Protection of Mouse Photoreceptors by Survival Factors in Retinal Degenerations

Matthew M. LaVail, Douglas Yasumura, Michael T. Matthes, Cathy Lau-Villacorta, Kazuhiko Unoki, Ching-Hwa Sung, and Roy H. Steinberg

PURPOSE. To examine the protective effect of a number of survival factors on degenerating photoreceptors in mutant mice with naturally occurring inherited retinal degenerations, including retinal degeneration (rd/rd), retinal degeneration slow (rds/rds), nervous (nr/nr), and Purkinje cell degeneration (pcd/pcd), in three different forms of mutant rhodopsin transgenic mice and in light damage in albino mice.

METHODS. Various survival factors were injected intravitreally into one eye of mice at or soon after the beginning of photoreceptor degeneration, with the opposite eye serving as the control, and the eyes were examined histologically at later ages. The survival factors included brain-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4, ciliary neurotrophic factor (CNTF), Axokine™ (a mutein of CNTF), leukemia inhibitory factor, basic fibroblast growth factor, and nerve growth factor and insulin-like growth factor II, either alone or in various combinations.

RESULTS. Photoreceptor degeneration was slowed in rd/rd and nr/nr mutant mice and in Q344ter mutant rhodopsin mice by certain forms of CNTF; the degeneration in Q344ter mice was slowed by Axokine and by leukemia inhibitory factor; and the degeneration in a few nr/nr mice was slowed by BDNF. The other agents were ineffective in these mice, and none of the agents were effective in the other mutants and other mutant rhodopsin transgenic mice. However, light damage experiments that compared agent effectiveness in albino mice versus rats suggested a significant delivery problem with the very small mouse eye, thereby making the interpretation of negative findings equivocal in mutant mice. Basic fibroblast growth factor failed to protect the mouse retina from the damaging effects of constant light, whereas it showed a strong protective effect in the rat, indicating an important species difference.

CONCLUSIONS. The slowing of degeneration in the rd/rd and Q344ter mutant mice demonstrated that intracocularly injected survival factors can protect photoreceptors from degenerating in animal models with the same or similar genetic defects as those in human inherited retinal degenerations. (Invest Ophthalmol Vis Sci. 1998;39:592-602)

Various neurotrophic factors, growth factors, and cytokines, known collectively as survival factors, protect neurons from cell death caused by different types of insults and different forms of degeneration. In the retina, basic fibroblast growth factor (bFGF) was shown to slow the progression of photoreceptor degeneration in Royal College of Surgeons (RCS) rats with inherited retinal dystrophy, which was the first time an inherited retinal degeneration had been slowed significantly by a pharmacologic agent. Subsequently, bFGF and several different survival factors were found to protect the normal albino rat retina from the damaging effects of constant light. These included brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), which gave robust protective responses, as well as neurotrophin-3 and several others that gave a less significant protective response. The functional rescue of photoreceptors from light damage in albino rats, as measured by electroretinography, also has been seen after protection with bFGF.

Inherited photoreceptor degenerations of the retinitis pigmentosa class are a major cause of blindness worldwide, and, except for the recent report of vitamin A therapy, there has been no pharmacologic means to treat these disorders. For this reason, we felt it important to test the efficacy of survival factors in animal models of these diseases. We turned to the mouse for a number of reasons. First, whereas the degenerations of the RCS rat and constant light in the albino rat have no currently identified direct human counterparts, two naturally occurring mouse mutations do have the same gene defects found in human patients with retinitis pigmentosa. Second, other naturally occurring inherited retinal degenerations are found in mice that allow us to test whether survival factors can rescue these different forms of retinal degeneration regardless of etiology. Third, several mutant rhodopsin transgenic mice...
are available with mutations identical or similar to those in some human forms of retinitis pigmentosa. Fourth, testing the agents in mice examines their effectiveness in a species other than the rat.

In this study we tested many of the survival factors that protected the albino rat from constant light damage, as well as some additional factors or combinations of factors, in the extensively studied, naturally occurring retinal degeneration mutations in mice. We attempted to protect photoreceptors (that is, slow the progression of degeneration) in the following mouse mutants: retinal degeneration (rd/rd), retinal degeneration slow (rds/rds), retinal degeneration slow heterozygotes (rds/+), nervous (nr/nr), and Purkinje cell degeneration (pcd/pcd). In the rd/rd mouse, defects in the β-subunit of the cyclic guanosine monophosphate-phosphodiesterase gene result in an autosomal-recessive disease in which a rapid loss of rods begins approximately on postnatal day 10, is almost complete by approximately postnatal day 20, and is followed by a slow loss of cones in the ensuing months. Mutations in the human homolog of this gene mostly result in autosomal-recessive retinitis pigmentosa, although one produces autosomal-dominant stationary night blindness. In rds/rds mice, photoreceptor cell loss is much slower than in rd/rd mice, and there is a relatively rapid phase between postnatal days 11 and 28 followed by a much slower phase that lasts 8 to 11 months. The gene defect in rds/rds mice leads to an absence of normal peripherin/RDS, a photoreceptor-specific outer segment protein, so that photoreceptor outer segments fail to elaborate but not organized into normal outer segments. Different mutations in the human peripherin/RDS gene produce strikingly varied forms of retinal degeneration. In the cerebellar mutant mice, nr/nr and pcd/pcd, both show a slow form of retinal degeneration, with a later phase of cell loss remarkably similar to that of rds/rds mice. Despite this similarity, each of these slow degenerations is a result of an unrelated mutant gene, and each degeneration is phenotypically distinct.

