Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina

Michael J. Belliveau and Constance L. Cepko*

Program in Neuroscience, Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA

*Author for correspondence (e-mail: cepko@rascal.med.harvard.edu)

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SUMMARY

The seven major classes of cells of the vertebrate neural retina are generated from a pool of multipotent progenitor cells. Recent studies suggest a model of retinal development in which both the progenitor cells and the environment change over time (Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M. and Ezzeddine, D. (1996). Proc. Natl. Acad. Sci. USA 93, 589-595). We have utilized a reaggregate culture system to test this model. A labeled population of progenitors from the embryonic rat retina were cultured with an excess of postnatal retinal cells and then assayed for their cell fate choices. We found that the postnatal environment had at least two signals that affected the embryonic cells' choice of fate; one signal inhibited the production of amacrine cells and a second affected the production of cone cells. No increase in cell types generated postnatally was observed. The source of the inhibitor of the amacrine cell fate appeared to be previously generated amacrine cells, suggesting that amacrine cell number is controlled by feedback inhibition. The progenitor cell lost its ability to be inhibited for production of an amacrine cell as it entered M phase of the cell cycle. We suggest that postmitotic cells influence progenitor cell fate decisions, but that they do so in a manner restricted by the intrinsic biases of progenitor cells.

Key words: Cell Fate, Retina, Rat, Neurogenesis, Amacrine, Cone cell

INTRODUCTION

The vertebrate CNS is composed of a vast array of neuronal cell types. In many regions of the nervous system, there is a particular order of production of cell types and different types of neurons are present in precise ratios with respect to one another. The mechanisms that regulate these two aspects of neural development are poorly understood. The neural retina has served as a model for the study of the genesis of neuronal diversity. The seven major cell types that compose the neural retina arise from a pool of multipotent progenitors (Turner and Cepko, 1987; Holt et al., 1988; Wets and Fraser, 1988; see below). There is a phylogenetically conserved order of birth of different cell types, but with considerable overlap among the different cell types (reviewed in Altschuler et al., 1991). Furthermore, the different cell classes are present in different proportions; in the mouse, rod photoreceptors constitute 70% of the adult retina, while retinal ganglion cells constitute less than 3% of all cells (Young, 1985).

The progeny of individual progenitors can comprise combinations of different cell types and a certain degree of multipotency has been shown to persist throughout retinal development. These observations prompted the suggestion that environmental factors influence the choice of cell fate (Turner et al., 1990; Reh, 1991). Results from recent in vitro studies using rhodopsin immunoreactivity as a marker for photoreceptor development have provided evidence in support of this hypothesis. For example, secreted factors have been shown to stimulate (Altschuler et al., 1993; Kelley et al., 1994; Levine et al., 1997) or inhibit (Lillien, 1995; Kirsch et al., 1996, 1998; Ezzeddine et al., 1997; Neophytou et al., 1997) rod development when added to rodent retinal cultures. The role of these factors in the regulation of the development of rod photoreceptors in vivo has not been elucidated. However, there exists within fractions of retinal extracts or medium conditioned by retinal cells both stimulatory and inhibitory activities when assayed in vitro for effects on rod development (Altschuler et al., 1993).

Although little is known concerning the mechanisms that regulate the production of neurons, induction is believed to be crucial for their proper development, whereby signals generated by one group of cells control the fate of neighboring cells. The spatiotemporal regulation of an induction process may be controlled by either the signaling cells, via limited production or range of action of the ligand, or the neighboring cells, via regulation of their competence to respond to the ligand (for review see Jessell and Melton, 1992). In non-neuronal tissues, there is evidence that there exists a feedback mechanism from differentiated cells to the stem cell population (Morrison et al., 1997). Similar studies of retinal development have been
performed in vivo in which ablation of particular cell classes induce the selective production of neurons of the same class as those ablated (Negishi et al., 1982; Reh and Tully, 1986; Reh, 1987). Waid and McLoon (1998) reported that older retinal cells secrete a factor that inhibits the production of ganglion cells. This was shown by culturing younger retinal cells adjacent to older retinal cells. Ganglion cell production was restored when ganglion cells were depleted from the older cell population. These results support the hypothesis that neuronal cell ratios are regulated in part via feedback from differentiated neurons onto undifferentiated progenitors.

Another unresolved issue is how the sequential production of cell types is regulated. Studies of the developing mammalian cortex revealed that cells generated early in development could be respecified to later fates upon transplantation into the ventricular zone of the older animals (McConnell, 1988). This respecification required that the progenitors underwent mitosis within the new environment (McConnell and Kaznowski, 1991). In contrast, in our recent study in which embryonic retinal progenitors were mixed with an excess of postnatal cells in vitro, there was no increase in the percentage of the cells born on E16 that adopted the rod cell fate, the principal fate choice of postnatal progenitors (Morrow et al., 1998). These data suggested that retinal progenitors are intrinsically limited in their ability to respond to cues that induce production of certain cell types. Here, the reaggregate culture system used in this earlier study was further explored as a means to address the roles of extrinsic and intrinsic cues that control production of retinal neurons.

MATERIALS AND METHODS

Animals
Timed pregnant Sprague-Dawley rats were purchased from Taconic Laboratories.

$[^3]$Hthymidine labeling
For all culture experiments, the mitotic E16 cells were pulse-labeled with $[^3]$Hthymidine. Retinae were dissected free of surrounding tissues and placed into DF10 (45% DME, 45% Ham’s F12 Nutrient Mixture (Gibco), 10% FCS and penicillin/streptomycin (100 U/mL)) containing 5 μCi $[^3]$Hthymidine/ml for 1 hour at 37°C. Retinae were subsequently rinsed by three media changes. Labeled cells were cultured as described below. In some experiments, retinae were dissociated and processed autoradiographically and immunocytochemically as described below. For pulse-chase experiments, 4 μM BrdU was added to the medium.

