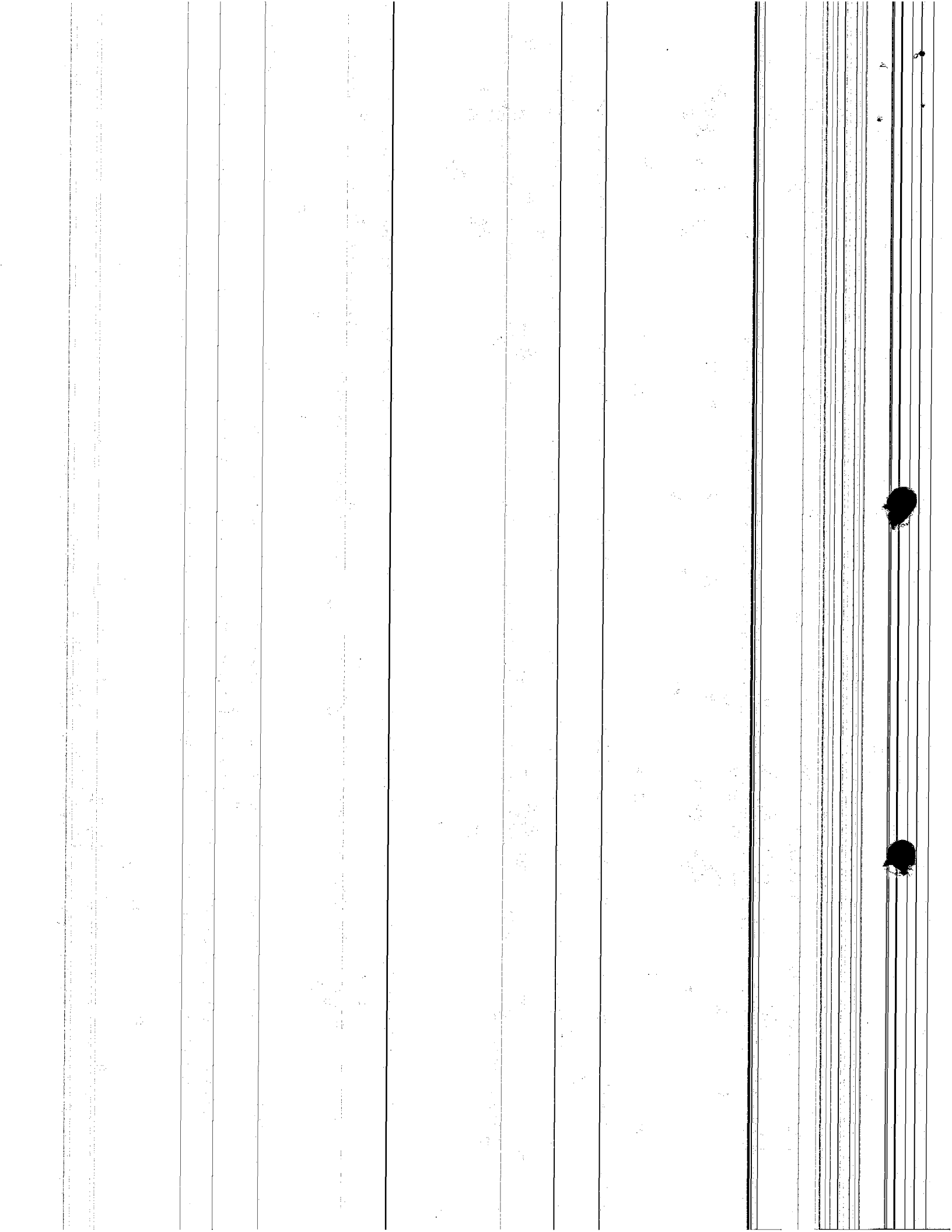
Materials and Methods

Animals

C3H/He/J mice homozygous for the *retinal degeneration* (*rd*) gene (*rd/rd* mice) and C57/BL6/J control mice aged 12, 15, 17, 19, and 24 days were obtained from single breeding colonies maintained exclusively for our laboratory by Charles River Animal Suppliers (St. Aubin-les-Elbeuf, France), maintained in clear plastic cages, and subjected to standard light:dark cycles of 12 hours All procedures con-



URE 2. Preservation of record-ERG in GDNF- compared with injected 22-day *rd/rd* mouse. (A) togram showing amplitudes of a- and b-waves from individual mals. (B) ERGs recorded from NF and PBS-injected eye. The re-dings were made from mouse 7 in and represent average traces three separate recordings.



