Retinoic acid promotes differentiation of photoreceptors in vitro

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SUMMARY

The results of several recent studies have demonstrated that cell commitment and differentiation in the developing vertebrate retina are influenced by cell-cell interactions within the microenvironment. Retinoic acid has been shown to influence cell fates during development of the nervous system, and retinoic acid has been detected in the embryonic retina. To determine whether retinoic acid mediates the differentiation of specific neuronal phenotypes during retinal histogenesis, we treated dissociated cell cultures of embryonic and neonatal rat retina with varying concentrations of all-*trans* or 9-*cis* retinoic acid and analyzed the effects on cell fate using neuron and photoreceptor-specific antibodies. Addition of exogenous retinoic acid caused a dose-dependent, specific increase in the number of cells that developed as photoreceptors in culture throughout the period of retinal neurogenesis. In the same cultures, retinoic acid also caused a dose-dependent decrease in the number of cells that developed as amacrine cells. Also, results of double-labeled immunohistochemical studies using bromodeoxyuridine demonstrated that the primary effect of retinoic acid was to influence progenitor cells to develop as newly generated rod photoreceptors. Since retinoic acid and at least one of the retinoic acid receptors (RARα) have been localized to the developing neural retina, these results suggest that retinoic acid may play a role in the normal development of photoreceptor cells in vivo.

Key words: rod photoreceptors, neural retina, neurogenesis, retinoids, rat development

INTRODUCTION

During the development of the vertebrate central nervous system, the proliferating cells of the germinal neuroepithelium generate a variety of different types of neurons and glia. A number of studies in both the central and peripheral nervous systems have shown that interactions between the progenitor cells and their developing microenvironment play an essential role in the determination of cell phenotype (see McConnell, 1992 and Bronner-Fraser, 1992 for reviews). In an effort to characterize further the nature of these intercellular interactions, we have studied the control of cell fate decisions during histogenesis of the rat neural retina in tissue culture. The rat retina is particularly attractive as a model for the study of the factors that control cell commitment in the nervous system, because the different neuronal cell types are well characterized and are generated in a well-defined sequence during retinal histogenesis (see Reh, 1992a for review). In most mammals, including rats, the generation of the different classes of retinal cells can be roughly divided into early and late phases (Robinson, 1991; LaVail et al., 1991). In the early phase, ganglion cells, horizontal cells, cone photoreceptor cells and most amacrine cells are born, while most rod photoreceptor cells, bipolar cells and Muller glia have birthdates in the later phase of histogenesis.

Recently, several laboratories have taken advantage of this defined sequence in retinal histogenesis to devise heterochronic co-culture experiments to partly characterize the nature of the interactions necessary for the differentiation of retinal cells in rodents (Watanabe and Raff, 1990, 1992; Reh, 1992a,b; Altshuler and Cepko, 1992). Using reaggregate cultures of dissociated retinal cells, Watanabe and Raff (1990) found that rat retinal cells from embryonic day 15 (E15) could be induced to form rod photoreceptor cells (a cell type generated primarily during the postnatal period of histogenesis) at a higher frequency when mixed with postnatal retinal cells. In a subsequent study (Watanabe and Raff, 1992), the same authors demonstrated that the rod promoting effect was not inhibited when E15 and postnatal cells were separated by a millipore filter. Based on these results, the authors concluded that the rod phenotype was promoted by a soluble factor. In a similar series of experiments, early embryonic mouse cells (E12) were shown to differentiate into rod photoreceptor cells with a higher frequency when co-cultured with either postnatal rat retinal cells (Reh, 1992a) or with later staged embryonic retinal cells that were competent to generate rod photoreceptors (Reh, 1992b). Overall, the results of these studies suggest that the differentiation of retinal progenitor cells as rod photoreceptors is influenced by locally diffusible signals in the extracellular environment, that are developmentally regulated during the period of retinal histogenesis.

Although the specific factors that promote differentiation of cells as rod photoreceptors are not known, several classes of molecules have been shown to influence cell determination in other developing systems and might also play a role in the developing retina. For example, small peptide growth factors have been shown to play a role in the developing nervous system (Anchan et al., 1991; Park and Hollenberg, 1989;

Pittack et al., 1991; Hicks and Courtois, 1992). Several laboratories have studied the effects of both epidermal growth factor (EGF) and fibroblast growth factor (FGF) in retinal cultures. Although these growth factors have been shown to promote proliferation of retinal progenitor cells and rod photoreceptor survival, none of the factors that were tested stimulated the specific differentiation of photoreceptor cells from embryonic retinal progenitors (Anchan et al., 1991; Hicks and Courtois, 1992; Lillien and Cepko, 1992).

Among the other classes of molecules that have been shown to influence cell fate in the developing nervous system is the vitamin A derivative retinoic acid. In particular, all-*trans* retinoic acid has been shown to alter cell fate decisions in the developing limb bud, hindbrain and inner ear (Tickle et al., 1982; Thaller and Eichele, 1987; Durston et al., 1989; Represa et al., 1990; Ruiz i Altaba and Jessell, 1991; Kelley et al., 1993). More recently, the 9-*cis* isomer of retinoic acid has been shown to have biological effects that are apparently mediated through the RXR family of nuclear receptors (Mangelsdorf et al., 1992; Thaller et al., 1993; Kraft and Juchau, 1993). In addition, several studies have demonstrated that both all-*trans* and 9-*cis* retinoic acid and at least one of the nuclear retinoic acid receptors (RARα) are present in the developing retina (McCaffery et al., 1992; Ruberte et al., 1991; Kraft and Juchau, 1993). Moreover, recent studies have found that retinoids have effects prior to, and following, the period of retinal histogenesis; retinoic-acid treatment of frog and zebrafish embryos at gastrulation results in subsequent changes in retinal patterning (Manns and Fritzsch, 1991; Hyatt et al., 1992); retinoids, including all-*trans* retinoic acid, can influence the survival of cells dissociated from E8 chick retina, a stage when neurogenesis is largely complete (Stenkamp et al., 1993). Thus, although none of these earlier studies have implicated this class of molecules in cell fate decisions in the developing retina, the retinoids and their receptors are present during the stages of retinogenesis when such decisions are thought to be made. Therefore, we determined the effects of retinoic acid on the process of retinal histogenesis by assaying the numbers of individual types of retinal neurons that were generated in vitro in the presence of various concentrations of all *trans* and 9-*cis* retinoic acid.

METHODS AND MATERIALS

Retinal cell culture

Timed pregnant Sprague-Dawley rats, as determined by presence of a vaginal plug, were obtained from Simonsen Laboratories. On embryonic day 15 (E15) or embryonic day 18 (E18), pregnant females were killed with CO_2 and the embryos were removed into sterile Hanks' buffered saline solution with HEPES buffer at 4°C. The neural retinas from all of the embryos in a single litter were dissected and then dissociated by incubation at 37°C in calcium-/magnesium-free saline with trypsin (0.025%). Total retinal cell number was determined with a hemacytometer and cells were then plated into 24-well plates (well diameter = 19 mm) (Falcon) at a density between 200,000 and 500,000 cells per well. Cells were plated onto coverslips (diameter = 12 mm) coated with polylysine/Matrigel (Collaborative Research). Since each coverslip covered only 64% of the total area of the bottom of each well, approximately 36% of the total number of cells that were plated adhered to the Matrigel-coated floor of each well rather than to the coverslip. The polylysine/Matrigel substrata were

prepared by incubating sterile 12 mm coverslips in a 10 μg/ml polylysine solution for 30 minutes or more, followed by washes in distilled water, and application of a thin film of Matrigel (diluted 1:100). In subsequent experiments, cultures were established from neonatal (P0) retinas. To obtain a higher percentage of single cells, these neonatal retinas were dissociated through three sequential, 10 minute incubations in trypsin. Between each enzyme incubation, the larger pieces of tissue were allowed to settle and the dissociated cells were removed and inactivated with fetal bovine serum; new trypsin was then added to the larger pieces of tissue. Dissociated postnatal cells were then plated into 24-well plates onto coverslips coated with a fibrillar collagen gel at 40,000 cells per well. The fibrillar collagen-coated coverslips were prepared using Vitrogen (Celtrix), according to the Manufacturer's specifications. All cultures were maintained at 37°C and 5% CO_2.

