Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment of the retina

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SUMMARY

Lineage analyses of vertebrate retinæ have led to the suggestions that cell fate decisions are made during or after the terminal cell division and that extrinsic factors can influence fate choices. The evidence for a role of extrinsic factors is strongest for development of rodent rod photoreceptors (‘rods’). In an effort to identify molecules that may regulate rod development, a number of known factors were assayed in vitro. Ciliary neurotrophic factor (CNTF) was found to have a range of effects on retinal cells. Addition of CNTF to postnatal rat retinal explants resulted in a dramatic reduction in the number of differentiating rods. Conversely, the number of cells expressing markers of bipolar cell differentiation was increased to a level not normally seen in vivo or in vitro. In addition, a small increase in the percentage of cells expressing either a marker of amacrine cells or a marker of Müller glia was noted.

It was determined that many of the cells that would normally differentiate into rods were the cells that differentiatated as bipolar cells in the presence of CNTF. Prospective rod photoreceptors could make this change even when they were postmitotic, indicating that at least a subset of cells fated to be rods were not committed to this fate at the time they were born. These findings highlight the distinction between cell fate and commitment. Resistance to the effect of CNTF on rod differentiation occurred at about the time that a cell began to express opsin. The time of commitment to terminal rod differentiation may thus coincide with the initiation of opsin expression.

In agreement with the hypothesis that CNTF plays a role in rod differentiation in vivo, a greater percentage of cells were observed differentiating as rod photoreceptors in mouse retinal explants lacking a functional CNTF receptor, relative to wild-type littermates.

Key words: rod, photoreceptor, CNTF, retina, vertebrate, postmitotic cell, bipolar cell

INTRODUCTION

The vertebrate retina has served as a model system for studies of development of the central nervous system (Harris and Hartenstein, 1991; Reh, 1991; Raymond, 1995; Cepko et al., 1996). During embryonic and perinatal development of rodents, the one glial and six neuronal retinal cell types are generated in a conserved order. Even though there is an order, at any one time more than one cell type is being generated (Altshuler et al., 1991). The different cell types are generated in numbers that vary greatly, with the final percentages ranging from 0.3% to 70% in the mature mouse retina (Young, 1985b). The mechanisms ruling the temporal and quantitative aspects of cell generation must be under tight regulation to account for the observed fidelity of this process.

Previous studies using a lineage tracing method have established that rodent retinal progenitors are multipotent (Turner and Cepko, 1987; Turner et al., 1990). The progeny of individual progenitor cells can comprise combinations of different cell types, and a certain degree of multipotency has been shown to persist throughout development. These observations prompted a suggestion that environmental factors influence the choice of cell fate. Studies of extrinsic cues that might regulate the development of retinal cell types have frequently focussed on rod photoreceptors (e.g. Altshuler and Cepko, 1992; Altshuler et al., 1993; Fuhrmann et al., 1995; Kelley et al., 1994; Kirsch et al., 1996; Watanabe and Raff, 1992).

Rod photoreceptors are the most abundant cell type in the rodent retina, accounting for approximately 70% of all cells at maturity (Young, 1985b). Studies conducted on cultured retinal cells have implicated diffusible molecules in the development of rods. For example, taurine (Altshuler et al., 1993) and retinoic acid (Kelley et al., 1994) stimulate, and TGFα, EGF and bFGF; (Lillien, 1995; Lillien and Cepko, unpublished) inhibit rod development when added to retinal cultures. In addition, fractions of retinal extracts or of medium conditioned by retinal cells have stimulatory or inhibitory effects on rod development (Altshuler et al., 1993).

Mature rods are highly specialized cells, with an elaborate membranous outer segment that is exquisitely sensitive to light (Rodieck, 1973). Despite the studies cited above, very little is known about the steps that lead to the formation of a terminally
differentiated rod. Outstanding questions include when commitment to the rod fate occurs and, in particular, whether commitment occurs in the mitotic progenitor cell or in postmitotic cells. It is also unknown how many steps occur subsequent to commitment. However, one step that has been studied is that in which photopigment synthesis begins. Many studies of rod development use as an assay the expression of rhodopsin, the photopigment specific to rods, comprising an apoprotein, opsin, and a chromophore, 11 cis-retinal. In many species, there is a significant lag between the time that a cell becomes postmitotic and the time that opsin expression begins (Knight and Raymond, 1990; Watanabe and Raff, 1990; Dorn et al., 1995, reviewed in Cepko, 1996). The lengthy delay makes the question of the relationship between birth of the rod progenitor and commitment to terminal differentiation a particularly intriguing one. The present study addresses this question.

In a search for factors that might act as extrinsic inducers or inhibitors of cell type specification, we examined the role of CNTF. CNTF is one of the members of a family of cytokines that includes interleukin-6 (IL-6), interleukin-11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), and the newly discovered cardiotrophin-1 (CT-1) (reviewed by Ip and Yancopoulos, 1996). These cytokines do not display extensive sequence homology; rather, their common feature is a shared receptor (Ip et al., 1993). CNTF has been known for years to have a wide range of effects on a variety of neuronal and glial cell populations: for example, it has been shown to promote the survival of embryonic chick ciliary ganglion neurons in vitro (Adler et al., 1979; Lin et al., 1989; Stockli, 1989), to inhibit the proliferation of sympathetic neuron progenitors (Ernsberger et al., 1989) and to promote the differentiation of glial progenitor cells into type-2 astrocytes (Hughes et al., 1988; Lillien et al., 1988). More recently, various effects of CNTF on the in vitro development, proliferation, or survival of chick, and rat, retinal neurons have been described (Fuhrmann et al., 1995; Kirsch et al., 1996).

Here we show that CNTF can modify the cell type composition of the rat retina in vitro. In retinal explants, cells normally destined to become rods are directly or indirectly prevented from differentiating into rods, but can initiate other differentiation programs, such as the bipolar one, after exposure to CNTF. This change in choice of differentiation pathway can be made by postmitotic cells. We thus propose that presumptive rods are not committed to terminal rod differentiation at the time that they are born. Resistance of rod progenitors to inhibition of differentiation by CNTF coincides with the initiation of opsin expression, and may reflect the timing of commitment to the rod fate. In correspondence with one of the effects of CNTF in vitro, namely, inhibition of rod differentiation, the signaling pathway shared by CNTF and other cytokines may be involved in retinal cell development in vivo, as demonstrated by an increase in the number of cells expressing opsin in explants derived from mice lacking a functional CNTF receptor.

