

Müller-cell-derived leukaemia inhibitory factor arrests rod photoreceptor differentiation at a postmitotic pre-rod stage of development

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SUMMARY

In the present study, we examine rod photoreceptor development in dissociated-cell cultures of neonatal mouse retina. We show that, although very few rhodopsin⁺ rods develop in the presence of 10% foetal calf serum (FCS), large numbers develop in the absence of serum, but only if the cell density in the cultures is high. The rods all develop from nondividing rhodopsin⁻ cells, and new rods continue to develop from rhodopsin⁻ cells for at least 6-8 days, indicating that there can be a long delay between when a precursor cell withdraws from the cell cycle and when it becomes a rhodopsin⁺ rod. We show that FCS arrests rod development in these cultures at a postmitotic,

rhodopsin⁻, pre-rod stage. We present evidence that FCS acts indirectly by stimulating the proliferation of Müller cells, which arrest rod differentiation by releasing leukaemia inhibitory factor (LIF). These findings identify an inhibitory cell-cell interaction, which may help to explain the long delay that can occur both in vitro and in vivo between cell-cycle withdrawal and rhodopsin expression during rod development.

Key words: mouse retina, rod photoreceptors, retinal development, LIF, Müller cells

INTRODUCTION

The major classes of cells in the vertebrate neural retina, including photoreceptors, neurons and Müller glial cells, develop from multipotential precursor cells (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). The different cell types develop in a reproducible sequence, with ganglion cells and cones developing early, for example, and bipolar cells and rod photoreceptors (rods) developing late (Harman and Beazley, 1987; La Vail et al., 1991; Young, 1985). It is not known how the individual retinal precursor cells choose between alternative developmental fates, but there is evidence that both the intrinsic character of the precursor cell (Cepko et al., 1996; Watanabe and Raff, 1990) and extracellular signalling molecules (Altshuler and Cepko, 1992; Altshuler et al., 1993; Austin et al., 1995; Guillemot and Cepko, 1992; Harris and Hartenstein, 1991; Harris and Messersmith, 1992; Kelley et al., 1994, 1995; Lillien, 1995; Watanabe and Raff, 1992) can influence the choice and that both of these change as the retina develops (Altshuler and Cepko, 1992; Cepko et al., 1996; Lillien and Cepko, 1992; Reh, 1992; Watanabe and Raff, 1990, 1992).

Rods constitute more than 70% of the cells in the adult rodent retina (Drager and Olsen, 1981; Young, 1985), which makes them an attractive retinal cell type with which to study the factors that influence cell-fate choice and differentiation, especially as rhodopsin can be used as an unambiguous marker of differenti-

ated rods. There is accumulating evidence that rod development depends on short-range cell-cell interactions. In two studies of retinal development in rats, for example, it was found that rods develop in explant (Sparrow et al., 1990) and pellet (Watanabe and Raff, 1990) cultures, but not in dissociated cell cultures. Moreover, using a mouse-rat co-culture system, Reh (1992) showed that rods differentiate in monolayer cultures only if they are in aggregates with other differentiated rods; and, in studies of *Xenopus* photoreceptor development, Harris and Messersmith (1992) found that cells acquire rod-specific antigens only if cultured as clumps and not if cultured as dissociated cells.

The signalling molecules that mediate these rod-promoting cell-cell interactions in culture remain to be determined, although at least some of them seem to be diffusible (Altshuler and Cepko, 1992; Watanabe and Raff, 1992). Several known molecules have been shown to promote rod development in vitro, including retinoic acid (Kelley et al., 1994), taurine (Altshuler et al., 1993), basic fibroblast growth factor (bFGF) (Hicks and Courtois, 1992) and S-laminin (Hunter et al., 1992). Others have been found to inhibit such development, including transforming growth factor α (TGF α) (Anchan et al., 1991; Lillien and Cepko, 1992), ciliary neurotrophic factor (CNTF) (Kirsch et al., 1996) and LIF (Ezzeddine et al., 1997), although CNTF has been reported to promote rod development in cultures of chick retinal cells (Fuhrmann et al., 1995). It is still uncertain, however, if any of these molecules regulate rod development in vivo.

The present study began with an attempt to understand why rods fail to develop in dissociated-cell cultures of neonatal rodent neural retina (Sparrow et al., 1990; Watanabe and Raff, 1990), even when cultured at high density, where most of the cells are in contact with other cells. The answer seems to be that the serum in such cultures arrests rod differentiation at a postmitotic, pre-rod stage of development. In exploring the mechanism by which serum exerts this effect, we uncovered an inhibitory interaction between Müller cells and rod precursor cells, which is probably mediated by LIF. Although it is uncertain whether this interaction is direct or indirect, it may help to explain why there is often a long delay, both *in vivo* and *in vitro*, between when precursor cells stop dividing and when they first express rhodopsin (reviewed by Cepko, 1996).

MATERIALS AND METHODS

Reagents

All reagents were from Sigma, unless otherwise stated. Rat CNTF was kindly provided by Michael Sendtner. Human LIF was purchased from Preprotech.