Cultures were maintained for periods of 2 to 8 days in low serum tissue culture medium. The culture medium contained DMEM:F12 (without glutamate or aspartate), 25 μg/ml insulin, 100 μg/ml transferin, 60 μM putrescine, 30 nM selenium, 20 nM progesterone, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.05 M Hepes, and 1% fetal bovine serum (FBS) (Gibco-BRL). One half of the media in each well was changed every 48 hours. All-*trans* retinoic acid was purchased from Sigma Chemical Corp. 9-*cis* retinoic acid was a generous gift from Hoffman-La Roche Pharmaceuticals. Stock solutions of 10 mM all-*trans* or 9-*cis* retinoic acid were made in dimethyl sulfoxide (DMSO) and stored in 5 μl aliquots at −20°C in the dark. Individual aliquots were thawed and diluted to the appropriate concentration in culture medium just prior to use.

Labeling of proliferative cells

To assay directly the effects of retinoic acid on the mitotically active progenitor cells in the retina, proliferating cells were labeled with the thymidine analog bromodeoxyuridine (BrdU) (Sigma). After dissection, whole neural retinas were incubated in culture medium containing 10% FBS and 10^{-4} M BrdU at 37°C for periods between 4 and 24 hours, with gentle agitation. At the end of the incubation, retinas were dissociated and plated as described above. To prevent the possible dilution of the BrdU signal as a result of continued proliferation in culture, 10^{-5} M BrdU was added to the culture medium for the duration of each experiment.

Determination of cell phenotypes

After survival periods of from 2 to 8 days, depending on the experiment, coverslips were fixed in 4% paraformaldehyde overnight and then rinsed in phosphate-buffered saline prior to processing for immunohistochemistry. For most experiments, to determine the effects of different treatments on cell number, the remaining cells in each of the culture wells (i.e., the 36% of the plated cells that adhered to the plastic surface of the well rather than to the coverslip) were dissociated with trypsin and counted with a hemacytometer. To identify specific types of retinal cells in each of the cultures, the coverslips were processed for immunohistochemistry using a previously published protocol (Reh and Kljavin, 1989; Kljavin and Reh, 1991). Primary antibodies used in these experiments were as follows: (1) rod-specific opsin, 4D2 monoclonal antibody from Dr. R. Molday and Dr. D. Hicks, U. British Columbia (Hicks and Barnstable, 1987); (2) recoverin, affinity-purified polyclonal rabbit antisera from Dr. J. Hurley, U. Washington (Dizhoor et al., 1991; Milam et al., 1993); (3) cellular retinoic acid binding protein (CRABP), C1 monoclonal antibody from Dr. J. Saari, U. Washington (Gaur et al., 1990); (4) HPC-1 monoclonal antibody from Dr. C. Barnstable, Yale University (Barnstable et al., 1985); (5) PCP-2 monoclonal antibody from Vandaele et al., U. Colorado (Vandaele et al., 1991); (6) nerve cell adhesion molecule (N-CAM) monoclonal antibody from Drs. Jessell and Dodd, Columbia University (Dodd et al., 1988); (7) nestin, Rat-401 monoclonal antibody from Dr. S. Hockfield (Hockfield and McKay, 1985); (8) neuron-specific enolase monoclonal anitbody

(Dako). Primary antibody labeling was detected using fluoresceinated secondary antibodies. Labeled cells were viewed on a Zeiss standard compound microscope with Epi-Fluorescent illumination, and the number of labeled cells on each coverslip was quantified in most cases by counting all of the labeled cells in either a vertical or horizontal strip across the entire coverslip. In some of the embryonic experiments, where the distribution of the cells was more uniform, the labeled cells in six to ten random fields were counted. All of the postnatal data was collected with the experimenter blind to the treatment condition. A minimum of three different coverslips were counted from each experiment and each experiment was repeated a minimum of three times.

RESULTS

Retinoic acid promotes photoreceptor differentiation in embryonic retinal cultures

Retinal cell cultures

Embryonic retinal cells established from either E15 or E18 embryos attached to the substratum and flattened within 4 to 8 hours of plating. At the initial time of plating, most of the cells were either single cells or members of small groups (fewer than 20 cells). Over the course of the experiment, both single cells and groups of cells passed through several rounds of mitosis to generate a monolayer of cells that covered the bottom of the culture well. After 6 to 8 days in vitro, the overall number of cells in each well increased by an average of 278%. Although it is difficult to resolve the individual cells within these high-density cultures, the majority of the cells in each culture have been characterized using one or more of the following methods: (1) cell-type-specific antibodies that label only a subpopulation of the total cells within each culture. These include antibodies that label rod and cone photoreceptors, amacrine cells, bipolar cells and ganglion cells; (2) redissociation of high-density cultures followed by replating at low density prior to fixation, and subsequent labeling with antibodies that recognize more ubiquitously expressed antigens; (3) parallel low-density cultures established from the same animals and at the same time as the high-density cultures in which many of the cells are either isolated or in small (fewer than 10 cells) clusters, facilitating analysis based on morphology and antigen expression.

Results indicate that after 7 days in vitro, cells in high-density cultures established from E18 retinas can be classified as follows: 99% of the cells are immunoreactive for N-CAM, which is expressed by neurons, glia and neuroepithelial progenitor cells (reviewed in Schachner, 1989; Rutishauser, 1993); 44% of the cells are immunoreactive for nestin, an antigen which is expressed by neuroepithelial progenitor cells throughout much of the central nervous system, including the retina (Lendahl et al., 1990); 38% of the cells are immunoreactive for neuron-specific enolase, an antigen that is expressed at high levels by various classes of large multipolar neurons (eg. ganglion cells and amacrine cells; Wilhelm et al., 1992). These data, along with the results of previously published double label experiments, using [³H]thymidine and antibodies that are specific for particular classes of retinal cells (Reh and Kljavin, 1989; Reh, 1992b; see also the data presented in this study), indicate that after one week in vitro, these cultures consist of both neuroepithelial progenitor cells and different types of retinal neurons that were generated both before and after the

cultures were established. Neuroepithelial progenitor cells, which are defined based on immunoreactivity for nestin and NCAM, on incorporation of [³H]thymidine or BrdU, and on a flattened or bipolar morphology, make up about 40-50% of the cells in these cultures. The remainder of the cells in these cultures can be classified into the different retinal cell classes based on labeling with specific and ubiquitous antibodies and on morphology (Reh and Kljavin, 1989; Anchan et al., 1991; Reh, 1992b). In addition, cells in these cultures frequently show a regular organization in which photoreceptor and bipolar cells develop in radial arrays, while amacrine cells and ganglion cells develop outside the radial portion of each rosette (Fig. 1A,C,E) (Reh, 1992b).

Rod photoreceptor cells were identified by their immuno-reactivity for rhodopsin (rho 4D2 monoclonal) and recoverin (a calcium-binding protein) (Hicks and Barnstable, 1987; Dizhoor et al., 1991). However, since recoverin also labels cone photoreceptor cells and cone bipolar cells, those cells that are immunoreactive for recoverin, but not opsin, may be either of these two cell types (Milam et al., 1993). In the embryonic cultures, three lines of evidence suggest that most of these cells (recoverin⁺, rho4D2⁻) are likely to be cones: (1) in high-density cultures, the recoverin immunoreactive cells have a distinctive morphology, similar to that observed in vivo, with one long terminal-like process and one thick, short, cone-shaped process; (2) bipolar cells are not normally generated until postnatal ages; (3) other bipolar antigens (PKC, PCP-2) do not label cells until postnatal day 6-7, consistent with their postnatal generation. Amacrine cells were identified with the C1 monoclonal antibody to CRABP or with HPC-1, two antigens that are confined to subsets of amacrine cells in the rat retina (Gaur et al., 1990; Barnstable et al., 1985). In postnatal cultures, bipolar cells were identified with the PCP-2 antibody, a monoclonal antibody directed against a protein expressed only in a subpopulation of bipolar cells in rodent retina (Vandaele et al., 1991).