**MATERIALS AND METHODS**

**Tissue culture**

**Collagen gels**

Collagen gel cultures, in which dissociated cells remain separated from each other throughout the culture period, were made from postnatal day 0 (P0) rat retinæ as described (Altshuler and Cepko, 1992). High density gels containing \(1.5 \times 10^6\) cells per 100 \(\mu\)l were used. Factors were added directly to media in the wells, in volumes of 10-20 \(\mu\)l.

**Explants**

Explants were made from P0 rat retinal cells based upon the protocol by Lillien and Cepko (1992) with the following modifications: retinæ were dissected in HBSS, then transferred into culture medium. Two types of media were used: either Sato’s medium without serum (Bottenstein and Sato, 1979), or a serum-containing medium (5% fetal calf serum in a 1:1 mix of DMEM and F12 containing Hapes, penicillin, streptomycin and insulin in the same amounts as for Sato’s medium). Tissue surrounding the optic nerve head was removed and retinæ were cut into ten sectors each, such that all pieces contained roughly equivalent proportions of central and peripheral retinal tissue. Every day, 20 \(\mu\)l of culture medium taken from the corresponding well were added directly on top of each explant to provide equal access to media and factors for both faces of the explant. Factors were added to explants by diluting them first in small volumes of media, which were then added to the well. 20 \(\mu\)l of well medium containing the added factor(s) were then placed directly on the explant, on top of the filter.

**Dissociation of retinal cultures**

After a specified number of days in culture, gels were dissociated to recover single cells for immunocytochemical analysis as described (Altshuler and Cepko, 1992) with slight modification. Medium from the wells was replaced with 100 \(\mu\)l of 1 \(\mu\)g/ml collagenase in medium, the plates were incubated for 1 hour at 37°C, then the suspension was triturated to separate the cells. Explants were dissociated by sinking the filters into the well medium to detach the explants, transferring the explants to Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS containing 1 mM EDTA, then processing them as described in the ‘Collagen gels’ section for the dissociation of fresh retinal tissue into single cells. For both gels and explants, once dissociated, the cells were plated on coated 8-well glass slides purchased from Cel-Line Associates (coating: 50-80 \(\mu\)l of 10 \(\mu\)g/ml poly-D-lysine for 30-120 minutes, followed by 3 HBSS washes), allowed to recover at 37°C for 1-2.5 hours, then fixed in 1% or 4% paraformaldehyde for 10-20 minutes. The slides were kept at 4°C overnight and processed for immunocytochemistry the next day. The same dissociation and fixation procedures were followed when processing freshly dissected retinæ for immunocytochemistry.

**Histology**

To obtain sections of fresh retina or of explants for immunocytochemistry, the retinæ were dissected (or the explants removed from the supporting filter), the tissue was fixed in 4% paraformaldehyde for 30 minutes, washed in PBS 3-5 times for 5-10 minutes each, put in 30% sucrose until sinking, swirled in a 1:1 mixture of 30% sucrose and OCT embedding medium (Tissue-Tek) for 1-2 hours, then embedded on dry ice in OCT. The blocks were stored at -70°C. Sections 8-20 \(\mu\)m thick were cut on a Jung CM3000 cryostat. Sections were collected onto Superfrost/Plus glass slides (Fisher Scientific). Once deposited onto the slides, the sections were air-dried for 10 minutes to an hour at room temperature, then postfixed in 4% formaldehyde for 5 to 10 minutes, and immediately processed for
immunocytochemistry, as described in the next paragraph for single-cell preparations. The only difference was in the length of incubation (typically, sections were kept in primary antibody solution overnight), and the amount of Triton X-100 added (generally higher for immunocytochemistry on sections, i.e., 0.2-0.5%).

Cytokines

Human recombinant (hr) CNTF was provided by Regeneron Pharmaceuticals (Tarrytown, NY). A 100 µg/ml aqueous stock solution was kept in aliquots at −70°C. Rat recombinant (rr) CNTF and hrCNTF were also purchased from Boehringer Mannheim. A 10 µg/ml aqueous solution was kept in aliquots at −20°C. Human recombinant interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and oncostatin M (OSM) were purchased from Boehringer Mannheim. Aqueous stock solutions of, respectively, 2 µg/ml, 5 µg/ml, and 10 µg/ml were kept in aliquots at −20°C. Once thawed, aliquots were kept at 4°C and used within one week.

Immunocytochemistry

Cells fixed onto 8-well glass slides were processed for immunocytochemistry as described by Altshuler and Cepko (1992) with slight modification: blocking solution was left on the slides overnight, primary antibody incubation was done for 2-5 hours for cells, and overnight for tissue sections, and secondary antibody incubation was done for 1 hour. After the final PBS washes, slides were processed for autoradiography, or were mounted with Gelvatol and air-dried overnight at room temperature in the dark. Table 1 summarizes the labeling protocols for the various antibodies used in these studies to distinguish the various retinal cell types. Labeled cells or tissue sections were viewed using a Zeiss Axiophot microscope, using a 40× or a 63× Plan NEOFluar objective.

Double labeling with two different markers was performed by combining two antibodies raised in different animal species (e.g., one in mouse, one in rabbit) at the primary antibody addition step and also combining two corresponding secondary antibodies.

For confocal microscopy, a BioRad MRC-600 attached to a Zeiss Axiophot microscope was used.

[3H]thymidine birthdating

To mark the cells that undergo their final S phase on P0 (also referred to as the cells born on P0), P0 retinal explants were prepared as described above, but before laying the tissue onto filters, the small explant pieces were floated in a 35 mm tissue culture dish containing 5 µCi/ml of [3H]thymidine in Sato’s medium for 1 hour at 37°C. [3H]thymidine was removed by washing the explant pieces in medium 3 times, for 10 minutes each, at 37°C. The explants were then processed as usual, and cultured for 10-11 days.

Immunocytochemistry was performed and, once completed, the slides were coated with autoradiographic emulsion (Kodak, prepared as recommended by the manufacturer), allowed to dry for 2-24 hours in the dark, and kept at 4°C for 3-5 days. The slides were developed in Kodak D-19 developer and fixer.

The relative quantity of [3H]thymidine per cell was estimated based on the number of silver grains, visualized by light microscopy, inside and around cell nuclei. The number of grains per cell was determined for 5-600 consecutively encountered cells in CNTF-treated and untreated samples. A corresponding histogram was produced and used to determine the maximum number of grains found in a cell, n, and the distribution of grain numbers. Cells displaying at least (n/2) grains were estimated to be ‘heavily labeled’ and, following, to have been born on P0. The identity later assumed by cells generated on P0 was assessed by scoring heavily labeled cells for expression of various cell type markers.

