Diffusible rod-promoting signals in the developing rat retina

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Summary

We previously developed a reaggregate cell culture system in which embryonic rat retinal neuroepithelial cells proliferate and give rise to opsin-expressing rod photoreceptor cells (rods) on the same schedule *in vitro* as they do *in vivo*. We showed that the proportion of neuroepithelial cells in the embryonic day 15 (E15) retina that differentiated into opsin$^+$ rods after 5-6 days in such cultures increased by ~40-fold when the E15 cells were cultured in the presence of an excess of postnatal day 1 (P1) neural retinal cells. In the present study, we have further analyzed this rod-promoting activity of neonatal neural retinal cells. We show that the activity is mediated by a diffusible signal(s) that seems to act over a relatively short distance. Whereas neonatal (P1-P3) neural retina has rod-promoting activity, E15 and adult neural retina, neonatal thymus, cerebrum and cerebellum do not. Finally, we show that neonatal neural retina promotes rod but not amacrine cell development.

Key words: rat retina, rod photoreceptor cell, cell fate determination, reaggregate culture, transfilter culture.

Introduction

The vertebrate retina contains Müller glial cells, photoreceptors (rods and cones) and a variety of neurons, all of which develop from retinal neuroepithelial cells (Rodieck, 1973). Clonal analyses in the developing rat (Turner and Cepko, 1987), mouse (Turner et al. 1990) and *Xenopus* (Holt et al. 1988; Wetts and Fraser, 1988) retina have demonstrated that various combinations of these cell types can develop from single precursor cells, indicating that at least some retinal neuroepithelial cells are multipotential, and that cell fate in the developing retina is probably not determined solely by an invariant program in each neuroepithelial cell. The challenge now is to determine how individual multipotential precursor cells decide what type of differentiated retinal cell to become.

In the rodent retina, the various cell types do not develop synchronously. The majority of ganglion cells, cones and horizontal cells, for example, develop before birth, whereas most rods develop after birth (Sidman, 1961; Weidman and Kuwabara, 1968; Kuwabara and Weidman, 1974; Hinds and Hinds, 1978, 1979; Carter-Dawson and La Vail, 1979; Young, 1985; Turner and Cepko, 1987; Turner et al. 1990). We have focused our attention on the development of rods in the rat retina because they are the main cell type in rat retina, they develop late, at a time when it is relatively easy to manipulate the retina, and monoclonal anti-opsin

antibodies can be used to identify them (Barnstable, 1980). In principle, at least two mechanisms, which are not mutually exclusive, could account for the late development of rods: environmental signals required for rod development might appear late, or the multipotential precursor cells might change their properties such that they are able to give rise to rods only relatively late in retinal development.

To help distinguish between these two mechanisms, we previously devised a cell pellet culture system in which proliferating neuroepithelial cells dissociated from embryonic rat neural retina generate opsin$^+$ rods on the same schedule *in vitro* as they do *in vivo*: embryonic day 15 (E15) neural retinal cell pellets, for example, begin to give rise to opsin$^+$ rods after 5 days *in vitro*, which is equivalent to the time that opsin$^+$ rods first appear *in vivo* (Watanabe and Raff, 1990). We showed that mixing the E15 cells with a 50-fold excess of postnatal day 1 (P1) neural retinal cells does not appreciably influence the time when the E15 cells first give rise to opsin$^+$ rods: even though precursor cells from the neonatal retina give rise to opsin$^+$ rods within 2 days in such mixed-age cultures, the E15 cells do not do so until 3 days later. This finding suggests that the normal late development of rods in the rat retina is not due solely to a lack of rod-inducing signals early in development. We also found that embryonic and neonatal neural retinal cells differ in their capacity to proliferate and/or survive *in vitro*, and that these

differences too persist in mixed-age cultures. We concluded from these two observations that the properties of retinal neuroepithelial cells change as retinal development proceeds (Watanabe and Raff, 1990).

Although, in these previous studies, the presence of a 50-fold excess of neonatal cells did not influence the *time* when E15 cells first gave rise to opsin$^+$ rods in mixed-age cultures, the presence of the neonatal cells did greatly increase the *proportion* of E15 cells that differentiated into opsin$^+$ rods. This finding suggested that cell-cell interactions might play an important part in rod development and raised the possibility that neonatal retinal cells produce a rod-promoting signal(s) that E15 cells cannot respond to until they are older (Watanabe and Raff, 1990). Earlier studies in goldfish (Negishi et al. 1982) and frog (Reh and Tully, 1986) had suggested that cell-cell interactions are important in amacrine cell development in the vertebrate retina.

In the present study, we have extended our observations on the rod-promoting signal(s) produced by neonatal rat retinal cells in culture. We show that the signal is diffusible but seems to act over a relatively short distance, it is made by neonatal retina but not E15 or young adult retina, neonatal thymus, cerebrum or cerebellum, and it influences rod but not amacrine cell development.