Antibody labeling of living cells
Five P0 retinae were harvested, dissected and dissociated as described above. Incubation in VC1.1 primary antibody and FITC-conjugated goat anti-mouse secondary were each performed in blocking solution (DMEM with 10% FCS, 3% BSA, and penicillin/streptomycin (100 U/mL)) for 30 minutes on ice. Two washes in blocking solution were performed on ice for 5 minutes following each antibody. Cells were sorted on a Cytofluorograf IIS (OrthoDiagnostic Systems Inc.) at a rate of 1000 cells/second. The brightest and dimmest 12% were collected on ice. Fidelity of sorting was verified by re-analyzing each population. There were no VC1.1-positive cells within the negative population, while 85.3±6.1% of the cells in the bright population were VC1.1-positive. Sorted cells were used in reaggregate cultures as described below. Some cells were plated immediately for immunocytochemistry as described below.

Reaggregate cultures
The reaggregate culture protocol was modified from Watanabe and Raff (1990) and is schematized in Fig. 1A. Dissociated, $[^3]$Hthymidine-labeled E16 cells were counted and pelleted in a microcentrifuge tube containing 20-fold more unlabeled P0 retinal cells by centrifugation for 7 minutes at 1150 g. Total cells per pellet was $5 	imes 10^5$ to $1 	imes 10^6$ cells. Pellets were transferred to nucleopore polycarbonate membranes, 0.2 μm pore size (Costar) and cultured for 3, 5, 7, 10, 12 or 15 days as described for explants (Lillien and Cepko, 1992) in DF10. At the end of the culture period, pellets were dislodged from the membranes, dissociated and processed autoradiographically and immunocytochemically as described below.

Explant cultures
Retinae previously labeled with $[^3]$Hthymidine were transferred to nucleopore polycarbonate membranes, 0.2 μm pore size and cultured for 5 or 15 days as described previously (Lillien and Cepko, 1992) in Sato’s defined medium without serum (Bottenstein and Sato, 1979). At the end of the culture period, explants were dislodged from the membranes, dissociated and processed autoradiographically and immunocytochemically as described below. For pulse-chase experiments, 4 μM BrdU was added to the medium.

Low-density cultures
Embryonic retinae were labeled with $[^3]$Hthymidine and dissociated as described above, then placed in DMEM onto bacterial Petri dishes which had been coated with 1 mg/mL BSA in PBS overnight at 4°C. After 6 hours, the medium was collected and adherent cells removed with washes of Ca$^{2+}$- and Mg$^{2+}$-free HBSS. Collected cells were resuspended in DF10 and placed into reaggregate cultures as described above.

![Fig. 1. Autoradiographic and immunofluorescent analysis of dissociated retinal cells following culture. (A) Outline of protocol for experiments using reaggregates (see Materials and methods). Freshly dissociated E16 retinal cells previously labeled with $[^3]$Hthymidine were reaggregated by centrifugation with 20-fold more unlabeled P0 cells. Reaggregates were transferred to filters floating on medium. Following culture, cells were dissociated, plated onto slides and processed for immunocytochemistry and autoradiography. Silver grains (B), above one of several DAPI-labeled nuclei (C), indicated the presence of $[^3]$Hthymidine in this cell. This cell, which had 24 silver grains, was also VC1.1-positive (D).](image-url)
Dissociation of cultures

Pellets or explants were dissociated by sinking the filters into the well to detach the cells, transferring the pellets to Ca\(^{2+}\) - and Mg\(^{2+}\)-free HBSS, then processing them as described previously (Altshuler and Cepko, 1992). Once dissociated, the cells were plated on coated 8-well glass slides (Cell-Line Associates) coated with 100 µl of 10 µg/ml poly-D-lysine for 20 minutes. The cells were allowed to attach at 37°C for 90 minutes, then fixed in 4% formaldehyde for 15 minutes. The slides were then processed for immunocytochemistry.

Immunocytochemistry

Slides were blocked for 1 hour in 2% donkey serum, 2% goat serum and 0.1% Triton X-100 detergent in PBS. Primary antibody incubation was performed as described previously (Ezzeddine and Cepko, 1997). Anti-rhodopsin staining with Rho4D2 (1:250, Molday, 1989), anti-calbindin (1:300; Sigma) and anti-CRALBP (1:10000; Jackson Immunoresearch). Nuclear staining was performed by adding DAPI to the wash solution at a final concentration of 0.0005%. Slides were then processed for autoradiography.

 Autoradiography

Cells becoming postmitotic the day of \[^{3}H\]thymidine pulse administration were identified as described previously (Young, 1985; Morrow et al., 1998). Slides with \[^{3}H\]thymidine-labeled cells were dipped in NTB2 autoradiography emulsion (Kodak). Slides were exposed for 5 days at 4°C in the dark. Slides were then developed for 5 minutes in D19 developer (Kodak), rinsed in distilled water, followed by 20 minutes in fixer (Kodak). Slides were washed with distilled water for 10 minutes, then mounted in gelvatol. Slides were examined using a Zeiss Axiophot using a 63\(\times\) Plan NEOFLUAR objective.

The relative quantity of \[^{3}H\]thymidine per cell was estimated based on the number of silver grains, visualized by light microscopy, inside and around the cell nucleus. Cells displaying at least \((n/2)\) grains (where \(n\) is the number of silver grains in the most heavily labeled cell) were estimated to be ‘birthdated’, and thus to have been born on the day of label (see Morrow et al., 1998). The identity later assumed by these cells was assessed by scoring individual heavily labeled cells for expression of cell type markers.