Animals

C/D rats were purchased from Charles River Laboratories. lifr β knockout mice (Ware et al., 1995) were purchased from Jackson Laboratories. Genotyping was performed as described by Ware et al. (1995) and phenotypic identification (lack of milk in the stomach) was used in conjunction with genotyping. ctnf α knockout mice (DeChiara et al., 1995) were provided by Regeneron Pharmaceuticals (Tarrytown, NY). Genotyping was either performed by Southern blot analysis or was done as described for lifr β+/− mice. As for ctnf α+/− mice, the same feature was used for phenotypic identification.

RESULTS

CNTF-induced changes in cell type composition of the retina in vitro

Preliminary results obtained by CNTF addition to dissociated

<table>
<thead>
<tr>
<th>Antibody name and specificity</th>
<th>Source</th>
<th>Type</th>
<th>Fixation</th>
<th>Dilution % TX-100</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RetP1 (opsin, rods)</td>
<td>C. Barnstable (Yale U.)</td>
<td>Mouse monoclonal</td>
<td>4% para</td>
<td>1:100K 0.02-0.2</td>
<td>Barnstable, C. (1980)</td>
</tr>
<tr>
<td>Rho4D2 (opsin, rods)</td>
<td>R. Molday (U. of British Columbia)</td>
<td>Mouse monoclonal</td>
<td>4% para</td>
<td>1:250 0.02-0.2</td>
<td>Hicks, D. and Barnstable, C. (1987)</td>
</tr>
<tr>
<td>115A10 (Ag unknown, bipolar)</td>
<td>N. Onoda and S. Fujita (Gunma U., Japan)</td>
<td>Mouse monoclonal</td>
<td>1% para</td>
<td>Neat** 0.02</td>
<td>Onoda, N. and Fujita, S. (1987)</td>
</tr>
<tr>
<td>PKC (protein kinase C α, bipolar)</td>
<td>Amersham</td>
<td>Mouse monoclonal</td>
<td>1% para</td>
<td>1:50-100 0.02-0.2</td>
<td>Negishi, K. et al. (1988); Greferath, U. et al. (1990); Zhang, D. R. and Yeh, H. H. (1991)</td>
</tr>
<tr>
<td>mGluR6 (metabotropic glutamate receptor, bipolar)</td>
<td>S. Nakanishi (Kyoto U., Japan)</td>
<td>Rabbit polyclonal</td>
<td>1% para</td>
<td>1:2-400 0.02-0.2</td>
<td>Nomura, A. et al. (1994)</td>
</tr>
<tr>
<td>VC1.1 (HNK-1/N-CAM, amacrines)</td>
<td>Sigma</td>
<td>Mouse monoclonal</td>
<td>4% para</td>
<td>1:1000 0</td>
<td>Arimatsu, Y. et al. (1987); Naegele, J. R. and Barnstable, C. J. (1991); Kosaka, T. et al. (1990)</td>
</tr>
<tr>
<td>GFAP (glial fibrillary acidic protein, Müller glia)</td>
<td>Sigma</td>
<td>Mouse monoclonal</td>
<td>1% para</td>
<td>1:400 0.02-0.2</td>
<td>Debus, E. et al. (1983); Franke, F. E. et al. (1991)</td>
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*Detergent: Triton X-100. **Hybridoma supernatant.
cells grown in collagen gel cultures had suggested a possible developmental role for CNTF (LoTurco and Cepko, unpublished results). To further assay for involvement of CNTF in retinal development, it was added to rat retinal explants at P0 and cultured for 10-11 days under conditions that allow for mitosis and differentiation at near normal levels (Sparrow et al., 1990). To confirm the similarity between development in vivo and in vitro, in explants, the relative proportions of four postnata-

<table>
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<th>Table 2. Effect of CNTF on cell type composition of the rat retina</th>
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<tr>
<td>Rods (Rho4D2)</td>
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<tr>
<td>Bipolars (115A10)</td>
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<tr>
<td>(PKC)</td>
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<tr>
<td>(mGluR6)</td>
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<tr>
<td>Amacrine (VC-1)</td>
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<tr>
<td>Müller glia (GFAP)</td>
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The percentage of cells expressing markers of each postnata-

| Fig. 2. Various patterns of α-PKC labeling observed in the presence or absence of CNTF. (A-C) Confocal photomicrographs of cells dissociated at 10-11 d.i.v. from CNTF-treated rat retinal explants cultured on P0. (A) Cytoplasmically localized α-PKC labeling normally observed in vivo and in vitro, in the absence of CNTF treatment, and also on some cells after CNTF treatment. (B, C) Anti-

CNTF was added at the onset of the culture of P0 retinae and left in for the entire culture period. After 10-11 days in vitro in the presence of CNTF, the percentage of cells expressing opsin was found to be significantly decreased (Table 2; Fig. 1). Given the dramatic reduction in the number of cells expressing opsin, the possibility of a compensatory increase in another postnata-

Table 2. Effect of CNTF on cell type composition of the rat retina

<table>
<thead>
<tr>
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<th>P 10-11 in vivo</th>
<th>No CNTF in vitro</th>
<th>+ CNTF in vitro</th>
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</thead>
<tbody>
<tr>
<td>Rods (Rho4D2)</td>
<td>54.0±11.7</td>
<td>46.6±14.1</td>
<td>2.8±2.5</td>
</tr>
<tr>
<td>Bipolars (115A10)</td>
<td>5.5±1</td>
<td>4.5±1.2</td>
<td>30.8±3.9</td>
</tr>
<tr>
<td>(PKC)</td>
<td>3.4±1.5</td>
<td>2.7±0.7</td>
<td>34.1±4.8</td>
</tr>
<tr>
<td>(mGluR6)</td>
<td>3.9±0.9</td>
<td>5.9±1.6</td>
<td>28.9±7.6</td>
</tr>
<tr>
<td>Amacrine (VC-1)</td>
<td>5.6±2.1</td>
<td>4.0±3.4</td>
<td>7.5±2.5</td>
</tr>
<tr>
<td>Müller glia (GFAP)</td>
<td>0.4±0.2</td>
<td>0.9±0.4</td>
<td>2.0±0.6</td>
</tr>
</tbody>
</table>

The percentage of cells expressing markers of each postnata-

| Fig. 2. Various patterns of α-PKC labeling observed in the presence or absence of CNTF. (A-C) Confocal photomicrographs of cells dissociated at 10-11 d.i.v. from CNTF-treated rat retinal explants cultured on P0. (A) Cytoplasmically localized α-PKC labeling normally observed in vivo and in vitro, in the absence of CNTF treatment, and also on some cells after CNTF treatment. (B, C) Anti-

To further investigate the differentiation of cells expressing bipolar markers, cells derived from explants treated with CNTF for 10-11 days were labeled with dual combinations of the three bipolar markers, 115A10, anti-PKCα and anti-mGluR6 (Fig. 3). On average, 96.2% of the cells expressing PKCα (either as bars or as cytoplasmic labeling) were also labeled by anti-mGluR6. 89% of the cells expressing mGluR6 were also
were used. For percentages of cells double-labeled with each combination of antibodies, see text.