Retinal cell cultures and blocking reagents

Balb/c × C57Bl/6 F₂ mice and Sprague Dawley rats were bred in the animal facility at University College London. To time the pregnancies, female mice (or rats) were caged with males overnight and then removed; this was taken as day 0 of the pregnancy. Peripheral neural retinae, free of pigment epithelium and astrocytes (see Watanabe and Raff, 1988), were removed from embryonic or neonatal mice (or rats) and dissociated into a single cell suspension with 0.125% trypsin (Boehringer Mannheim) in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (CMF PBS, Gibco/BRL), and triturated in DMEM/20% FCS/0.04% deoxyribonuclease (Gibco/BRL) as previously described (Watanabe and Raff, 1990). Cells were centrifuged at 200 g, washed once in serum-free culture medium and seeded at 450,000 cells in 0.5 ml of culture medium in 24-well tissue culture plates (Falcon), precoated with poly-D-lysine (PDL). In some experiments about 6 × 10⁶ cells were cultured in a 24 cm² culture flask (Falcon). In most experiments the cultures were maintained for up to 7 days without changing the medium. The culture medium consisted of DMEM/F-12 (Gibco/BRL, 1:1), supplemented with 100 µg/ml apo-transferrin, 20 nM progesterone, 30 nM sodium selenite, 60 µM putrescine, 10 µg/ml bovine serum albumin (BSA), 10 µg/ml insulin and 40 µg/ml gentamycin (Gibco/BRL). In some experiments 250,000 newborn retinal cells were centrifuged into pellets, which were then cultured on floating Nucleopore filters, as previously described (Watanabe and Raff, 1990).

To label cells in the S phase of the cell cycle, bromodeoxyuridine (BrdU) was added to the cultures at 4 µM for the same period. To

block cell division *in vitro*, cytosine arabinoside (Ara-C) was added at 1 µM for the 7-day period of the experiment. To block signalling by LIF and related cytokines in the cultures, we added a mutant form of LIF (hLIF05, 2 µg/ml) (Hudson et al., 1996), which binds to the LIF receptor but not to the transducing subunit gp130 and therefore acts as antagonist to the action of LIF and related cytokines that use the LIF receptor, including CNTF, oncostatin M and cardiotrophin-1 (CT-1) (A. B. Vernallis, K. Hudson and J. Heath, unpublished). hLIF05 was added every 2 days. To block signalling by LIF, we used rabbit antibodies raised against recombinant mouse LIF purified from transfected cos cell supernatants (Nichols et al., 1990), which were added (2 µg/ml) every 3 days. In assays on embryonic stem cells, these antibodies neutralised the activity of mouse LIF, but not human LIF (hLIF) or mouse CT-1 (A. Smith, unpublished observations).

Müller cell cultures and conditioned medium

Cultures of partially purified Müller cells were prepared by growing neonatal mouse neural retinal cells in 24 cm² tissue culture flasks (Falcon) for 7 days in 10% FCS and 20 ng/ml TGFα (Preprotech). The cells were then shaken on a rotary shaker overnight at room temperature, causing the photoreceptors and neurons to lift off, leaving a near-confluent monolayer of Müller cells. The purity of the cultures (>90%) was confirmed by staining with CRALBP antibodies. Conditioned medium was obtained from such cultures by maintaining the cells in serum-free culture medium without TGFα for a further 3-6 days, harvesting the medium and centrifuging it at 200 g for 10 minutes. Conditioned medium from 3T3 cells was used as a control and was obtained in the same way by culturing confluent monolayers of Balb/c 3T3 cells in serum-free culture medium for 3-6 days.

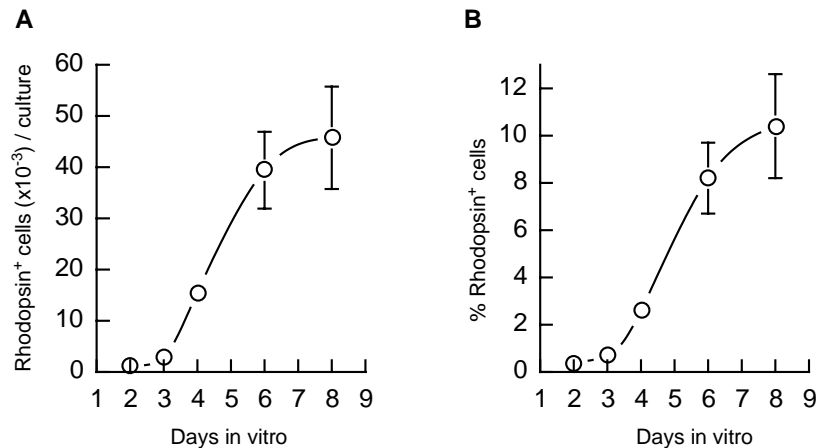
Immunocytochemistry and nuclear labelling

The antibodies used in this study are listed in Table 1. Cell suspensions were prepared with trypsin from freshly dissected neural retinae or from cultures of newborn neural retinal cells. The cells were counted and 120,000 cells were plated in 20 µl of phosphate-buffered saline (PBS) containing 10 µg/ml insulin, on poly-D-lysine (PDL)-coated 13 mm glass coverslips and left for 1 hour at room temperature to allow the cells to adhere. The cells were then fixed in either 70% ethanol for 10 minutes at -20°C when staining with B6-30, CRALBP, HPC-1, calbindin, GFAP or A9C6 antibodies, or 4% paraformaldehyde in 0.1 M phosphate buffer for 5 minutes at room temperature when staining with CD90 or 115A10 antibodies. For staining with recoverin antibodies, cells were fixed in paraformaldehyde and permeabilised with 0.1% Triton X-100 in PBS for 20 minutes. Fixed cells were incubated with primary antibodies, followed by fluorescein-conjugated goat anti-mouse immunoglobulin (Ig) or fluorescein-conjugated goat anti-rabbit Ig (Jackson, diluted 1:100). The antibodies were diluted in Tris-buffered saline containing 0.5% BSA, 100 mM L-lysine and 10% goat serum, and all incubations were for 1 hour at room temperature. In some experiments the cells were fixed in paraformaldehyde, stained on their surface with 115A10 and CD90 antibodies, postfixed in 70% ethanol/20% acetic acid for 10 minutes at room temperature, and then