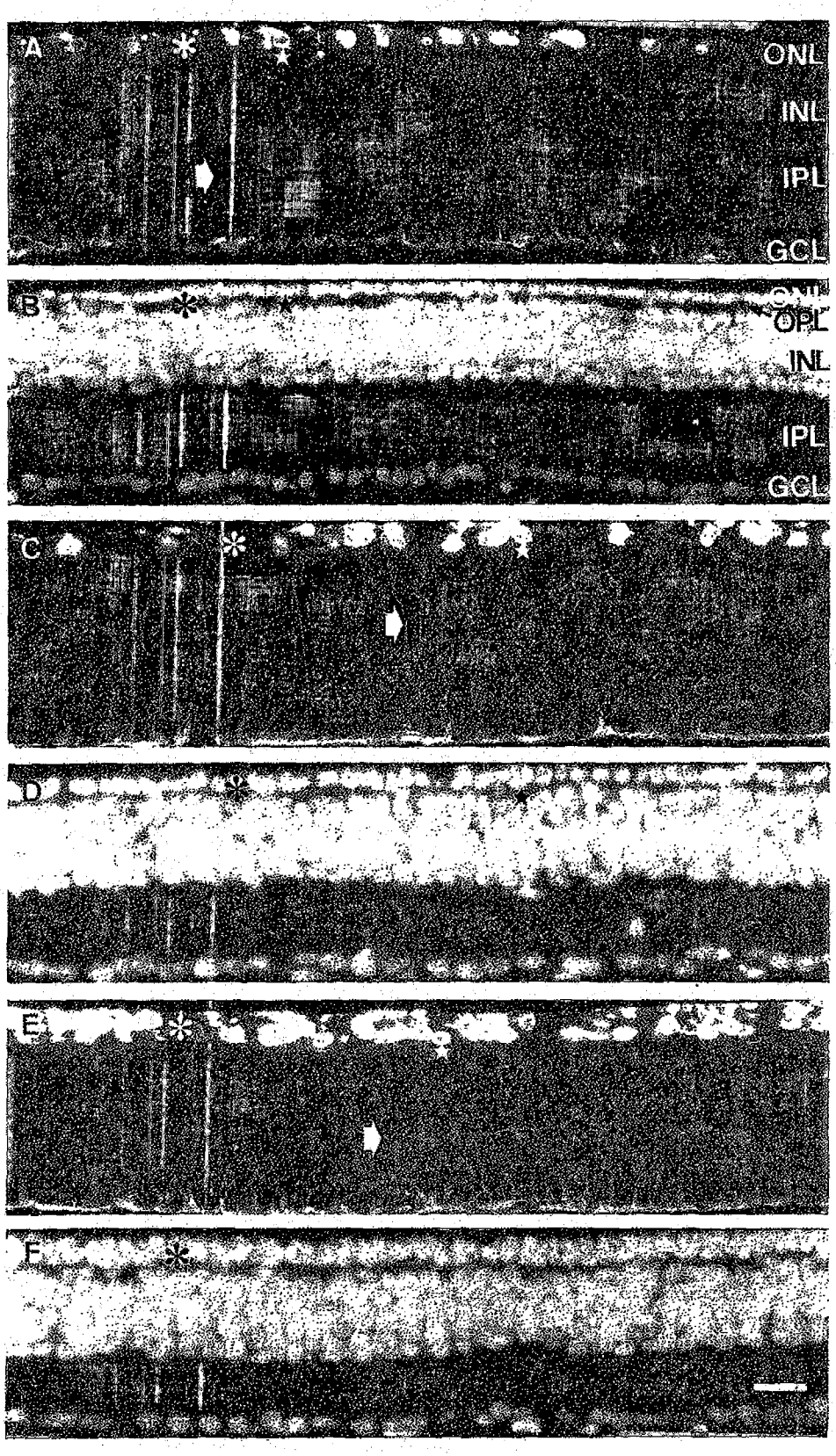
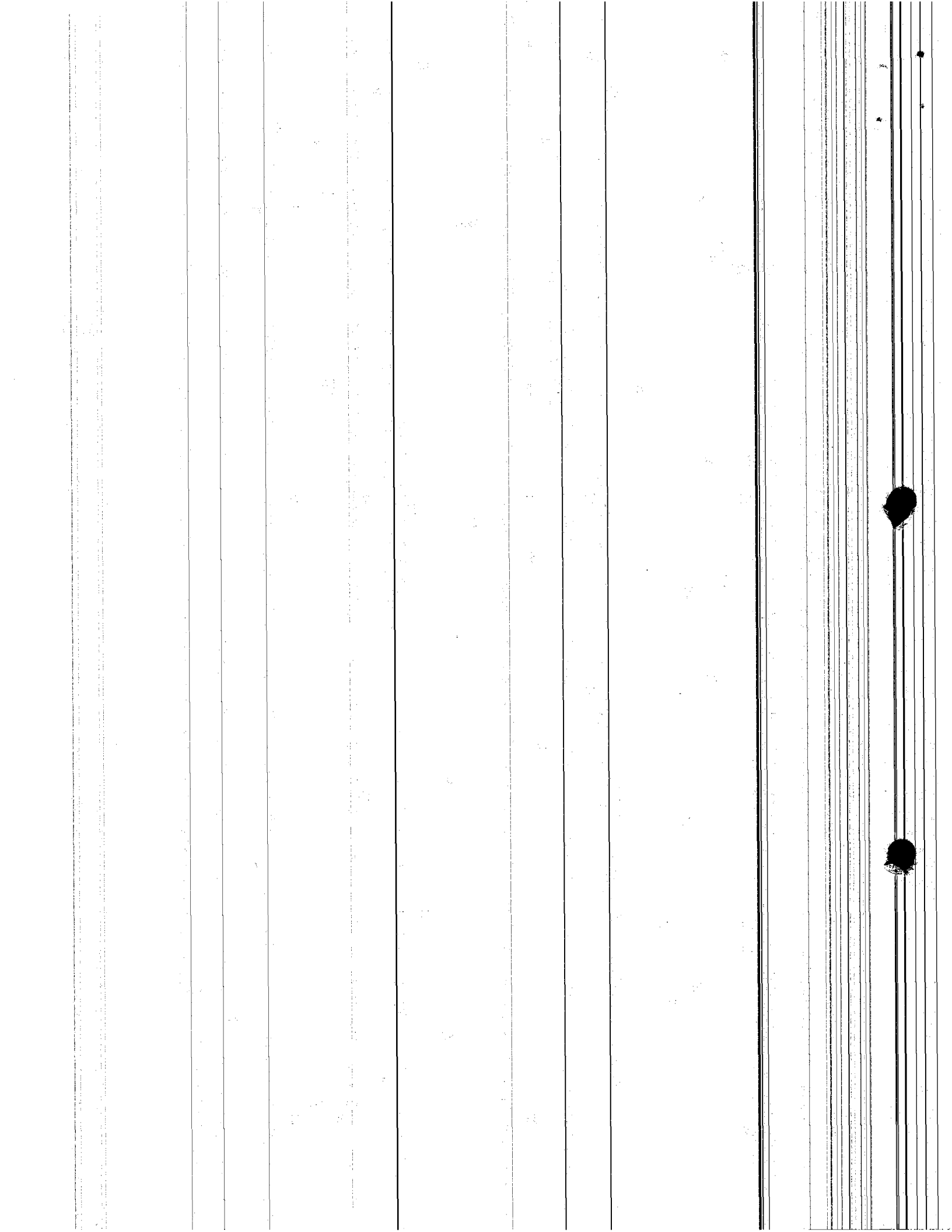