Thin sections

E16 retinae were labeled with 100 µCi \[^{3}H\]thymidine/ml in DF10 for 1, 3, 5 or 7 hours. After several rinses, retinæ were fixed in 2% formaldehyde, 2.5% glutaraldehyde and processed for Epon embedding. Sections (0.5 µm) were processed for autoradiography. Following developing, slides were counterstained with 1% toluidine blue.

RESULTS

Cultured progenitor cells from E16 rat retina produce, in addition to rods, two other major cell types, amacrine cells and cone photoreceptors. We thus examined the role of the environment in the production of these cell types by performing reaggregate culture experiments. Reaggregate cultures derived from either embryonic or postnatal rat retinæ were previously shown to generate rod photoreceptors with kinetics similar to those in vivo (Watanabe and Raff, 1990; Morrow et al., 1998). Moreover, several other retinal cell types, such as amacrine cells, horizontal cells, cone photoreceptors, bipolar cells and Müller glial cells have been shown to be produced in these cultures (see below, also Watanabe et al., 1997).

Amacrine cell production by E16 retinal progenitors is reduced in the postnatal environment

To label cycling progenitor cells, E16 or P0 rat retinae were labeled for 1 hour with \[^{3}H\]thymidine. The retinae were then dissociated and cultured as reaggregates for 3 to 15 days, after which the cells were dissociated, plated and processed for immunocytochemistry and autoradiography. Cells were assayed for the presence of silver grains and antibody labeling (Fig. 1). Cells containing >50% of the maximum grain count were regarded as having undergone their terminal mitosis immediately following labeling (Morrow et al., 1998) and were termed ‘birthdated’. This subset was selected for analysis. The production of amacrine cells was assayed using two amacrine cell markers, VC1.1 (Arimatsu et al., 1987) and anti-mGluR2 antibodies (Koulen et al., 1996). For both antibodies, the percentage of marker-positive cells reached its plateau between 5 and 7 days in vitro (DIV; data not shown). Thus 5 DIV was chosen as the harvest point for assay of the amacrine cell fate. As VC1.1 also immunolabels horizontal cells (Arimatsu et al., 1987), we also examined the production of horizontal cells using a horizontal cell-specific antibody, calbindin (see below).

The percentage of birthdated VC1.1-positive cells from E16 cells cultured alone as reaggregates and P0 cells cultured alone as reaggregates after 5 DIV was 36.7±5.5% and 10.3±1.3%, respectively. Similarly, the percentage of birthdated mGluR2-positive cells was 29.5±5.0% and 8.5±0.7% (Fig. 2A). These values correlate well with amacrine cell production in retinal explants and in vivo (Fig. 3; Alexiades and Cepko, 1997; Ezzeddine and Cepko, 1997).

In the presence of a P0 environment, \[^{3}H\]thymidine-labeled E16 progenitors produced fewer amacrine cells. When E16 retinal cells were reaggregated with a 20-fold excess of P0 retinal cells and cultured for 5 days, the percentage of birthdated VC1.1-positive or mGluR2-positive cells was 11.7±2.5% and 11.5±2.1%, respectively (Fig. 2A). This percentage is significantly lower than that of the E16 control reaggregate; there appears to be a threefold decrease in the percentage of cells born from E16 cells that expressed markers of amacrine cells. Moreover, the percentage of marker-positive cells produced by E16 progenitors was similar to that produced by the P0 cells in the control reaggregate (Fig. 2A).

Cone photoreceptor production by E16 retinal progenitors is increased in the postnatal environment

To determine if the reduction in the amacrine cell fate was accompanied by a concomitant increase in photoreceptors, we assayed reaggregate cultures with antibodies to cone opsins (Wang et al., 1992; Chiu and Nathans, 1994), recoverin, a pan-
photoreceptor antigen (Dizhoor, 1991) and rhodopsin (Molday, 1989). Reaggregate cultures were maintained for either 5 or 15 days. After 5 DIV, 27.3±3.5% of the birthdated E16 cells cultured alone were recoverin-positive. (Fig. 2B). A similar percentage of birthdated E16 cells cultured alone were cone opsin-positive (27.0±2.7), while no birthdated cells were rhodopsin-positive (0/500). Hence these recoverin-positive cells were presumed to be cones (Fig. 2B). These results correlate well with previous findings that cells born around E16 that are fated to become rod photoreceptors do not express rhodopsin for an extended period either in vitro or in vivo (Watanabe and Raff, 1990; Morrow et al., 1998) and that recoverin onset immediately precedes or coincides with rhodopsin expression (Kirsch et al., 1998). Among P0 cells cultured alone, 33.0±1.8% of the birthdated cells were recoverin-positive (Fig. 2B). No birthdated cells from P0 retinae were cone opsin-positive (0/300), consistent with the notion that cone production has been completed (Young, 1985; M. M. LaVail, personal communication). In addition, 25.8±2.8% of birthdated P0 cells cultured alone for 5 days were rhodopsin-positive (Fig. 2B). Thus, these recoverin-positive cells were presumed to be rods.
When E16 cells were cultured with 20-fold excess P0 cells for 5 DIV, there was an increase in the percentage of postmitotic progeny adopting the cone cell fate (Fig. 2B), as determined by recoverin (57.3±1.5%) or cone opsins (56.0%±4.6) expression. There was no precocious rhodopsin expression by the embryonic cells resulting from coculturing with postmitotic retinal cells (0/500), consistent with previously published findings (Watanabe and Raff, 1990; Morrow et al., 1998).

After 15 DIV, the percentage of birthdated E16 or P0 cells expressing rhodopsin had reached its plateau (Morrow et al., 1998). The total percentage of birthdated E16 cells cultured alone that became photoreceptors was 52.7±3.8% (Fig. 2C). Birthdated E16 cells at this age were composed of both rod photoreceptors (19.2±2.1%) and cone photoreceptors (25.3±3.2%). The total percentage of birthdated P0 cells cultured alone that became photoreceptors was 71.0±3.8% (Fig. 2C). The birthdated P0 cells appeared to be exclusively rod photoreceptors (Fig. 2C).