Table 1. Antibodies used in this study

Antibody	Antigen	Cell type	Used as	References
B6-30, mouse monoclonal	Rhodopsin	Rods	Purified antibody, 1:200	Adamus et al., 1991
Rabbit antiserum	Recoverin	Rods, cones, and rod bipolar cells	1:5000	Dizhoor and Stryer, 1991
A9C6, mouse monoclonal	Arrestin	Rods	Purified antibody, 1:200	McGinnis et al., 1992
HPC-1, mouse monoclonal	Syntaxin	Amacrine cells	Supernatant, 1:100	Barnstable et al., 1985
Mouse monoclonal	Calbindin	Horizontal cells	Purified antibody, 1:100	Rabie et al., 1985
Rabbit antiserum	CRALBP	Müller cells	1:1200	De Leeuw et al., 1990
Mouse monoclonal	PKC	Rod bipolar cells	Purified antibody, 1:100	Zhang and Yeh, 1991
115A10, mouse monoclonal	Surface antigen	Bipolar cells	Supernatant, 1:1	Onoda and Fujita, 1987
CD90, mouse monoclonal	Thy1.2	Retinal ganglion cells	Ascites fluid, 1:500	Barnstable and Drager, 1984
Rabbit antibodies	Mouse LIF	?	(NH ₄) ₂ SO ₄ cut, 2 µg/ml	A. Smith, unpublished
K10, rabbit antiserum	CNTF	Müller cells and astrocytes	1:500	Stockli et al., 1991

Fig. 1. Development of rods in vitro. Newborn mouse neural retinal cells were cultured in serum-free medium. After various times the cells were removed from the dish and counted, and the proportion of rhodopsin⁺ cells was determined by immunostaining. Both the total number (A) and the proportion (B) of rods progressively increased over the period of the culture and reached a plateau value by about 7 days in vitro.



stained simultaneously with B6-30, HPC-1, CRALBP, calbindin and GFAP antibodies, followed by a mixture of the two fluorescent conjugates. For double labelling with B6-30 and anti-BrdU antibodies, cells were stained with B6-30 antibody as described above, then postfixed in 70% ethanol/20% acetic acid for 10 minutes at room temperature, and treated with 2 M HCl for 20 minutes to denature the nuclear DNA and then with 0.1 M Na₂B₄O₇ (pH 8.5) for 5 minutes to neutralise the acid. Cells were then incubated with Bu20a anti-BrdU antibody (Magaud et al., 1989), followed by rhodamine-conjugated goat anti-mouse Ig (Jackson, diluted 1:100).

To visualise the nuclear morphology of cultured retinal cells, we incubated the fixed antibody-stained cells with the fluorescent DNA-binding dye bisbenzimidazole (Hoechst 33358, 5 µg/ml) for 10 minutes at room temperature and then postfixed them with either 4% paraformaldehyde or 70% ethanol/20% acetic acid.

Stained cells on coverslips were mounted in Citifluor (Citifluor, Ltd), sealed with nail varnish, examined on a Zeiss Axioskop fluorescence microscope and photographed with Tmax 100 film, rated at 100 ASA (Kodak).

RESULTS

Rhodopsin⁺ rods first develop in the mouse retina at embryonic day 18

To identify rods, we used the B6-30 monoclonal anti-rhodopsin antibody, which does not recognise cone opsins (Adamus et al., 1991). Whereas rhodopsin is expressed exclusively in the outer segments of mature rods, it is diffusely distributed over the entire surface of newly formed rods (Barnstable, 1980; Fekete and Barnstable, 1983). To determine when rods first develop in the mouse retina, we used the anti-rhodopsin antibody in indirect immunofluorescence assays on cells dissociated from the neural retina of embryonic mice at different ages. Rhodopsin⁺ cells were first detected in small numbers (0.03±0.005%; mean±s.e.m. of 6 embryos) at embryonic day 18 (E18), and their numbers increased rapidly during the first postnatal week. Similar results were obtained using antibodies that detect either of two other largely rod-specific proteins, recoverin and arrestin: the first immunopositive cells were found at E18, when 0.23±0.04% were recoverin⁺ and 0.05±0.01% were arrestin⁺.

Rhodopsin⁺ rods develop from postmitotic rhodopsin⁻ cells in serum-free dissociated-cell cultures

To determine if rods can develop in dissociated-cell culture, we

prepared cell suspensions from newborn mouse neural retina and cultured the cells in serum-free medium. After various times, we harvested the cells, counted them and determined the proportion of rhodopsin⁺ cells. As shown in Fig. 1, both the proportion and total number of rhodopsin⁺ cells progressively increased for the 6-8 day culture period, indicating that rhodopsin⁺ rods continually developed from rhodopsin⁻ cells in these cultures. When the cell density in the cultures was decreased ninefold (to 50,000 cells in 500 µl), however, very few rods developed (not shown).