We also examined three different transgenic mice with retinal degenerations in which mutations are present in the rhodopsin gene, because more than 70 different rhodopsin mutations have been discovered in patients with retinitis pigmentosa and rhodopsin mutations account for approximately 20% to 25% of dominant retinitis pigmentosa. The mutant transgenes included a P23H single amino acid transposition in which most photoreceptors are missing by postnatal day 20; three altered amino acids, V20G, P23H and P27L, near the N terminus of the molecule (VPP mice) showing a slower loss of photoreceptors, with approximately 20% missing by postnatal day 32 and with approximately 2 to 3 rows still surviving at 7 months of age; and a truncated carboxyl terminus (Q344ter-1) in which the last five amino acids of rhodopsin are removed, showing a loss of photoreceptors beginning at postnatal day 10 with most missing by approximately postnatal day 21. The rates of rod cell degeneration in these transgenic mice depend on the degree of expression and, presumably, the nature of the mutant transgene. Humans carrying the P23H and Q344ter-1 rhodopsin mutations have autosomal-dominant forms of retinitis pigmentosa. Our strategy with each of the mutants was to inject, as we have done previously, the survival factors intravitreally into one eye immediately before or just after photoreceptor degeneration and then to compare both eyes of individual animals at a later time to determine whether a slowing of degeneration (protection) had occurred. In the case of the rd/rd mouse, however, the differential rate of rod and cone cell degeneration allowed a second series of experiments with injections at a later age in an attempt to rescue cones. This work was stimulated by a preliminary report that the transplantation of neonatal photoreceptors from normal mice into the subretinal space in the peripheral retina of rd/rd mice at 3 weeks of age (when most rods had disappeared) resulted in the rescue of many cones at 2 to 4 months of age compared with unoperated or sham-operated retinas. Our goal was to determine whether survival factors might also enhance cone survival in rd/rd mice.

We demonstrate here that survival factors can protect retinal photoreceptors in three different types of inherited retinal degeneration in mice, two of which have similar gene defects as human forms of retinitis pigmentosa. However, in early experiments, most of the retinas showed little or no protection with the survival factors. For this reason, we questioned whether mice, in general, were refractory to the protective effects of the survival factors. To test this hypothesis, we compared the effectiveness of several survival factors to protect the retinas from the damaging effects of constant light in albino mice and albino rats. These results, which show significant species differences, are also included. The findings of this study have been presented elsewhere in abbreviated form.

**Materials and Methods**

**Animals and Husbandry**

All mice were born in our laboratory and were maintained in a 12-hour on/12-hour off cyclic light environment of less than 15 foot-candles (ft-c) in-cage illumination. Breeder rd/rd mice were of the C3H/HeOuJ strain, and, as described previously, the albino nr/nr mice were on the BALB/c genetic background. albino pcd/pcd mice were on the C57BL/6j background, and albino rds/rds mice were on the O20/A strain. Heterozygote rds/+ mice were produced by crossing rds/rds mice with inbred albino BALB/cByJ mice. The transgenic mice were maintained by crossing affected heterozygotes with wild-type C57BL/6j (Q344ter and VPP) or C57BL/6NCr1BR (P23H), which produced approximately 50% affected heterozygote progeny and 50% normal wild-type progeny. All procedures involving the mice adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the UCSF Committee on Animal Research.

**Factor Injection and Histologic Procedures**

In the experiments with mutants, we made injections just before, at, or just after the onset of photoreceptor degeneration (Table 1). For the experiments attempting to rescue cones in rd/rd mice, the animals were injected at postnatal days 18 to 21 (Table 2), when most of the rods had disappeared and while most of the cones still remained. The injections were made with a 32-gauge Hamilton syringe into the vitreous of the superior hemisphere of one eye. The volume of injection was 0.5 μl in most cases, but a few mice of most of the mutations...
TABLE 1. Photoreceptor Protection\* in Mutant Mice with Inherited Retinal Degenerations by Different Survival Factors