Fig. 3. Confocal photomicrographs of cells double-labeled with different anti-bipolar antibodies after CNTF treatment. Cells were dissociated at 10-11 d.i.v. from rat retinal explants cultured on P0 and treated with 100 ng/ml CNTF beginning at 0 d.i.v. (A) α-PKC labeling. Note the bar structure. (B) α-mGluR6 labeling (Texas Red-conjugated secondary antibody); (C) double-labeled cell (merged photographs); (D) 115A10 labeling; (E) anti-mGluR6 labeling; (F) double-labeled cell (merged photograph). (A,D) FITC-conjugated secondary antibody; (B,E) Texas Red-conjugated secondary antibody. (A) 115A10 labeling; (B) α-mGluR6 labeling; (C) double-labeled cell (merged photograph). (A,D) FITC-conjugated secondary antibody; (B,E) Texas Red-conjugated secondary antibody were used. For percentages of cells double-labeled with each combination of antibodies, see text.

positive for 115A10. Since the percentage of mGluR6-positive cells is roughly equivalent to that of PKCα-positive cells, it can be inferred that a major proportion of cells express all three markers.

In addition to rods and bipolar cells, two other cell types are born postnatally: amacrine cells and Müller glia. The effect of CNTF on the differentiation of these two cell types was assessed using the antibody VC1.1 (Naegle, 1991; Arimatsu et al., 1987) as a marker of amacrine cells and anti-glial fibrillary acidic protein (GFAP; Bignami et al., 1972) as a marker of Müller glial cells. The experimental conditions were identical to those described above. A small increase in the percentage of VC1.1-positive cells and GFAP-positive cells was observed following CNTF treatment (Table 2).

These results indicate that CNTF addition at P0 causes changes in the cell type composition of the rodent retina in vitro. It directly or indirectly leads to a lack of rod differentiation and the initiation of bipolar differentiation in an abnormally large percentage of cells, and perhaps leads to the initiation of amacrine or glial differentiation in a slightly increased percentage of cells.

Members of the CNTF family of cytokines, LIF and OSM, can also alter retinal cell type composition

CNTF belongs to a family of cytokines that share various combinations of receptor components, as well as an intracellular signaling pathway (reviewed by Ip and Yancopoulos, 1996). Like CNTF, IL-6, LIF and OSM require the shared signal transducer, gp130. CNTF, LIF and OSM (but not IL-6) also require an additional signal transducer, LIFRβ. The IL-6 receptor includes IL-6 receptor α (IL-6Rα), a specificity-conferring component that restricts the site of action of this cytokine to cells expressing it, few of which, if any, are in the nervous system. CNTF signaling through its receptor also requires a specificity-conferring component, CNTFRα, which is present in neural tissue, including the retina (Ip et al., 1993). It was therefore expected that LIF and OSM, but not IL-6, would mimic the effects of CNTF.

The three factors LIF, IL-6 and OSM were assayed for an effect on cell type composition of the retina in vitro and, as predicted, results similar to those described for CNTF on rods were obtained for LIF and OSM, but not for IL-6 (Fig. 1). An increase in the percentage of cells expressing bipolar markers, including the appearance of PKC bars in cells after treatment with either LIF or OSM, was also noted (data not shown).

These results demonstrate that the subset of CNTF family members that require only the widely distributed gp130 and LIFRβ components of the common receptor system can mimic the effects of CNTF on cell type composition of the retina.

Similarity of dose-response and temporal characteristics for the effects on rods and bipolar cells

The simultaneous occurrence of rod inhibition and initiation of bipolar differentiation raised the possibility that the two effects were the result of CNTF directly or indirectly affecting the same population of cells. To begin to examine this possibility, the dose-response curves for each effect was assessed. The dose-response curves for inhibition of opsin expression and stimulation of bipolar differentiation (Fig. 4) had nearly identical EC50s, of 2-5 ng/ml in explants, where retinal tissue remains essentially intact (Fig. 4B), and 0.2-0.5 ng/ml in gels, for which the retina is initially dissociated into single cells that are maintained apart from the culture period by embedding in a semisolid collagen gel matrix (Fig. 4A).

To define the window of time in which CNTF could cause either effect, the cytokine was added to P0 explants at progressively later times: 1, 3, 5, 7 or 9 d.i.v. (days in vitro, day 0 being defined as the day the cultures were initiated). The results (Fig. 5) indicate that both rod inhibition and initiation of bipolar differentiation decrease in magnitude with progressively later addition.

The similarity in the EC50s for rod inhibition and initiation of bipolar differentiation, as well as the nearly identical temporal sensitivity of the two effects, are consistent with the hypothesis that the cells that do not differentiate into rods initiate a program of bipolar differentiation.

Rod progenitors give rise to excess bipolar cells in the presence of CNTF

To further explore whether the excess cells expressing bipolar markers are derived from the cells expected to differentiate into rods, we asked whether CNTF addition could result in the expression of bipolar markers by cells that are known, by virtue of their ‘birthdate’, to normally adopt a rod fate. The birthdate of a cell is the day of its terminal S phase, and this has been shown to be highly correlated with the identities that a cell can adopt. The cell types born each day during retinal development have previously been quantified in the rodent retina using the [3H]thymidine birthdating technique (Sidman, 1961; Young, 1985b). Cells born on P0 in the rat retina reproducibly give rise to the combination of cell types shown in Fig. 6A (LaVail, personal communication). The overwhelming majority normally become rods (80%) and only a small percentage become bipolar cells (6.1%), making P0 an adequate birthday
for the assessment of bipolar hallmarks in cells normally destined to become rods. The remainder of the cells born on P0 comprise amacrine cells (9.5%), and Müller glia (1.8%). Fewer than 2% of the cells born on P0 are of unknown fate.

The effect of CNTF addition on the identity of cells born on P0 was examined by pulse-labeling retinal explants at P0 with $[^3]H$thymidine, then treating the explants with CNTF for 10-11 days, performing immunocytochemistry followed by autoradiography on the dissociated cells and assessing changes in the percentage of cells born on P0 that display each cell type marker assayed. The cells born on P0 were identified by the large number of silver grains in them and will be referred to as ‘heavily labeled cells’ (see Materials and Methods).