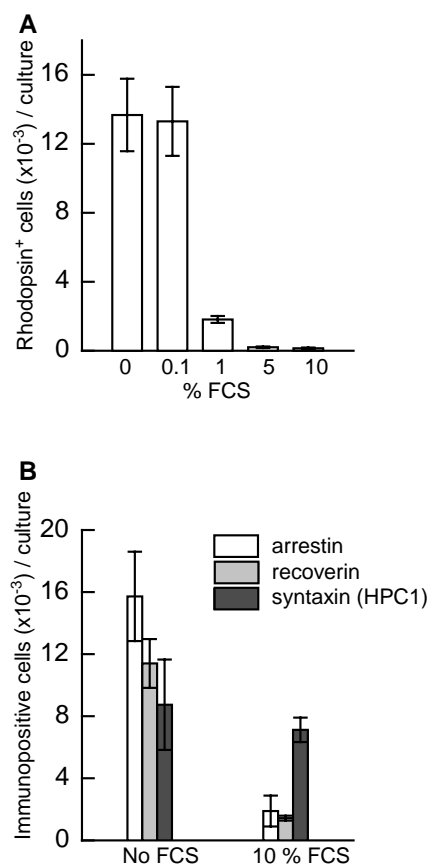
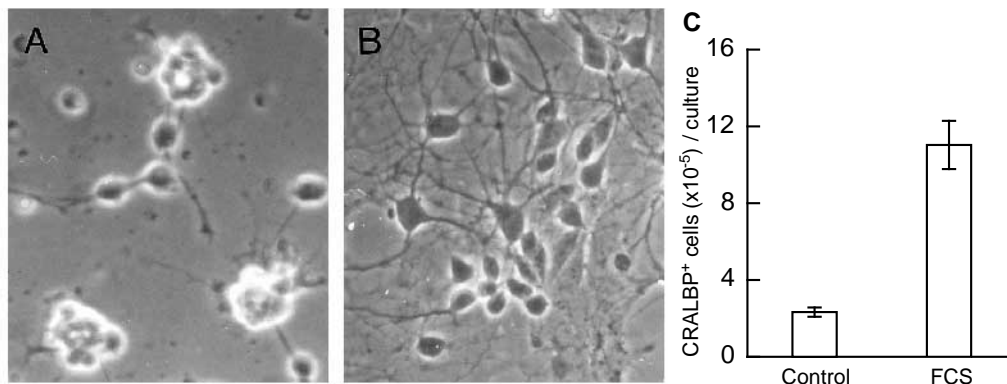


Fig. 2. Inhibition of rod development in culture by FCS. Cells were cultured for 7 days as in Fig. 1 and then stained for rhodopsin (A) or arrestin, recoverin or syntaxin (B).

Fig. 3. Increased number of Müller cells in FCS. Cells were cultured for 7 days as in Fig. 1, in the absence (A) or presence (B) of 10% FCS. They were then photographed (A,B) or dissociated and stained for CRALBP (C).



To determine if the rods developed from dividing precursor cells in these cultures, we added BrdU to the cultures from the start. After 7 days we double-labelled the cells with both anti-BrdU and anti-rhodopsin antibodies. None of the rhodopsin⁺ cells were BrdU⁺ (not shown), indicating that all of the increase in rods reflected the differentiation of nondividing rhodopsin⁻ cells into nondividing rhodopsin⁺ cells. Furthermore, the addition of cytosine arabinoside to the cultures from the start to inhibit cell proliferation had no influence on the number of rhodopsin⁺ rods that developed by 7 days in culture (not shown), confirming that the rhodopsin⁺ rods developed from postmitotic rhodopsin⁻ cells.

Serum inhibits rod development in dissociated-cell cultures

When newborn neural retinal cells were cultured in 10% FCS, very few rhodopsin⁺ cells appeared, even after 10 days. The inhibitory effect of FCS was dose-dependent, with as little as 1% having a significant effect (Fig. 2A). A similar inhibitory effect of 10% FCS was seen when rods were identified with antibodies against arrestin or recoverin, while FCS had no appreciable effect on the number of amacrine cells, identified with the HPC-1 monoclonal anti-syntaxin antibody (Barnstable et al., 1985) (Fig. 2B). Both goat and horse sera had a similar inhibitory effect on rod development and FCS also inhibited rod development in dissociated-cell cultures of neonatal rat neural retina (not shown).

Serum is mitogenic for Müller cells, which block rod development

Retinal cultures maintained in 10% FCS contained many more Müller cells than did serum-free cultures: by 3-4 days in FCS-containing cultures there was usually a near-confluent monolayer of Müller cells, whereas only scattered islands of Müller cells were seen in serum-free cultures (Fig. 3A,B). Immunolabelling with anti-CRALBP antibodies to identify Müller cells (De Leeuw et al., 1990) after 7 days in culture indicated that there were 4-5 times more Müller cells in 10% FCS than in serum-free cultures (Fig. 3C), although the total number of cells was only about 20% higher in 10% FCS (not shown).

To test whether FCS inhibited rod differentiation indirectly by increasing the number of Müller cells, we prepared monolayers of partially purified Müller cells and cultured newborn neural retinal cells on top of them in serum-free medium. As shown in Fig. 4A, even fewer rhodopsin⁺ rods developed in these conditions than when the retinal cells were cultured in 10% FCS. Conditioned medium from partially purified Müller cell cultures (MCM) had a similar effect, while conditioned medium from 3T3 cells did not (Fig. 4B). These findings suggest that FCS inhibits rod cell development by increasing the number of Müller cells. Consistent with this possibility, transforming growth factor- α (TGF α), which is mitogenic for Müller cells and greatly increased the numbers of Müller cells in serum-free cultures (not shown), was as effective as FCS in inhibiting rod cell development (Fig. 4C).