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Age most litters injected</th>
<th>Postinjection interval</th>
<th>P7–10†</th>
<th>P6–11*</th>
<th>P6–11†</th>
<th>P8–12‡</th>
<th>P13–17%</th>
<th>P8–11+</th>
<th>P8–11+</th>
<th>P15–21**</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 week</td>
<td>3 weeks</td>
<td>3 weeks</td>
<td>3 weeks</td>
<td>3 weeks</td>
<td>10 days</td>
<td>10 days</td>
<td>3 weeks</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bCNTF</td>
<td>4, 0, 11</td>
<td>0, 0, 6</td>
<td>2, 2, 15</td>
<td>0, 0, 11</td>
<td>0, 0, 15</td>
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<td>0, 0, 1</td>
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<td>12, 0, 1</td>
<td>0, 0, 7</td>
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<td>Axokine</td>
<td>0, 1, 24</td>
<td>0, 1, 2</td>
<td>0, 0, 8</td>
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<td>5, 2, 13</td>
<td>0, 0, 8</td>
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<td>LIF</td>
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<td>0, 0, 6</td>
<td>7, 0, 0</td>
<td>0, 0, 13</td>
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<tr>
<td>BDNF</td>
<td>(1, 2, 5)</td>
<td>(0, 0, 3)</td>
<td>0, 0, 12</td>
<td>0, 0, 13</td>
<td>0, 0, 12</td>
<td>0, 1, 10</td>
<td>0, 0, 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and 10 mg/ml</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NT-3</td>
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<td>0, 0, 0</td>
<td>0, 0, 0</td>
<td>0, 0, 7</td>
<td>0, 0, 4</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
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<td>0, 0, 5</td>
<td>0, 0, 12</td>
<td>0, 0, 10</td>
<td>0, 0, 9</td>
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<td>bFGF</td>
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<td>0, 0, 7</td>
<td>0, 0, 6</td>
<td>0, 0, 4</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
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<td></td>
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<tr>
<td>bFGF + NGF</td>
<td>0, 0, 5</td>
<td>0, 0, 0</td>
<td>0, 0, 5</td>
<td>0, 0, 6</td>
<td>0, 0, 6</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BDNF + hCNTF</td>
<td>0, 0, 5</td>
<td>0, 0, 0</td>
<td>0, 0, 7</td>
<td>0, 0, 6</td>
<td>0, 0, 6</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BDNF + NT-3</td>
<td>0, 0, 5</td>
<td>0, 0, 0</td>
<td>0, 0, 7</td>
<td>0, 0, 6</td>
<td>0, 0, 6</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BDNF + NGF</td>
<td>0, 0, 5</td>
<td>0, 0, 0</td>
<td>0, 0, 7</td>
<td>0, 0, 6</td>
<td>0, 0, 6</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDNF + bFGF + IGF-II</td>
<td>0, 0, 5</td>
<td>0, 0, 0</td>
<td>0, 0, 7</td>
<td>0, 0, 6</td>
<td>0, 0, 6</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>hCNTF</td>
<td>0, 0, 4</td>
<td>0, 0, 4</td>
<td>0, 0, 4</td>
<td>0, 0, 10</td>
<td>0, 0, 12</td>
<td>0, 0, 11</td>
<td>0, 0, 11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of mutants showing definite protection, number showing possible or equivocal protection, number showing no protection.  † Mutant rhodopsin transgenic mice.  ‡ Age at which most litters were injected. Few litters were injected at postnatal day (P) 11, 12, or 13; one at P15; §few at P7 or 13; ¶few at P8, 9, 12, 15, or 23.  9 few at P8, 9, 12, 13, or 23.  99 The mice with definite and possible protection each were administered 1 mg/ml.  hCNTF, human ciliary neurotrophic factor; rCNTF, rat ciliary neurotrophic factor; LIF, leukemia inhibitory factor; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; IGF, insulin-like growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4.

Table 2. Counts* of Surviving Cones in rd/rd Mice at P60-66 after Injection of One Eye with One or a Combination of Survival Factors at Postnatal Days 18 to 21

<table>
<thead>
<tr>
<th>Agent</th>
<th>n</th>
<th>Uninjected</th>
<th>Injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF (1 mg/ml)</td>
<td>6</td>
<td>38.7 ± 10.8</td>
<td>37.5 ± 15.0</td>
</tr>
<tr>
<td>BDNF (5 mg/ml)</td>
<td>6</td>
<td>41.2 ± 19.3</td>
<td>48.0 ± 14.3</td>
</tr>
<tr>
<td>BDNF (10 mg/ml)</td>
<td>1</td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td>hCNTF</td>
<td>3</td>
<td>33.0 ± 11.8</td>
<td>34.3 ± 9.3</td>
</tr>
<tr>
<td>rCNTF</td>
<td>10</td>
<td>49.4 ± 8.8</td>
<td>32.7 ± 13.8</td>
</tr>
<tr>
<td>bFGF</td>
<td>6</td>
<td>28.5 ± 9.8</td>
<td>29.7 ± 9.6</td>
</tr>
<tr>
<td>NT-4</td>
<td>4</td>
<td>40.2 ± 16.2</td>
<td>45.0 ± 5.4</td>
</tr>
<tr>
<td>BDNF + CNTF</td>
<td>5</td>
<td>39.6 ± 11.5</td>
<td>38.2 ± 19.5</td>
</tr>
<tr>
<td>BDNF + bFGF + IGF-II</td>
<td>8</td>
<td>43.6 ± 10.4</td>
<td>33.3 ± 17.8</td>
</tr>
</tbody>
</table>

* Mean ± SD of counts from 1-μm sections (from one section) of all surviving cone cell nuclei in the uninjected control eye and the injected experimental eye of a single mouse. Each agent was used with a single litter of C3H mice.

† BDNF, brain-derived neurotrophic factor; hCNTF, human ciliary neurotrophic factor; rCNTF, rat ciliary neurotrophic factor; bFGF, basic fibroblast growth factor; and IGF, insulin-like growth factor; NT-4, neurotrophin-4.

AND of most of the agents were administered either 1.0 μl or 2.0 μl; because there was no difference in the results, the data were pooled. In the constant-light experiments (described below), 1.0 μl was injected into the rat eyes and 0.5 μl was injected into the mouse eyes. In the experiments with the mutants, the opposite eye was uninjected in most cases. When photoreceptor rescue was seen with a given factor, we studied additional mice in which phosphate-buffered saline was injected as a surgical control. To make the injection in mice before their eyes were open (at approximately postnatal days 12 and 13), we performed manual retraction to open the eyelids. All injections were made by the same person (DY). Mice younger than postnatal day 10 were anesthetized with ice, and those older were anesthetized with Avertin.

