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Ganglion cells influence the fate of dividing retinal cells in culture

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SUMMARY

The different retinal cell types arise during vertebrate development from a common pool of progenitor cells. The mechanisms responsible for determining the fate of individual retinal cells are, as yet, poorly understood. Ganglion cells are one of the first cell types to be produced in the developing vertebrate retina and few ganglion cells are produced late in development. It is possible that, as the retina matures, the cellular environment changes such that it is not conducive to ganglion cell determination. The present study showed that older retinal cells secrete a factor that inhibits the production of ganglion cells. This was shown by culturing younger retinal cells, the test population, adjacent to various ages of older retinal cells. Increasingly older retinal cells, up to embryonic day 9, were more effective at inhibiting production of ganglion cells in the test cell population. Ganglion cell production was restored when ganglion cells were depleted from the older cell population. This suggests that ganglion cells secrete a factor that actively prevents cells from choosing the ganglion cell fate. This factor appeared to be active in medium conditioned by older retinal cells. Analysis of the conditioned medium established that the factor was heat stable and was present in the <3 kDa and >10 kDa fractions. Previous work showed that the neurogenic protein, Notch, might also be active in blocking production of ganglion cells. The present study showed that decreasing *Notch* expression with an antisense oligonucleotide increased the number of ganglion cells produced in a population of young retinal cells. Ganglion cell production, however, was still inhibited in cultures using antisense oligonucleotide to *Notch* in medium conditioned by older retinal cells. This suggests that the factor secreted by older retinal cells inhibits ganglion cell production through a different pathway than that mediated by Notch.

Key words: Chick, Determination, Induction, Notch, Ganglion cell, Retina

INTRODUCTION

The vertebrate central nervous system is composed of a great diversity of cell types. These cell types arise from a seemingly homogenous population of cells that makes up the neural tube. The fates of individual cells appear to be determined by a variety of mechanisms, which are poorly understood. Being composed of just seven major cell types, the neural retina is a valuable model used to study the mechanisms that determine the fate of cells in the developing nervous system. The retina arises as an outpocketing of the neural tube early in development (Dowling, 1987; Rodieck, 1973). The progeny of any one retinal progenitor cell in this outpocketing can differentiate into any one of the major cell types that comprise the mature retina (Turner and Cepko, 1987; Turner et al., 1990; Holt et al., 1988; Wetts and Fraser, 1988). Current evidence suggests that the fate of individual retinal cells is determined by a combination of cell-intrinsic mechanisms and of various cell-cell interactions, which are not yet fully characterized.

The ganglion cell phenotype appears to be the default state of all progenitor cells in the early developing retina. When retinal progenitor cells are allowed to differentiate isolated from other retinal cells in vitro, most of the cells differentiate as ganglion cells (Reh, 1992; Austin et al., 1995). Furthermore, ganglion cells start to differentiate within minutes following mitosis (Waid and McLoon, 1995), which suggests that the ganglion cell fate is inherited by cells rather than acquired during postmitotic events. Finally, the ganglion cell is one of the first cell types to be produced in vertebrate retina, and few ganglion cells are generated late in development (Rubinson and Cain, 1988; Snow and Robson, 1994; Belecky-Adams et al., 1996). These results suggest that cells will differentiate into ganglion cells in the absence of instructive signals from differentiated cells. The question remains as to why all retinal cells do not differentiate as ganglion cells. One possibility is that, as the first cells differentiate into ganglion cells, they express factors that actively inhibit production of more ganglion cells and promote determination of other cell types.

Several studies provide evidence that cell-cell interactions play a major role in determining cell fate (reviewed by Altshuler et al., 1991). Studies in which cells from early developing retina were cultured with cells from older retina showed an increased production of rod cells in the younger cell population than would have appeared if cells from the younger retina were cultured alone (Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992). Rod cells are normally produced late in development (Carter-Dawson and LaVail, 1979; Altshuler et al., 1991). These findings can be interpreted to mean that

differentiated cells present in older retina, which would include ganglion cells, produce factors that promote development of late developing cell types. A similar result was obtained when a porous membrane that did not allow cells to touch separated the cells of the two ages. This indicates that the active factors are secreted and are soluble in the extracellular compartment. Several secreted factors have been identified that are expressed in developing retina and that promote development of the rod cell type. These include taurine, CNTF, bFGF and Sonic Hedgehog (Hicks and Courtois, 1992; Altshuler et al., 1993; Fuhrmann et al., 1995; Kirsch et al., 1996; Levine et al., 1997). A prediction suggested by these findings is that, in mixed cell cultures, while secreted factors released by older retinal cells promote development of late developing cell types in the young cell population, a concomitant reduction in production of early developing cell types, such as ganglion cells, should result.

In addition to findings that suggest a role for secreted factors, recent findings indicate that signaling through cell-cell contact mediated by Notch is involved in directing cell fate decisions. The *Notch* genes encode transmembrane, cell surface receptors that are involved in development of the *Drosophila* and vertebrate eye. In *Drosophila* and in vertebrates, the Notch proteins are activated by cell surface ligands, Delta and Serrate/Jagged (Arvantis-Tsakonas et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996). When expression of *CNotch-1* was blocked in early developing chick retina by application of antisense oligonucleotide, an increase was observed in the number of ganglion cells that developed and, conversely, when Notch was constitutively active, a decrease in ganglion cell production was observed (Austin et al., 1995). Similar results were obtained by manipulating expression of *CDelta-1* (Ahmad et al., 1997). One interpretation of this result is that activation of Notch by certain cell-cell contacts blocks cells from assuming the ganglion cell phenotype. This suggests the possibility that cell-cell-contact-mediated signaling is important in early cell fate decisions and that secreted factors are important in late phenotypic decisions. If this is the case, then it may be that factors that affect rod cell production have no effect on ganglion cell production.

The study reported here assessed the relative roles of secreted factors and Notch-mediated cell-cell signaling in regulation of ganglion cell production in the developing retina. The results showed that older retinal cells produce a secreted factor or factors that inhibit development of additional ganglion cells and that ganglion cells themselves may be a major source of the factor. The findings of this study also suggest that, among the factors regulating production of ganglion cells, the secreted factor acts at a higher level or in a separate pathway than does Notch.