Effects of all-*trans* retinoic acid

Addition of all-*trans* (AT) retinoic acid to the culture medium had a striking effect on the development of photoreceptors. After 8 days in vitro, cultures that were maintained in medium that contained either 100 or 500 nM AT retinoic acid developed a significantly greater number of photoreceptor cells in comparison with controls (Fig. 1). The morphology of these cells was reminiscent of developing photoreceptors in vivo, including a thin axonal-like process and a wider process more similar to the inner segment and developing outer segment. AT retinoic acid-treated cultures developed a greater number of photoreceptors both inside (Fig. 1C,D) and outside of rosettes (Fig. 1A,B). Fig. 2 summarizes the results from three experiments using retinal cells from E18 embryos, as a function of AT retinoic acid concentration. The number of both recoverin immunoreactive cells and rhodopsin immunoreactive cells was significantly greater ($\alpha=0.025$) in cultures treated with AT retinoic acid at concentrations of 100 or 500 nM (approximately 210% and 360% of control values, respectively). The number of rod photoreceptors was also elevated in cultures treated with 10 nM AT retinoic acid as compared with control values, but the difference was not significant. The greatest increase in the number of photoreceptor cells was in culture wells treated with 500 nM AT retinoic

acid, while the number of photoreceptor cells in cultures maintained in medium with 1.0 µM AT retinoic acid was approximately equal to the value for cultures treated with 100 nM AT retinoic acid (Fig. 2). The observed increase in the number of photoreceptor cells in cultures treated with AT retinoic acid was not due to stimulation of cell proliferation since the total number of cells in each culture well did not increase with different concentrations of AT retinoic acid (Fig. 2). In addition, we assayed for the specificity of the effect of AT retinoic acid by determining the number of cells that

developed as amacrine cells at each concentration of retinoic acid (Fig. 2). Results indicate that AT retinoic acid caused a significant decrease in the number of cells that developed as amacrine cells in cultures treated with AT retinoic acid at concentrations of 100 nM or greater (Fig. 2).

Cultures established from E15 embryos responded to AT retinoic acid in a manner similar to that observed for cultures established from E18 embryos. A progressively greater number of cells developed as photoreceptor cells with increasing concentrations of AT retinoic acid (Fig. 3). In cultures
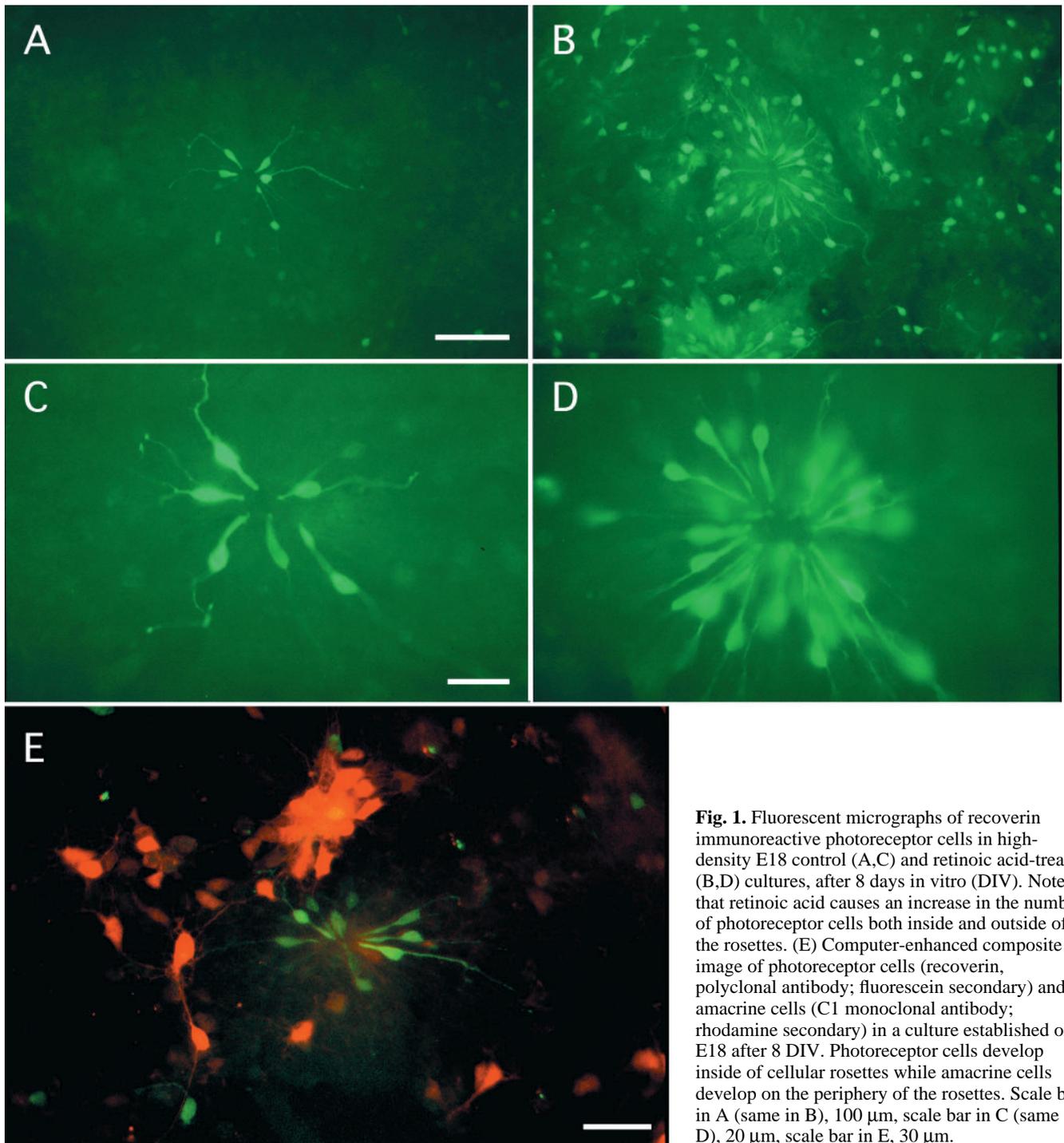
Fig. 1. Fluorescent micrographs of recoverin immunoreactive photoreceptor cells in high-density E18 control (A,C) and retinoic acid-treated (B,D) cultures, after 8 days in vitro (DIV). Note that retinoic acid causes an increase in the number of photoreceptor cells both inside and outside of the rosettes. (E) Computer-enhanced composite image of photoreceptor cells (recoverin, polyclonal antibody; fluorescein secondary) and amacrine cells (C1 monoclonal antibody; rhodamine secondary) in a culture established on E18 after 8 DIV. Photoreceptor cells develop inside of cellular rosettes while amacrine cells develop on the periphery of the rosettes. Scale bar in A (same in B), 100 µm, scale bar in C (same in D), 20 µm, scale bar in E, 30 µm.
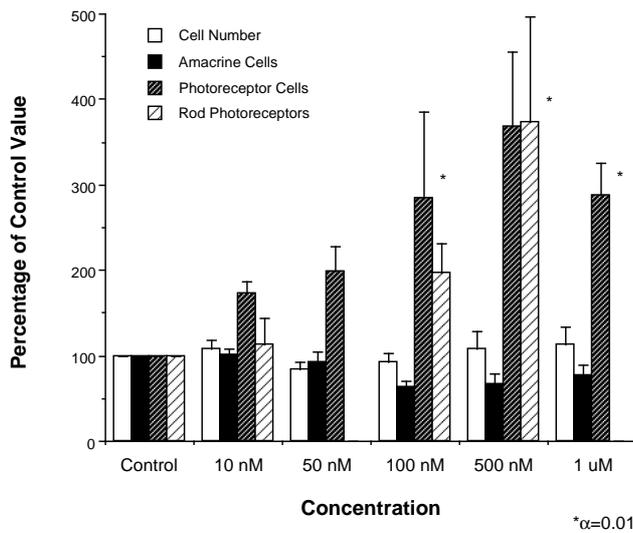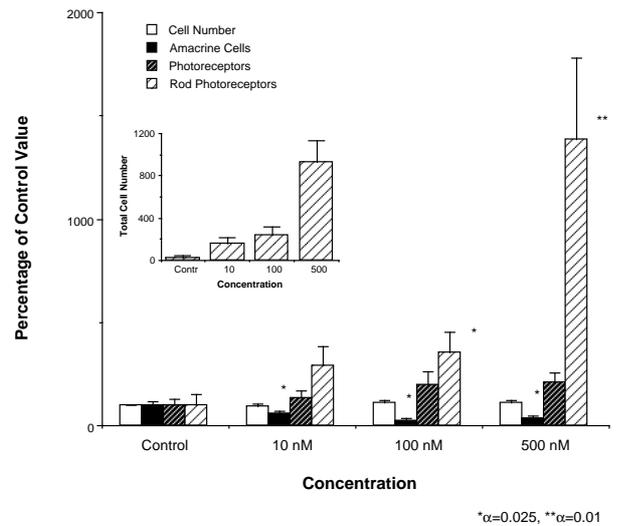
Fig. 2. Response of retinal cultures established on E18 to different concentrations of all-*trans* retinoic acid after 8 days in vitro. Retinoic acid caused a significant dose-dependent increase in the number of cells that developed as photoreceptor cells and in particular, in the number of cells that developed as rod photoreceptors. The total cell number did not change between cultures with different concentrations of retinoic acid but the number of cells that differentiated as amacrine cells decreased significantly in cultures with high concentrations (100 or 500 nM) of retinoic acid ($\alpha=0.025$). Data are from the results of three separate experiments. Error bars are standard error of the mean (s.e.m.).