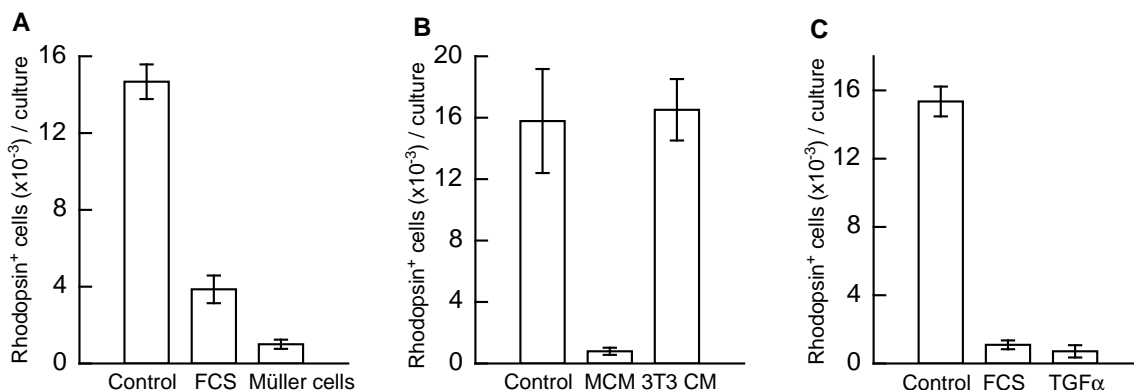


Fig. 4. Müller cells inhibit rod cell development in serum-free cultures. In all cases the cells were cultured for 7 days and then stained for rhodopsin. (A) Newborn mouse neural retinal cells were cultured either on their own in the presence or absence of FCS or on top of a monolayer of partially purified Müller cells in the absence of FCS. (B) Cells were cultured in conditioned medium obtained from cultures of either partially purified Müller cells (MCM) or 3T3 cells (3T3 CM). In this and subsequent Figs, MCM was used at a final dilution of 1:100. (C) Cells were cultured without serum, or in the presence of either 10% FCS or TGF α (20 ng/ml).

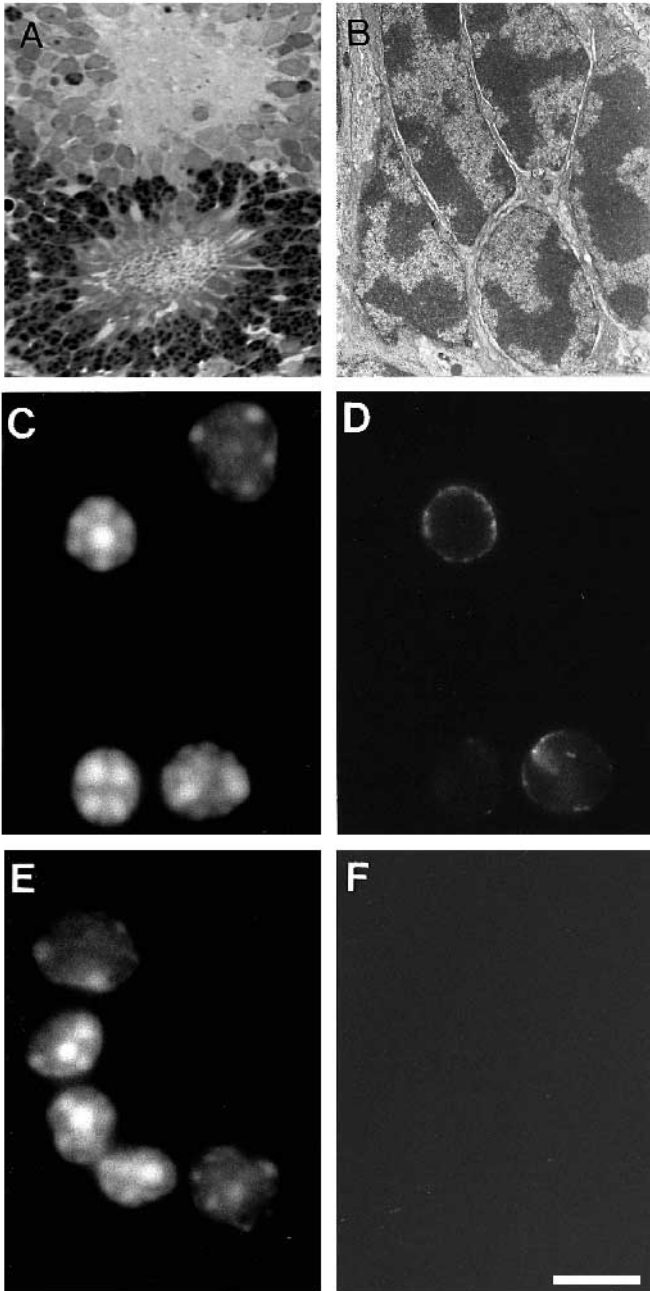


Fig. 5. The nuclear morphology of rods and pre-rod cells in vitro. In pellet cultures of E15 rat neural retinal cells, rods and interneurons form separate aggregates, or rosettes (Watanabe et al., 1997). As shown here in a light micrograph of a toluidine-blue-stained semi-thin plastic section (A) and in an electron micrograph of an ultra-thin section (B) of such cultures, the rods can be readily distinguished by their characteristic dark nucleus, which contains several large clumps of heterochromatin (B). This morphology is also apparent in monolayer cultures of newborn mouse neural retinal cells grown for 7 days in the absence (C,D) or presence (E,F) of 10% FCS, where the cells were stained for rhodopsin (D,F) and with bisbenzimidide (C,E). The characteristic pattern of chromatin staining seen in the rhodopsin⁺ rods that develop in the serum-free cultures (C,D) is also seen in many rhodopsin⁻ cells (putative pre-rods) in the FCS-containing cultures (E,F). A and B are from Watanabe et al. (1997). Scale bar, 32 μm (A), 2 μm (B) and 8 μm (C-F).

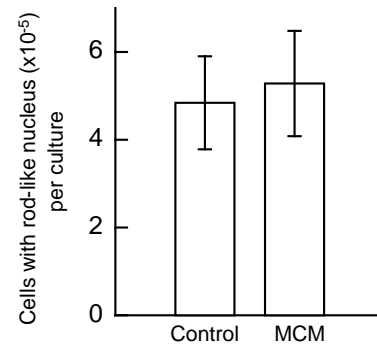


Fig. 6. The number of cells with a characteristic rod-like nucleus is the same in the presence or absence of MCM. Cells were cultured for 7 days in 25 ml Falcon tissue culture flasks (6.1×10^6 cells/flask) before they were dissociated, fixed and stained with bisbenzimidide. Cells with a rod-like nucleus, which include both rods and putative pre-rods, made up about 20% of the cells in either the presence or absence of MCM.