MATERIALS AND METHODS

Animals

Fertilized chicken eggs, pathogen-free White Leghorn crossed with Rhode Island Red, were obtained from the University of Minnesota Poultry Research Center. The eggs were incubated at 37°C in an egg incubator. Chicks with retinas depleted of ganglion cells were prepared using a technique described by Hughes and McLoon (1979). Briefly, the embryos were removed from the shells after 3 days of incubation and placed in culture chambers. The primordial optic tecta were destroyed by applying heat to the tectal surface by electrocautery. Cultured embryos with tectal ablations were maintained in a forced-draft tissue culture incubator at 37°C, 95% relative humidity and 1% CO_2. The retinas from some of the embryos were processed for immunohistochemistry with the RA4 antibody to confirm the loss of ganglion cells by E14.

Cell culture

The cell culture technique used in this study was adapted from Watanabe and Raff (1992). Embryonic day 4 (E4) chick retinas were dissected in culture medium and dissociated by gentle trituration. Cells were pelleted by centrifugation at 500 ***g*** for 4 minutes. This pellet, the test population, was placed on a polycarbonate membrane with a maximum pore size of 0.01 μm and covered with another membrane. Approximately 10^7 cells were used for each test cell pellet. A pellet of cells, the conditioning cell population, prepared the same way from E4, E7, E9, E14 or E14 ganglion cell-depleted retinas, was placed directly adjacent to the test cell population. Approximately three times the number of cells used in the test cell pellets was used in the conditioning cell pellet. The culture sandwich was maintained in F12 medium supplemented according to Bottenstein et al. (1980) with the addition of 2 mM glutamate and 1.5 μg/ml bromodeoxyuridine (BrdU). Cultures were incubated at 37°C and 5% CO_2 for 24 hours.

Immunocytochemistry

Cultures were fixed in 70% EtOH for 10 minutes and cryoprotected in 20% sucrose/phosphate buffer for 1 to 2 hours. The intact culture sandwiches were embedded in 10% tragacanth gum/20% sucrose/0.1 M phosphate buffer and sectioned at 10 μm with a cryostat. The sections were mounted on chrome alum/gelatin-coated glass slides. Sections were fixed to the slides with 1% paraformaldehyde in phosphate buffer for 2 minutes and rinsed in phosphate-buffered saline (PBS). DNA in the sections was denatured by incubation in 0.3 M NaOH for 2 minutes. The sections were rinsed in PBS and nonspecific antibody binding was blocked by incubation in 10% normal goat serum/ 0.3% Triton X-100/ PBS. These sections were incubated for 1 hour in an antibody to BrdU (1:10; Becton Dickinson), rinsed in PBS and incubated for 1 hour in goat anti-mouse IgG affinity-purified Fab fragment conjugated to fluorescein isothiocyanate (1.8 μg/ml; Jackson ImmunoResearch Labs Inc.). The sections were again rinsed in PBS and then incubated for 1 hour in goat anti-mouse IgG affinity-purified Fab fragment (80 μg/ml; Jackson ImmunoResearch Labs Inc.) to block any remaining binding sites. Sections were rinsed in PBS, incubated for 1 hour in an antibody to ganglion cells, RA4 (hybridoma culture supernatant; McLoon and Barnes, 1989), rinsed in PBS again and incubated for 1 hour in affinity-purified goat anti-mouse IgG conjugated to lissamine rhodamine B sulfonyl chloride (0.8 μg/ml; Jackson ImmunoResearch Labs Inc.). Following another rinse in PBS, sections were counterstained for 60 seconds with 1.5×10^{-6} μM DAPI. The stained sections were rinsed again in PBS and coverslipped with buffered glycerin mounting media (pH 7.0). These sections were viewed and photographed with an epiflourescence microscope, and the number of cells expressing particular labels was counted in the test cell populations. At least five fields were counted from each culture. At least five cultures were used for each datum point. All data are expressed as the mean ± s.e.m. The results of different experimental groups were compared using the Student's *t*-test.

Dying cells were identified in the sections of cultures using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling, TUNEL, (Gavrieli et al., 92). Sections were rinsed in buffer and incubated for 1 hour at 37°C in digoxigenin-labeled UTP and TdT. Following three rinses, the sections were incubated for 30 minutes in an antibody to digoxigenin conjugated to fluorescein. Following further rinses, the sections were covered with a glass coverslip and SlowFade mounting media (Molecular Probes). Analysis was done as described above. In some cases, the same section after TUNEL analysis or adjacent sections were stained with Thionin. Pyknotic cells were counted in these sections as an indicator of cell death.

Conditioned medium

For conditioned medium studies, E9 chick retinas were dissociated and the cells were plated onto laminin-coated coverslips at a density of 1×10^5 cells/mm^2. Cultures were incubated at 37°C and 5% CO_2. After 24 hours, the medium was removed from the culture dishes, centrifuged at 500 ***g*** for 5 minutes and filtered through a low protein binding 0.22 μm PVDF membrane to remove cell debris. In some cases, the conditioned medium was size-fractionated prior to use with 3 kDa and 10 kDa Centriprep filters (Amicon), according to the manufacturer's instructions. Fresh medium was also size-fractionated by the same method as the conditioned medium and the corresponding missing fractions were added to the fractions of conditioned medium. This gave three differently sized fractions: >10 kDa, 3-10 kDa and <3 kDa. Immediately following preparation, conditioned medium was used to culture E4 retinal cell pellets as described above.

Antisense oligonucleotide

Antisense oligonucleotide was added to cultures to reduce expression of Notch. The sequence for the antisense oligonucleotide was taken from Austin et al. (1995). The oligonucleotide was designed to hybridize with the *lin-12/Notch* repeat region of *CNotch-1* and had the following sequence: 5′-CCAGCACTGCAGTGACTGTGAGC-3′. The 23-mer oligonucleotide was synthesized with phosphorothioate linkages between all bases. The oligonucleotide was extracted and precipitated to remove salts and organics. A missense oligonucleotide was used as a control. Cultures of the retinal cells, prepared as described above, were incubated in medium containing 25 μM oligonucleotide for 24 hours.