The percentage of birthdated E16 cells that became photoreceptors when cultured with a 20-fold excess of P0 cells for 15 DIV was higher than when cultured alone (Fig. 2C). As previously reported, there was no increase in bipolar cell production by birthdated E16 cells at this age (Table 1). Both bipolar cells and Müller glial cells were among the birthdated E16 cells (0.8±1.3%) compared to control (0.4±0.8, statistically significant increase in bipolar cell production by birthdated P0 cells in the P0 control either culture condition (Table 1). Both bipolar cells and Müller glial cells were among the birthdated P0 cells cultured alone that became photoreceptors (21.0±3.8%) compared to control (6.5±1.4, P=0.03).

Postnatal retinal extract contains activity that inhibits production of amacrine cells

To further characterize the mechanism of the apparent cell fate change from amacrine cell to cone photoreceptor that is described above, the following experiment was performed. Retinae from E16 rats were labeled with [3H]thymidine as in the previous experiments. The retinae were not dissociated, however, but cultured as organ explants in a defined medium. To these organ explant cultures, a <10 kDa retinal extract from postnatal retinae was added. Previously, this extract was shown to contain a factor or factors that stimulated rod photoreceptor differentiation in rat retinal collagen gel cultures (Altshuler et al., 1993). Moreover, preliminary experiments had identified the <10 kDa extract as having an activity that influenced E16 progenitors (see below).

Explant cultures of E16 retina were maintained for 5 days, processed for immunocytochemistry and autoradiography, and the percentage of birthdated cells that expressed either VC1.1 or cone opsins was determined. In the absence of postnatal retinal extract, the percentage of VC1.1-positive and cone opsins-positive cells produced was 31.0±3.0% and 22.0±4.1%, respectively (Fig. 3A), correlating well with reaggregate culture (Fig. 2) and in vivo data (M. M. LaVail, personal communication). In the presence of extract, there was a decrease in birthdated VC1.1-positive cells to 9.3±2.5% but recoverin-positive cells were unchanged at 21.0±3.0% (Fig. 3A). As discussed previously, VC1.1 immunolabels both amacrine cells and horizontal cells. Since the decrease in VC1.1-labeled cells following administration of extract is greater than the total percentage of horizontal cells, we conclude that there must be a decrease in amacrine cells following treatment with extract. At present, whether horizontal cells are also decreased is undetermined.

One way to explain the alteration in the percentage of amacrine cells is that there was differential proliferation or cell death in the explants treated with extract. To examine this, histogram profiles of the number of silver grains quantified for a large number of cells from E16 explants cultured for 5 days in either the presence or absence of <10 kDa postnatal retinal extract were examined (Fig. 3B,C). If there was a significant alteration in either proliferation or death, this would be revealed in the comparison of silver grain histograms from extract-treated and untreated explants (Ezzeddine et al., 1997). For example, a significant decrease in the survival or proliferation of mitotic cells due to addition of the extract would have resulted in a larger fraction of heavily labeled cells. Similarly, a decrease in the proportion of heavily labeled cells would have resulted from a decrease in the survival of postmitotic cells. We saw, however, no evidence that there was a decrease.

### Table 1. Quantification of antigen expression by heavily labeled cells after 15 DIV

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Antibody</th>
<th>E16</th>
<th>P0</th>
<th>E16:P0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aamacrine</td>
<td>mGluR2</td>
<td>25.7±2.3</td>
<td>8.0±1.7</td>
<td>9.8±1.6</td>
</tr>
<tr>
<td>Horizontal</td>
<td>Calbindin</td>
<td>6.5±1.4</td>
<td>0.0*</td>
<td>3.4±0.9</td>
</tr>
<tr>
<td>Cone</td>
<td>Cone Opsins</td>
<td>25.3±3.8</td>
<td>0.0*</td>
<td>53.3±2.5</td>
</tr>
<tr>
<td>Rod</td>
<td>Rhodopsin</td>
<td>20.0±1.7</td>
<td>68.7±2.2</td>
<td>20.3±3.1</td>
</tr>
<tr>
<td>Bipolar</td>
<td>115A10</td>
<td>0.4±0.8</td>
<td>8.0±1.4</td>
<td>0.8±1.3</td>
</tr>
<tr>
<td>Müller</td>
<td>CRALBP</td>
<td>0.0*</td>
<td>2.5±0.7</td>
<td>0.0*</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td>22.1</td>
<td>12.8</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Reaggregate cultures of E16, P0 or E16:P0 (1:20) were dissociated after 15 DIV. Following processing for immunocytochemistry and autoradiography, birthdated cells were scored for their labeling by the indicated antibodies. Values represent the mean ± SD of 3-5 trials. For each trial, ≥100 heavily-labeled cells were assayed.

*0 out of 300 heavily labeled cells were marker-positive.
either differential proliferation or death due to administration of the postnatal retinal extract.

Postnatal amacrine cells inhibit the production of amacrine cells from embryonic progenitors in vitro

A possible source for the factor or factors that inhibit the production of amacrine cells is the differentiated amacrine cells themselves. The influence of amacrine cells on the cell fate choices of embryonic progenitors was examined by performing an experiment that utilizes the fact that one of the markers for amacrine cells, VC1.1, is an extracellular epitope. Dissociated, living, P0 retinal cells were processed for immunocytochemistry. (E) The VC1.1-enriched population contained 5-fold more mGluR2-positive cells and 5-fold fewer recoverin-positive cells, while the VC1.1-depleted population contained 7-fold fewer mGluR2-positive cells.

dimmest fraction were found to contain 85.3±6.1% and 0.0±0.0% (0/1500) VC1.1-positive cells (Fig. 4E). This confirmed that the labeling and sorting could provide both a VC1.1-depleted and a VC1.1-enriched population.