For each mutant, postinjection intervals were specifically set to be long enough for degeneration to advance to the point at which protection could be distinguished from degeneration (in the opposite, uninjected eye), but short enough to fall within the suspected effective protection time of the agent in the retina (see Discussion). These intervals are given in Tables 1 and 2. The mice were killed by an overdose of carbon dioxide followed immediately by vascular perfusion of mixed aldehydes, the eyes were embedded in epoxy resin, and 1-μm thick histologic sections were taken along the vertical meridian to allow the comparison of all regions of the eye in the superior and inferior hemisphere, as described elsewhere. 40

Entire litters of mice were injected. In the case of rd/rd, rds/rds, and rds/+ mice, all were affected and all displayed retinal degeneration. With the cerebellar mutants, nr/nr and pcd/pcd, heterozygotes were bred so that approximately 25% of the pups from each litter were affected (that is, 107 of 388 were nr/nr; 90 of 313 were pcd/pcd). With the transgenic animals, the transgene was inherited as autosomal dominant, so approximately 50% of the offspring were affected. The affected cerebellar mutants and transgenic mice were identified histologically by the presence of...
TABLE 3. Comparison of Photoreceptor Rescue* from Constant Light Damage in BALB/c Albino Mice and Sprague-Dawley Albino Rats by Different Survival Factors

<table>
<thead>
<tr>
<th>Agent</th>
<th>BALB/c Mouse</th>
<th>Sprague-Dawley Rat</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Rescue</td>
<td>No Rescue</td>
</tr>
<tr>
<td>BDNF†</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>NT-4</td>
<td>8</td>
<td>3</td>
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<tr>
<td>hCNTF</td>
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<td>3</td>
</tr>
<tr>
<td>rCNTF</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Axokine</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>bFGF‡</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>IGF-II</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>BDNF + hCNTF</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>BDNF + rCNTF</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>bFGF + BDNF</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>BDNF + bFGF + IGF-II</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No rescue (excluding bFGF in mouse)</td>
<td>31.5%</td>
<td>7.9%</td>
</tr>
</tbody>
</table>

* Number of mice; degree of rescue shown in Figure 4. Except where indicated, each agent or combination of agents was used with a single group of mice and rats, and in all cases the rats and mice were injected on the same day from the same agent lot.
† Results of 1, 2, and 5 mg/ml were indistinguishable and were thus combined.
‡ Sum of five separate experiments using two different sources of bFGF.

Survival Factors

The agents injected were the following: recombinant human BDNF (1, 2, 5, and 10 mg/ml; Amgen/Regeneron Partnership, Tarrytown, NY); recombinant human or rat ciliary neurotrophic factor (hCNTF and rCNTF, respectively; 0.5 or 0.68 mg/ml; Regeneron Pharmaceuticals, Tarrytown, NY); recombinant human neurotrophin-3 (0.75 mg/ml; Amgen/Regeneron Partnership); recombinant human neurotrophin-4 (0.6 or 1.0 mg/ml; Regeneron Pharmaceuticals); Axokine, a mutant form of human neurotrophin-3 (0.4 or 1.0 mg/ml) modified to enhance its specific activity 41 and to increase its solubility and stability (Regeneron Pharmaceuticals); human bFGF (1 mg/ml; a gift from Dr. Denis Gospodarowicz, University of California at San Francisco School of Medicine); human leukemia inhibitory factor (1.0 mg/ml; R & D Systems); mouse nerve growth factor (0.5 mg/ml; a gift from Dr. William Mobley, University of California at San Francisco School of Medicine); and human leukemia inhibitory factor (1.0 mg/ml; R & D Systems). Each lot of each of the agents except nerve growth factor was made using the Student's f-test between the eyes that received the agents and the opposite uninjected eyes from the same mice.

Assessment of Protection by Survival Factors

Photoreceptor protection in all cases was scored by comparing the retina of the injected eye with that of the uninjected or buffer-injected opposite eye of the same animal. For the mutants, the retinas were scored by four or five independent observers as either definite, equivocal, or no protection. Definite protection was defined as the presence of two to three more rows of photoreceptor nuclei in the outer nuclear layer of the treated eye than in the control eye, either with or without greater photoreceptor inner segment and outer segment integrity, alone or in combination. Rescue was considered equivocal if the outer nuclear layer of the injected eye had only one to two more rows of nuclei than the control eye.

For quantification of the number of surviving cone cells in rd/rd mice, we counted all the surviving cone nuclei using criteria described elsewhere 43 using a single section from each eye. Photoreceptor nuclei in the outer nuclear layer were omitted from the counts. Statistical comparisons of the cone counts were made using the Student's t-test between the eyes that received the agents and the opposite uninjected eyes from the same mice.

For an assessment of protection in the light-damage experiments, the injected eyes were scored on a scale of rescue of 0 to 4 by four or five observers; 4 was the maximal protection, and 0 was no protection, as described elsewhere. 8 Statistical analysis of the absence of protection by bFGF in mice was made by chi-square analysis using the Yates correction for continuity.
FIGURE 1. Light micrographs of plastic-embedded sections of mouse retinas. (A) Postnatal day 21, a normal mouse retina in which the outer nuclear layer (asterisk) consists of eight to nine rows of photoreceptor nuclei. Photoreceptor inner segments (is) and outer segments (os) are shown, as is the retinal pigment epithelium (rpe). (B, C) Postnatal day 21, retinas from a Q344ter rhodopsin mutant transgenic mouse, one of which was uninjected (B) and the other of which was administered ciliary neurotrophic factor (CNTF) intravitreally at postnatal day 12 (C). In the control retina (B), the outer nuclear layer consists of less than one complete row of nuclei (asterisk), whereas in the CNTF-injected eye (C), the outer nuclear layer consists of three to four rows of nuclei (asterisk); and some photoreceptor inner and outer segments are present (arrowhead). Toluidine blue stain was used. Scale bar, 25 \( \mu \)m.