RESULTS

Mixed cell cultures

The main goal of this study was to examine environmental factors that influence ganglion cell determination in the developing retina. More specifically, the aim of this study was to determine whether differentiated cells in the developing retina release factors that influence the fate of cells that differentiate later. Young embryonic retinal cells at embryonic day 4 (E4), an age when ganglion cells are just starting to be generated, were cultured adjacent to other embryonic retinal cells taken from various ages, particularly ages at which ganglion cells had already developed. The two populations of cells were separated by a membrane that prevented cell-cell contact but that allowed soluble molecules to pass (Fig. 1), an approach used previously by Watanabe and Raff (1992). The thymidine analogue, bromodeoxyuridine (BrdU), was added to the culture medium to label dividing cells. This allowed the cells that divided in culture to be distinguished from those that differentiated in vivo. After 24 hours, the co-cultures were fixed and processed for immunohistochemistry using an antibody to BrdU and an antibody to RA4, which recognizes ganglion cells within minutes after mitosis (Fig. 2; McLoon and Barnes, 1989; Waid and McLoon, 1995). Cells that were double labeled with RA4 and BrdU were considered to be ganglion cells that developed in the culture system. This culture system was used to address the question of whether older retinal cells released a factor that prevents development of more ganglion cells.

More ganglion cells developed in the E4 retinal test population cultured adjacent to other E4 cells than when cultured adjacent to older populations of retinal cells (Fig. 3). When E4 cells were cultured adjacent to other E4 cells, 24% of the test population of cells that divided in culture, as evident by BrdU labeling, was also labeled with RA4. However, when E7

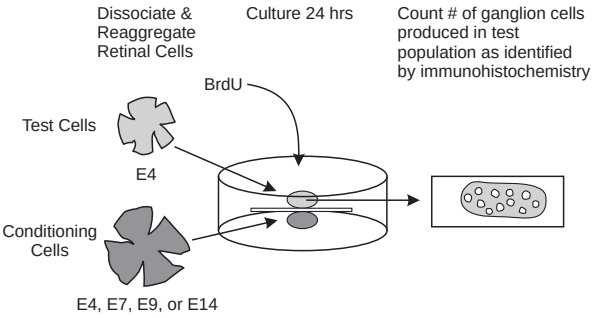

Fig. 1. Schematic of the mixed cell culture design. A test population of cells from E4 chick retina was dissociated, reaggregated and cultured opposite a similarly prepared population of cells from either E4, E7, E9 or E14 retina. The two populations were separated from each other by a membrane with pore size of 0.01 μm and cultured in medium containing BrdU. After 24 hours, the cultures were fixed and sectioned. Immunohistochemistry was used to quantify the number of ganglion cells produced in culture in the E4 test population.

retinal cells, an age at which more than half the ganglion cells have been produced, were cultured adjacent to E4 cells, 10% of the E4 test population that underwent division in culture was labeled by the RA4 antibody. When E4 cells were cultured adjacent to E9 or E14 cells only 5% of the test population that divided in culture were labeled with the RA4 antibody.

The effect of different aged conditioning populations on E4 test cells could have been due to a decrease in the rate of cell division in the younger population or to an increase in death of selected cells in the younger population. Cell division and cell death were quantified in the test cell population with each co-culture pairing. To assess differences in cell division, the

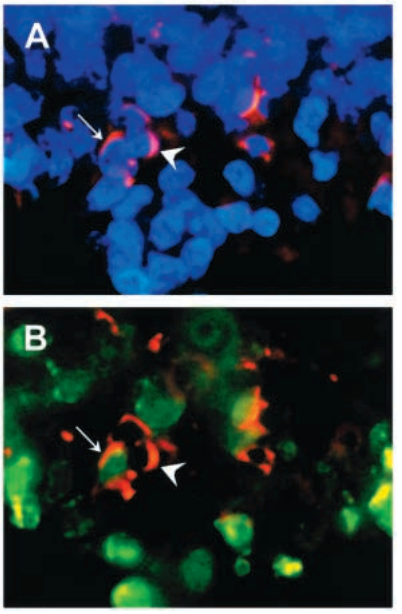

Fig. 2. Micrographs of a section from an E4/E4 co-culture stained (A) with an antibody to RA4 (red) to show ganglion cells and with DAPI (blue) to show all cells or (B) with an antibody to RA4 (red) and an antibody to BrdU (green) to show cells that divided in vitro. The arrow points to an RA4$^+$ cell with BrdU label in its nucleus, indicating a ganglion cell that underwent division in culture. The arrowhead points to a ganglion cell that did not undergo division in culture.

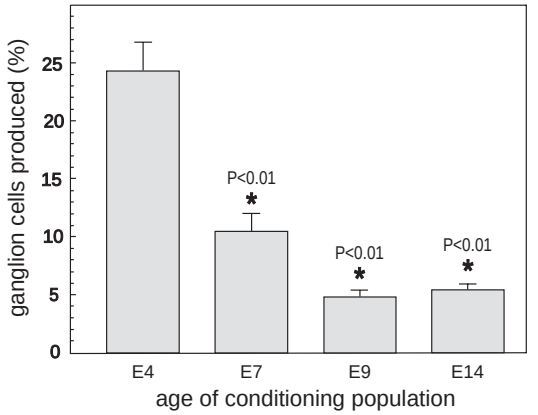

Fig. 3. Ganglion cell production was decreased in young retinal cell populations cultured adjacent to older retinal cells. The graph compares the percent of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the E4 test population when cultured adjacent to E4, E7, E9 or E14 retinal cells. Increasingly older conditioning cells, up to E9, were more effective at reducing ganglion cell production in the test cell population. Asterisks indicate ganglion cell production significantly different than in the E4/E4 co-cultures.