Müller cells arrest rod differentiation at a postmitotic pre-rod stage

It was shown previously (Watanabe et al., 1997) that rhodopsin⁺ rods that develop in pellet cultures of embryonic rat neural retinal cells have a characteristic small, dark nucleus, with one or more large clumps of heterochromatin, while the various types of interneurons have a larger and paler nucleus, with smaller clumps of heterochromatin (Fig. 5A,B). When we stained our serum-free, dissociated-cell cultures with cell-type-specific antibodies (see Table 1) and then with a dilute solution of the DNA-binding dye bisbenzimidide to visualise chromatin, all of the rhodopsin⁺ rods had a characteristic small, dark nucleus containing one or more large clumps of heterochromatin, whereas all of the CRALBP⁺ Müller cells, GFAP⁺ astrocytes, 115A10⁺ or PKC⁺ bipolar cells, HPC-1⁺ amacrine cells and Calbindin⁺ horizontal cells had a pale nucleus, with one or more smaller clumps of heterochromatin (Fig. 5C,D). Some rhodopsin⁻ cells, however, also had a rod-like nucleus, which was indistinguishable from that in the rhodopsin⁺ rods (Fig. 5E,F). We shall refer to these cells as pre-rods.

The total number of cells with such a rod-like nucleus (i.e. rods plus pre-rods) after 7 days in culture was not significantly different in the presence or absence of MCM (Fig. 6), even though there were at least sevenfold more rhodopsin⁺ rods in the absence of MCM (see Fig. 4B). In 7-day cultures maintained in FCS or MCM, the cells with a rod-like nucleus did not stain with any of the cell-type-specific antibodies listed in Table 1 (except for a very small proportion that stained with antibodies against rhodopsin, arrestin or recoverin), and none of these cells incorporated BrdU when it was added from the start of the culture (not shown). Taken together, these findings suggest that Müller cells block the differentiation of postmitotic rhodopsin⁻, arrestin⁻, recoverin⁻ pre-rods into rhodopsin⁺, arrestin⁺, recoverin⁺ rods.

CNTF and LIF arrest rod differentiation

It has been reported that CNTF and LIF inhibit the development of rods in dissociated-cell cultures of rat neural retinal cells (Cepko et al., 1996; Kirsch et al., 1996). We tested these cytokines in our serum-free cultures of newborn mouse neural

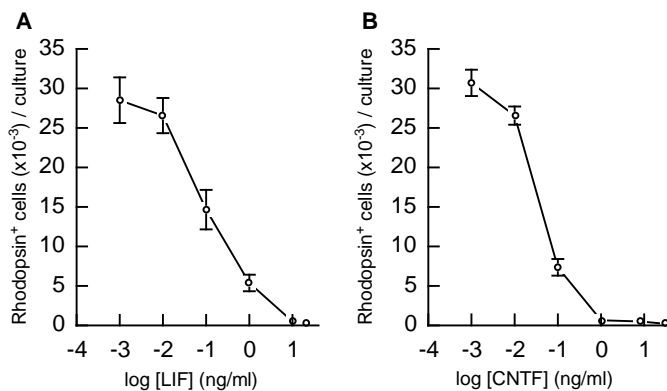


Fig. 7. LIF (A) and CNTF (B) inhibit rod development with similar efficiency. Cells were cultured for 7 days as in Fig. 1.

retinal cells. Even though CNTF and LIF did not increase the number of Müller cells (not shown), they were as effective as FCS and MCM in inhibiting rod cell development: as little as 1 ng/ml of either completely blocked the development of rhodopsin⁺ cells (Fig. 7). Recombinant human IL-6 (Preprotech; 8 ng/ml), a related cytokine, had no effect on rod development (not shown). As in the case of cultures treated with FCS or MCM, CNTF- or LIF-treated cultures contained as many cells with a rod-like nucleus as untreated cultures, and these cells did not stain with antibodies against other retinal cell types (not shown). Thus these cytokines, like FCS and MCM, apparently blocked rod differentiation at a pre-rod stage.

It was shown previously that rhodopsin⁺ cells develop in large numbers in pellet cultures of perinatal rat retinal cells in the presence of 10% FCS (Watanabe and Raff, 1990). Why does FCS block rod cell development in dissociated-cell cultures but not in pellet cultures? As shown in Fig. 8A, 10% FCS increased CRALBP⁺ Müller cells by only twofold in pellet cultures of newborn mouse retinal cells (compared to four- to fivefold in dissociated cell cultures) and had only a small inhibitory effect on the development of rhodopsin⁺ cells. By contrast, CNTF (Fig. 8A) and LIF (Fig. 8B) inhibited rod development in such pellets, even though they did not affect the number of Müller cells in the cultures (Fig. 8A and not shown). These findings are consistent with the possibility that FCS inhibits rod differentiation in dissociated-cell cultures by stimulating the proliferation of Müller cells, which release CNTF or LIF or a related cytokine, which in turn inhibit rod differentiation.

It has been suggested that CNTF and LIF inhibit rod development in rat retinal cultures by diverting precursor cells that were fated to become rods to become bipolar cells instead (Cepko et al., 1996; Ezzeddine et al., 1997). As shown in Fig. 8A, concentrations of CNTF that greatly inhibited rod development in pellet cultures of mouse retina did not increase the number of bipolar cells identified by staining with the 115A10 antibody (or with antibodies against protein kinase C; not shown). Similar results were obtained with LIF (not shown). Thus, the inhibitory effect of CNTF and LIF on rod development in mouse retinal cultures is unlikely to be due to the diversion of precursor cells from rod development to bipolar cell development.