RESULTS

Survival Factor Protection in Mice with Inherited Retinal Degenerations

Photoreceptor protection was seen in three mutants, rd/rd, nr/nr, and Q344ter mutant rhodopsin transgenic mice, after the injection of one or more of several of the survival factors, as described in detail below. No protection was seen with any of the survival factors in rds/rds, rds+, and pcd/pcd mutants, or with the P23H or VPP mutant rhodopsin transgenic mice (Table 1).

Q344ter Mutant Rhodopsin Transgenic Mice

In control Q344ter rhodopsin transgenic mice, the outer nuclear layer was reduced to less than one complete row of photoreceptor nuclei as a result of the death and loss of cells by postnatal day 21 (Fig. 1B), compared with eight to nine rows in normal mice of this age (Fig. 1A). However, when rCNTF was injected at ages up to postnatal day 12, and when Axokine or leukemia inhibitory factor were injected at ages up to postnatal day 11, far less degeneration was seen in the injected eyes than in the control eyes. For example, in mice injected with rCNTF at 12 days of age, three to four rows of nuclei (that is, 30-40\% of normal) were still present, as were some photoreceptor inner and outer segments (Fig. 1C). Thus, not only did more cells survive at this age, the cells that survived had better structural integrity than the photoreceptors in the more severely degenerated control retinas. The degree of protection seen in those animals scored as definite protection was fairly consistent from animal to animal. It should be noted, however, that a large number of Q344ter mice that received these same factors showed no protection (Table 1).

None of the other factors gave definite protection of photoreceptors in Q344ter mice, including hCNTF. One combination of agents, BDNF and rCNTF, showed definite rescue in one mouse, which was not surprising given the protection afforded by rCNTF alone.

FIGURE 2. Postnatal day 21, retinas from a retinal degeneration (rd/rd) mouse, one of which was uninjected (A) and the other of which received ciliary neurotrophic factor (CNTF) at postnatal day 12 (B). In the control retina (A), the outer nuclear layer consists of less than one complete row of nuclei (asterisk), whereas in the CNTF-injected eye (B), the outer nuclear layer consists of one to two rows of nuclei (asterisk). Toluidine blue stain was used. Scale bar, 25 \( \mu \)m.
Retinal Degeneration Mice

In the rd/rd mouse, rCNTF reduced the loss of photoreceptors from that seen in the control retinas, although the protective effect was less in rd/rd mice than in Q344ter rhodopsin transgenic mice. For example, in control eyes of rd/rd mice at postnatal day 21, the outer nuclear layer consisted of less than one complete row of photoreceptor nuclei (Fig. 2A), whereas the outer nuclear layer in the eyes of nine mice injected with rCNTF at postnatal day 12 consisted of one to two rows of nuclei (Fig. 2B). In these mice, the degree of protection was fairly consistent from animal to animal. None of the other survival factors slowed the rate of degeneration in rd/rd mice, although several showed equivocal protection with hCNTF and Axokine (Table 1).

When survival factors were injected at approximately 3 weeks of age to examine the protective effect on cones in rd/rd mice, no agent alone or in combination increased cone numbers when compared with the opposite uninjected eye (Table 2). Although 12 of the 49 mice in this study showed higher numbers of surviving cones in the injected eyes, with a few higher, an almost equal number showed substantially fewer cones. Indeed, the variability in numbers of surviving cones was great, not only in the injected eyes but also in the uninjected control eyes (Table 2), as described elsewhere.44

Nervous Mice

In a few nr/nr mice, a definite protective effect by BDNF and hCNTF was seen (Table 1). For example, in a nr/nr mouse injected with BDNF at postnatal day 12 and killed at postnatal day 32, the outer nuclear layer was reduced to five to six rows of photoreceptor nuclei in the phosphate-buffered saline-injected control eye, whereas approximately eight rows of nuclei remained in the BDNF-injected eye. In a nr/nr mouse injected with hCNTF at postnatal day 9 and killed at postnatal day 40, the outer nuclear layer was reduced to five to six rows of nuclei in the uninjected control eye, whereas six to seven rows of nuclei remained in the hCNTF-injected eye. In addition, the photoreceptor inner and outer segments were longer and were arranged in a more orderly array in this hCNTF-injected eye than in the control eye. None of the other agents showed a clear protective effect in nr/nr mice. Moreover, even with the agents that did slow the degeneration, an absence of protection was observed in far more mice (Table 1).

In some of the experiments with nr/nr mice using BDNF, hCNTF, rCNTF, or Axokine, one to several of the mice seemed to show a reversal, that is, a slightly greater degree of degeneration in the factor-injected eye. Because none of these agents showed harmful effects to the retina in our previous studies, we considered the possibility that variability may exist between the two eyes in this mutant. Thus, we compared histologic sections from both eyes of 14 nr/nr mice at postnatal days 36 to 41 that were administered no intraocular injections. The fact that bFGF did not protect the mouse retina from light damage in BALB/c albino mice (solid bars) and Sprague-Dawley albino rats (shaded bars). Values are the means ± SD of the number of mice and nits that showed rescue in Table 5; those showing no rescue were omitted from the calculations.

FIGURE 3. Comparison of the degree of photoreceptor rescue from light damage in BALB/c albino mice (solid bars) and Sprague-Dawley albino rats (shaded bars). Values are the means ± SD of the number of mice and nits that showed rescue in Table 5; those showing no rescue were omitted from the calculations.