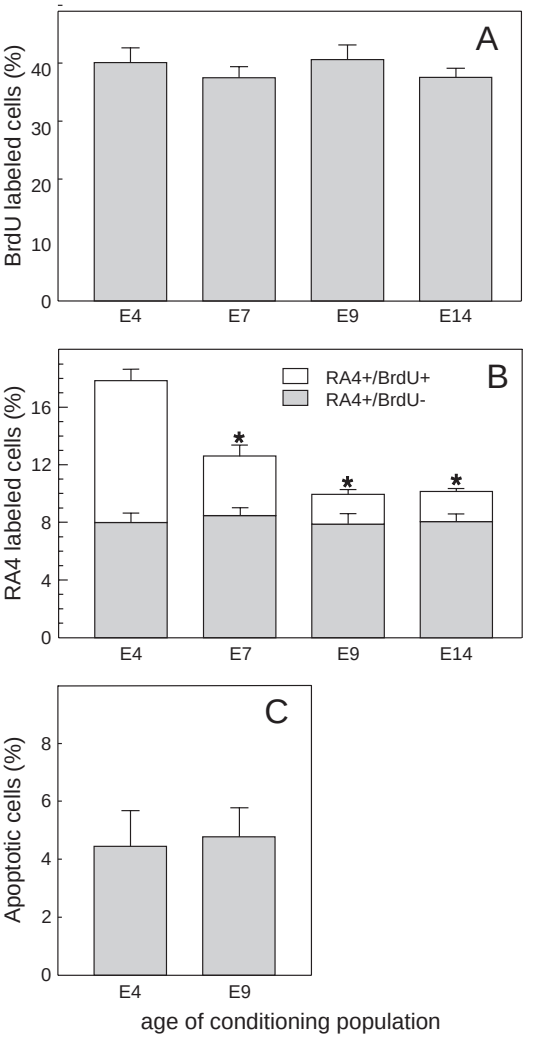

Fig. 4. Cell division and cell death in the test population were not affected by different-aged conditioning cells. (A) The graph compares the percentage of all cells in the E4 test population that divided in culture ($BrdU^+$). There were no statistically significant differences among the different experimental groups. (B) The graph compares the number of ganglion cells produced while in culture and produced in vivo in the test population for each conditioning population. The open bar represents ganglion cells that were produced in the culture ($RA4^+/BrdU^+$), and the solid bars represent the ganglion cells that were produced in vivo ($RA4^+/BrdU^-$). The differences among the different experimental groups in the number of ganglion cells were only in those produced in vitro; there were no statistically significant differences in the number of ganglion cells produced in vivo among the different experimental groups. This suggests that death of ganglion cells was not responsible for the differences in the number of ganglion cells. Ganglion cell death would be expected to affect ganglion cells produced in vivo and in vitro. (C) The graph compares the percentage of apoptotic cells in the E4 test population for each conditioning population. There were no statistically significant differences in the percentage of apoptotic cells among the different experimental groups.

percentage of cells that incorporated BrdU was determined in the test cell population for each culture condition. There was no statistical difference in the percentage of BrdU-labeled cells between any of the culture conditions examined (Fig. 4A). This excludes the possibility that the decrease in the percentage of $RA4^+$ cells observed, when E4 cells were cultured adjacent to older retinal cells, was due to a change in cell division. Cell death in the cultures was examined in two ways. First, if cell death targeted ganglion cells in certain conditions, then one would expect an overall reduction in the total number of ganglion cells in the test population including those produced in vitro and in those produced in vivo prior to removing the retinas to culture. The number of ganglion cells produced prior to culturing was quantified in the test population under each condition by counting the $RA4^+/BrdU^-$ cells. There was no significant difference in this population of cells between any of the culture conditions examined (Fig. 4B). The only significant differences in the number of ganglion cells among the different conditions were in those cells produced in vitro (i.e. $RA4^+/BrdU^+$, Fig. 4B). Second, the percentage of dying cells in the E4 test population was also compared among the various conditions. Dying cells were identified by end labeling DNA with a histochemical marker. Dying cells with fragmented DNA were heavily labeled. Again, no statistical difference was found in the percentage of dying cells between any of the culture conditions (Fig. 4C). Similarly, the number of pyknotic cells per square millimeter identified in Thionin-stained sections of the E4 test population from E4/E4 and E4/E9 cocultures were statistically the same. These data do not support the possibility that differences in cell death were responsible for the different number of $RA4^+$ cells observed in the different culture conditions. This suggests that older retinal cells secrete some factor that blocks development of ganglion cells.

A major change in retina from E4 to E7 to E9 is the progressive addition of more ganglion cells. It is possible that, once ganglion cells differentiate, they produce a signal that inhibits development of more ganglion cells. To examine this possibility, E4 retinal cells were cultured adjacent to an older population of cells that had very few ganglion cells. Retinas 'depleted' of ganglion cells were made by removing the tectum from embryos at a very young age and allowing the embryos to develop until E14. In the absence of the central target for their axons, the ganglion cells degenerate

prior to this age (Hughes and McLoon, 1979). Immunohistochemistry with the RA4 antibody confirmed the loss of retinal ganglion cells in these embryos by E14. When E4 cells were cultured opposite E14 retinal cells depleted of ganglion cells, 20% of the cells that divided in culture were $RA4^+$ (Fig. 5). However, when E4 cells were cultured opposite normal E14 retinal cells, only 5% of the cells that divided in culture were $RA4^+$. Both of these conditions showed similar rates of cell division and cell death in the test cell population. Thus, the results obtained with E14 retinal cells depleted of ganglion cells as the conditioning population looked similar to results obtained from E4/E4 co-cultures. In fact, there was no statistical difference between the number of ganglion cells produced in E4 cells when cultured opposite either other E4 cells or E14 cells depleted of ganglion cells (P=0.2). This result suggests that the ganglion cells in the older cultures are the source of a diffusable factor that blocks development of more ganglion cells.