Materials and methods

Animals

Timed pregnant Sprague-Dawley rats were obtained from the breeding colony of the Imperial Cancer Research Fund, South Mimms, or from Charles River, Ltd. To time the pregnancies, female rats were caged with males overnight and then removed; this was taken as day 0 of pregnancy. Birth normally occurred at E22, which was taken as postnatal day 0 (P0).

Cell pellet and explant cultures

Neural retinae were dissected from embryonic day 15 (E15) or neonatal (P1-P3) rats, while cerebrum, cerebellum and thymus were dissected from P2 rats. To prepare pellet cultures, E15 and neonatal neural retinae were dissociated into single-cell suspensions with trypsin as described previously (Watanabe and Raff, 1988). After a wash with minimum Eagle's medium (MEM) with 0.02 M Hepes buffer (MEM-HEPES) containing 10% foetal calf serum (FCS), approximately 150,000 E15 cells, 1,000,000 neonatal cells, or a mixture of E15 and neonatal cells, were centrifuged for 7 min at 420 g to produce a pellet (Watanabe and Raff, 1990). At the start of each experiment, three E15 pellets were dissociated into single cells and counted in a haemocytometer to determine the initial cell number per pellet. The remaining E15 and the mixed-age cell pellets were transferred onto floating 13 mm polycarbonate filters (Nuclepore) with 0.8 μm diameter pores. About 3-4 pellets were cultured on each filter.

To prepare explant cultures, E15, neonatal or P45 neural retinae, or P2 cerebrum or cerebellum (dissected free of meninges), or P2 thymus were cut into small pieces (0.5-1 mm), which were transferred onto a polycarbonate filter with either 3.0 μm or 0.8 μm diameter pores. For medium conditioning experiments, pieces of an entire neonatal retina were placed onto a single filter.

Polycarbonate filters with cell pellets or tissue explants were floated in Dulbecco's modified Eagles medium without glutamate (DMEM), supplemented with 10% FCS (DMEM-FCS), in a Petri dish (Falcon) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air as previously described (Watanabe and Raff, 1990).

Transfilter experiments

About 20 pieces of E15, neonatal or P45 retinal explants, or P2 cerebral, cerebellar or thymic explants were placed as a group on a floating filter with either 0.8 μm or 3.0 μm diameter pores and were then covered by a small piece of filter (7 mm × 7 mm) with either 0.8 μm or 0.01 μm diameter pores. A single E15 retinal cell pellet was then placed on top of the small piece of filter, either directly over the underlying explants, or displaced about 0.5-1 mm away from them. In those experiments where filters with 0.01 μm diameter pores were used, the uppermost E15 pellet was covered by a similar filter (2 × 2 mm) with 0.03 μm diameter pores, which was then covered with a drop of culture medium to prevent the pellets from drying.

Pellet cultures containing a mixture of E15 cells and lightly fixed neonatal cells

Mixed-age pellet cultures were carried out as previously described (Watanabe and Raff, 1990), except that the older cells were lightly fixed with glutaraldehyde. To label dividing E15 neural retinal cells, pregnant rats at 15 days gestation were injected intraperitoneally with bromodeoxyuridine (BrdU, Boehringer) at a dose of 0.1 mg per g of body weight. Two hours later, neural retinae were dissected from embryos and dissociated into single-cell suspensions.

To prepare lightly fixed P3 neural retinal cells, dissociated P3 cells were suspended in 1 ml of 0.05% glutaraldehyde (Fluka) in phosphate-buffered saline (PBS) and incubated for 30 sec at room temperature. Two ml of lysine (0.2 M, pH 8.0) were then added for 3 min at room temperature to stop the fixation reaction, and the cells were washed 3 times with MEM-HEPES containing 10% FCS. The cells were counted, and approximately 20,000 cells from the BrdU-labelled population of E15 cells (of which 30-35% had incorporated BrdU) were mixed with either 140,000 unlabelled E15 cells, 10^6 unfixed P3 cells, or 10^6 fixed P3 cells. The mixtures were centrifuged into cell pellets, which were transferred onto floating filters (with 0.8 μm diameter pores) and cultured as described above.

Immunofluorescence staining

Retinal cell pellets were dissociated into single-cell suspensions as previously described (Watanabe and Raff, 1988). After counting the number of cells in each pellet, 100,000 cells were plated in 10 μl of DMEM-FCS on poly-D-lysine-coated 13 mm glass coverslips and incubated for 2 h at 37°C to allow the cells to adhere. The cells were then fixed for 5 min at room temperature in 2% paraformaldehyde in PBS, followed by 70% ethanol for 10 min at −20°C. The fixed cells were then incubated with either RET-P1 anti-opsin monoclonal antibody (Barnstable, 1980: ascites fluid, diluted 1:100 in MEM-HEPES containing 10% FCS and 0.1% [w/v] sodium azide) or HPC-1 monoclonal antibody (Barnstable, 1980: culture supernatant; diluted 1:20 in MEM-HEPES containing 10% FCS, 0.1% [w/v] sodium azide and 1% Triton X-100) for 1 h at room temperature, followed by sheep anti-mouse immunoglobulin coupled to biotin (Sh anti-MIg-Bt, diluted 1:50; Amersham) and then streptavidin coupled to Texas Red (SA-

TR, diluted 1:100; Amersham), both for 30 min at room temperature.