Constant Light Damage

In experiments to compare the effectiveness of survival factors in the protection of photoreceptors from constant light in mice and rats, BDNF, neurotrophin-4, and insulin-like growth factor II were equally potent in the two species (although insulin-like growth factor II was less effective than the other two factors in both species; Fig. 3). All the other agents or combinations of factors were less effective in the mouse than in the rat (Fig. 3).

In addition to the generally lower degree of protection, many more mice than rats failed to show any protection (Table 3). In our experience, a small percentage of injections is apparently ineffective with constant-light rat experiments, and this seems to occur irrespective of the presence or absence of obvious backflow or leakage of the agent from the eye at the time of injection (LaVail et al., unpublished observations). This is borne out by the present study in which approximately 8% of the rats failed to show protection (Table 3). Almost 32% of the mice, however, failed to show protection, four times the incidence in rats (Table 3, not counting the bFGF-injected mice; see below). We surmise that this higher incidence represents a delivery problem caused by some aspect of the small size of the mouse eye, not by a property of the survival factors. Even among those factors that gave comparable protection in the rat, there was the same high incidence of eyes that had no protection as there were agents that were less effective (Table 3). It should be noted that during the study we attempted to use different needle sizes and devices to alter the delivery rates in other mice (data not shown) and that we were unsuccessful in decreasing the incidence of mice that showed no protection.

Surprisingly, bFGF did not protect the mouse retina from constant light (Table 3), and this was observed in all 31 mice studied in five separate experiments with two sources of bFGF, which protected all but 1 of 20 animals (Table 3). Because the overall failure to protect the retina from light damage in the mouse with other factors was 31.5% (Table 3), we would have expected approximately 10 mice to show failure of protection with bFGF and 21 mice to be protected by bFGF. The fact that
no mice were protected (100% failure to protect; Table 3) is different from the 31.5% failure rate to protect with all the other agents with a high level of statistical significance (χ² = 42.03; P < 0.000005). Thus, it is likely that the bFGF results indicate a failure of the agent to protect the mouse retina from constant light, and the results are not a consequence of the apparent delivery problem that caused a 31.5% incidence of failure with the other agents.

**Absence of Adverse Morphologic Effects of Survival Factors**

In mice injected at postnatal day 14 or later, there were no obvious adverse morphologic effects of the survival factors in either the mutant mouse eyes or the normal mouse eyes (that is, nonhomozygous mice in the nr/nr and pcd/pcd lines and nontransgenic mice from the mutant rhodopsin transgenic lines) (Table 1).

In a few mice injected at postnatal day 13, but in many mice injected at postnatal day 10 and earlier, the retinas showed one to several rosettes or folds in the outer nuclear layer of individual sections (data not shown). This was observed in mutant retinas and in normal retinas with all agents, as well as in some of the phosphate-buffered saline-injected control eyes. Thus, the presence of rosettes depended on the age of the mouse at injection and did not seem to be caused by the factor itself. Our findings are consistent with the conclusion of Tansley, who indicated that rosettes were formed by lowering the intraocular pressure during the period of histogenetic movement of photoreceptors in the developing outer nuclear layer in the rat eye (before postnatal day 15) and not at older ages.45

**DISCUSSION**

**Photoreceptor Protection in Mice with Inherited Retinal Degenerations**

The successful in vivo experiments with rd/rd and Q344ter mice represent the first demonstration that survival factors can protect photoreceptors from degenerating in animal models with the same or similar gene defects as those in human retinal degenerations. We have shown that photoreceptor degeneration can be slowed in rd/rd, nr/nr, and Q344ter mutant rhodopsin mice by the intravitreal injection of certain forms of CNTF and that the degeneration in Q344ter mice also can be slowed by Axokine and leukemia inhibitory factor, whereas the degeneration in some nr/nr mice can be slowed by BDNF. Cayouette and Grave46 have recently reported a similar experiment in which rd/rd mouse photoreceptors were protected by the injection of rat CNTF, and Caffé et al.17 have demonstrated the slowing of photoreceptor degeneration in rd/rd mice by a combination of bFGF and nerve growth factor in organ culture, although the same combination of factors was not effective in vivo with intravitreal injections.

The actual mechanism of photoreceptor protection by these survival factors is unknown. Indeed, the site, or sites, of the action of CNTF and BDNF in the retina is unknown. Although photoreceptor protection by these agents seems to be mediated by specific receptors in the retina,48,49 CNTF-α receptors are present in the inner retina, particularly in the inner nuclear layer, but are apparently absent on photoreceptor cells,48,49 as are BDNF (trkB) receptors.50-54 This implies the involvement of an intermediate cell type, or types, in the protective response to the factors.

It is significant that CNTF protects photoreceptors from degenerating in three different inherited degenerations in mice, each with a unique gene defect. In addition, CNTF protects photoreceptors from constant light damage in the mouse (Fig. 3) and the rat,8 and rCNTF protects photoreceptors from degenerating in RCS rats (LaVail et al., unpublished observations). That these five different degenerations, inherited and environmentally induced, can be protected by rCNTF strongly suggests a common underlying molecular mechanism of protection. One candidate is apoptosis, a pathway of cell death. It has been shown that the RCS55 and constant-light-damaged56-59 rats display features of apoptosis during photoreceptor cell death, as do rd/rd60-63 and mutant rhodopsin transgenic60-65 mice. Whether nr/nr mice follow the apoptotic cell death pathway has not been studied, but it seems likely because virtually all types of photoreceptor cell death studied—ranging from death caused by the expression of the SV40 T antigen64 to retinitis pigmentosa in human patients65—have been shown to do so. Thus, the modulation of apoptosis by CNTF (and perhaps by other survival factors) is a possible common mechanism of action that must be explored. It should be noted, however, that rd56/rd56 mice also undergo apoptosis during cell death,60-63 as presumably do the pcd/pcd mutants and P23H and VPP mutant rhodopsin transgenic mice that we examined, yet these did not show protection by any of the factors. If these negative findings represent an inability of the factors to protect these mutants, then the failure to rescue would argue against the modulation of apoptosis as a common protective mechanism. Before these data can be interpreted in relation to common mechanisms, however, the cause of negative findings in these studies must be resolved (see below).