Conditioned medium

The evidence points to a factor released from older retinal cells that inhibits ganglion cell development in this paradigm. It is possible that the older conditioning cells release enough factor that the culture medium would have sufficient activity to affect developing cells. If true, this would facilitate characterization of the factor. To test this hypothesis, culture medium was conditioned for 24 hours by cultures of dissociated E9 retinal cells. The medium was removed from the conditioning cultures, filtered and added to cultures of E4 retinal cells with BrdU to label dividing cells. After 24 hours, the cultures were fixed and assayed for ganglion cell production as described above. Fewer ganglion cells developed in the E4 retinal test population of cells cultured in E9 conditioned medium than when cultured in fresh medium. When E4 retinal cells were cultured in fresh medium, approximately 21% of the cells that divided in culture labeled with the RA4 antibody, compared to only 5% when cultured in conditioned medium (Fig. 6). It is possible that developing neuronal cells deplete conditioned medium of some needed nutrient for ganglion cell production. To test this, E6 forebrain cells were used to condition medium and E4 retinal cells were cultured in this conditioned medium. There was no significant difference in ganglion cell production between E4 cells cultured in fresh medium or forebrain-cell-conditioned medium (Fig. 6). Thus, E9 retinal cells apparently release a factor into medium with sufficient activity to alter cell fate. Furthermore, since E6 forebrain cells did not exhibit this activity, it suggests that the factor produced by retinal cells is not produced by all CNS neurons, or at least not at all stages of development.

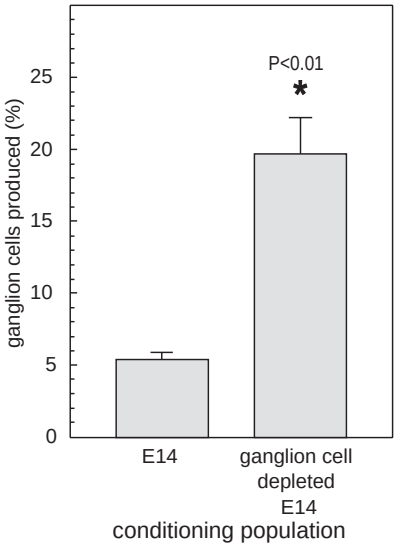

Fig. 5. Conditioning cell populations depleted of ganglion cells lost the ability to inhibit ganglion cell production in the E4 test cell population. The graph compares the percentage of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the test cell population when cultured adjacent to E14 retinal cells or E14 retinal cells depleted of ganglion cells. Ganglion cell production in the test population was restored to that seen with E4 conditioning cells when older retina depleted of ganglion cells was used for the conditioning population. The asterisk indicates ganglion cell production significantly different than the E14 control.

In order to further characterize the factor, conditioned medium was heated to 70°C for 15 minutes, a treatment that denatures many proteins. E4 retinal cells were cultured in the heat-treated conditioned medium and, after 24 hours, were fixed and assayed for the production of ganglion cells. Approximately 6% of the cells that divided in culture were $RA4^+$, which is similar to the percentage of $RA4^+$ cells produced in untreated conditioned medium (Fig. 6). This indicates that the factor responsible for inhibiting ganglion cell production is heat stable.

In order to characterize the approximate size of the factor produced by older retinal cells that inhibits ganglion cell production, conditioned medium was filter fractionated into high (>10 kDa), medium (3-10 kDa) and low (<3 kDa) molecular mass components. Each conditioned medium fraction was combined with its missing fractions prepared with fresh medium. Each reconstituted medium was used to culture E4 retinal cells. After 24 hours, ganglion cell production was assessed in these cultures. The medium-sized fraction did not affect ganglion cell production significantly (Fig. 6). Whereas, E4 retinal cells cultured with the <3 kDa and >10 kDa fractions had a significant reduction in the percentage of ganglion cells that was produced in the culture compared to fresh medium. There was no significant difference between the number of ganglion cells produced when the E4 cells were cultured in the low molecular mass fraction or whole conditioned medium. This suggests that the major factor or factors produced by the older retinal cells, and possibly by ganglion cells, that inhibits ganglion cell production is <3 kD in size.

Notch

A previous study reported that activation of the Notch protein in progenitor cells inhibited ganglion cell production in developing chick retina (Austin et al., 1995). It is possible that the factor partially characterized in the present study activates Notch, or it may be part of a separate parallel pathway for controlling cell fate. If the latter is true, then one mechanism may dominate. To test the relationship between these factors, E4 retinal cells were cultured in medium conditioned by E9 retinal cells or in an antisense oligonucleotide to *CNotch-1*, as used in Austin et al. (1995) or in a combination of both. In fresh medium, the addition of antisense oligonucleotide to block *Notch* expression resulted in an increased number of ganglion cells produced in culture, as reported previously (Fig. 7). Conditioned medium, together with the antisense oligonucleotide, had no effect on the number of ganglion cells produced in the E4 population compared to E9 conditioned medium alone (Fig. 7). Cultures treated with

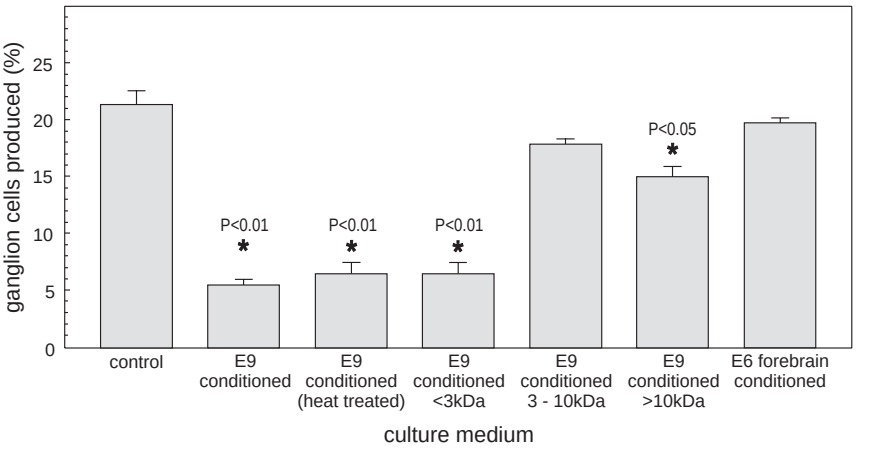

Fig. 6. The ganglion cell inhibitory factor recovered in medium conditioned by older retinal cells was <3 kDa and heat stable. Conditioned medium was harvested from 24 hour cultures of dissociated E9 retinal cells. The conditioned medium was used to culture reaggregated E4 retinal cells, the test cells. The graph compares the percentage of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the E4 test cell population in fresh medium (control), conditioned medium, conditioned medium heated to 70°C for 15 minutes, conditioned medium size fractionated and in medium conditioned by embryonic forebrain cells. Asterisks indicate ganglion cell production significantly different than the control.

missense oligonucleotides showed no change in ganglion cell production compared to cultures maintained in normal medium. This indicates that the factor produced by older retinal cells prevents cells from differentiating as ganglion cells even when *Notch* expression is reduced. This suggests that Notch may either play some other role than specifically directing the ganglion cell fate pathway or that its role is secondary and independent to the secreted factor produced by older retinal cells.