FIGURE 7. Transverse sections taken from wholemounts of 23-day *rd/rd* retinas prelabeled with rho-4D2 and relabeled with anti-GFAP. (A) and (B) are the same section from noninjected, (C) and (D) from PBS-injected, and (E) and (F) from GDNF-injected retinas. (A, C, E) Simultaneous visualization of rod opsin and GFAP immunoreactivity; (B, D, F) DAPI labeling of all nuclei. The rho-4D2–immunopositive rods are visible as brightly labeled, small, round cells lying along the vestigial ONL (*stars* in A, C, E, with the corresponding cell indicated by *stars* in B, D, F). Rho-4D2–immunonegative cells can be seen as unlabeled areas in the ONL (*asterisks* in A, C, and E, with corresponding cells indicated by *asterisks* in B, D, and F). The different number of immunolabeled rods after no injection ($n = 16$), PBS alone ($n = 22$), or GDNF treatment ($n = \sim 40$) can be clearly seen (A, C, E, respectively). Also, the increasing intensity of radial Müller glial fibers labeled by anti-GFAP antibody can be seen in the order GDNF-injected > PBS-injected > noninjected (E, C, and A, respectively, *arrows*), whereas astrocyte staining by the same antibody remains approximately constant along the vitreal border. All micrographs were taken in proximity to the optic nerve head and exposed for the same times. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 25 μm.