Double labelling with RET-P1 and anti-BrdU antibodies was performed as described previously (Watanabe and Raff, 1990). In brief, dissociated cells on poly-D-lysine-coated glass cover slips were fixed in 70% ethanol at −20°C for 30 min, and stained with RET-P1 antibody as described above. The cells were then treated with 2 M HCl for 20 min to denature the nuclear DNA and then with 0.1 M Na$_2$B$_4$O$_7$ (pH 8.5) for 5 min to neutralize the acid. Cells were then incubated with Bu20a anti-BrdU antibody (Magaud et al. 1988), followed by goat anti-MIg coupled to fluorescein (Cappel).

After staining, cells were mounted in Citifluor (Citifluor Ltd.), examined in a Zeiss Universal fluorescence microscope, and photographed with Tri-X film rated at 400 ASA.

Immunofluorescence staining of frozen sections of pellet cultures

E15 neural retinal cell pellets were cultured with neonatal neural retinal explants, separated by a polycarbonate filter with 0.8 μm diameter pores. After 7-8 days *in vitro*, the pellets were fixed in 4% paraformaldehyde in PBS at 4°C overnight, cryoprotected in 30% sucrose in PBS, embedded in OCT compound (Miles), and frozen in liquid nitrogen. Frozen sections (15-20 μm) were cut with a Bright cryostat, transferred to gelatin-coated slides, and air dried for 2 h at room temperature. The sections were incubated with RET-P1 antibody (ascites fluid, diluted 1:100 in MEM-HEPES containing 10% FCS, 0.1% [w/v] sodium azide, and 1% Triton X-100) overnight at 4°C, followed by Sh anti-MIg-Bt and then SA-TR, both for 1 h at room temperature. The sections were then washed in PBS, mounted and examined as described above.

Results

Lack of rod-promoting activity in culture fluid containing neonatal neural retinal explants

We previously showed that the proportion of E15 retinal neuroepithelial cells that develop into opsin$^+$ rods after 5-6 days in pellet cultures is increased about 40-fold if a large excess of neonatal neural retinal cells is present in the same pellet (Watanabe and Raff, 1990). To test whether this rod-promoting activity of the neonatal retinal cells is mediated by long-range diffusible molecules, pellets of E15 neural retinal cells were cultured on floating polycarbonate filters as previously described, but in the presence of a large excess of neonatal neural retinal explants on separate filters floating in the same culture medium. In these experiments, a single filter carrying 3 or 4 E15 cell pellets, each containing about 150,000 cells, was cultured with 8-10 filters, each of which carried the equivalent of an entire neonatal neural retina (about 20×10^6 cells). As postnatal neural retinal cells do not survive in culture as well as embryonic ones (Watanabe and Raff, 1990), in some experiments half of the filters carrying the neonatal explants were replaced every 2-3 days. When the E15 pellets were dissociated into single cells and immunostained with the RET-P1 anti-opsin antibody after 7-8 days *in vitro*, the proportion of opsin$^+$ rods was not significantly changed by the presence of the neonatal explants, although the total number of cells
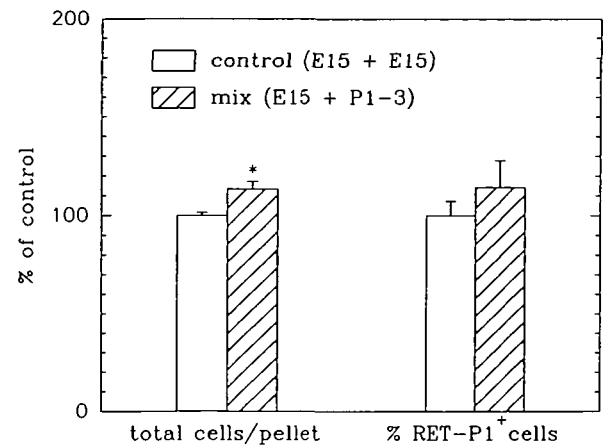

Fig. 1. Lack of effect of medium conditioning by neonatal retinal explants on the development of RET-P1$^+$ rods in pellet cultures of E15 neural retinal cells. Three to four E15 retinal cell pellets were cultured on a single polycarbonate filter floating in the same culture medium as 8-10 other filters, each carrying the pieces of an entire P1-P3 neural retina (or of E15 neural retina in control dishes). After 7-8 days *in vitro*, the E15 pellets were dissociated into single cells, counted and immunostained with RET-P1 monoclonal antibody as described in Materials and Methods. In this and the other figures, the total number of cells and the proportions of RET-P1$^+$ cells in mixed-age (E15 + P1-P3) cultures are compared with those in control (E15 + E15) cultures, which are taken as 100%; except for Fig. 3, the data are graphed as the means ± s.e.m. of at least three separate experiments, each done at least in triplicate, and, in all figures, * indicates a significant difference (*P*=0.001) compared with control cultures, when analyzed by Student's *t*-test. In the experiments shown here, the total number of cells per E15 pellet at the start of each culture was (14 ± 3) × 10^4, and in the control cultures after 7-8 days *in vitro* the total number rose to (121 ± 10) × 10^4. (Note that these values, and those in all the other figure legends, are given as means ± s.d.) The number of RET-P1$^+$ cells per 100,000 total cells at this time was 9 ± 2. Although these numbers varied considerably from experiment to experiment (hence the large s.d.s), in this and in all of the other figures (except for Fig. 3), the proportional changes compared with control did not.