Fig. 3. Response of retinal cultures established on E15 to different concentrations of all-*trans* retinoic acid after 8 DIV. Similar to the effects of retinoic acid on cultures established on E18, retinoic acid caused a significant increase in the number of cells that developed as photoreceptor cells and rod photoreceptors. The total cell number did not change between retinoic acid concentrations, but the number of cells that developed as amacrine cells decreased significantly at high concentrations. Inset graph shows the average number of rod photoreceptors per 1×10^6 retinal cells under each culture condition. Data are from the results of three separate experiments. Error bars are s.e.m.

treated with concentrations of 500 nM AT retinoic acid, the total number of photoreceptor cells increased to as much as 280% of the control level. The same concentration of AT retinoic acid also caused an increase in the number of cells that developed as rod photoreceptor cells, to as high as 1400% of the control level. The scale of this increase was due, in part, to the low number of cells that differentiated as rod photoreceptor cells in control cultures of E15 retina after 6 days. The average frequency of rod photoreceptor cells in E15 cultures after 6 days in vitro was 1 per 38,000 cells. By contrast, in cultures treated with 500 nM AT retinoic acid, the frequency of rod photoreceptor cells increased to 1 per 1000 cells (Fig. 3 inset). Therefore, AT retinoic acid treatment caused an increase of approximately 15-fold in the number of rod photoreceptors. As described for cultures established on E18, the total number of cells per well did not change between different AT retinoic acid or control treatments in the E15 cultures. In addition, the number of amacrine cells from cultures established on E15 showed a decline in response to AT retinoic acid that was similar to the decline described for cultures established on E18.
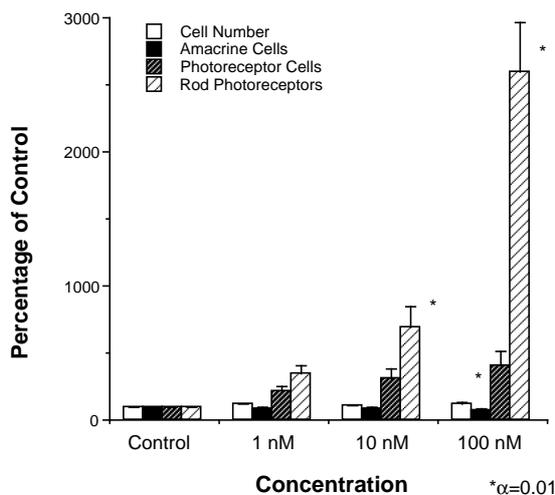
Effects of 9-*cis* retinoic acid

Recent studies have demonstrated that the preferred ligand for the retinoid X receptors (RXR) is the 9-*cis* isomer of retinoic acid (Heyman et al., 1992). Since a percentage of AT retinoic acid can be isomerized to 9-*cis* retinoic acid in vivo, it is possible that the observed effects of AT retinoic acid in these cultures were actually mediated by 9-*cis* retinoic acid.

Therefore, the E18 experiments were repeated using exogenous 9-*cis* rather than all-*trans* retinoic acid. Cultures of E18 retinal cells that were exposed to 9-*cis* retinoic acid for 6 days responded similarly to E18 cultures that had been exposed to AT retinoic acid (Fig. 4). There was a dose-dependent increase in the number of cells that developed as photoreceptor cells. However, the magnitude of the increase in the number of photoreceptors was significantly higher in cultures treated with 9-*cis* retinoic acid. In cultures treated with 100 nM 9-*cis* retinoic acid, the total number of rod photoreceptors increased by as much as 2300% over control values, while the number of cells that developed as photoreceptor cells increased by 450%. By comparison, the maximum effects of AT retinoic acid (at 500 nM) were increases of approximately 350% for either photoreceptor marker. Addition of 1 nM or 10 nM 9-*cis* retinoic acid caused an increase in the number of photoreceptor cells that was comparable to the increase observed with 10 nM or 100 nM AT retinoic acid. Also, the number of amacrine cells showed a dose-dependent decline in response to 9-*cis* retinoic acid, similar to that observed for the AT retinoic acid.

The results of these experiments strongly suggest that the observed effects of AT retinoic acid are mediated through RXR receptors. Recent studies have demonstrated that 9-*cis* retinoic acid and AT retinoic acid bind RAR receptors with equal efficiency while 9-*cis* retinoic acid binds RXR receptors 10 to 100 times more efficiently than AT retinoic acid (Heyman et al., 1992). Since the results of these experiments indicate that an equivalent increase in the number of cells that develop as pho-
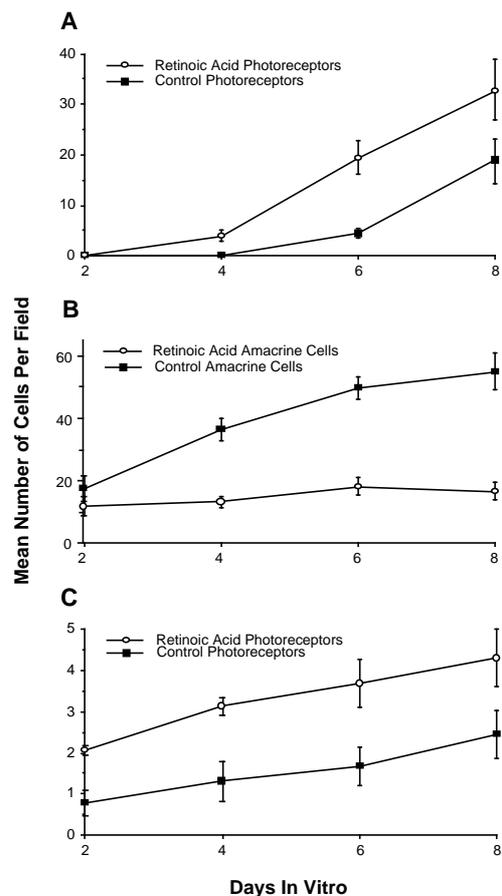
Fig. 4. Response of retinal cultures established on E18 to different concentrations of 9-*cis* retinoic acid after 6 DIV. 9-*cis* retinoic acid caused a signficant dose-dependent increase in the number of cells that developed as photoreceptor cells and in the number of cells that developed as rod photoreceptors. However, the magnitude of the increase in the number of photoreceptors was approximately 10 times greater than the response to an equivalent concentration of all-*trans* retinoic acid. Total cell number did not change between cultures with different concentrations of 9-*cis* retinoic acid and the number of amacrine cells decreased with high concentrations of 9-*cis* retinoic acid. Data are from the results of at least three separate experiments except for the values for amacrine cells which are from the results of a single experiment. Error bars are s.e.m.