were subsequently immunolabeled with anti-GFAP antibody and the two labels visualized simultaneously with a double-band-pass fluorescence filter. GFAP immunoreactivity was visible as bright labeling of astrocytes within the nerve fiber layer and faint transverse fibrous labeling of retinal Müller glia. In noninjected retinas, GFAP immunoreactivity was mostly confined to the astrocytes, with only rare GFAP-immunopositive fibers (Fig. 7A). PBS-injected retinas showed increased numbers of such fibers (Fig. 7C), and GDNF-injected retinas contained large numbers (Fig. 7E). Quantitative analysis of rod



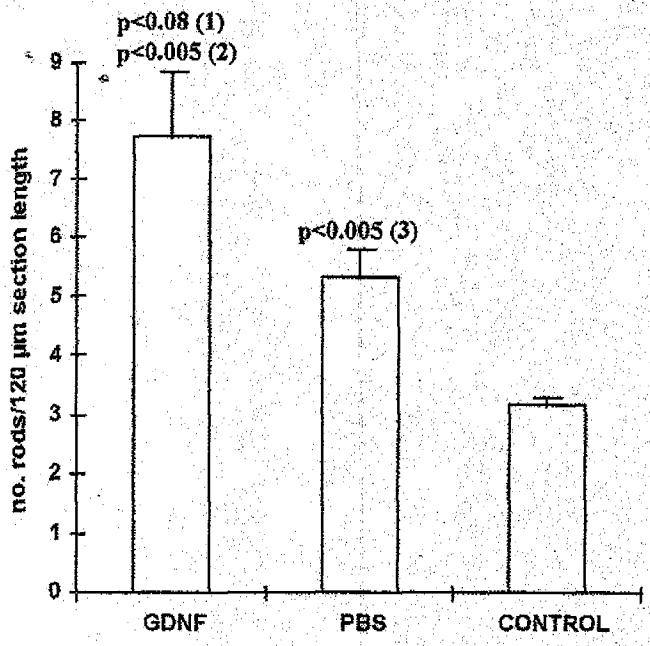


FIGURE 8. Rod numbers determined from transverse sections of GDNF-, PBS-, and noninjected *rd/rd* retinas. Average numbers (mean ± SEM) of immunolabeled rods were calculated from the entire width of single section passing through the optic nerve head from retinas of each experimental group ($n = 5$). Rod numbers were not statistically different between GDNF- and PBS-treated retinas (1: $P < 0.08$). Rod numbers were significantly higher in GDNF- compared with noninjected retinas (2: $P < 0.005$) and in PBS- compared with noninjected retinas (3: $P < 0.005$).

numbers from transverse sections revealed an average (±SEM) of 7.70 ± 1.10 rods/120 µm ($n = 5$) for GDNF-injected, 5.30 ± .48 rods/120 µm ($n = 5$) for PBS-injected, and 3.14 ± 0.13 ods/120 µm ($n = 5$) for noninjected retinas (Fig. 8). The ifference in rod numbers between GDNF-treated, PBS-treated, nd nonsurgical retinas was not significant between GDNF and BS ($P < 0.08$), but was significant between GDNF and non-rgical control eyes ($P < 0.005$) and between PBS-injected nd nonsurgical control eyes ($P < 0.005$).