per E15 pellet was increased by a small, but statistically significant, amount (Fig. 1). This result suggests that the rod-promoting activity of neonatal retinal cells when they are present in the same pellet as E15 retinal cells might not be mediated by stable, long-range, diffusible signals.

Lightly fixed neonatal retinal cells do not promote rod cell development in mixed-age pellets

To test whether cell-surface-bound signals are responsible for the rod-promoting activity of neonatal retinal cells in mixed-age pellet cultures, we mixed E15 neural retinal cells, which had been labelled with bromodeoxyuridine (BrdU) as previously described (Watanabe and Raff, 1990), with a 50-fold excess of unlabelled P3 neural retinal cells, which had been fixed with 0.05% glutaraldehyde for 30 sec at room temperature; these

fixation conditions were chosen because antigen-presenting cells fixed in this way are still capable of presenting peptides to appropriate T lymphocytes (Shimonkevitz et al. 1984). The mixed cells were centrifuged into a pellet and cultured on a floating filter; after 6 days, the pellets were dissociated into single cells and immunostained with both RET-P1 and anti-BrdU monoclonal antibodies. The presence of the lightly fixed neonatal retinal cells decreased rather than increased the proportion of BrdU-labelled E15 cells that developed into opsin$^+$ rods. In a typical experiment, whereas $0.008 \pm 0.004\%$ of the BrdU$^+$ cells were RET-P1$^+$ in control (E15 + E15) pellets, and $0.41 \pm 0.12\%$ of the BrdU$^+$ cells were RET-P1$^+$ in pellets where the E15 cells were mixed with live P3 cells, only $0.001 \pm 0.001\%$ of the BrdU$^+$ cells were RET-P1$^+$ in pellets where the E15 cells were mixed with fixed P3 cells (in each case the results are expressed as the mean $\pm$ s.d. of three cultures). These findings suggest that if surface-bound signalling molecules are responsible for mediating the rod-promoting activity of neonatal retinal cells, then the molecules either must have been inactivated by the brief fixation, or they must have to be present on living cells in order to function.

The rod-promoting activity can operate across polycarbonate filters

To test whether the rod-promoting activity of neonatal retinal cells can operate across a small-pore filter, neonatal neural retinal explants were cultured on a floating polycarbonate filter with a pore diameter of 0.8 μm. The explant was then covered by a similar filter, and a single E15 neural retinal cell pellet was placed on top, so that the pellet and explants were directly opposite each other but separated by a single polycarbonate filter (Fig. 2, mix-1). When the E15 pellets were dissociated into single cells after 7-8 days and immunostained with RET-P1 antibody, there was about a 6-fold increase in the proportion of cells that were opsin$^+$ rods compared to when the E15 cells were cultured over E15 neural retinal explants (Fig. 2). Similar results were obtained when pellets, rather than explants, of neonatal neural retina were used (not shown). When frozen sections of E15 pellets that had been cultured over neonatal explants were cut and immunolabelled with RET-P1 antibody in order to study the distribution of the opsin$^+$ rods in relation to the underlying neonatal explant, the rods were not preferentially clustered in the region facing the neonatal explant (Fig. 3). When the same transfilter experiments were performed, but with the E15 pellet placed 0.5-1 mm away from the edge of the group of underlying neonatal explants, no significant effect of the neonatal pellet was observed on the proportion of opsin$^+$ rods that developed in the E15 pellet (Fig. 2, mix-2), suggesting that the rod-promoting activity might operate over only a relatively short distance. The presence of the neonatal cells did, however, cause a small, but significant, increase in the total number of cells found in the displaced E15 pellet (Fig. 2).
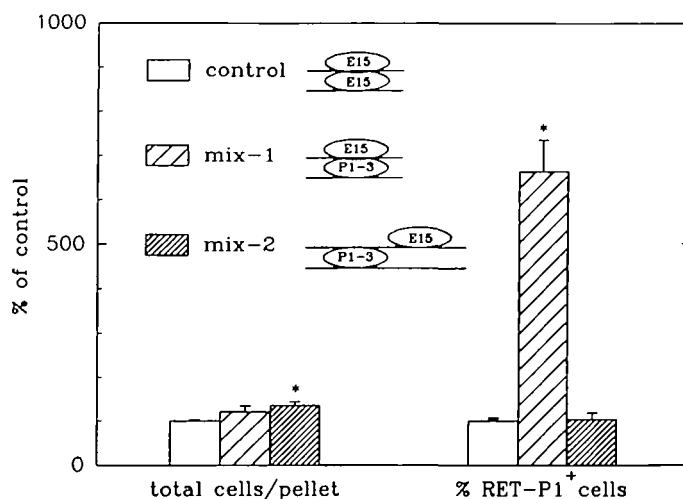
Because cell processes could extend through the 0.8