To verify that the VC1.1 labeling of living cells had maintained its fidelity, immunocytochemistry on the sorted populations was performed. Another antibody for amacrine cells, anti-mGluR2, labeled 8.2±2.5% of the unsorted P0 cells (Fig. 4E). In the VC1.1-enriched population, 75.7±4.9% of the cells were anti-mGluR2-positive, while in the VC1.1-depleted population, this value was 1.1±0.1% (Fig. 4E). This confirmed that a population of cells highly enriched for amacrine cells as well as a population almost completely depleted of this cell type could be produced. To further examine the purity of the two populations, the presence of recoverin-positive cells, which constituted 7.9±2.1% of the total P0 population, was analyzed. In the VC1.1-enriched fraction, recoverin-positive cells were 2.0±0.4% of all cells, while the depleted fraction contained 9.1±1.7% recoverin-positive cells (Fig. 4E). Thus we concluded that, while the VC1.1-enriched fraction was composed primarily of amacrine cells, there existed within this population a small percentage of other cell types.

Reaggregates of E16 cells cultured with a 20-fold excess of either VC1.1-enriched or VC1.1-depleted P0 cells for 5 days were assayed for their cell fate choice (Fig. 5). In the VC1.1-enriched environment, there was a reduction in birthdated E16 cells expressing VC1.1 (10.7±3.8%). Unlike previous reaggregates, but similar to retinal explants treated with postnatal retinal extract, there was no increase in recoverin-positive cells (29.3±2.1%). In the VC1.1-depleted environment, there was an increase in birthdated E16 cells expressing VC1.1 (55.7±3.2%), and a reduction in recoverin-positive cells (8.3±2.1%), compared to control (Fig. 5).
Temporally restricted sensitive period exhibited by E16 progenitors

To determine at what point during the 5 day culture period the embryonic cells were being influenced by the postnatal signal, reaggregates were formed at progressively later times following \[^{3}H\]thymidine labeling. Preliminary experiments suggested that, within 24 hours, the embryonic cells were no longer sensitive to the postnatal extract (see below).

To more precisely delineate the period of plasticity, E16 retinae were labeled with \[^{3}H\]thymidine for one hour, and then rinsed repeatedly to remove residual label. The retinae were left intact for 4, 10 or 24 hours, then dissociated, reaggregated with P0 retinal cells and cultured for 5 days. Reaggregates were then dissociated and processed for immunocytochemistry and autoradiography.

Embryonic progenitors left in explant culture for 24 hours prior to dissociation and heterochronic reaggregation were similar to E16 control reaggregates for both VC1.1 (40.8±13.5% versus 36.7±5.5%) and anti-recoverin labeling (30.7±3.1% versus 27.3±3.5%; Fig. 6A). Likewise, progenitors left in situ for 10 hours prior to dissociation and reaggregation also behaved similarly to E16 control reaggregates for both VC1.1 (32.2±1.8%) and anti-recoverin (38.0±2.7%). If, however, the progenitors were dissociated and reaggregated after 4 hours, a smaller percentage of birthdated cells was VC1.1-positive (21.3±2.1%) and a larger percentage was recoverin-positive (44.5±2.2%; Fig. 6A). These values were also significantly different from those when E16 progenitors were immediately dissociated and reaggregated with P0 cells for both VC1.1 and recoverin expression (Fig. 6A).

Experiments similar to the ones described above were performed on E16 retinal explant cultures. Explants were \[^{3}H\]thymidine labeled as described previously. At 0, 4, 10 or 24 hours following label, <10 kDa postnatal retinal extract was added to the culture. After 5 DIV, the explants were dissociated, processed for immunocytochemistry and autoradiography, and scored for VC1.1 immunoreactivity.

Embryonic progenitors cultured as explants for either 10 or 24 hours prior to addition of the retinal extract behaved similarly to E16 explants cultured without extract (29.3±0.6%...
of S phase when \[^{3}H\]thymidine labeled. Progenitors from E16 BrdU-negative represented a cohort that were in the last hour of specification that was identified as late S/early G2 (McConnell, 2001). In the ferret neocortex, in which early progenitors could be modified by environmental cues, but only during a sensitive period for specification that was identified as late S/early G2 (McConnell and Kaznowski, 1991). We wished to examine more closely how the phase of the cell cycle in which E16 cells lost their ability to be influenced by the postnatal environment. For this analysis, a cohort in a particular portion of a cell cycle phase was identified and followed. The \[^{3}H\]thymidine-labeling protocol used for previous experiments was not suitable for this; all or most of the S phase cells become labeled, and as S phase occupies 8 of the 18 hour cell cycle length (Alexiades and Cepko, 1996), the labeled cohort would be highly unsynchronized. A smaller, synchronized cohort was labeled by immediately following the thymidine pulse with a continuous exposure to BrdU (Belecky-Adams et al., 1996). Following culture, cells that were \[^{3}H\]thymidine-positive and BrdU-negative represented a cohort that were in the last hour of the S phase when \[^{3}H\]thymidine labeled. Progenitors from E16 retinas were labeled as described above, and postnatal extract was added after 0, 2, 4 or 6 hours. After 5 DIV, the cultures were processed for immunocytochemistry and autoradiography.