Expression of GDNF mRNA in *rd/rd* Mice

o study the possible involvement of GDNF as an endogenous ophic factor for rods, we analyzed the expression of endog-nou[redacted]DNF mRNA in mouse retina (Fig. 9A). Lane 1 shows hat GDNF mRNA was expressed in higher steady state mounts in retina compared with the remainder of the eye atio 6:4). Twelve-day-old *rd/rd* mice expressed rod-specific odopsin mRNA at levels comparable to 35-day-old C57 mice ig. 9B, lanes 1, 10, and 11). Large decreases in rhodopsin RNA expression were observed in *rd/rd* mice between days 2 and 120 (lanes 1 through 9). During the whole period, levels f GDNF mRNA (lanes 12 through 20) and those of the house-eeping enzyme G6PDH (lanes 23 through 31) remained un-hanged and were similar to their respective expression inten-ties in C57 mouse eye at 35 days (lanes 21, 22, 32, and 33).

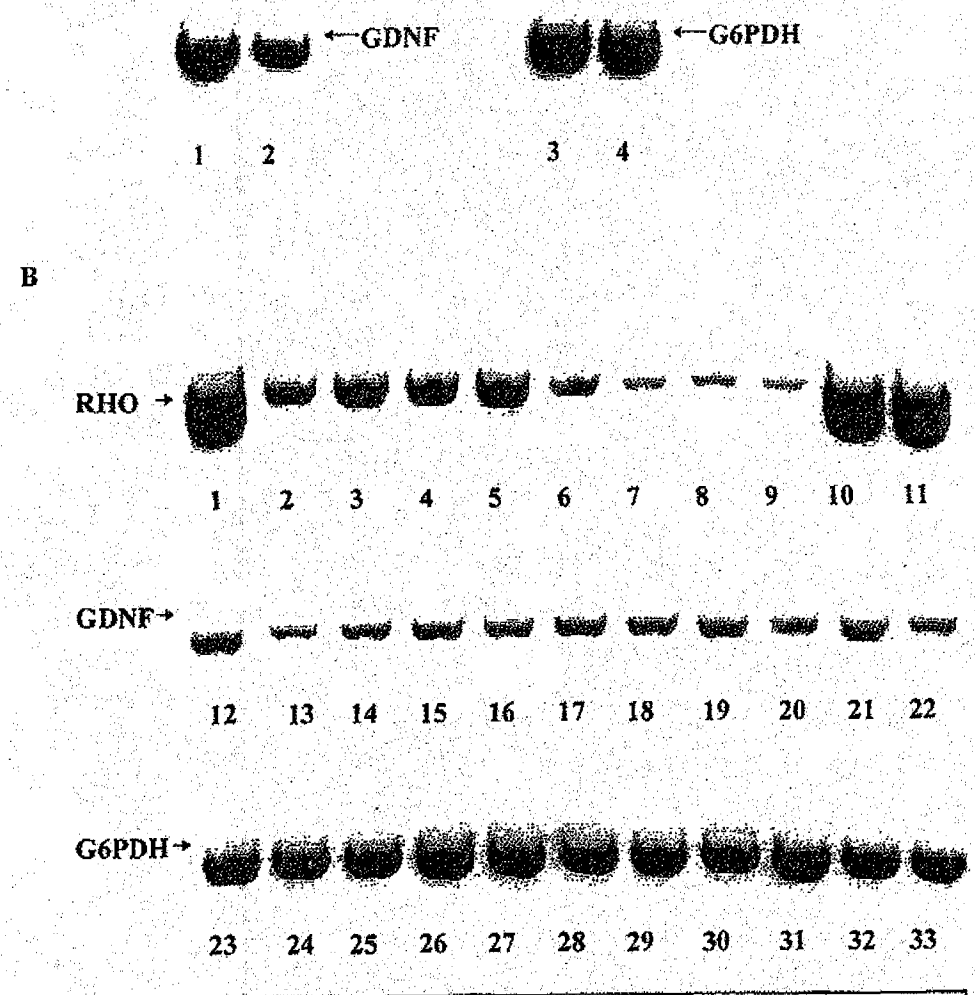
DISCUSSION

ur data show a stimulatory effect of subretinal injections of e neurotrophic factor GDNF on rod photoreceptor survival in the *rd/rd* mouse. This effect was demonstrated through immunohistochemical and morphometric analyses and through measurement of light-evoked responses. To the best of our knowledge this is the first report in which injection of a neurotrophic growth factor has been shown to promote improvement of retinal function in an animal model of retinal degeneration closely resembling human RP.