Fig. 5. Time course of all-*trans* retinoic acid effects on differentiation of photoreceptors and amacrine cells. (A) Addition of 500 nM retinoic acid results in a significant increase in the number of cells that differentiate as photoreceptors. The time course of photoreceptor differentiation in control cultures is similar to the time course during development of the retina in vivo (see text for details). In retinoic acid-treated cultures, the development of photoreceptors appears to be shifted ahead by approximately 48 hours. (B) Addition of 500 nM retinoic acid inhibits the differentiation of new amacrine cells between 2 and 8 days in vitro. The decrease in the number of cells that differentiate as amacrine cells is sufficient to account for the observed increase in photoreceptors. (C) Time course of the effects of 100 nM retinoic acid on retinal cultures established on P0. The number of cells that develop as rod photoreceptors is increased after 2 DIV and this increase persists at 4, 6 and 8 DIV. Data are from triplicate coverslips from a representative experiment. Error bars are s.e.m.

toreceptors could be induced by addition of either 10 nM 9-*cis* retinoic acid or 500 nM AT retinoic acid, it seems likely that this response is regulated through RXR rather than RAR receptors.

Time course of retinoic acid effects

The time course of the effects of retinoic acid on the differentiation of photoreceptor cells was determined by fixing retinal cells after progressively longer periods of time in culture. E18 retinal cells were dissociated and maintained in culture at high density with 500 nM AT retinoic acid. Individual culture wells were fixed after 2, 4, 6, or 8 days in vitro. The results of these experiments are shown in Fig. 5. In both retinoic acid-treated and control cultures, there were no rhodopsin immunoreactive rods after 2 days of culture; however, by four days in vitro, there was already a significant increase over control wells in the number of rods in the retinoic acid-treated wells (Fig. 5A). This difference increased after 6 and 8 days in vitro, as the number of photoreceptors continued to increase in both control and treated wells.

In the same experiment, the time course of the effects of retinoic acid on development of amacrine cells was also examined (Fig. 5B). In the control wells, the number of amacrine cells steadily increased during the 8-day culture period, while wells treated with retinoic acid contained nearly the same number of amacrine cells throughout the duration of the experiment. This result suggests that retinoic acid inhibits cells from developing as amacrine cells at the same time that it promotes cells to differentiate as photoreceptor cells.

Retinoic acid acts on the progenitor cells to direct cell fate

On embryonic day 18, approximately 70% of the cells in the rat retina are mitotically active progenitors, while the remaining cells are postmitotic cells that are differentiating as photoreceptors and neurons of several different phenotypes (Hinds and Hinds, 1978, 1979). We took advantage of this sequence to determine whether retinoic acid directly influences the proliferating progenitor cells to adopt a photoreceptor cell fate, or alternatively, acts primarily to promote the differentiation of postmitotic photoreceptors, concomitantly increasing their expression of photoreceptor-specific antigens. The mitot-

ically active cells in E18 retinas were labeled with bromod-eoxyuridine (BrdU) for between 6 and 24 hours prior to dissociation and preceding exposure to retinoic acid. After 6 or 8 days in vitro, cultures were fixed and labeled with anti-BrdU and photoreceptor-specific antibodies. Fig. 6 shows an example of a rod photoreceptor cell that is also labeled with BrdU and the number of both single and double-labeled rods in the control and retinoic acid-treated cultures is shown in Fig. 7. Both single- and double-labeled rods were present in control as well as retinoic acid-treated cultures; however, virtually all of the difference between the experimental and control cultures could be accounted for by the change in the number of double-labeled rods. These results indicate that retinoic acid acts directly on the newly generated cells, or those that are still in S-phase, rather than on postmitotic rods that had not yet expressed detectable levels of rhodopsin.

The promotion of the photoreceptor cell fate by AT and 9-*cis* retinoic acid is quite specific. Table 1 lists several other factors that are known to be present in the retina during histogenesis, some of which have been found to be active in assays of retinal cell proliferation, differentiation or survival in cell culture. We have assayed these factors for their effects on photoreceptor differentiation in embryonic rat retinal cell cultures, and found that none of these factors cause a significant increase in the number of photoreceptors in high-density cultures, over a wide range of concentrations. While some of these molecules may be important for cell survival and differentiation in low-density culture assays, they are not limiting for the generation of new photoreceptors in high-density cultures, where their concentrations are likely to be similar to that in the intact retina. By contrast, the number of photoreceptors that are generated in high-density cultures is limited by the concentration of retinoic acid, either the AT or 9-*cis* isomers, consistent with a role in the regulation of photoreceptor number in vivo.

Table 1. Factors that do not increase the number of rod photoreceptor cells in high density embryonic day 18 rat retinal cultures after 7 days in vitro

Factor	Concentration
Epidermal growth factor	0.01-100 ng/ml
Transforming growth factor alpha	0.01-100 ng/ml
Fibroblast growth factor, acidic	0.01-10 ng/ml
Fibroblast growth factor, basic	0.01-10 ng/ml
Platelet derived growth factor	1-10 ng/ml
Transforming growth factor beta 1	0.01-100 ng/ml
Transforming growth factor beta 2	0.01-100 ng/ml
Transforming growth factor beta 3	0.01-100 ng/ml
Amphiregulin	0.01-100 ng/ml
Neu Differentiation Factor	Cos cell CM
Oncostatin-M	0.01-100 ng/ml
CDF/LIF	1-10 ng/ml
IL-2	10-1000 U/ml
Dopamine	1-10 mM
Taurine	1-10 mM

Factors were obtained from commercial sources, with the exception of oncostatin-M, amphiregulin and TGF-alpha, which were generously provided by Dr M. Shoyab of Bristol-Meyers Squibb. Neu differentiation factor was obtained from media conditioned by Cos cells that were stably transfected with this gene and were known to produce functionally active protein (provided by Dr C. Clegg; Bristol-Meyers Squibb). All peptide factors were tested for activity in independent assays, with the exception of CDF/LIF and IL-2.

Retinoic acid promotes photoreceptor differentiation in early postnatal retinal cultures

The differentiation of postnatal retinal cells into rod photoreceptors is also thought to be regulated by diffusible factors in the microenvironment (Altshuler and Cepko, 1992). To determine whether retinoic acid could promote development of rod photoreceptors in postnatal retina, we established cultures from neonatal retinas and then treated the cultures with AT retinoic acid. In order to dilute the endogenous rod-promoting factors, neonatal cells were dissociated to single cells or small clumps containing fewer than 10 individual cells and then plated at low density (40,000 cells per culture well) onto collagen-coated coverslips. Individual cells were unable to move through the collagen matrix, so cells remained isolated throughout the duration of the experiment. Cell survival was assayed by determining the number of single cells in each culture after 2, 4, 6 and 8 days in vitro. Results indicate no significant change in the number of single cells between 2 and 8 days in vitro (Table 2). After 6 or 8 days in vitro, the number of single isolated cells that developed as rod photoreceptors was approximately 8.0% of the total number of isolated cells (Fig. 8). Similar levels of photoreceptor differentiation have been reported in previous experiments with newborn rat retinal cells in low-density collagen gel cultures (Reh and Kljavin, 1989; Altshuler and Cepko, 1992)

Effects of retinoic acid

In low-density cultures of neonatal cells maintained in medium with 10 nM AT retinoic acid, the number of isolated cells that differentiated as rod photoreceptors was approximately 14% of the total cell number and, in cultures treated with 100 nM AT retinoic acid, the number of rods was approximately 17% of the total cells. These values represent increases of 175% (10 nM) and 210% (100 nM) of the control value (Fig. 9). The number of cells that differentiated as single rod photoreceptors in cultures treated with 500 nM AT retinoic acid was only 12% of the total cell number. In addition to single, isolated cells, the cultures in these experiments also contained small aggregates of 5 to 10 cells. Treatment with AT retinoic acid also caused an increase in the number of rod photoreceptors in these small aggregates. The increases in the number of rod photoreceptors in small aggregates were similar to the increases that were observed for single cells (Fig. 9).