Fig. 2. Transfilter effects of neonatal retinal explants on the development of RET-P1$^+$ rods in E15 neural retinal cell pellets *in vitro*. E15 retinal cell pellets were cultured with neonatal (or E15 in controls) retinal explants, separated by a polycarbonate filter with 0.8 μm diameter pores as described in Materials and Methods. In each case, a single E15 pellet was cultured over a group of about 20 pieces of neonatal retina, which, for simplicity, are shown in the figure as a single entity. After 7-8 days *in vitro*, the uppermost E15 pellets were processed as in Fig. 1. The total number of cells per E15 pellet at the start of the culture was $(16 \pm 3) \times 10^4$, and in the control cultures after 7-8 days *in vitro* the total number rose to $(96 \pm 21) \times 10^4$. The number of RET-P1$^+$ cells per 100,000 total cells at this time was 19 ± 13.

μm pores in the filters used in the above experiments (Wartiovaara et al. 1974; Saxén et al. 1976; Saxén and Lehtonen, 1978), it was possible that the transfilter rod-promoting effect of neonatal explants involved direct contact between the neonatal and embryonic cells. To test this possibility, the experiments were repeated with polycarbonate filters with 0.01 μm diameter pores (Table 1). Although the rod-promoting effect of neonatal explants was less than that seen with filters with 0.8 μm diameter pores, there was a significant effect across these small-pore filters (Fig. 4); out of the 9 experiments using filters with 0.01 μm pores summarized in Fig. 4, a significant rod-promoting effect was seen in 6. Because this pore size is too small to permit any cell process to pass through, we conclude that cell-cell contact is not required for the rod-promoting effect.

Specificity of rod-promoting activity

To test whether other tissues besides neonatal retina were able to promote rod development in E15 neural retinal cell pellets, E15 pellets were cultured over explants of neonatal rat cerebrum, cerebellum or thymus, or E15 or P45 retina, with the two tissues separated by a polycarbonate filter with 0.8 μm pores. As shown in Fig. 5, all of the explants increased the total number of cells in the E15 pellets. None of these tissues, however, increased the proportion of opsin$^+$ rods in E15 pellets, and thymus and P45 retina
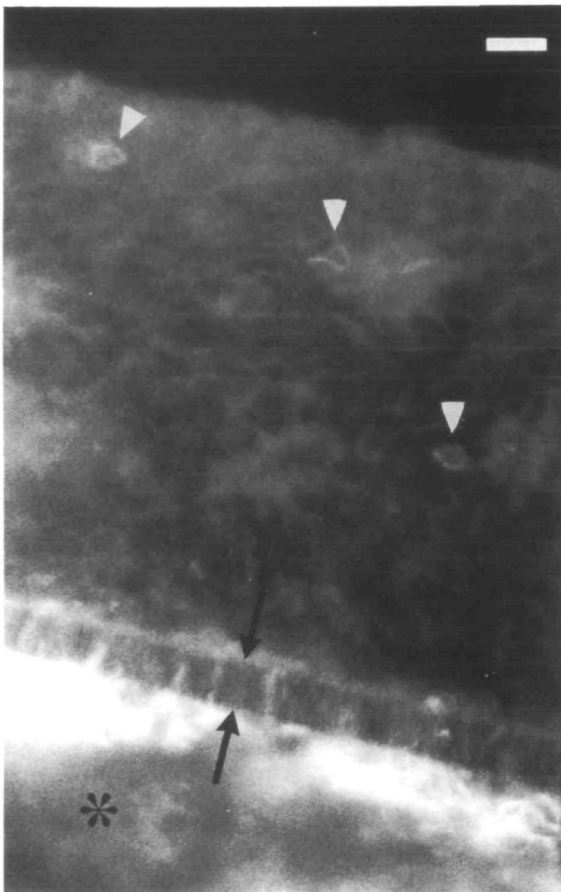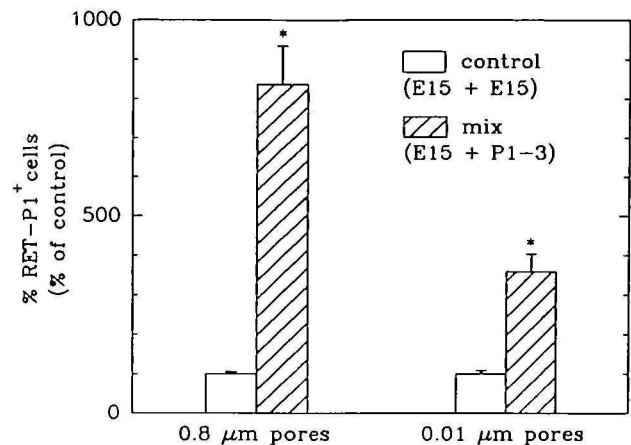
Fig. 4. Rod-promoting effects of neonatal neural retinal explants across polycarbonate filters with different pore diameters. E15 neural retinal cell pellets were cultured with either E15 or P1-P3 neural retinal explants, separated by a filter with either 0.01 μm or 0.8 μm diameter pores, as shown in Fig. 2 (control and mix-1). After 7-8 days *in vitro*, the uppermost E15 pellets were processed as in Fig. 1. The total number of cells per E15 pellet at the beginning of the culture was $(15 \pm 3) \times 10^4$, and the numbers of cells per pellet in the control cultures after 7-8 days *in vitro* with 0.01 μm and 0.8 μm diameter pores rose to $(84 \pm 27) \times 10^4$ and $(85 \pm 28) \times 10^4$, respectively. The numbers of RET-P1$^+$ cells per 100,000 total cells at this time were 19 ± 19 and 8 ± 4, respectively. Data shown in the figure are the mean $\pm$ s.e.m. of 9 separate experiments, each done in triplicate.