Faktorovich et al.[10] first showed the feasibility of using polypeptide growth factors to slow photoreceptor loss in inherited retinal degeneration. Using a rat model of RP they showed that intraocular injections of FGF-2 delayed rod photoreceptor loss. They also reported recently the effects of intraocular injection of multiple neurotrophic factors (CNTF, brain-derived neurotrophic factor, FGF-2, leukemia inhibitory factor, insulin-like growth factor II, neurotrophin-3 and -4, and nerve growth factor) on slowing rod photoreceptor loss in several mouse models of retinal degeneration including the *rd/rd* mouse.[6] These molecules were chosen because they are known to exhibit neuroprotective effects in other retinal models or elsewhere in the nervous system.[5,30,31] Of all these factors, only rat CNTF injected between postnatal days 7 and 10 had a protective effect 1 week later in *rd/rd* mice. In an independent study, CNTF delivered by adenoviral vectors showed histologic and functional protection of rod photoreceptors in another mutant, the retinal degeneration slow (*rds*) mouse.[32] Rescue effects of PBS injections alone have not been previously observed in mouse *rd* mutants, whereas in our study PBS injection alone induced a significant rescue effect at the histologic level, but did not lead to detectable functional improvement in any animal examined. Transient rescue effects of PBS injections have been previously reported in the rat[33] and are thought to result from local release of endogenous trophic factors as a result of tissue damage.

GDNF was one of the few neurotrophic factors not tested in the exhaustive recent trials by LaVail et al.[6] It was shown recently, when used at relatively high concentrations, to have protective effects against photoreceptor outer segment collapse in short-term in vitro assays.[24] It is impossible to know the final concentration of the subretinally injected GDNF used in the present study, although the dose administered lies in the range normally used for intraocular growth factor injections.[6,10] Although it is possible that GDNF may constitute one of the few identified neurotrophic factors influencing rod photoreceptor survival in inherited retinal degenerations in which the gene defect is localized to the rod cell, other criteria such as timing or ocular site of injection and approach were probably critical. Our injections were performed at relatively late times, after the initial onset of rod loss in this strain.[14] These ages were chosen to permit injection into the subretinal space, which was not routinely possible in younger animals. The stereological method of counting, originally developed for assessment of neuronal numbers in brain regions,[27,34] allows an accurate unbiased estimation of total cell numbers, reducing greatly the variability induced by regional differences (superior versus inferior hemisphere, site of injection). Nevertheless, increased rod numbers were also reliably scored in transverse retinal sections as routinely used by other investigators.[6,10]

GDNF may be a particularly interesting neuroprotective molecule, because unlike FGF-2 it is not reported to be angiogenic and thus should not lead to neovascular complications.[35] Histologic observations in the present study also did not record the presence of new retinal blood vessels or invading macro-