We examined the specificity of the effect of retinoic acid by comparing the number of rod bipolar cells in treated and

Table 2. The overall number of single cells in low density P0 cultures does not change significantly over the in vitro period

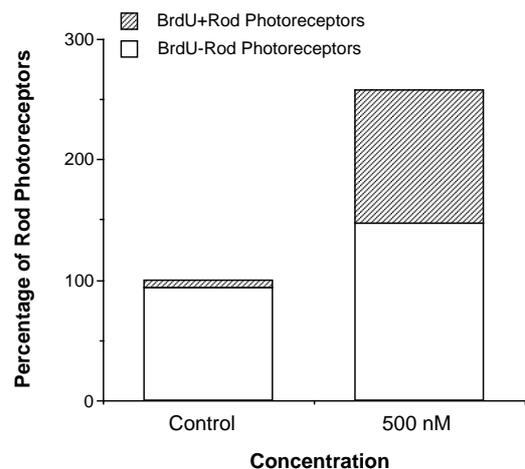| Days in vitro	Control	100nM retinoic acid
2	41±6	43±2
4	43±6	57±17
6	39±8	43±12
8	46±7	58±9

The total number of single cells in P0 cultures was determined after 2, 4, 6 and 8 days in vitro by counting 10 fields on each coverslip from three separate experiments with a 40× phase objective. The data show that the number of cells does not decline over the period of cell culture and the number of cells in the retinoic acid-reated cultures is not significantly different from that in the control sister cultures. Variance is s.e.m.
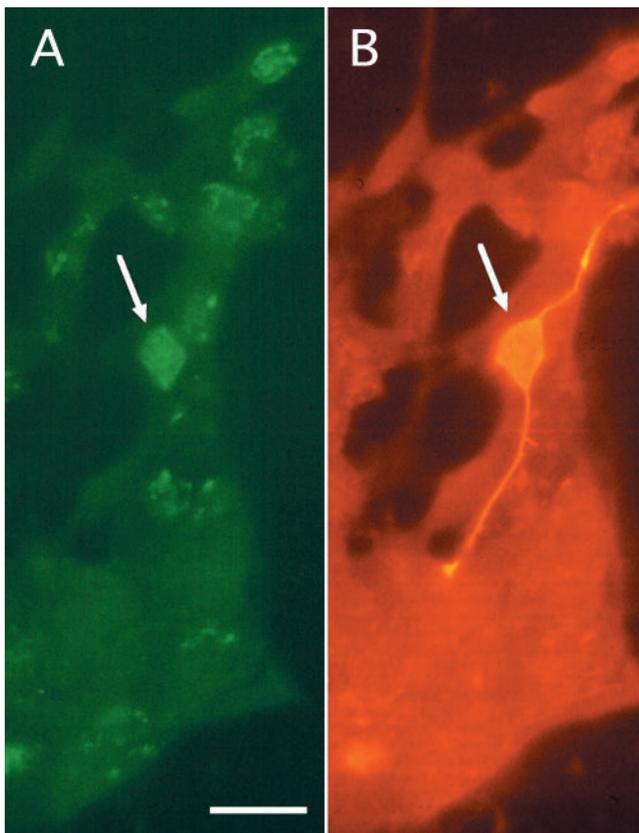
Fig. 6. Fluorescent micrograph of a rod photoreceptor from a culture that was established on E18 and maintained in culture medium with 500 nM retinoic acid for eight days. The pictured cell is immunoreactive for both rho-4D2 (rhodamine secondary) (B), and for BrdU (fluorescein secondary) (A). Scale bar in A (same in B), 20 μm.

Fig. 7. Comparison of opsin[+]/BrdU[−] and opsin[+]/BrdU^{+} cells from retinal cultures established on E18 after 6 DIV. In control cultures less than 10% of opsin^{+} cells are also positive for BrdU. In retinoic acid-treated cultures, there is a significant increase in the number of opsin[+]/BrdU^{+} cells, as expressed as a percentage of total cell number. Data are from the results of three separate experiments.

control wells. We chose to examine rod bipolar cells in postnatal cultures, rather than amacrine cells as in embryonic cultures, because rod bipolar cells are generated after birth in the rat retina. Results indicate that the number of rod bipolar cells did not differ significantly between control culture wells and those treated with 10 nM or 100 nM AT retinoic acid (Fig. 9); however, there was a 30% decrease in the number of bipolar cells in cultures treated with 500 nM AT retinoic acid.

To determine whether the increase in rod photoreceptor cells caused by retinoic acid was due to a selective survival effect on photoreceptors, we assayed the total number of retinal cells in each culture well by counting the number of isolated cells on control and retinoic acid-treated coverslips (Table 2; Fig. 9). We found no evidence that retinoic acid increased cell survival in the cultures. There was no change in the number of single cells in cultures that were treated with 10 nM or 100 nM retinoic acid from that in control wells, however at the highest concentration of retinoic acid, 500 nM, there was a 25% decrease in the number of cells in the treated wells. The decrease in overall cell number, as well as the decrease in number of rod photoreceptors and rod bipolar cells in wells treated with 500 nM retinoic acid, suggests that high concentrations of this compound are toxic to P0 retinal cells in low-density cultures after several days in vitro. The effects of 9-*cis* retinoic acid on P0 retinal cells were not determined.

To determine the time course of the effects of all-*trans* retinoic acid on the differentiation of photoreceptors cells in neonatal cultures, retinal cells from postnatal day 0 rat pups were plated at low density and fixed after 2, 4, 6 or 8 days in vitro, and the number of rod photoreceptor cells was quantified (Fig. 5C). The total number of rods in the control cultures increased steadily over the 8 days of the experiment; however, as early as 2 days in vitro, there was a marked increase in the total number of rods in the treated cultures as compared with controls. The margin of difference between experimental and control cultures was maintained or grew wider during the remaining 6 days of the experiment.

DISCUSSION

Retinoic acid promotes photoreceptor differentiation in embryonic retinal cells

In recent years, several studies have shown that cell-cell interactions are necessary for the appropriate differentiation of vertebrate retinal neurons, both in vivo (Negishi et al., 1985; Reh and Tully, 1986; Reh, 1987) and in vitro (see below). As noted in the Introduction, the rat retina has been extensively utilized as a model for the study of the factors that control cell commitment in the nervous system. The heterochronic co-culture experiments of three laboratories have demonstrated that the differentiation of retinal progenitor cells as rod photoreceptors is influenced by locally diffusible signals in the extracellular environment. These signals are developmentally regulated during the period of retinal histogenesis (Watanabe and Raff, 1989, 1992; Reh, 1992a,b; Altshuler and Cepko, 1992), however the specific factors that determine or promote the rod photoreceptor phenotype have not been identified.