Fig. 3. Distribution of RET-P1$^+$ rods in a 20 μm frozen section of an E15 neural retinal cell pellet that was cultured over neonatal neural retinal explants (asterisk) for 7-8 days. Only about 10-15 opsin$^+$ rods were seen per section (three of which are seen here; arrowheads), and these cells were not concentrated close to the polycarbonate filter (arrows), which separated the E15 pellet from the underlying neonatal explants. Scale bar=15 μm.

Table 1. *Relationship of filter characteristics and rod-promoting effect*

Pore size (μm)	Pore density (pores cm^{-2})	Total pore area (mm^2 cm^{-2})	Thickness of filter (μm)	Fold increase in rods*
0.8	3×10^7	15.07	10	8.4
0.01	6×10^8	0.05	6	3.6

*Taken from Fig. 4.

significantly decreased it. Thus, the rod-promoting activity seems to be specific to neonatal retina.

To test whether neonatal neural retina promoted the development of cell types other than rods, E15 neural retinal pellets were again cultured over E15 or neonatal neural retinal explants, with the two tissues separated by a filter with 0.8 μm pores. After 7-8 days *in vitro*, the E15 pellets were dissociated and immunolabelled with

RET-P1 and the HPC-1 monoclonal antibody, which specifically labels amacrine cells (Barnstable, 1980). Although, as expected, there was a 6- to 7-fold increase in the proportion of opsin$^+$ rods when E15 pellets were cultured over neonatal retinal explants (compared to when they were cultured over E15 explants), there was no significant difference in the proportion of HPC-1$^+$ amacrine cells under these two conditions (Fig. 6).

Discussion

We showed previously that neonatal neural retinal cells, when added to pellet cultures of E15 neural retinal cells, greatly increase the proportion of the E15 cells that give rise to opsin$^+$ rods after 5-6 days *in vitro* (Watanabe and Raff, 1990). To demonstrate this rod-promoting influence of neonatal retinal cells, we prelabelled the dividing E15 cells with BrdU in order to distinguish them and their progeny from the neonatal cells and their progeny; at the end of the culture period, the cells were immunolabelled with both anti-opsin and anti-BrdU antibodies, and the proportion of BrdU$^+$ cells that expressed opsin was determined. In the present study, we have shown that the rod-promoting effect can operate across a polycarbonate filter that separates the embryonic and neonatal neural retinal cells; in this way the effect can be measured much more readily, without the need to prelabel the E15 cells. We
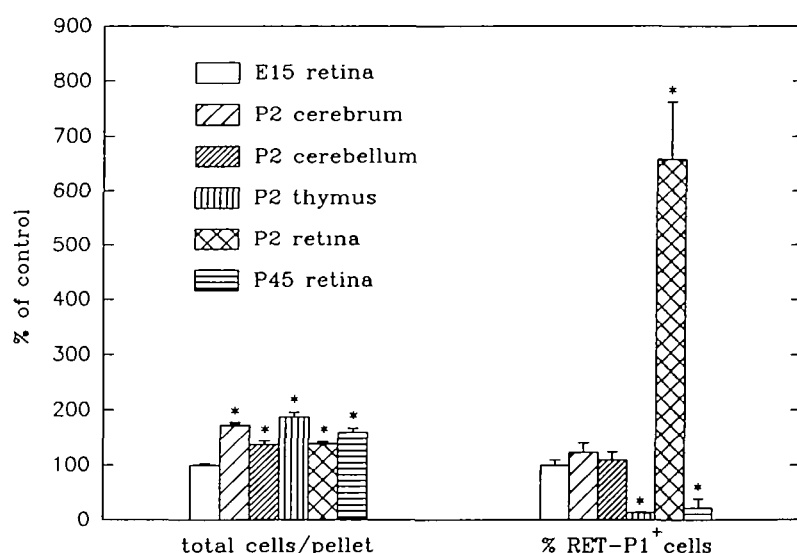
Fig. 5. Tissue and age specificity of the rod-promoting activity. E15 neural retinal cell pellets were cultured with explants of P2 cerebrum, cerebellum, thymus, or retina, or of E15 or P45 retina, using the transfilter arrangement shown in Fig. 2 (control and mix-1). After 7-8 days *in vitro*, the uppermost E15 pellets were processed as in Fig. 1. The total number of cells per pellet at the beginning of the culture was (16 ± 4) × 10^4 cells, and the total number of cells per pellet in the control cultures after 7-8 days *in vitro* rose to (99 ± 17) × 10^4. The number of RET-P1$^+$ cells per 100,000 total cells at this time was 22 ± 24.