Embryonic progenitors cultured for either 4 or 6 hours prior to addition of the retinal extract behaved similarly to E16 explants cultured without extract (33.3±2.5% and 38.0±1.0%, respectively, versus 37.5±3.0%; Fig. 6C). In contrast, progenitors cultured for 2 hours prior to addition of the retinal extract behaved similarly to E16 explants cultured with extract added immediately after labeling (13.7±3.5% vs. 10.3±1.3%; Fig. 6C). Thus between 2 and 4 hours after \[^{3}H\]thymidine labeling, the E16 cells lose their ability to respond to the P0 signal. To determine the cell cycle location of the cells during the interval when cells ceased to be sensitive, E16 retinal explants were labeled with \[^{3}H\]thymidine for 1 hour. The explants were then cultured for 0, 2, 4 or 6 hours, in the presence of \[^{3}H\]thymidine. At the indicated time, explants were washed, fixed and processed for thin sectioning and autoradiography. M phase cells, from early prophase to telophase, could be identified by the appearance of their chromatin appearance as well as by their position. These cells were scored for silver grains (Fig. 6D). Using this method, the entry into M phase could be determined. There was a large increase in the percent of labeled M phase cells between 2 hours (6.25%) and 4 hours (63.87%). Hence, since the embryonic cells labeled with \[^{3}H\]thymidine during the end of their last S phase lost their ability to respond to the postnatal activity after 4 hours, we conclude that this loss occurred prior to or concurrently with the entry to the terminal M phase of the E16 progenitor cell (Fig. 6C).

**Evidence for feedback loop in embryonic retina**

The previous data indicating a feedback inhibition on the production of amacrine cells by amacrine cells was performed by culturing E16 cells with amacrine cells isolated from the P0 population. The observation that more amacrine cells were produced when E16 cells were cultured with a pool of P0 cells depleted of amacrine cells than when cultured on their own suggested that the embryonic environment might provide some feedback inhibition to limit the production of amacrine cells. To determine if embryonic cells were influencing the cell fate choice of E16 progenitor cells in situ, the following experiment was performed. E16 retinae were labeled with \[^{3}H\]thymidine for one hour and dissociated. To minimize cell-cell communication, the dissociated cells were cultured at a density of 1000 cells/mm² for 6 hours in DMEM without serum or hormones (Fig. 7A; Bohner et al., 1997). To reduce adhesivity, bacterial plates preblocked with high-grade bovine serum albumin were used. Following the culture, the cells were reaggregated and cultured for 5 DIV and then assayed for their cell fate choice. There was a larger percentage of birthdated VC1.1-positive cells (50.2±4.2%) and a smaller percentage of recoverin-positive cells (11.4±1.4%) following 6 hours at low density compared to control cultures (Fig. 7B). This alteration in fate choice was not due to selective death or proliferation (Fig. 7C). These data suggest that, during retinal development, cells that may have been generated previously influence the cell fate decisions of progenitor cells.

**DISCUSSION**

**Extrinsic cues alter production of amacrine and cone cells by embryonic progenitor cells**

Classical studies have revealed that multiple cell types are generated at any one time during retinal development and the different numbers of each cell type are produced. How might these processes be regulated? A model in which there are multipotential, yet distinct, progenitors coexisting in the retinal neurepithelium at each time in development has been proposed (Austin et al., 1995; Cepko et al., 1996; Alexiades and Cepko, 1997; and see below). These progenitors have been hypothesized to interact with environmental cues to direct the fate of their progeny. The evidence for such extrinsic cues comes primarily from experiments where rod photoreceptor or ganglion cell development has been examined (Altschuler et al., 1993; Kelley et al., 1994; Austin et al. 1995; Lilien, 1995; Kirsch et al., 1996, 1998; Ezzeddine et al., 1997; Levine et al., 1997; Neophytou et al., 1997; Waid and McLoon, 1998). Here we present evidence that both amacrine and cone cell fate can be regulated by extrinsic cues. There was also a small but significant decrease in the percent of cells born from E16 progenitor cells that became horizontal cells when cultured with P0 cells than when cultured alone.

The fates of postmitotic cells born from E16 progenitors could be altered by culturing them in the presence of 20-fold more P0 cells; fewer amacrine and horizontal cells and more
cone photoreceptors were produced compared with control. This could result from the loss of a signal in the embryonic environment that stimulates amacrine development, and/or the presence of a signal in the postnatal environment that inhibits amacrine development. Support for the latter possibility comes from the observation that postnatal retinal extract applied to embryonic retinal explants reduced the number of amacrine cells produced. In addition, culture of embryonic cells at low density failed to reveal a requirement for an embryonically produced stimulator of amacrine development. The identity of the postnatal activity is currently undetermined, but it is <10 kDa. There are two other reports of a <10 kDa factor that alters the percentage of rods (Altshuler et al., 1993) or ganglion cells in retinal cultures (Waid and McLoon, 1998). It is intriguing to speculate that the same or similar activity may be utilized at several points during retinal development and that the differential responses are due to intrinsic differences in the progenitors. Alternatively, each of the three activities could be due to unique small molecules, such as neurotransmitters or neuropeptides.

Embryonic progenitors cultured with 20-fold more postnatal cells produced fewer amacrine cells and more cone photoreceptors. However, when embryonic progenitors were exposed to the <10 kDa postnatal retinal extract, there was no increase in cone photoreceptors, despite a decrease in amacrine cells similar to that observed in the reaggregate system. This observation suggests that a second factor is required for the stimulation of cone development, and that the second factor is not in the <10 kDa extract. This second factor may be present in the >10 kDa fraction (Altshuler et al., 1993). A more thorough characterization of the biochemical nature of the signal(s) is currently in progress.