DISCUSSION

The primary aim of this study was to determine whether differentiated cells in the developing retina express factors that reduce further production of ganglion cells, one of the first cell types to develop in the retina. This was studied by culturing very young retinal cells, the test population, adjacent to retinal cells of various ages, the conditioning population, and then quantifying subsequent ganglion cell production in the test population. The presence of older retinal cells resulted in production of fewer ganglion cells in the test population. There was a greater effect with older conditioning cells up to embryonic day 9 (E9).

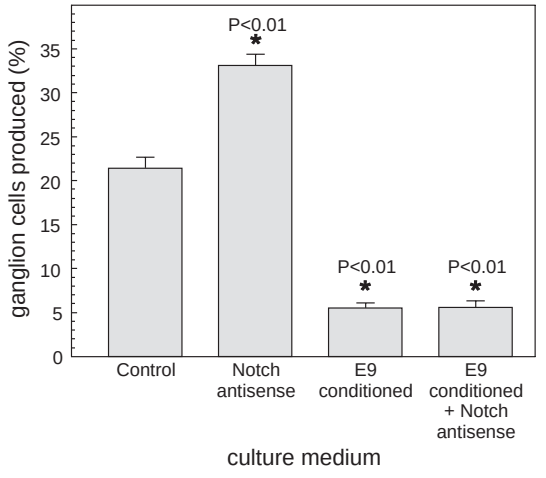

Fig. 7. Reducing Notch expression in cultures of E4 retinal cells with antisense oligonucleotides resulted in increased ganglion cell production, while medium conditioned by older retinal cells together with Notch antisense oligonucleotides resulted in reduced ganglion cell production. The graph compares the percentage of cells produced in vitro ($BrdU^+$) that differentiated as ganglion cells ($RA4^+$) in the test cell population in fresh medium (control), medium with Notch antisense oligonucleotides added, medium conditioned by E9 retinal cells and in conditioned medium supplemented with antisense oligonucleotides. Asterisks indicate ganglion cell production significantly different than the control.

The effect of older retinal cells on the younger cell population was due to a secreted factor. The test cell population was separated from the conditioning cell population by a porous membrane. This prevented any direct cell-cell contact between the two populations but allowed soluble molecules to diffuse between the two populations. Medium conditioned by older retinal cells and then used to culture younger retinal cells also affected the number of ganglion cells that developed in the younger cell population. This also indicates that a factor secreted by older retinal cells blocked development of ganglion cells in the younger cell population. These findings are complementary to previous studies that showed older retinal cells secrete factors that promote production of rod cells in populations of younger retinal cells (Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992). The approaches used in these previous studies were similar to those used in the present study. It is possible that the older retinal cells blocked expression of certain ganglion-cell-specific genes in the younger retinal cell population rather than reducing commitment of cells to the ganglion cell phenotype. These studies together, however, suggest that older retinal cells secrete factors that cause progenitors in the younger population to produce rods and possibly other cell types instead of producing ganglion cells.

The factor produced by older retinal cells that altered ganglion cell production most likely acted only on cells undergoing division rather than switching the fate of cells that were postmitotic. The presence of the conditioning population resulted in changes in the number of ganglion cells in the test population that divided in culture. BrdU labeling was used to identify the cells that divided in culture. There was a change with different aged conditioning cells only in the number of ganglion cells labeled with BrdU; the number of ganglion cells not labeled with BrdU in the test population was statistically the same for all experimental conditions. Previous studies linked environmentally induced changes in cell phenotype to cell division. Cell cycle progression has been shown to be coupled to expression of *even-skipped*, a gene required for neuronal specification in *Drosophila* (Weigmann and Lehner, 1995; Cui and Doe, 1995). Blocking the

cell cycle also prevented the expression of *even-skipped* and neuronal development, suggesting that neuronal differentiation is linked to the cell cycle. Work on vertebrates also suggests that cell division is required for cells to acquire a phenotype appropriate for their environment (McConnell and Kaznowski, 1991). Half of the cells transplanted from embryonic ferret cortex to postnatal cortex migrated to laminar positions appropriate for cells born in postnatal embryos while the remainder migrated to positions appropriate for cells born in embryonic cortex. Those cells that migrated to positions appropriate for their new environment underwent at least one round of cell division after transplantation. Thus, it may be a general phenomenon in the central nervous system that induction of specific cell phenotypes is intimately linked to the cell cycle.

Differentiated cells in the conditioning cell population in the present study most likely secreted the factor that altered cell determination of younger retinal cells. The main difference in the retina with increasing age is the addition of more differentiated cells. Furthermore, it is likely that differentiated ganglion cells specifically are responsible for secreting the factor. The majority of ganglion cells in the chick retina are produced between the ages of E3 and E9 (Fujita and Hori, 1963; Kahn, 1974; Spence and Robson, 1989; Snow and Robson, 1994; Waid and McLoon, 1995). The progressive addition of ganglion cells with increasing age paralleled the effectiveness of different aged conditioning cells in reducing ganglion cell production in the test population. The effectiveness of older conditioning cells in reducing ganglion cell production plateaued at E9, the same age at which ganglion cells reach their peak number. Tectal ablations were used to generate older conditioning cell populations depleted of ganglion cells (Hughes and McLoon, 1979). Older conditioning cell populations depleted of ganglion cells did not alter the number of ganglion cells that developed in the young test population. This suggests that as ganglion cells develop in the normal retina, they release a factor that blocks development of more ganglion cells.