have used this transfilter culture system to characterize further the rod-promoting activity.

Our most important finding is that the rod-promoting effect can operate across a filter with a pore diameter as small as 0.01 μm. As the plasma membrane alone is at least 0.005 μm thick, it seems highly unlikely that a cell process could penetrate through a 0.01 μm pore, as it would have to do so without cytoplasm or a cytoskeleton. Thus, at least part of the rod-promoting effect appears not to require contact between the neonatal and embryonic cells and is, therefore, presumably
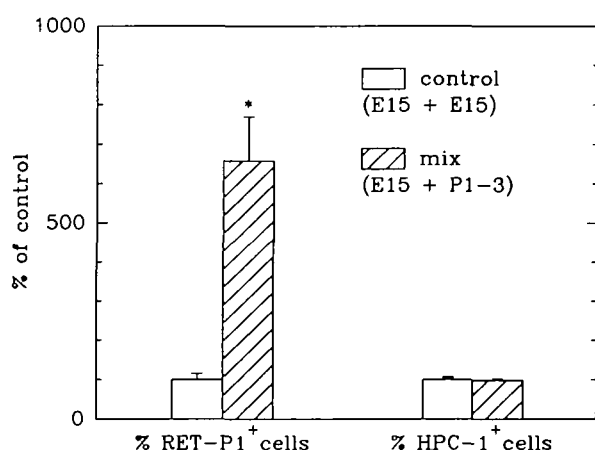

Fig. 6. Cell-type specificity of the rod-promoting activity. E15 neural retinal cell pellets were cultured with either E15 or P1-P3 neural retinal explants using the transfilter arrangement illustrated in Fig. 2 (control and mix-1). After 7-8 days *in vitro*, the E15 pellets were dissociated into single cells, counted, and immunostained with RET-P1 monoclonal antibody to identify the rod cells, and with HPC-1 monoclonal antibody to identify the amacrine cells. The total number of cells per E15 pellet at the beginning of the culture was (20 ± 2) × 10^4, and the total number of cells per pellet in the control cultures after 7-8 days *in vitro* rose to (110 ± 19) × 10^4. At this time, the number of RET-P1$^+$ cells per 100,000 total cells was 24 ± 9, while the proportion of HPC-1$^+$ cells was 13 ± 3%.

mediated by diffusible molecules. Our finding that lightly fixed neonatal retinal cells do not have rod-promoting activity when mixed in the same pellet as E15 retinal cells is consistent with this conclusion. Similar transfilter experiments have been used extensively to study the nature of the signals involved when salivary mesenchyme or spinal cord induces metanephric mesenchyme to form kidney tubules (Wartiovaara et al. 1974; Saxén et al. 1976; Saxén and Lehtonen, 1978). In this system, an interposed polycarbonate filter with 0.5 μm diameter pores completely blocks the inductive effect of salivary mesenchyme, while a filter with 0.05 μm pores completely blocks the effect of spinal cord, suggesting that, in both cases, cell contact is required for the induction. Although the rod-promoting effect clearly occurred across filters in our experiments, it was less dramatic than when the neonatal and embryonic retinal cells were mixed in the same pellet (Watanabe and Raff, 1990). Moreover, the magnitude and consistency of the effect decreased with decreasing pore size, which is perhaps not surprising, as the total pore area in these filters decreases greatly as the pore diameter decreases; the total pore area is 300-fold less in a filter with 0.01 μm diameter pores than it is in one with 0.8 μm diameter pores (Table 1).