For untreated explants, the composition of the population born on P0, after 10-11 days in culture, closely matched the composition of the population born on P0 in vivo (Fig. 6A,B). In contrast, the composition of the population born on P0 in the presence of CNTF dramatically differed from that of the control (Fig. 6C). The percentage of cells born on P0 that expressed opsin at 10-11 d.i.v. was significantly reduced, whereas the percentage of cells expressing bipolar markers was greatly increased. A slight increase in the percentage of cells expressing either the amacrine marker, VC1.1, or the Müller glial marker, GFAP, was also noted.

**Developmental plasticity of rod progenitors extends into the postmitotic period**

The above results are consistent with a change in the choice of differentiation pathway made by cells going through their terminal S phase in the presence of CNTF. To determine whether this effect could also be exerted on postmitotic cells, CNTF was added to cultures at 2 d.i.v., when the pulse of $[^3]H$thymidine had been given on day 0. This delay between $[^3]H$thymidine labeling and CNTF addition should be sufficient for all cells born on P0 to complete their final M phase (Alexiades and Cepko, 1996). The birthdating experiment described above was repeated to determine the composition of the cell population born on P0 and exposed to CNTF after exit from the cell cycle. The results were nearly identical to those obtained for day 0 addition of CNTF (Fig. 6D). One small difference was that with day 2 addition of CNTF, no increase in the percentage of cells expressing amacrine or Müller glial markers was observed.

With the percentage of amacrine cells and Müller glia essentially unchanged, and the low initial percentage of unidentified cells, the only possible source of excess bipolar-like cells is rod progenitors which do not differentiate in the presence of CNTF. These results suggest that CNTF can halt the progression of rod differentiation in postmitotic cells, in at least a subset of rod progenitor cells, in a manner that preserves the ability of those cells to take an alternate differentiation pathway, namely, the bipolar pathway.

**CNTF effects cannot be explained by changes in proliferation and survival patterns in vitro**

The observed changes in the composition of the cell population born on P0 in the presence of CNTF could be the result of a change in proliferation and/or survival rates of groups of cells, rather than in choice of cellular identity as postulated.

**Fig. 4.** Dose response to CNTF on gels (A) and explants (B). Human recombinant CNTF was used in these experiments. (A) Gels from P0 rat retinae were cultured for 9 days. Estimated EC$_{50} = 0.2$-0.5 ng/ml for both effects (on rods and bipolar cells) (B) Explants from P0 rat retinae were cultured for 10 days. Estimated EC$_{50} = 2$-5 ng/ml for both effects

**Fig. 5.** Time course of CNTF effect on rods and bipolar cells. Explants were made from P0 rat retinae and 100 ng/ml hrCNTF were added to different cultures at gradually later times. All explants were dissociated at 10 d.i.v. and immunocytochemistry was performed.
Given the magnitude of the effect of CNTF on rods and bipolar, a detectable proliferation or survival effect would be needed to account entirely for it. A number of methods were available to investigate whether CNTF effects proliferation or survival of retinal cells in vitro. Most of them rely on periodic measurements of the number of cycling cells and/or the number of dead cells, and only allow detection of overall cell population effects. We sought a method that takes into account the dynamics of the two processes, proliferation and survival, and which allows one to detect both overall population effects and effects on subsets of cells. The method chosen relies upon analysis of the data from the birthdating experiment described above. The analysis was designed to look for differences in the number of times cells had divided in the presence of CNTF and in the survival of cycling and postmitotic cells in the presence of CNTF.

The number of silver grains per cell was counted for 500-600 cells from untreated and CNTF-treated explants. The data were compiled to produce a histogram of the number of grains per cell. As shown in Fig. 7, the cells were grouped into three categories: (i) unlabeled, (ii) lightly labeled and (iii) heavily labeled, corresponding respectively to (i) cells that were postmitotic at P0 or were mitotic but not in S phase during the pulse, (ii) cells that continued to divide after P0, and (iii) cells that divided on P0 for the last time. Most of the possible deviations from the normal proliferation and survival rates have predictable consequences on the structure of the histograms. For example, a significant increase in overall rate of proliferation, or in survival of mitotic cells, would have resulted in an increase in the relative proportions of labeled to unlabeled cells, as well as a shift from heavily labeled to lightly labeled cells. Another example: a change in survival of postmitotic cells would have resulted in a change in the proportion of heavily labeled cells. Only small variations in proliferation and survival, or changes that would be precisely reciprocal, would go undetected by this method.

As shown in Fig. 7, no significant difference was found in either the ratio of labeled to unlabeled cells, or the size of any particular category, indicating that CNTF had no significant effect on proliferation or survival of retinal cells in explants. Examination of the detailed histograms used to produce the summary shown in Fig. 7 where subtle changes would have been detected also indicate that there were no differences (data not shown).

**Commitment to the rod fate may coincide with opsin expression**

The time at which a progenitor cell commits to the rod fate has not yet been defined in any vertebrate retina. The suggestion that cells destined to become rods can be induced to acquire hallmarks of bipolar identity after they become postmitotic raises the question of when rod progenitors become

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**Fig. 6.** Cell type composition of the population born on postnatal day 0. Retinæ were dissected from P0 rats, pulse-labeled with [3H]thymidine, washed, then cultured as explants. hrCNTF (100 ng/ml) was added either at 0 or 2 d.i.v. The explants were dissociated at 10 d.i.v. Immunocytochemistry and autoradiography were performed successively to determine the percentage of cells born on P0 that expressed markers of each of the four postnatally born cell types: rods, bipolar cells, amacrine cells and Müller glia. The cell type composition of the population born on P0 in vivo (A) (unpublished, from Matt LaVail) and in vitro (B) are compared. (C,D) Demonstrate the change in cell type composition produced by CNTF exposure at, respectively, 0 d.i.v. and 2 d.i.v. (E) Table of the cell type composition of the population born on postnatal day 0 (n=2).

**Fig. 7.** Lack of effect of CNTF on survival or proliferation in vitro: the experiment was performed as described in the legend to Fig. 6. The graph shows the percentage of [3H]thymidine-labeled cells in each category of silver grain numbers either in the absence of CNTF, or after exposure to CNTF beginning at 0 or 2 d.i.v. To obtain these data, the number of silver grains per cell was counted for 500-600 consecutive cells, and the cells were split into three categories of grain numbers corresponding to unlabeled, lightly labeled and heavily labeled cells. These categories reflect the number of times a cell has divided since the [3H]thymidine pulse.
committed to their fate. Expression of opsin is considered to be the earliest specific marker of rod differentiation, but it is unclear whether or not rods expressing opsin are already committed to the rod differentiation pathway. In order to determine whether opsin-expressing cells continue to express in the presence of CNTF, the percentage of opsin-expressing cells at 10 d.i.v., when CNTF had been administered at day N, was compared to the number of cells that were already opsin-positive in explants on day N.