Intrinsic properties of progenitor cells influence choice of cell fate

The model discussed above in which there are distinct progenitor cells during retinal development is supported by previous work on amacrine and horizontal cell genesis from our laboratory. Alexiades and Cepko (1997) reported that there are at least two types of progenitor cells present during retinal development and that they can be distinguished by the expression of two markers, the VC1.1 epitope and syntaxin. These two markers are normally expressed by mature amacrine and horizontal cells. However, they can also be co-expressed by retinal progenitor cells that are in S phase. The expression of these markers indicates a bias to produce amacrine and horizontal cells. However, VC1.1-positive progenitor cells were shown to be plastic in that they could produce mitotic daughter cells that did not express the VC1.1 epitope or syntaxin, and other retinal cell types later in development. These data indicated that progenitor cells vary in both their gene expression profile and their competence to make different retinal cell types. The current work shows that the pool of progenitor cells present at E16 and P0 is complex, comprising what appears to be distinct types of cells competent to make different cell types in response to extrinsic cues.

During the neonatal period of retinal development, rod photoreceptors are by far the most abundantly produced cell type, e.g. constituting 80% of the daughters made at P0 (M.M. LaVail, personal communication; Ezzeddine et al., 1997). One might postulate that P0 cells present a rod-inducing environment and, indeed, it does appear that neonatal cells require signals present in the neonatal environment to develop as rods (Altshuler and Cepko, 1992). It is rather surprising then that postnatal cells do not induce embryonic progenitors to make additional rod cells, nor do they alter the kinetics of rhodopsin expression by E16-born cells (Morrow et al., 1998). In the current study, we found that the P0 environment did induce production of photoreceptor cells. However, the induction was of cone photoreceptors, not rod photoreceptors. This result is all the more surprising as no cone cells are made

![Fig. 7. Evidence for feedback inhibition in embryonic retina. E16 retinas were labeled with [³H]thymidine and dissociated as described previously. The cells were reaggregated immediately, or following 6 hours at a density of 1000 cells/mm² in DMEM. (A) Representative field of E16 cells placed into low-density culture for 6 hours. (B) Following 5 DIV, the reaggregates were dissociated and processed immunocytochemically and autoradiographically. Heavily labeled cells were scored for immunoreactivity to either VC1.1 or anti-recoverin. Values represent the mean ± s.d. of 3 trials. For each trial, >100 heavily labeled cells were assayed. (C) Lack of an effect of low-density culture on survival or proliferation of E16 cells. The number of silver grains per cell was counted for 500 consecutive cells. The data from the histogram were then placed into bins consisting of unlabeled (0 grains), lightly labeled (1-9 grains) and heavily labeled (10-18 grains) cells.](image-url)
in the postnatal period. Furthermore, there was no statistically significant increase in the percent of E16-born cells expressing markers for two other fates made in the postnatal period, Müller glial cells and bipolar neurons. Similarly, there was no increase in the percentage of rod photoreceptors produced from E16 progenitor cells (Morrow et al., 1998). These data suggest that retinal progenitor cells of different ages have different intrinsic biases towards certain cell fates and that these biases are temporally regulated. Intrinsic differences among chick progenitor cells of different ages placed into low-density collagen gel culture have also been observed (Austin et al., 1995). While nearly 70% of E4 progenitors cultured in this manner adopted the ganglion cell fate, only 5% of E7 progenitors did so when cultured similarly. Moreover, when late chick progenitors were transplanted into early developing retina, they did not adopt early fates, but rather remained restricted to later ones (D. Fekete and C. L. C., unpublished results). Similarly, when late rat retinal progenitors were mixed with 20-fold more embryonic retinal cells in vitro, there was no production of early cell types (M. J. B. and C. L. C., unpublished results). The mechanisms that regulate the restriction in competence and the movement from one competence state to another are currently unknown. In other regions of the nervous system, this type of temporal restriction has not been observed. For example, when early ferret cortical progenitors were transplanted into a later developing ferret cortical ventricular zone, these cells could be respecified to later fates (McConnell, 1988). Similarly, when early-migrating avian neural crest progenitors were substituted heterochronically for their late-migrating counterparts, these cells behaved like late-migrating crest cells (Baker et al., 1997). Interestingly, while in these two systems the ‘early-to-late’ transplants provided similar results, the corresponding ‘late-to-early’ transplants did not. Later cortical progenitor cells transplanted into early ferret ventricular zone did not adopt the early fates, but rather adopted laminar fates appropriate for their age (Frantz and McConnell, 1996). In contrast, the late-migrating neural crest population substituted for the early-migrating population behaved like early-migrating crest (Baker et al., 1997). An intriguing addition to these observations comes from the work of Raible and Eisen (1996). In this study, the authors showed that, in zebrafish, late-migrating crest cells did not form neurons, either during normal development or when transplanted into an early environment. These cells could form neurons, however, if the early-migrating cells were ablated, suggesting that the early-migrating cells inhibit the late-migrating cells from adopting the neuronal fate (Raible and Eisen, 1996).

In the interpretation of changes in cellular composition as changes in the choices of cell fate, it is important to consider the effects of differential proliferation or cell death. We present several observations that argue against these possibilities as underlying the changes that were observed. Only cells that underwent their terminal mitosis were included in this analysis. Thus, one can rule out the possibility that cells were scored that divided in response to the postnatal environment. Furthermore, as the mitotic index of P0 cells is lower than that of E16 cells (Alexiades and Cepko, 1996), it may be expected that fewer progenitors would choose to divide in the postnatal environment, thus altering the heavily labeled fraction of cells. However, when this was directly assayed, following treatment of E16 explants with postnatal extract, there was no change in the percentage of cells that were heavily labeled (see below).