In the present study, we have found that retinoic acid can mimic the effects of older environments on early embryonic rat retinal cells that were reported in the studies of Watanabe

and Raff (1992) and Reh (1992b). In reaggregate cultures of dissociated retinal cells, Watanabe and Raff (1989, 1992) found that cells isolated from embryonic day 15 rat retinas differentiated as rod photoreceptor cells at a higher frequency when co-cultured with postnatal retinal cells; millipore filters failed to block the effect, indicating that the effect was mediated by a soluble factor. In the present study, we have found a retinoic acid dose-dependent increase in the number of opsin immunoreactive cells in our high-density E15 cultures after six days in vitro, with the maximum response nearly a 15-fold increase over sister control cultures. In addition, the
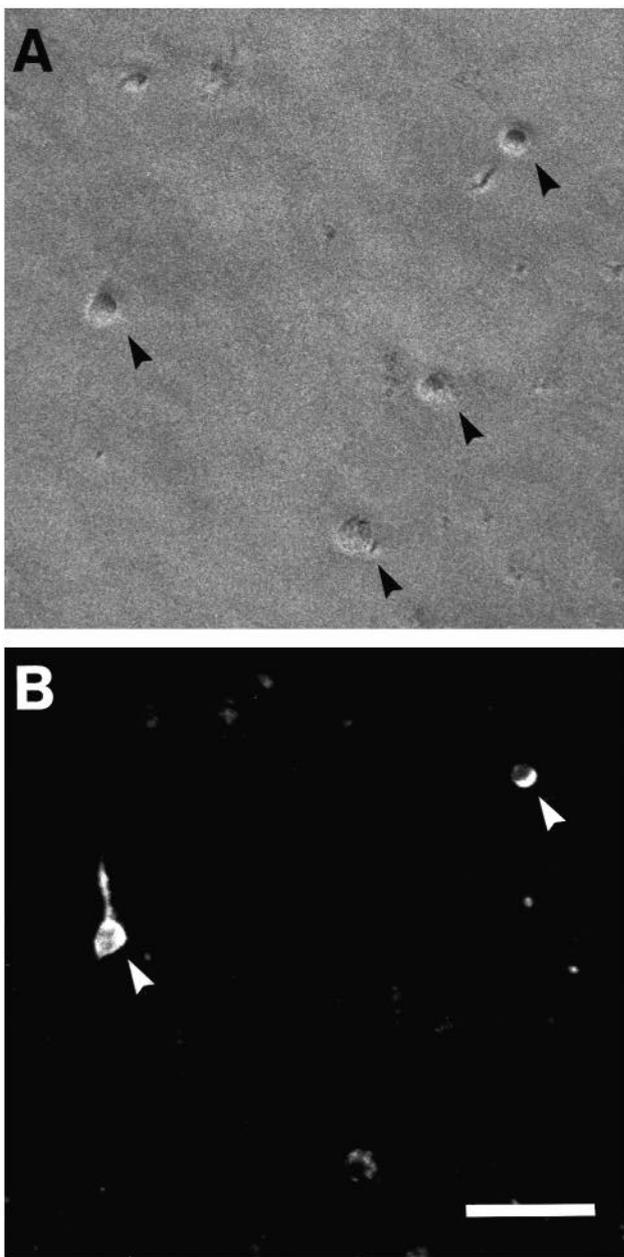

Fig. 8. Photomicrographs showing rod photoreceptor differentiation in low-density collagen gel cultures. (A) Phase micrograph showing four isolated retinal neurons (arrowheads). (B) Fluorescent micrograph showing the same field as in A. Two of the isolated retinal neurons are labeled as rod photoreceptors with an antibody

effects of retinoic acid on the E15 cells further mimic the effects of postnatal co-culture in the specificity of its effects. Neither the number of amacrine cells nor the overall cell number was increased by the treatment with retinoic acid.

The total number of cells that were induced to differentiate as photoreceptors as a result of the retinoic acid treatment was much lower in E15 cultures than in cultures established on E18 or P0. These results suggest that retinoic acid is probably not the only factor necessary for photoreceptor differentiation. However, a recent hypothesis has suggested that as development proceeds the potential phenotype choices of individual progenitor cells may be influenced based on changing sensitivities to different factors (Reh and Cagan, 1994). If this hypothesis is correct, then retinoic acid might only have an effect on those progenitor cells that have become restricted to differentiate as later retinal phenotypes such as amacrine cells or rod photoreceptors.

In some of the experiments using the embryonic cells, we also assayed for the differentiation of photoreceptor cells with an antibody to recoverin, a protein that is expressed in both rod and cone photoreceptors. We found that retinoic acid treatment also caused a marked increase in the number of recoverin immunoreactive cells in both E15 and E18 cultures. Many of these cells exhibited morphological features of cone photoreceptor cells rather than rod photoreceptor cells; moreover, in double label experiments in which the cultures were labeled with both recoverin and opsin antibodies, not all of the recoverin immunoreactive cells were also immunoreactive for
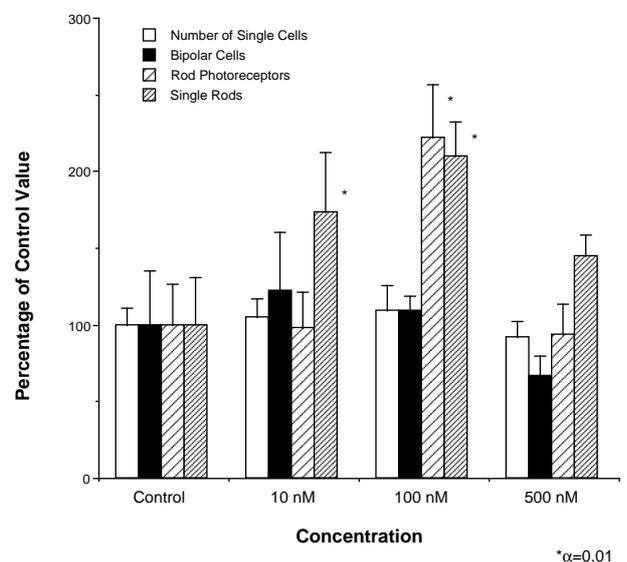
Fig. 9. Response of retinal cultures established at low-density on P0 to different concentrations of all-*trans* retinoic acid after 6 DIV. The total number of cells that developed as rod photoreceptors, as well as the number of isolated cells that developed as rod photoreceptors, increased in cultures treated with retinoic acid. The number of isolated cells in each culture and the number of cells that developed as bipolar cells did not change with retinoic acid treatment. High concentrations of all-*trans* retinoic acid (500 nM) appeared to be toxic to cells in low-density cultures. Many cells in the 500 nM cultures appeared abnormal and the cell number, number of rod photoreceptors and number of bipolar cells was reduced. Data are from the results of three separate experiments. Error bars are s.e.m.

opsin. It is important to consider that antibodies to recoverin also label cone bipolar cells and that, at this time, we cannot eliminate the possibility that the retinoic acid-induced increase in recoverin staining includes an increase in the number of cells that develop as cone bipolar cells. However, based on the birth-dates of cone photoreceptors and cone bipolar cells (early versus late phases of histogenesis) and on the morphology of the recoverin-positive cells, it seems unlikely that a significant number of the recoverin-positive cells in retinoic acid-treated cultures are cone bipolar cells. Therefore, it seems likely that the number of cone photoreceptor cells is also increased in embryonic cultures treated with retinoic acid.

Retinoic acid acts on the progenitor cells to direct cell fate

The results of these experiments demonstrate that retinoic acid can influence the development of rod photoreceptor cells in vitro. In the embryonic cultures, retinoic acid appears to act directly on the mitotically active progenitor cells to direct their fate to the rod photoreceptor phenotype. In cultures established on E18, the increase in the number of rod photoreceptors after 6 days in vitro was almost entirely due to an increase in the number of double-labeled, not single-labeled, photoreceptor cells. Therefore, in the embryonic cultures, retinoic acid appears to act on the newly generated cells, i.e. those that are still in S-phase, rather than on rod photoreceptors that were postmitotic prior to the culture period but had not yet expressed detectable levels of rhodopsin. Moreover, in cultures established on E18, along with the increase in the number of photoreceptors, there was a concomitant decrease in the number of cells that differentiated as amacrine cells with retinoic acid treatment, even though the overall cell number remained constant. This suggests that, in the presence of retinoic acid, some of the uncommitted cells in each of the cultures that would have differentiated as amacrine cells may have been influenced to change their fates and to differentiate as photoreceptor cells. Also, retinoic acid is capable of promoting rod differentiation in cultures from E15 rat retinas, when thymidine birthdating analysis indicates that few, if any, cells have become committed to the rod fate in vivo (LaVail, personal communication).