The inhibitory effect of MCM depends on a LIF-like cytokine and is reversible

To determine whether CNTF, LIF or a related cytokine was

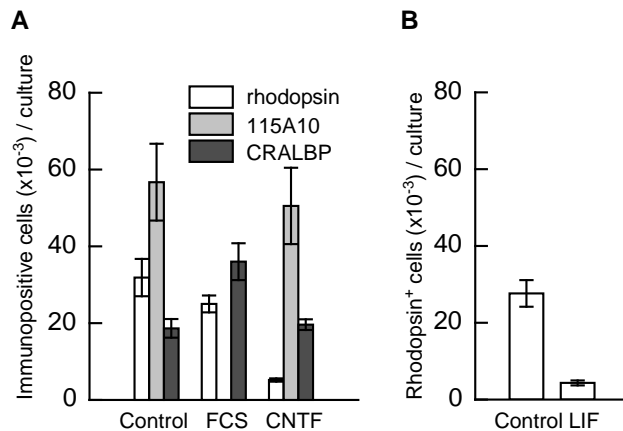
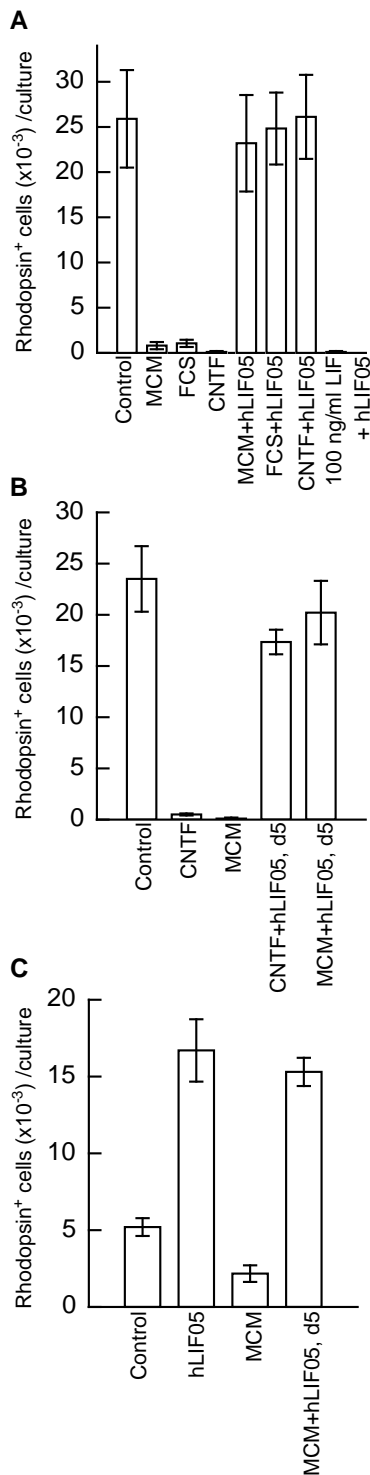


Fig. 8. LIF and CNTF are more effective than FCS at inhibiting rod development in pellet cultures. Newborn mouse neural retinal cells were cultured as pellets on floating Nucleopore filters for 7 days in serum-free medium (Control), in 10% FCS, or in serum-free medium with LIF or CNTF, both at 8 ng/ml. The cells were then dissociated and stained with the 115A10 antibody, or for rhodopsin or CRALBP.

responsible for the rod-inhibitory activity of MCM, we used the LIF receptor antagonist, hLIF05, which is a mutated form of human LIF (hLIF). By blocking the LIF β receptor, hLIF05 antagonises the action of LIF (Hudson et al., 1996), as well as of other cytokines that utilise the LIF β receptor, including CNTF, oncostatin-M and CT-1 (A. B. Vernallis, K. Hudson and J. Heath, unpublished). As shown in Fig. 9A, when this protein was added every 3 days from the start of 7-day cultures, it completely blocked the inhibitory effects of CNTF, FCS and MCM. The addition of 100 ng/ml of LIF completely neutralised the blocking effect of the hLIF05, suggesting hLIF05 was acting via the LIF β receptor (Fig. 9A). These results demonstrate that the inhibitory effects of both FCS and MCM depend on a cytokine that binds to the LIF β receptor.

To determine whether the inhibitory effect of MCM and CNTF was reversible, we added hLIF05 after 5 days *in vitro* to cultures treated from the start with either MCM or CNTF. As seen in Fig. 9B, almost as many rhodopsin⁺ rods were present after 12 days *in vitro* as in control cultures that received no treatment. This result suggests that the inhibitory effects of MCM and CNTF are largely reversible after 5 days, when the number of rhodopsin⁺ rods in untreated control cultures would be expected to have reached about 70% of the plateau value (see Fig. 1A).

To determine whether newborn rat retinal cultures behaved in the same way as mouse cultures, we added hLIF05 after 5 days *in vitro* to cultures of newborn rat neural retinal cells treated from the start with MCM. As seen in Fig. 9C, the late addition of hLIF05 greatly increased the number of rhodopsin⁺ rods that were present after 12 days *in vitro* in the MCM-treated cultures, so that their number was even greater than in control cultures that were not treated with MCM, suggesting that MCM also arrests rat rod development at a pre-rod stage of development. As in mouse cultures, the arrested pre-rods had the same characteristic nuclear morphology as rhodopsin⁺ rods (not shown). The addition of hLIF05 from the beginning of newborn rat cultures that were not treated with MCM also greatly increased the number of rhodopsin⁺ rods that were present after 12 days *in vitro* (Fig. 9C), suggesting that



cytokine-mediated inhibition normally holds back rod differentiation even in serum-free cultures of rat retinal cells, although it appears to do so to a lesser extent in similar cultures of mouse cells (not shown).