In central nervous system neuronal degeneration, survival factors typically are only effective, or are much more effective, when administered before cells have begun to degenerate,66-68 which is also seen in light damage in the rat retina (LaVail et al., unpublished observations). The slowing of photoreceptor degeneration by CNTF in Q344ter rhodopsin mutant mice, however, was observed when the injection was given either before or after photoreceptor degeneration began at postnatal day 10. Similarly, protection was seen when CNTF was injected at postnatal day 12 in rd/rd mice, 2 days after rod photoreceptor degeneration began.59 Although the slowing in rd/rd mice was less than in Q344ter mice, that CNTF slows the degeneration at all after injection at postnatal day 12 is remarkable in this very rapid degeneration. This is because CNTF requires approximately a day to reach its full potential effect (at least in the light-damaged rat; unpublished observations); approximately 50% of the outer nuclear layer is already gone and most of the remaining cells are pyknotic by postnatal day 1369; most of the rod photoreceptors disappear by postnatal day 17.13 Thus, CNTF and BDNF probably can act on injured or dying cells, although the relative effectiveness on healthy versus dying cells cannot be determined from these studies.

**Interpretation of Negative and Positive Findings**

Why do some retinal degeneration mutants show no protection by any of the survival factors or show protection by some factors but not by others (Table 1)? It is tempting to conclude that those factors that give no protection are ineffective in slowing these particular degenerations. Several possibilities
should be considered, however, before making this conclusion. Moreover, these same possibilities may explain why many of the mutants fail to show consistent protection by some survival factors (Table 1).

Another consideration is species differences of the survival factor sources and their specificity of action. It is known, for example, that rat CNTF has four to five times the biologic activity of human CNTF in some systems.41 Many of the agents we used were from different species. However, the light-damage experiments (Fig. 3) demonstrate that all the factors, except bFGF, can protect mouse photoreceptors at least from constant light damage, albeit to a lesser degree than in the rat in most cases. Thus, species differences of the factor sources would not explain the high degree of variability and the perplexing negative results in some cases in which several factors protect in one form of mutant rhodopsin transgenic mouse (Q344ter), but not in two others (Table 1); rat CNTF, Axokine, and leukemia inhibitory factor, all which act through members of the CNTF receptor family, protect photoreceptors in Q344ter mice, but only one of these agents (rat CNTF) protected in the rd/rd mouse; and human CNTF protected in some nr/nr mice, but the generally more potent rat CNTF did not.

Another possible cause of different protective responses is genetic background based on strain difference. This is possible in the case of rds/rds mice because they are on the O20/A background, but the three mutant rhodopsin transgenic mice and pcd/pcd mice are on the C57Bl/6 background, and only one of these mutants (Q344ter) showed protection by survival factors. Thus, strain differences are unlikely to explain differences in the effectiveness of the factors in different mutants, and they do not explain the variable response of a given mutant to a given factor.

Another possible explanation for the many negative findings is that the biologic life of the agents may not be adequate for single-injection experiments. In RCS rats with inherited retinal dystrophy, bFGF protects photoreceptors for at least 60 days.6 In addition, BDNF and CNTF protect photoreceptors for at least 15 days in constant-light experiments in which the insult is extremely rapid and severe.70 Thus, because most of the mutant mice were examined at 1- to 3-week postinjection intervals, biologic half-life should not have been a major consideration, although in the rd/rd cone experiments a 39- to 48-day postinjection interval may have been too long. Biologic half-life, though, also would not explain the variable response of a given mutant to a given factor.

These considerations, either separately or combined, may explain the variability seen in the study of mutant mice with retinal degenerations. As a result of the number of issues, we caution that until they are resolved, particularly the delivery problem to the very small, young mouse eye, only positive results should be considered in drawing firm conclusions about whether a particular form of degeneration is responsive to a particular factor, especially if that form of degeneration has not yet been shown to be amenable to survival factor protection. However, it is reasonable to accept positive results as indicative of protection by survival factors, even if only in a fraction of cases, because in all the years that these animals have been studied, neither we nor others have seen inter-eye variability in these mutants that would give false-positive results. The only other known potential source of confounding positive results, that of injury-induced protection seen in the rat,67 does not occur in the mouse.38 For this reason, our findings of variability and high incidence of failure to protect caused by technical reasons are significant considerations regarding the mouse as an experimental model for survival factor research. They do not, however, compromise the central positive finding of the study, that some survival factors can protect photoreceptors from degenerating in an animal with gene defects that model human retinal degeneration.