The results of another study suggested that differentiated amacrine cells might also inhibit further development of their own cell type (Reh and Tully, 1986). When dopaminergic amacrine cells were depleted in developing retina, the next cells to differentiate included an abnormally high percentage of dopaminergic amacrine cells. When these animals were allowed to survive longer, the period of increased production of dopaminergic amacrine cells was followed by a period of reduced production of this cell type. An alteration in the number of dopaminergic amacrine cells was not accompanied by a change in the number of other amacrine cell types. Thus, it was suggested that cells were recruited to the dopaminergic amacrine cell type from an uncommitted pool of cells rather than causing a switch in the transmitter type within a pool of amacrine cells. The interpretation of these findings is that dopaminergic amacrine cells produce a factor that acts locally to reduce further production of the same cell type. It may be a general mechanism in nervous system development that differentiated cells inhibit further production of more of their same cell type.

The identity of the factor secreted by older retinal cells that blocks development of more ganglion cells is not yet known. The factor, as present in medium conditioned by older retinal cells, was partially characterized. The factor was heat-stable, and the majority of the activity remained in a <3 kDa fraction. A previous study with rat suggested that the amino acid, taurine, was responsible for promoting rod cell production in medium conditioned by older retinal cells (Altshuler et al., 1993). Taurine is heat-stable and <3 kDa. Taurine is expressed transiently in high levels by ganglion cells shortly after they differentiate in rat retina (Lake, 1994). It is possible that taurine is responsible for switching cells from the ganglion cell fate in the present study as well. Another study, however, failed to show a similar effect of taurine on chick retinal cells, even though it showed that taurine increased the number of rods that developed in rat retina (Kirsch et al., 1996). In chick, taurine may block development of ganglion cells while increasing production of cell types other than rods. A small but significant reduction in ganglion cell production was also observed with the >10 kDa fraction of conditioned medium. A previous study observed that a >10 kDa fraction of medium conditioned by older retinal cells inhibited rod cell production (Altshuler et al., 1993). This activity could be related to the high molecular mass activity observed in the present study. Further work is needed to identify the factors secreted by older retinal cells in developing retina.

The finding that older retinal cells secrete a factor that blocks development of ganglion cells is somewhat at odds with recent findings, which indicate that the Notch protein has a similar function in chick retina (Austin et al., 1995; Ahmad et al., 1997; Henrique et al., 1997). *Notch-1* is expressed in the proliferative zone of the developing retina. When Notch activity was increased by transfection of a constitutively active form of *Notch-1* or with a Notch ligand, Delta, there was a reduction in ganglion cell production. Conversely, blocking *Notch-1* or *Delta-1* expression with antisense oligonucleotides resulted in increased ganglion cell production. One interpretation of these results is that Notch specifically regulates ganglion cell production. The Notch protein is a transmembrane receptor that is activated by the cell surface ligands, Delta and Serrate/Jagged (Arvantis-Tsakonas et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996). Thus, Notch is believed to function via cell-cell contact. In the present study, the ability of older retinal cells to regulate ganglion cell genesis appears to be mediated by a secreted factor. It could be that this secreted factor represents a novel Notch ligand or a completely independent regulatory mechanism. To test this, young retinal cells were cultured with both C*Notch-1* antisense oligonucleotides and medium conditioned by older retinal cells. While the antisense oligonucleotide alone increased ganglion cell production, in combination with medium conditioned by older retinal cell, there was a reduction in ganglion cell production. This suggests that the secreted factor and Notch regulate ganglion cell production through separate pathways.

Another possibility is that Notch plays a more general role in retinal cell differentiation. Activation of Notch may prevent differentiation of any cell type and differentiation into specific cell types may be controlled by other factors (Bao and Cepko, 1997; Henrique et al., 1997). In *Drosophila*, activation of the Notch protein in progenitor cells appears to prevent the cells from committing to any particular fate (Fortini et al., 1993). R8 photoreceptors are normally the first cell type to differentiate in the *Drosophila* eye. Blocking Notch expression in the *Drosophila* eye caused most of the cells posterior to the morphogenetic furrow to differentiate immediately as R8 cells (Cagan and Ready, 1989). Similarly, overexpression of Notch or the Notch ligand, Delta, in developing *Xenopus* retina resulted in an increase in the number of progenitor cells and a

decrease in all types of differentiated cells (Dorsky et al., 1995, 1997). Thus, factors secreted by differentiated ganglion cells may promote differentiation of other retinal cell types, but only in cells in which Notch is not active.

In summary, this study suggests that as cells differentiate in the developing retina, they secrete factors that prevent development of ganglion cells and may possibly promote development of other cell types. Ganglion cells appear to be the likely source of the factors. The ganglion cell phenotype appears to be the default pathway for differentiation in the developing retina. In the absence of any environmental signal, retinal cells will differentiate as ganglion cells. It is likely that the secreted factor produced by ganglion cells prevents all cells in the developing retina from differentiating into ganglion cells. A similar function has been attributed to cell-contact-mediated signaling through Notch and Delta. It appears that secreted factors and Notch signaling represent separate control mechanisms. It may be that the secreted factors directly influence cell fate decisions, while the Notch pathway controls a more general decision of whether or not to differentiate.

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REFERENCES

Ahmad, I., Dooley, C. M. and Polk, D. L. (1997). Delta-1 is a regulator of neurogenesis in the vertebrate retina. *Dev. Biol.* **185**, 92-103.

Altshuler, D. M., Turner, D. L. and Cepko, C. L. (1991). Specification of cell type in the vertebrate retina. *In Development of the Visual System*, (ed. D. M. K. Lam and C. J. Shatz). pp 37-58. Cambridge: MIT Press.

Altshuler, D. and Cepko, C. (1992). A temporally regulated, diffusible activity required for rod photoreceptor development in vitro. *Development* **114**, 947-957.

Altshuler, D., LoTurco, J. J., Rush, J. and Cepko, C. (1993). Taurine promotes the differentiation of a vertebrate retinal cell type in vitro. *Development* **119**, 1317-1328.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Austin, C. P., Feldman, D. E., Ida, J. A. Jr. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of *Notch*. *Development* **121**, 3637-3650.