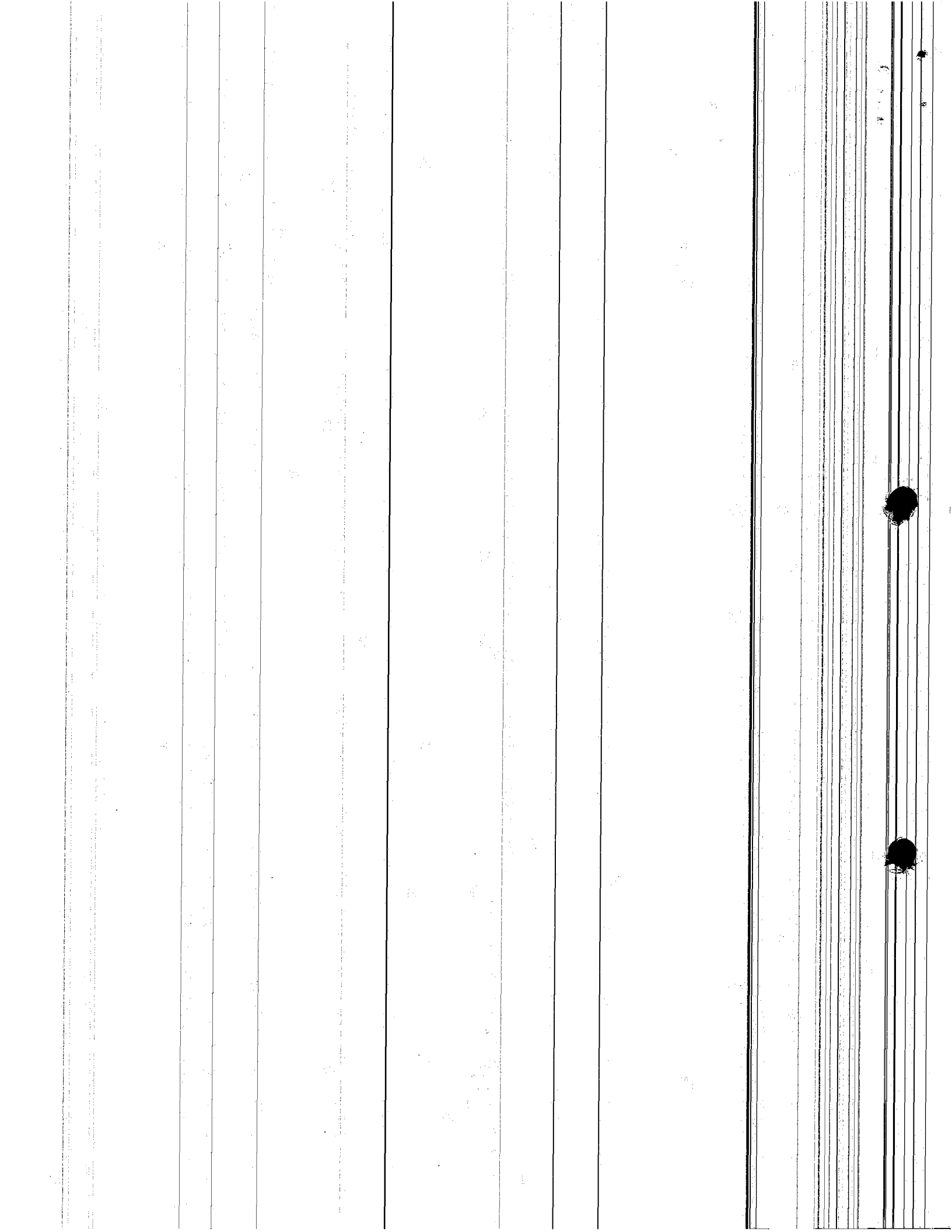


Age (days)	12	22	28	35	120	35
Strain	C3H	C3H	C3H	C3H	C3H	C57Bl6

FIGURE 9. GDNF mRNA expression was not altered during photoreceptor degeneration in the *rd/rd* mouse. GDNF mRNA expression was studied by semiquantitative RT-PCR. (A) GDNF expression was compared between retina and the remainder of the eye. Total RNA was isolated from *rd/rd* retina (*lanes 1, 3*) and the remainder of the eye (*lanes 2, 4*) and analyzed with primers specific for GDNF[1,2] and G6PDH.[3,4] (B) Total RNA isolated from whole eyes of 12- (*lanes 1, 12, 23*), 22- (*lanes 2, 3, 13, 14, 24, 25*), 28- (*lanes 4, 5, 15, 16, 26, 27*), 35- (*lanes 6, 7, 17, 18, 28, 29*) and 120- (*lanes 8, 9, 19, 20, 30, 31*) postnatal day *rd/rd* mice, and from 35-day postnatal normal mice (C57Bl6) (*lanes 10, 11, 21, 22, 32, 33*) was analyzed with primers specific for rhodopsin (RHO, *upper row*), GDNF (*middle row*), and the housekeeping gene glucose-6-phosphate dehydrogenase (G6PDH, *lower row*). The reduction in opsin mRNA levels during the course of rod degeneration and the uniform profile of steady state mRNA levels for GDNF and G6PDH can be seen.