The heavily labeled E16-born cells in the reaggregate cultures constituted less than 0.2% of the cells after 5 DIV; we could not arrive at a suitable experiment to specifically assay their survival as they were too small a component of the culture. Nonetheless, as both the control embryonic and the postnatal reaggregates produced the proper ratios of cell types with the proper kinetics, there is no reason to suppose that a subset of embryonic progenitors would die in the postnatal environment. Moreover, embryonic progenitors left for several hours in situ prior to dissociation and reaggregation with postnatal cells behaved similarly to reaggregates of embryonic cells alone. Thus for cell death to explain all of the reaggregate culture results, one would have to invoke a selective cell death, which occurred in only a subset of mitotic cells, an event that we find to be unlikely. Finally, embryonic progenitors in retinal explants treated with postnatal retinal extract also produced a smaller percentage of amacrine cells compared with their untreated counterparts. In this culture method, it was straightforward to assay for differential proliferation or death. The silver grain histogram profiles of these two conditions appeared identical, thus excluding any major alterations in proliferation or survival in these cultures. The sum of these observations leads us to favor the interpretation that changes in cell fate, rather than effects of differential survival or proliferation, were manifest.

Cell cycle dependence of plasticity

One aspect of the cell fate determination process that is of interest is its relationship to the cell cycle. Analysis of clones produced following infection of rodent progenitor cells revealed that two different cell types could arise from a terminal cell division (Turner and Cepko, 1987; Turner et al. 1990). The cell fate decision was thus proposed to occur during or after the terminal cell cycle (Turner and Cepko, 1987). This issue was examined for rod photoreceptors born on P0, where it was found that the cells were still plastic following the terminal cell cycle (Ezzeddine et al. 1997). Here, we examined this issue for amacrine cell genesis and found that the ability of embryonic progenitor cells to respond to extrinsic cues was limited to a discrete window of time. Analysis of a cohort of cells in a particular phase of the cell cycle allowed for determination of a sharp alteration in the ability of E16 progenitor cells to respond to the postnatal retinal extract. Cells receiving extract 2 hours after $[^3]$H]thymidine labeling responded identically to those that received it immediately. In contrast, cells that received extract 4 hours after $[^3]$H]thymidine labeling responded identically to those that never received extract. Examination of the kinetics of the cell cycle revealed that this alteration was most likely occurring before, or upon entry into, the terminal M phase. Similar findings were reported previously for cerebral cortical progenitor cells making laminar fate decisions (McConnell and Kaznowski, 1991). Cortical cells generated early in development were respecified to later fates upon transplantation into the ventricular zone of the older animals (McConnell, 1988), and this respecification required that the progenitors underwent mitosis within the new environment (McConnell and Kaznowski, 1991).
Amacrine cell production is controlled by feedback inhibition

An appealing model for the regulation of the number of each cell type is one that invokes feedback inhibition. If progenitor cells competent to make a particular cell type can sense when the plateau number of that cell type is present, they can then refrain from making more of that cell type. We observed that E16 cells in an explant treated with postnatal extract, or E16 cells cocultured with P0 cells, made many fewer amacrine cells relative to controls. The source of the activity within the postnatal environment appeared to be amacrine cells. When cultured with a 20-fold excess of P0 cells highly enriched in amacrine cells, E16 cells produced fewer amacrine cells. Similarly, when cultured with P0 cells depleted of amacrine cells, E16 cells produced more amacrine cells. This increase in amacrine cells was accompanied by a decrease in the production of cone cells. These data suggest that P0 amacrine cells produce a factor that inhibits the production of amacrine cells from E16 progenitors. Interestingly, there was no increase in the production of cone photoreceptors. Likewise, there was no increase in cone photoreceptors when E16 cells were cultured with a <10 kDa fraction of extract made from postnatal retina. These observations further suggest that a second factor is required for induction of cone cells and this factor is not present in the amacrine cell-enriched fraction.

The evidence cited above concerning feedback inhibition is from heterochronic mixtures in which the amacrine cells were from P0 retina and the progenitor cells from E16 retina. The fact that more amacrine cells were made when E16 cells were cultured with the amacrine cell-depleted pool of P0 cells than when E16 cells were cultured on their own was consistent with the notion that the amacrine cells present in the E16 environment provided some feedback inhibition to limit the production of amacrine cells at E16. To more directly address whether feedback inhibition existed in situ, labeled E16 cells were placed into low-density culture for 6 hours, then reaggregated without P0 cells and cultured. Low-density culturing minimized cell-cell contact and diluted secreted molecules. If there existed within E16 retina an inhibitor of the amacrine cell fate, cells placed in low density during the sensitive period would be uninhibited and, thus, more amacrine cells would be produced. An increase in amacrine cell production was observed following the low-density culture period, consistent with the notion that feedback inhibition operates during embryonic development (Negishi et al., 1982; Reh and Tully, 1986; Reh, 1987; Waid and McLoon, 1998). These low-density cultures also provided evidence that no positive-acting secreted molecules are required for the production of amacrine cells.

A model for the sequential production of retinal neurons

The data presented here can be interpreted to produce the following model. There are at least two extrinsic cues that operate on progenitor cells that are intrinsically different with respect to their abilities to make amacrine cells, horizontal cells, and cone photoreceptors. The extrinsic regulation could occur at several levels (Fig. 8). Factors could act to specifically promote or inhibit the differentiation of a postmitotic daughter into a particular cell type. Alternatively, or additionally, the factors could act to regulate the progression of a progenitor cell through its phases of competence, e.g. move it from the competence to make an amacrine cell into a state where it is competent to make a cone photoreceptor. Since it was previously shown that there appeared to be heterogeneity in the progenitor cell population, and that one population displayed a bias to produce amacrine and horizontal cells, an intriguing possibility is that the regulation of these two fates, as shown here, is through the regulation of the competence state of the progenitor cells (Fig. 8). By moving cells out of a particular phase of competence, there would be a decrease in the production of cells appropriate for that phase.

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have equivalent developmental potential in vivo. Development 124, 3077-3087.