It is important, however, to find an explanation for the negative findings in the present study, because there are significant ramifications if the negative results indicate no protective response (that is, if they are not caused by any of the confounding causes, as mentioned above). For instance, this would mean that in certain forms of degeneration, such as in rd/rd and Q344ter mice, only certain agents are protective, whereas others are not. Similarly, if none of the agents truly are protective in other forms of degenerations, such as in rds/rds, pcd/pcd, VPP, and P23H mice, then these forms of retinal degeneration may not be amenable to this form of pharmacologic therapy, at least with these specific agents. Furthermore, if the agents truly do not protect in VPP and P23H rhodopsin mutant mice, yet they do in Q344ter rhodopsin mutants, it suggests different pathologic mechanisms in different rhodopsin mutations that would result in different susceptibilities to survival factor protection. These important conclusions await resolution of the cause, or causes, of the high incidence of negative findings in the present study.

Effectiveness of Factors in Light Damage in the Mouse and Rat

In attempting to explain the lower effectiveness of survival factors in the mouse to protect from light damage, several considerations should be noted. First, the degree of variance of each of the groups of mice was similar to those of the rats (Fig. 3). Second, many of the mice showed only moderate light damage in the un.injected control eyes, and in many studies in light damage in the rat, we have found that the less severe the damage, the greater the protective effect of the factor.71 Thus, if the mouse responded in the same way as the rat, these moderately damaged retinas should have been well protected, assuming that a potent factor had reached the retina. Third, the generally lower effectiveness of most of the factors in the mouse was presumably not caused by the smaller volume of agent injected. Although a 0.5-μl agent was injected into the mouse eyes and 1.0 μl was injected into the rat eyes, a factor of 2, the approximate volume of the rat eye at postnatal day 90 (calculated from the external diameter of fresh eyes) is approximately 6.6-fold that of the mouse eye at the same age. Thus, a relatively larger volume of agent was injected into the mouse eye. (It should be noted that this same argument fails to explain...
the absence of protection in many of the mutant mouse eyes because the rat eye at postnatal day 90 is approximately 12.3-fold greater than the very small eyes of the mutant mouse eyes on postnatal days 8 to 12 (Table 1), most of which also were administered 0.5 μl.

Absence of Protection by Basic Fibroblast Growth Factor in Light Damage

One form of degeneration that is clearly responsive to many survival factors in the rat and the mouse (Table 3) is light damage (albeit the mouse somewhat less effectively than in the rat; see above). Thus, we think that the remarkable finding that bFGF failed to protect photoreceptors in constant-light experiments in albino mice is significant because other survival factors protected the mouse from light damage and because of the large number of bFGF experiments in the mouse (Fig. 3). In addition, one of us (KU) found the same negative result in albino mice in Japan (unpublished observations). Thus, there seems to be a clear species difference between the mouse and the rat in the protective effect of bFGF against light damage.

The absence of protection from light damage by bFGF in the mouse may be caused by the failure of the mouse to show significant upregulation of bFGF receptor after initial damage by light, unlike that seen in the rat. There is a growing body of evidence that the molecular events are similar after injury of various kinds. In the rat retina, mechanical injury and light damage lead to a rapid and significant upregulation of bFGF, and mechanical injury and light damage lead to a comparable upregulation of bFGF receptor mRNA. In the mouse, however, mechanical injury leads to a much smaller upregulation of bFGF mRNA than in the rat, and to very little, if any, upregulation of bFGF receptor mRNA. It is likely, therefore, that constant-light damage also produces little or no upregulation of bFGF receptor mRNA. Such a failure to respond in this way may lead to minimal binding of exogenous bFGF in the mouse, and thus to little or no protective action of the peptide. This remains to be demonstrated.

Implications for Human Retinal Degeneration

Survival Factor Therapy

We have demonstrated that survival factors can protect photoreceptors from degenerating in animal models with the same or similar genetic defects as those in human retinal degenerations. Moreover, the findings demonstrate that survival factors are effective in a species other than the rat. Although our observations demonstrate the potential efficacy of several factors for several forms of retinal degeneration, a number of questions must be answered before human therapy with survival factors can be considered. Relating specifically to the data reported here are the following: Would injections at different stages of degeneration be more or less effective? Would multiple injections slow the degeneration further? What are the dose-response relationships of the agents in slowing the degeneration? Unfortunately, the technical delivery problem and variability we encountered preclude studies answering these questions with the very small mouse eye because it is not possible to predict whether, or to what degree, a given eye will respond to an injection of survival factor. Such studies require a larger eye in which consistent responses to injected survival factors can be obtained and, indeed, our experience with mice has led us to begin the development of transgenic rats with retinal degenerations, which should be useful in this respect.

More general problems that must be overcome for consideration of survival factors as potential therapeutic agents for human diseases are those of toxicity and delivery. We have used relatively high concentrations of survival factors compared with those that achieve biologic responses in vitro, including BDNF up to 10 mg/ml, and we have not seen adverse affects when the retina is relatively mature at the time of injection. We do not know, however, whether repeated doses of survival factors at high concentrations would be equally benign. Another general problem is that of delivery, recognizing that the survival factors are peptides that do not cross the blood-retinal barrier. Several possibilities exist experimentally to circumvent the blood-retinal barrier, including the use of biodegradable or replenishable sustained release systems or the transduction of cells in the eye to cause them to produce biologically relevant agents, such as missing gene products or survival factors, but they are not yet developed for human use. Although the problems of toxicity and delivery are significant hurdles to overcome, we are hopeful that our present findings will provide the impetus to explore the possibility of treating inherited retinal degenerations with survival factors.

Acknowledgments

The authors thank Nancy Lawson and Gloria Riggs for technical and secretarial assistance, Muna Naash for the VPP transgenic mice, and Thaddeus Dryja for the P23H transgenic mice.

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