phages. Although this distant member of the transforming growth factor-β superfamily was originally thought to be a specific survival factor for dopaminergic neurons,[17] GDNF is now known to stimulate a wide variety of autonomic, sensory, and motoneurons[21,36,37] and has an important role in kidney development.[38] Previous studies have indicated the potential value of GDNF as a neuroprotective agent for the retina through the demonstration that subnanomolar concentrations of GDNF increase cell survival in enriched photoreceptor cultures prepared from newborn mice and that rat photoreceptor cultures possess high-affinity GDNF receptors.[23] GDNF is expressed in the rat retina from embryonic day (E) 15 to E19, mostly in the innermost layer.[22] In the mouse embryo, it is expressed from E8.5 in the neuroectoderm surrounding the optic vesicle and later in the mesenchymal components of the developing eye.[39] Another member of the GDNF subfamily, neurturin and its receptor components GFRα2 and Ret, were also recently detected in normal and *rd* mouse retina throughout the life span.[40] In the present study, two histologic observations may be of particular importance. The first is that GDNF injection was effective at slowing rod loss even though treatment was begun after the initiation of rod degeneration in this strain.[41] Such findings may be of clinical significance, because they suggest growth factor treatment could be effective after the onset of photoreceptor death in RP patients. Enhanced photoreceptor survival after gene transfer of CNTF in *rds* mice,[32] gene transfer of the normal *PDE* gene in *rd* mice,[42] or targeted digestion of mutant opsin mRNA by adenovirally delivered ribozymes in transgenic retinal degeneration rats[43] have all been obtained when treatment began long before photoreceptor loss. The second is that intraocular injection of GDNF (and to a lesser extent PBS) led to upregulation of GFAP expression, indicating that, similar to FGF-2,[44] this growth factor may regulate phenotypic expression in retinal Müller glia and that its effects on rod survival may be mediated through an indirect pathway.[45] We are currently exploring this hypothesis.

The detection of an ERG in GDNF-injected eyes is an especially important finding in the present study, indicating that visual function was extended in treated *rd/rd* retina. Our



ata on ERG decrease in young *rd/rd* mice are in agreement ith several previous studies.[46–48] The persistence of recordble ERG correlated in three of four cases, with the largest elative increases in rod cell numbers. However, because ERG ere absent in several *rd/rd* retinas containing at least as many ods as one animal in which signals were detected, recording as probably also influenced by anesthesia and cataract formaon. Experimental constraints (dark adaptation) prevented onitoring of ocular status before recording, but overt cataract rmation was noted in both eyes of two animals that did not roduce a detectable ERG. In addition, under the experimental onditions used, rods and cones would both have been stimlated and would have contributed to the signal. It should be ressed that ERG recordings and stereological assessment ere performed by a naive observer unaware of which eye had eceived GDNF.

We showed previously in the *rd/rd* mouse model the rotective effects of rod photoreceptor transplantation on host one cell survival and suggested that this effect was mediated y a diffusible factor released by rods.[49] Additional evidence or the existence of such diffusible signals produced by normal etina and influencing cone survival was obtained using cocul[illegible]re models.[50] Although exogenous GDNF protects rod photore[illegible]ors, it is unlikely to represent this endogenous signal or s[illegible]l reasons. GDNF is expressed in the *rd/rd* mouse etina even after complete loss of rods, suggesting that GDNF s not expressed in these cells (although it is possible that its xpression may be upregulated in inner retinal cells during hotoreceptor degeneration as has been shown to occur for GF-2 and CNTF during retinal injury[51]). Because the cone-urvival–promoting effect observed in our previous coculture tudies was dependent on the presence of rods,[50] GDNF is nlikely to constitute this diffusible molecule. Endogenous GDNF is not efficient in counteracting the apoptotic process in utant rods, and it may have functions other than photorecepor cell survival in the mouse retina.

In conclusion, although the endogenous expression of GDNF was not sufficient to prevent photoreceptor loss in the *rd/rd* mouse, intraocular injections were able to delay such osses significantly. Administration of GDNF using injections or ene delivery systems may be very useful to protect against hotoreceptor degeneration in humans.

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