As shown in Fig. 8, the number of cells already expressing opsin at the time of CNTF addition was found to be nearly identical to the number of cells which, at 10 d.i.v., expressed opsin despite exposure to CNTF. Cells expressing opsin appear to be resistant to CNTF, while those that did not express opsin at the time of CNTF addition do not appear to be able to express opsin. The absence of a significant time lag, which would have been indicated by a gap between the two curves, is indicative that the postulated resistance, which may correlate with commitment to the rod fate, is acquired just before, or concomitant with, opsin expression.

Abnormal development of rods in explants from mice lacking a functional CNTF receptor

Mice in which the cntf gene has been inactivated by targeted disruption were analyzed and found to have no detectable retinal phenotype (Masu et al., 1993). The cntfr α knockout mouse strain (DeChiara et al., 1995) displays a significantly more severe phenotype than the cntf knockout strain, which suggests redundancy in ligand, but not receptor function. The cell type composition of the retina in animals lacking a functional CNTF receptor, namely, cntfr α−/− and lifr β−/− mice (Ware et al., 1995) was therefore analyzed. In both lines, animals homozygous for the defect normally die in the early postnatal period, but explants can be made from their retinæ prior to death.

Explant were made from P0 animals homozygous for either defect, using retinæ from their wild-type littermates as a control. The explants were analyzed immunocytochemically after 10 d.i.v. to assay for any differences in the cell type composition of the mutant retinæ. For the cntfr α−/− explants, an increase in the percentage of opsin-positive cells was observed compared to explants from cntfr α+/+ animals (Fig. 9). No difference was found in the number of bipolar cells, amacrine cells, or Müller glia, as judged by the respective percentages of 115A10-positive, VC1.1-positive, and GFAP-positive cells (data not shown).

To determine whether the inhibitory effect of CNTF on rods in vitro requires CNTFRα, CNTF was added to explants from cntfr α−/− mice. An explant from one eye of cntfr α−/− animal was treated with 100 ng/ml of CNTF beginning at 0 d.i.v. The percentage of opsin-positive cells at 10 d.i.v. was determined and compared to the number of opsin-positive cells in an untreated explant from the other eye of the same animal. The ratio of the percentages of opsin-positive cells in a treated compared to an untreated explant in sets of explants from 12 animals was 1.0±0.15 (s.e.m.). These data indicate that CNTF requires CNTFRα to lead to the reduction in rod differentiation in vitro.

For lifr β−/− mice, the percentage of cells expressing opsin was found to be 40% higher in the mutants than in the wild-type explants (Fig. 9). The mean for the mutants was at or above the final percentage of rods expected in the adult retina. The difference between mutant and wild-type explants was found to be statistically significant using a Student’s t-test (P value <0.001). The percentages for three other cell types, amacrine cells, bipolar cells and Müller glia, were nearly identical for mutant and wild-type explants (Fig. 9). The total number of cells present after 10 days in culture was comparable for mutants, heterozygotes and wild-type explants (data not shown).

Fig. 8. Apparent resistance to CNTF effect on opsin expression. The broken line represents the percentage of cells expressing opsin on the day indicated on the X axis. This percentage was determined by dissociating explants made on P0 into single cells on the day indicated and performing immunocytochemistry. The solid line represents the percentage of cells expressing opsin after 10 d.i.v. when 100 ng/ml of CNTF were added on the day indicated on the X axis.

Fig. 9. Percentage of cells expressing various markers in 10-day explants made on P0 from cntfr α or lifr β homozygous knockout mouse retinae. Wild-type littermates were used as controls. Each retina was cut into four fragments, and the fragments from both eyes of each animal were mixed. Two explants, each composed of four fragments, were made for each individual animal. Cell type percentages from each animal were averaged for the two explants and counted as one data point. Explants from homozygous mutant and wild-type mice are being compared. For cntfr α−/− mice, n=4; for cntfr α+/+, n=2. For lifr β−/− mice, n=7 and for lifr β+/+, n=3.
DISCUSSION

We show that, when CNTF is added to postnatal rat retinal cultures, the expression of markers of two cell types, rod photoreceptors and bipolar neurons, is dramatically affected. Opsin expression does not occur in rod progenitors which have not yet started expressing this gene at the time of CNTF addition. Concurrently, bipolar cell differentiation is initiated in a large number of these cells. This change in choice of differentiation pathway can be made by rod progenitors even after exit from the cell cycle, thus demonstrating that the developmental plasticity of retinal progenitors can, in some cases, extend into the postmitotic period. For rod progenitors, plasticity seems to end at the onset of opsin expression: apparent resistance to inhibition by CNTF, manifest in the persistent expression of opsin despite CNTF, is in close temporal correlation with the onset of opsin expression. As will be discussed further, this result suggests that commitment to rod differentiation is reached concomitantly with the initiation of opsin expression.

As predicted by the ability of CNTF to mediate significant changes in cell type composition of the retina in vitro, the signaling pathway shared by CNTF and other members of its family may be involved in the development of retinal cells in vivo. In support of this hypothesis, an increase is observed in the percentage of cells differentiating as rods in retinal explants of LIFRβ−/− and CNTFRα−/− mutant animals compared to their wild-type littermates.

Plasticity of rod progenitors

When birthdating studies are conducted in virtually any area of the central nervous system, it is noted that there is an order of generation of cell types (Carter-Dawson and LaVail, 1979; Young, 1985a; Jacobson, 1991). Further, the elaboration of specific antigens and/or morphological characteristics frequently occurs immediately upon exit from the cell cycle (Waid and McLoon, 1995). Finally, lineage analyses have demonstrated that progenitors in most areas are multipotent (Cepko et al., 1997). All of these observations lead to the hypothesis that commitment to a particular cell fate occurs at about the time that a cell is born. In direct support of this hypothesis, ferret cortical cells are able to change their fate when transplanted to a new environment, but only if they go through their terminal S or G2 phase in the new environment (McConnell and Kaznowski, 1991).

In the vertebrate retina, clones of two cells marked by infection with a retrovirus vector can comprise two different cell types, such as a rod and a Müller glial cell, or a single cell type, most frequently rods (Turner and Cepko, 1987; Turner et al. 1990). These observations also have led to the suggestion that retinal cell fates are established at about the time a postmitotic cell is produced. This question is particularly interesting with respect to rod progenitors given the time lag reported between birth of a cell and overt differentiation, as characterized by opsin expression (Morrow and Cepko, unpublished data; Cepko, 1996). The present finding that cells which are fated to be rods express markers of another cell type in the presence of CNTF is a clear demonstration that at least some rod progenitors remain uncommitted until after their final division.