Although the results of the transfilter experiments suggest that at least part of the rod-promoting effect is mediated by secreted, diffusible molecules, it seems that these molecules might not act as stable, long-range signals. We were unable to demonstrate the effect when E15 neural retinal cells were cultured together with a large excess of neonatal neural retinal cells growing on separate filters. Nor have we been able to demonstrate an effect of extracts of neonatal neural retina, even when they were added daily to the culture medium (unpublished observation). Moreover, although the rod-promoting effect was seen in transfilter experiments in which the E15 neural retinal cell pellet was placed directly over neonatal neural retinal explants, it was not seen if the E15 pellet was displaced 0.5-1 mm from the underlying neonatal explants. We found previously that

the presence of a 50-fold excess of E15 neural retinal cells greatly decreased the proportion of neonatal retinal neuroepithelial cells that developed into opsin$^+$ rods in pellet cultures (Watanabe and Raff, 1990). As a possible explanation for this finding, we suggested that the rod-promoting signal(s) produced by neonatal retinal cells might act only at close range, so that its ability to influence neighbouring neonatal cells was decreased by the presence of intervening E15 cells; the finding that the presence of a 50-fold excess of meningeal cells or embryonic brain cells had an even greater inhibitory effect than E15 neural retinal cells was consistent with this explanation: whereas E15 retinal cells might themselves eventually produce the rod-promoting signal(s), nonretinal cells might not (Watanabe and Raff, 1990). Our transfilter experiments, as well as our finding that lightly fixed neonatal cells decreased the proportion of embryonic cells that developed into opsin$^+$ rods in mixed pellet cultures, are consistent with this explanation and suggest that at least part of the rod-promoting effect is mediated by a short-range diffusible signal(s). This mechanism of signalling is different from the best-studied example of cell-cell signalling in the developing *Drosophila* retina, where the development of the R7 photoreceptor depends on the R7 precursor cell binding a non-secreted signal bound to the surface of a neighbouring cell (R8) (Krämer et al. 1991).

In principle, there are a number of ways in which a secreted signalling molecule could act over only a short distance. It could be released in very small amounts, as seems to be the case for some signalling molecules that operate in the perivitalline space during the early development of the *Drosophila* embryo (Stein et al. 1991). It could be broken down, or otherwise inactivated, soon after it is released, as is the case for acetylcholine at the vertebrate neuromuscular junction (Kuffler et al. 1984). It could bind to the extracellular matrix, as is thought to be the case for basic fibroblast growth factor (Rifkin and Moscatelli, 1989). It could self-inactivate by oligomerization, as is the case for hydra head activator (Schaller et al. 1989). It is not clear which, if any, of these mechanisms is responsible for the apparent short-range action of the rod-promoting signal(s). There are other hints that, if extracellular signals play a part in cell fate determination in the vertebrate retina, as seems highly likely, then at least some of them probably act over short distances: there are well-described gradients of cell differentiation across the developing vertebrate retina (Sidman, 1961; Donovan, 1966; Carter-Dawson and La Vail, 1979; Young, 1985; Turner et al. 1990), for example, and clonal analyses have suggested that two daughter cells might differentiate into different cell types at or after their final cell division (Turner and Cepko, 1987; Holt et al. 1988; Wetts and Fraser, 1988; Turner et al. 1990), although the extensive cell death that normally occurs in the developing retina (Young, 1984) makes it difficult to interpret the latter findings. In our transfilter experiments, we were surprised to find that the opsin$^+$ rods that developed in E15 pellets cultured over

neonatal explants were not concentrated on the filter side of the E15 pellet. This finding suggests that the rod-promoting signal(s) emanating from the neonatal explant is able to diffuse for a distance that is at least equal to the thickness of the E15 pellet, which was about 200-300 μm; alternatively, the signal might induce neighbouring cells to secrete more signal, which would thereby be relayed from cell to cell across the pellet, as occurs in chemotactic signalling among *Dictyostelium* amoebae (Gerisch, 1982).

The transfilter culture system facilitated the study of the specificity of the rod-promoting activity, in terms of the tissues that make it, the time that it is produced, and the differentiation pathways that are affected by it. Of the tissues tested, we found that only neonatal retina, in which rods are developing, produce the rod-promoting signals; explants of P2 thymus, cerebrum or cerebellum, or E15 or P45 retina did not have such activity. All of the tissues tested demonstrated a small increase in the total number of cells in the overlying E15 retinal cell pellet, but the meaning of this finding is unclear. It is also unclear which cell type(s) in the neonatal retina produces the rod-promoting signal(s). The rod-promoting activity also showed some specificity in the differentiation pathways that are affected by it: whereas neonatal retina increased the proportion of opsin$^+$ rods that developed in an overlying E15 pellet, it did not increase the proportion of amacrine cells that developed in the same E15 pellet, suggesting that the signals produced by neonatal retina do not act non-specifically to increase cell differentiation.

Our findings do not suggest how the diffusible signal(s) produced by neonatal retina acts to increase the number of rods found in E15 pellets - hence our use of the phrase "rod-promoting activity". In principle, it could act by stimulating multipotential precursor cells to commit to rod development, by promoting the differentiation of committed rod precursors, by promoting the survival of rods or their immediate precursors, or even by inhibiting multipotential precursor cells from committing to non-rod fates, thereby increasing commitment to rod development by default. Recent independent experiments by Altshuler and Cepko (1992), however, suggest that the diffusible rod-promoting signal(s) produced by neonatal retinal cells influences cell fate determination rather than cell survival or just terminal differentiation. In their experiments, the signalling and responding cells were embedded in collagen gels and could communicate over distances of at least 5 mm. The reason for this difference in signalling range in the two experimental systems is unclear.

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