Retinoic acid promotes photoreceptor differentiation in postnatal retinal cells

Recent studies have shown that the differentiation of rod photoreceptor cells in retinal cell cultures from embryonic (Reh, 1992b) and postnatal (Akagawa, 1990; Altshuler and Cepko, 1992) rat or from embryonic frog (Harris and Messersmith, 1992) is dependent on cell density. Rod photoreceptor cells will only differentiate in embryonic cultures when the cells are cultured at high density while, in postnatal cultures, the percentage of cells that will differentiate as rod photoreceptor cells increases as the density of the cells is increased. Conditioned medium experiments have indicated that this effect may also be mediated in part by soluble factors (Altshuler and Cepko, 1992), and that one of these factors may be taurine (Altshuler et al., 1993). In our second series of experiments, we tested the effects of retinoic acid in low-density dissociated cell cultures from newborn rat retinas. We found that treatment of postnatal retinal cells with retinoic acid could induce a greater number of these cells to differentiate as rod photoreceptors than in

control cultures. This effect was observed for both single isolated cells and for cells in small clusters. Again, the effect appears to be specific for photoreceptors, since the retinoic acid treatment did not induce a corresponding increase in overall cell number and did not cause an increase in the number of bipolar cells, another late generated retinal cell class.

The results of these experiments and the results presented in Altshuler et al. (1993) indicate that retinoic acid and taurine have similar effects on the development of rod photoreceptors in low-density cultures of postnatal rat retinal cells. It seems unlikely that these two factors could function through similar signalling pathways, however Altshuler et al. (1993) demonstrated that conditioned medium promoted the development of rod photoreceptors more efficiently than taurine, suggesting that more than one factor may play a role in photoreceptor development. Therefore, it seems possible that both retinoic acid and taurine may both play important roles in the development of rod photoreceptors.

While the results from the embryonic cultures demonstrate that retinoic acid influences cell fate, the results from the postnatal cultures are also consistent with the possibility that both taurine and retinoic acid selectively support photoreceptor survival in the low-density cultures. Taurine has been shown in a number of studies to be essential for photoreceptor survival (Hayes et al., 1975; Lombardini, 1991) and, as noted in the Introduction, Stenkamp et al. (1993) have recently shown that retinoids also have a positive effect on survival of photoreceptors in postmitotic chick retinal cell cultures. However, there were no [^{3}H]thymidine or BrdU-labeled photoreceptors present in the cultures of either Stenkamp et al. (1993) or Altshuler et al. (1993). Therefore, the effects of both retinoic acid and taurine in these cultures may be to influence cell survival or opsin expression in cells that are already committed to develop as rod photoreceptors. Although overall cell number does not differ between control cultures and cultures with either retinoic acid or taurine, in both of these reports there is a 50-80% loss of cells in low-density retinal cell cultures over the course of the experiment (Stenkamp et al., 1993; Altshuler et al., 1993). Therefore, it is possible that addition of either retinoic acid or taurine might provide a selective survival advantage to rod photoreceptor cells in the low-density dissociated cultures (Stenkamp et al., 1993). In our experiments with postnatal retinal cells, overall cell number did not change over the course of the experiment in either control or retinoic acid-treated cultures, suggesting that an additional effect of retinoic acid may be to promote opsin expression in cells that are committed to differentiate as rod photoreceptors; however, the effects of retinoic acid on cell survival or enhancement of antigen expression do not appear to be significant in the high-density embryonic cultures, since treatment of these cultures with retinoic acid did not cause a significant increase in the number of opsin$^+$BrdU$^-$ (i.e. cells that were generated prior to the establishment of the culture) rod photoreceptors. Thus, the effects of taurine and retinoic acid may be distinguished by the response of high-density embryonic retinal cultures to these factors. While both factors may be important for cell survival and differentiation in low-density culture assays, taurine is not limiting for the generation of new photoreceptors in high-density cultures (Anchan and Reh, unpublished results), where its concentration is likely to be similar to that in the intact retina. By contrast, the number

of new photoreceptors that are generated in high-density cultures is limited by the concentration of retinoic acid.

Do retinoids control phenotypic choice in the developing retina in vivo?

The results presented in this study indicate that retinoic acid promotes the development of the photoreceptor phenotype in retinal cell cultures. Several lines of evidence indicate that retinoic acid may have a similar role in vivo. First, several studies have shown that retinoic acid can be synthesized by embryonic retinal cells (McCaffery et al., 1991) and that at least four dehydrogenases related to retinoic acid synthesis are present in the developing mouse retina (McCaffery et al., 1993). Second, there is evidence from experiments using a retinoic acid reporter cell line, transgenic mice with a reporter gene driven by an retinoic acid response element (RARE), and high performance liquid chromatography that both all-*trans* and 9-*cis* retinoic acid are normally present in the developing retina (McCaffery et al., 1992; Balkan et al., 1992; Kraft and Juchau, 1993). Third, in situ localization studies of retinoic acid receptors and retinoid X receptors have reported expression of the RAR-α and apparently RXR-β in the developing neural retina (Ruberte et al., 1991; Mangelsdorf et al., 1992).

If retinoic acid does control the differentiation of photoreceptor cells in the developing retina, then one prediction would be that the levels of retinoic acid should increase steadily during histogenesis, since as development of the retina proceeds, an increasing fraction of the retinal progenitor cells differentiate as photoreceptor cells (Carter-Dawson and LaVail, 1979). Less than one third of the cells generated at E15 become photoreceptors, and those that do are almost exclusively cone photoreceptor cells. However, by postnatal ages, more than 90% of the newly postmitotic cells differentiate as rod photoreceptors (Carter-Dawson and LaVail, 1979). At present, we do not know if the levels of different retinoic acid isomers change during retinal histogenesis as this model would predict. In addition, it is not known which cells of the developing retina produce retinoic acid. Our hypothesis would be that the newly differentiating cells of one of the other retinal cell classes, such as amacrine cells or horizontal cells, secrete retinoic acid; so, as the number of this cell class increases, the increased level of retinoic acid stimulates a greater percentage of the progenitor cells to differentiate as photoreceptor cells.

The potential involvement of retinoids in mammalian retinal development has an interesting parallel in the development of the *Drosophila* eye. Several aspects of the development of the *Drosophila* eye and the vertebrate retina are similar (see Reh and Cagan, 1994, for review). In both tissues, the differentiation of the different cell phenotypes occurs over a well-defined sequence; cells of different phenotypes are thought to arise from progenitor cells independent of their lineage; and a series of sequential cell inductive events is thought to control the choice of cell fate in both systems. While several of the molecules that participate in the inductive interactions in the *Drosophila* eye have been characterized, much less is known about these events in the vertebrate retina. Two types of receptor tyrosine kinases have been shown to influence cell fate decisions in the *Drosophila* eye: the sevenless kinase and the EGF receptor kinase. Although a homolog for sevenless has not yet been implicated in vertebrate retinal development, both

EGF and TGFa have been shown to influence cell fate decisions in rat retinal cell cultures (Reh, 1992), and it is likely that receptor tyrosine kinases will have an important role in cell fate decisions in the vertebrate retina. In addition to the receptor tyrosine kinases, however, another signalling pathway that appears to be involved in cell fate decisions in the *Drosophila* eye is the steroid hormone receptor system. Two members of the steroid hormone receptor superfamily have been implicated in these cell-cell inductive events involved in cell differentiation in the *Drosophila* eye. Seven-up, a steroid hormone receptor with an unknown ligand is necessary for commitment to the R7 fate (Mlodzik et al., 1990), and ultra-spiracle, the *Drosophila* homologue to the RXR receptor, has also been recently shown to be necessary for normal ommatidial development (Oro et al., 1992). Therefore, a further parallel between the vertebrate retina and the *Drosophila* eye may be in the molecular mechanisms by which cell fates are specified during development.

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