Not all rod progenitors express the bipolar markers examined when exposed to CNTF. There may be heterogeneity in the rod progenitor pool that reflects their plasticity and/or responsiveness to the ultimate effects of CNTF. To help resolve this issue, it would be useful to find markers of rod developmental milestones preceding opsin expression and bipolar cell markers preceding those tested. If such markers were to exist, they might help resolve the state of all cells in the rod progenitor pool. Alternatively, perhaps all prospective rods differentiate as bipolar cells, but we lack markers of all bipolar cell types (see below).

Inhibition of rod differentiation by CNTF was found to decrease with time. When the timing of the apparent resistance and of immunocytochemically detectable opsin expression were compared, they were shown to coincide. It seems reasonable to propose that the apparent resistance to CNTF is a proxy for rod commitment, since it correlates with a moment at which opsin expression, a recognized hallmark of rod differentiation, appears inexorable. However, by labeling opsin-expressing cells in CNTF-treated explants as resistant to CNTF, it is assumed, and not proven, that opsin expression is necessarily followed by terminal rod differentiation, an assumption which will need to be established as fact experimentally. It is not excluded that inhibition of opsin expression might occur in the opsin-positive cells at the transcriptional or translational level, and that the cells merely appear to resist the effects of CNTF as opsin is very stable and perhaps still detectable immunocytochemically. The ability of CNTF (and LIF) to maintain embryonic stem cells in an undifferentiated state (Conover et al., 1993) makes it tempting to postulate instead a common mechanism acting further upstream of specific differentiation hallmarks such as opsin. Further, the correlation between the appearance of bipolar markers and the decrease in opsin expression supports the notion that opsin expression does indicate a commitment, rather than simply an effect on differentiation.

Developmental significance of bipolar marker expression induced by CNTF

As postulated above, the initiation of bipolar differentiation in cells that do not fully differentiate into rods indicates that CNTF can influence the choice of differentiation pathway in postmitotic retinal cells. Alternatively, cells may express random markers of different cell types without being committed to any particular fate. CNTF-treated cells express at least three distinct markers of bipolar identity, which argues against non-specific stimulation of gene expression by CNTF. Also, increases in the proportions of cells expressing any one of several markers of amacrines or Müller glia were small, similarly indicating that markers of bipolar identity were preferentially turned on as a result of CNTF.

There is evidence to support the hypothesis that bipolar differentiation occurs via a default pathway. Reduction of rod differentiation is accompanied by an increase in the number of cells expressing the bipolar marker, 115A10, when retinal cells are cultured at low density (Altschuler and Cepko, 1992). In addition, when a particular fraction from crudely fractionated retinal extract is added to high density collagen gel cultures, the numbers of rods and bipolar cells are similarly inversely affected (Altschuler et al., 1993; unpublished observations). Although both results might support the existence of a default bipolar pathway, alternative explanations, such as dilution of a
bipolar inhibitor in low density gels, or presence of a bipolar inducer in the extract fraction, are not excluded.

The cells that express bipolar markers after treatment with CNTF may be incompletely formed rods which retain some rod features. The unusual pattern of PKCα labeling resembles the labeling of a cone photoreceptor outer segment structure by anti-PKCα (Okhi et al., 1994; see also Fig. 2). As rods also have outer segments, they may make a structure similar to the one decorated by PKCα in cones. In contrast to some cones, rods do not express PKCα, the isoform of PKC recognized by the antibody used in these studies. However, in developmentally arrested rod progenitors induced to express PKCα by CNTF, the postulated outer segment structure, if maintained intact, might be decorated by PKCα. The result would be the observed bar pattern of PKC staining after CNTF treatment. These data might also indicate that the photoreceptor structure decorated by PKCα is very stable.

A large number of cells born on P0 in the presence of CNTF remain unidentified. Two of the three bipolar markers used, mGluR6 and PKCα, are specific for ON bipolar cells, which includes all rod bipolar cells, and a subset of cone bipolar cells (Euler and Wässle, 1995; Nakajima et al., 1993). 115A10 marks a large subset of bipolar cells, and there are indications that its specificity may cover all bipolar subtypes (Onoda and Fujita, 1987; Euler and Wässle, 1995). Nonetheless, it remains possible that some of these unidentified cells differentiate into OFF bipolar cells, for which there is no known specific marker.

In contrast to what was observed in rat retinal cultures, an increase in the number of cells expressing bipolar markers following CNTF treatment has not been observed to date in mouse cultures. Differences between rat and mouse retinas exist: for example, the spatial distribution of the various cone types is dissimilar in mouse and rat (Szelen et al., 1992). Differences in the timing of developmental events, or in the quantity of a bipolar inducer released in vitro could account for the lack of detection of an effect on murine bipolar cells. It is also possible that the molecular mechanism which links CNTF to stimulation of the bipolar phenotype in rat in vitro is independent of the rod inhibition mechanism, and altogether absent in mouse. Alternatively, it may be that CNTF is not the true effector, and in mouse can only substitute for one of the two functions of the cognate factor.

Effects of CNTF on other cell types

The small increase in the number of GFAP-positive cells born on P0 in the presence of CNTF is either due to increased survival or proliferation of a small number of retinal astrocytes, which also express GFAP (Björklund et al., 1985; Schnitzer, 1988), or to acceleration of Müller cell differentiation by CNTF. To distinguish between those two interpretations, the effect of CNTF on the expression of other Müller markers is currently under investigation, and the results obtained should allow us to confirm the identity of the GFAP-expressing cells.

A small increase in the number of VC1.1 positive amacrine cells born on P0 in the presence of CNTF was noted. In contrast, no increase was observed when CNTF was added two days after the [3H]thymidine pulse. It might be that a progenitor cell’s competence to make amacrine cells only overlaps with its competence to make rods up to about P0. Addition of CNTF to explants from embryonic animals, at the time of peak amacrine cell genesis, is being tested to see if it would result in a larger increase in the number of cells expressing amacrine markers.

No increase in the percentage of positive cells was detected using another antibody, anti-CRABP (Milam, 1990), which marks a subset of amacrine cells, raising the possibility that generation of the subset of amacrine cells marked by CRABP ends before the entire amacrine population has been produced. In agreement with this proposition, none of the cells born on P0 in the birthdating experiment were CRABP-positive, and the majority of CRABP-positive cells had no silver grains in them, suggesting they were born before P0 (data not shown). The possibility that amacrine cells fall into more than one developmental category merits further investigation.