Bao, Z.-Z. and Cepko, C. L. (1997) The expression and function of Notch pathway genes in the developing rat eye. *J. Neurosci.* **17**, 1425-1434.

Belecky-Adams, T., Cook, B. and Adler, R. (1996). Correlations between terminal mitosis and differentiated fate of retinal precursor cells in vivo and in vitro: analysis with the 'window-labeling' technique. *Dev. Biol.* **178**, 304-315.

Bottenstein, J. E., Skaper, S. D., Varon, S. and Sato, G. H. (1980). Selective survival of neurons from chick sensory ganglionic dissociates utilizing serum-free supplemented medium. *Exp. Cell Res.* **125**, 183-190.

Cagan, R. and Ready, D. (1989). *Notch* is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* **3**, 1099-1112.

Carter-Dawson, L. D. and LaVail, M. M. (1979). Rods and cones in the mouse retina: II. Autoradiographic analysis of cell generation using tritiated thymidine. *J. Comp. Neurol.* **188**, 263-272.

Cui, X. and Doe, C. Q. (1995). The role of the cell cycle and cytokinesis in regulating neuroblast sublineage gene expression in the *Drosophila* CNS. *Development* **121**, 3233-3243.

Dorsky, R. I., Chang, W. S., Rapaport, D. H. and Harris, W. A. (1995). *Xotch* inhibits cell differentiation in the *Xenopus* retina. *Neuron* **14**, 487-496.

Dorsky, R. I., Rapaport, D. H. and Harris, W. A. (1997). Regulation of neuronal diversity in the *Xenopus* retina by Delta signaling. *Nature* **385**, 67-70.

Dowling, J. E. (1987). *The Retina – An Approachable Part of the Brain.* Cambridge, MA: Harvard University Press.

Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S. (1993). An activated *Notch* receptor blocks cell-fate commitment in the developing *Drosophila* eye. *Nature* **365**, 555-557.

Fuhrmann, S., Kirsch, M. and Hofmann, H-D. (1995). Ciliary neurotrophic factor promotes chick photoreceptor development in vitro. *Development* **121**, 2695-2706.

Fujita S. and Horii, M. (1963). Analysis of cytogenesis in the chick retina by tritiated thymidine autoradiography. *Arch. Histol. Jap.* **23**, 259-366.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.

Henrique D., Adam, J., Myat, A., Chitnis, A., Lewis J. and Ish-Horowicz, D. (1995). Expression of a *Delta* homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Henrique D., Hirsinger, E., Adam, J., LeRoux, I., Pourquie, O., Ish-Horowicz, D. and Lewis J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embyonic chick retina. *Curr. Biol.* **7**, 661-670.

Hicks, D. and Courtois, Y. (1992). Fibroblast growth factor stimulates photoreceptor differentiation in vitro. *J. Neurosci.* **12**, 2022-2033.

Holt, C. E., Bertsch, T. W., Ellis, H. M. and Harris, W. A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. *Neuron* **1**, 15-26.

Hughes, W. F. and McLoon, S. C. (1979). Ganglion cell death during normal retinal development in the chick: comparisons with cell death induced by early target field destruction. *Exp. Neurol.* **66**, 587-601.

Kahn, A. J. (1974). An autoradiographic analysis of the time of appearance of neurons in the developing chick neural retina. *Dev. Biol.* **38**, 30-40.

Kirsch, M., Fuhrmann, S., Wiese, A. and Hofmann, H. (1996). CNTF exerts opposite effects on in vitro development of rat and chick photoreceptors. *NeuroReport* **7**, 697-700.

Lake, N. (1994). Taurine and GABA in the rat retina during postnatal development. *Vis. Neursci.* **11**, 253-260.

Levine, E. M., Roelink, H., Turner, J. and Reh, T. A. (1997). Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. *J. Neurosci.* **17**, 6277-6288.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995) Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917.

McConnell, S. K. and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-285.

McLoon, S. C. and Barnes, R. B. (1989). Early differentiation of retinal ganglion cells: An axonal protein expressed by premigratory and migrating retinal ganglion cells. *J. Neurosci.* **9**, 1424-1432.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of *Serrate* and its relationship with *Notch* and *Delta* homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.

Reh, T. A. and Tully, T. (1986). Regulation of tyrosine hydroxylase-containing amacrine cell number in larval frog retina. *Dev. Biol.* **114**, 463-469.

Reh, T. A. (1992). Cellular interactions determine neuronal phenotypes in rodent retinal cultures. *J. Neurobiol.* **23**, 1067-1083.

Rodieck, R. W. (1973). *The Vertebrate Retina: Principles of Structure and Function*. New York: Freeman.

Rubinson, K. and Cain, H. (1989). Neural differentiation in the retina of the larval sea lamprey (Petromyzon marinus). *Vis. Neurosc.* **3**, 241-248.

Snow, R. L. and Robson, J. A. (1994). Ganglion cell neurogenesis, migration and early differentiation in the chick retina. *Neuroscience* **58**, 399-409.

Spence, S. G. and Robson, J. A. (1989). An autoradiographic analysis of neurogenesis in the chick retina in vitro and in vivo. *Neuroscience* **32**, 801-812.

Turner, D. L. and Cepko, C. L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. *Nature* **328**, 131-135.

Turner, D. L., Snyder, E. Y. and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron* **4**, 833-845.

Waid, D. K. and McLoon, S. C. (1995). Immediate differentiation of ganglion cells following mitosis in the developing retina. *Neuron* **14**, 117-124.

Watanabe, T. and Raff, M. C. (1990). Rod photoreceptor development in vitro: intrinsic properties of proliferating neuroepithelial cells change as development proceeds in the rat retina. *Neuron* **4**, 461-467.

Watanabe, T. and Raff, M. C. (1992). Diffusible rod-promoting signals in the developing rat retina. *Development* **114**, 899-906.

Weigmann, K. and Lehner, C. F. (1995). Cell fate specification by *even-skipped* expression in the *Drosophila* nervous system is coupled to cell cycle progression. *Development* **121**, 3713-3721.

Wetts, R. and Fraser, S. E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. *Science* **239**, 1142-1145.