Possible in vivo role of the signaling pathway shared by CNTF and other cytokines

When CNTF is injected into mature retinas in vivo, it can protect rods from light-induced degeneration (Unoki and LaVail, 1994). Recently, Fuhrmann et al. (1995) reported that CNTF addition results in an increase in the number of rods in chick retinal cultures. The effect was transient and the increase did not lead to a number of rods in excess of the percentage found in vivo at the equivalent age. These data did not allow one to distinguish between a developmental effect and an effect on rod survival. Using a similar monolayer culture system, the same group also reported observing an inhibitory effect of CNTF on rat rod photoreceptors (Kirsch et al., 1996). It is possible that CNTF leads to increased survival in both chick and rodent rods, but in developing cells, it leads to a block in development. Alternatively, chick and rodent rods differ in their response to CNTF during development.

All members of the CNTF family of cytokines have pleiotropic effects, and have been shown to substitute for each other in many, but not all, in vitro assays (reviewed by Ip and Yancopoulos, 1996). Their ability to substitute for each other can be predicted from the distribution of the various receptor subunits in the cells being assayed. CNTFRα has been shown to be expressed in the inner nuclear layer and ganglion cell layer of the retina, and possibly weakly in a subset of progenitors at P0 (Kirsch et al., 1995; Yang and Cepko, unpublished), while LIFRβ and gp130 have been found on Northern blots to be expressed in the retina (Ip and Yancopoulos, 1993). This leaves open the question of whether the action of CNTF is direct or indirect. Further, it is not clear where the various cytokines of the CNTF family are expressed. In situ hybridization using a probe for CNTF did not give a signal, but RT-PCR of P0 retinal RNA did (Yang et al., unpublished). Given the potency of CNTF, and the fact that an in situ signal was not detected elsewhere in the brain by our group or others, we presume that the factor is present in very low amount. We are currently investigating the expression of other family members.

Given the numerous activities of CNTF in vitro, it was surprising to learn that elimination of its expression via targeted disruption of the gene in mice did not result in any developmental defects (Masu et al., 1993). A null mutation has also been described for the human cntf gene (Takahashi et al., 1994), affecting 2.3% of the Japanese population. Homozygosity for the mutation in these Japanese individuals has not been correlated with any neurological disease, confirming that in humans as well, CNTF function is either unessential for normal neural development, or redundant. In contrast with the
results from knock-out of the gene encoding the factor, elimination of CNTFRα expression in mice was found to be perinatally lethal, and homozygous mutant animals display severe motor neuron deficits at birth (DeChiara et al., 1995). This suggests that one or more ligands requiring CNTFRα for signaling must exist, and have yet to be isolated.

The EC50 in explants for the CNTF effect on rods and bipolar cells (2-5 ng/ml) was slightly higher than that observed for other assays involving CNTF, as could be caused by CNTF signaling through an unknown receptor. However, when the effects of CNTF on rod development were assayed on mouse explants of CNTFRα−/− animals, the effect was not seen, indicating that most of the signaling goes through the known receptor (data not shown). The low level of inhibition observed in some animals may be the consequence of low affinity signaling by CNTF via the gp130-LIFRβ receptor combination. To test whether CNTF is capable of signaling through an incomplete receptor consisting of gp130 + LIFRβ, we are now attempting to breed cntfr α/lifr β− double knockout mice.

The gp130-linked cytokine receptor system and the intracellular JAK/STAT signaling pathway shared by the CNTF family of cytokines possibly play a role in the development of rods in vivo, as suggested by the 40% increase in the number of cells expressing opsin at 10 days in vitro, in the absence of a functional receptor. Since the respective percentages of the other postnatally born cell types remains relatively unchanged, the increase in the number of opsin-positive cells may represent a reduction of the long lag normally observed for the onset of opsin expression. Acceleration of opsin expression is a phenotype which has been observed in other mutant mice, notably the mice heterozygous for targeted disruption of the hairy and Enhancer of Split homolog-1 (hes-1) gene (Tomita et al., 1996). The physiological consequences of such an acceleration have not yet been characterized.

It remains possible that the greater percentage of rods produced in explants from mice lacking a functional CNTF receptor, as compared to their wild-type littermates, is attributable to a tissue culture phenomenon. We have observed that at 10-11 d.i.v., wild-type mouse explants, and to a lesser extent wild-type rat explants, make fewer opsin-positive cells than retinas of corresponding age in vivo (data not shown). This might be due to an excess of free CNTF in cultured retinae as a result of explantation and/or culture conditions. For example, ganglion cells, which normally die within a few days of explant or gel culturing, may be releasing CNTF or a homologous factor. Müller glia, whose numbers are often increased in the presence of specific inducers, these postulated factors appear to be in excess in rat explants or low density collagen gel cultures, but appear to be limiting in mice.

An alternative and perhaps simpler model in which CNTF effects the choice made by a bipotential cell poised to make either a rod or a bipolar may account for some of our observations. However, such a model would be in apparent disagreement with the absence of an effect on bipolar differentiation in mice, either in wild-type or mutant mice. Furthermore, there is an increase in the number of Müller glia and amacrine cells after CNTF treatment, which would complicate the simplest version of this model. We favor the concept of sequential states of competence as it is well-supported by studies of retinal ganglion cell (Austin et al., 1995) and amacrine cell development (Alexiades and Cepko, unpublished data).

Finally, there are reasons why the rodent retina might use inhibitors of commitment or differentiation that affect the rod pathway. As rods significantly outnumber all other retinal cell types, strong rod inducers might exist, which need to be controlled to prevent depletion of the pool of progenitors available for generating later-born cell types. Inhibition also may occur to prevent a conflict between rod differentiation and mitotic activity in the retina. Opsin synthesis, which is coincident with the initial phase of outer segment growth, enters its exponential phase during the first postnatal week (Watanabe and Raff, 1990; Morrow and Cepko, unpublished data), which is about the time that mitotic activity begins to dramatically decrease (Alexiades and Cepko, 1996; Young 1985a,b). The reason to restrain the growth of the outer segments until mitotic activity is over is that the M phase of mitotic cells and the outgrowth of outer segments occur at the same surface and might be incompatible. The retina may have evolved to regulate the timing of commitment, rather than of differentiation (i.e., growth of outer segments), so that differentiation can proceed immediately after commitment. It is interesting to note that, in many species, opsin synthesis and final photoreceptor differentiation do not occur until mitotic activity has ceased, despite the fact that photoreceptor birthdays begin weeks to months earlier (reviewed in Altshuler et al., 1991; Cepko, 1996).

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