The inhibitory effect of MCM is neutralised by anti-LIF antibodies

As shown in Fig. 10, rabbit anti-LIF antibodies, which block signalling by mouse LIF but not human LIF or mouse CT-1

Fig. 9. The effect of blocking signalling by LIF and LIF-like cytokines with a mutant form of human LIF, hLIF05. Newborn mouse (A,B) or rat (C) neural retinal cells were cultured for 7 days (A) or 12 days (B,C) as in Fig. 1, before they were dissociated and stained for rhodopsin. (A) The neutralising effect of hLIF05 on the inhibitory effects of FCS, MCM and CNTF (1 ng/ml), all added from the start in mouse cultures. Note that a high concentration of human LIF (hLIF) overcomes the neutralising effect of hLIF05. (B) The neutralising effect on mouse cultures of hLIF05 added after 5 days in culture, rather than at the start; the cultures were maintained for a total of 12 days in vitro. (C) The effect of hLIF05 on rat cultures; the hLIF05 was added on its own from the start or after 5 days when MCM was present, and the cultures were maintained for a total of 12 days.

(A. Smith, unpublished), almost completely neutralised the inhibitory effect of MCM. They did not neutralise the inhibitory effect of rat CNTF or human LIF (Fig. 10), attesting to the specificity of the antibodies. These findings suggest that LIF is made by Müller cells, at least in culture, and is largely responsible for the rod-inhibitory activity of MCM.

DISCUSSION

Delay between birthday and rhodopsin expression in rod development

We show here that rhodopsin⁺, arrestin⁺, recoverin⁺ rods develop in serum-free dissociated-cell cultures of neonatal mouse neural retinal cells, as long as the cell density is high, consistent with previous evidence that cell-cell interactions are required for rod development (Harris and Messersmith, 1992; Reh, 1992). There is little precursor cell proliferation in our cultures, and the rhodopsin⁺ rods develop exclusively from postmitotic rhodopsin⁻, arrestin⁻, recoverin⁻ cells. The increase in rods continues for at least 6-8 days, indicating that there can be a long delay from the time a precursor withdraws from the cell cycle (its birthday), to the time it differentiates into a rhodopsin⁺ rod, at least in vitro.

A similar delay can also occur in vivo (reviewed by Cepko, 1996). Birthdating studies in developing mouse retina, for example, indicate that the first rod precursor cells stop dividing at E13 (Young, 1985), while we show here that the first cells expressing rhodopsin, arrestin or recoverin appear in small numbers in the mouse retina at E18, suggesting that the minimum time from cell-cycle withdrawal to rod differentiation in the embryonic mouse retina is 5 days. We showed previously that, when a cohort of retinal precursor cells was labelled with BrdU in a newborn rat, the first BrdU⁺, rhodopsin⁺ rods were seen in small numbers after 54 hours, which indicates the minimum delay between the final S phase and rhodopsin expression in the neonatal rat retina (Watanabe and Raff, 1990). Similar experiments in goldfish indicated a minimum delay of 4 days (Knight and Raymond, 1990). These kinds of experiments, however, do not indicate what the maximum delay time can be. Nor do they indicate whether the delay occurs before or after a cell commits to rod development. Our present results, however, suggest that a long delay can occur after commitment to a rod fate (discussed below).

Experiments in *Xenopus* (Harris and Messersmith, 1992) suggest that photoreceptor development occurs in at least two

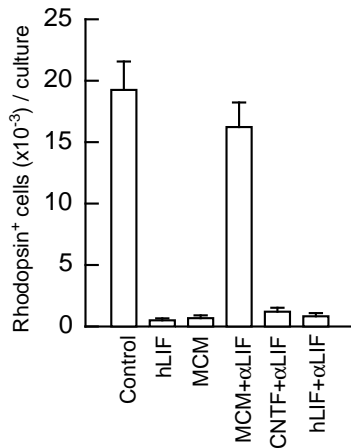


Fig. 10. The neutralising effect of anti-LIF antibodies on the inhibitory effect of MCM. Cells were cultured for 7 days as in Fig. 1. Anti-LIF antibodies (α LIF) were added every 3 days to a final concentration of 2 μ g/ml. hLIF and rat CNTF were used at 2 ng/ml, and their inhibitory effects were not neutralised by the anti-LIF antibodies.

steps: in the first step a cell commits to becoming a photoreceptor; in the second it commits to becoming a rod or a cone. Both steps seem to require short-range cell-cell interactions, as they occur when cells are cultured as clumps, but not when they are cultured as dissociated cells. Moreover, the second step still occurs if the cells are cultured in a dissociated state for 3 days and then as clumps for 3 days, suggesting that photoreceptor development can be temporarily arrested for a few days between steps 1 and 2 (Harris and Messersmith, 1992). Although there is no evidence that mammalian retinal precursor cells commit to a photoreceptor fate before deciding whether to become a cone or a rod, the long delay that can occur between a precursor cell withdrawing from the cell cycle and its expressing rod-specific proteins suggests that early mammalian rod development may also occur in multiple steps.

Inhibition of rod development by serum-induced Müller cell proliferation

The original goal of the present study was to determine why rods failed to develop in dissociated-cell cultures of neonatal rodent neural retina, even when the cells were cultured at high density, although they developed in large numbers in explant or pellet cultures (Sparrow et al., 1990; Watanabe and Raff, 1990). Since in these previous experiments the cells were cultured in 10% serum, our present findings that serum greatly inhibits rod development in dissociated-cell cultures but has much less effect in pellet cultures, provides a likely solution to the puzzle.

We provide four lines of evidence that serum acts indirectly to inhibit rod development by stimulating the proliferation of Müller cells in such dissociated cell cultures. First, there are many more Müller cells in these cultures if serum is present. Second, TGF α , which stimulates the proliferation of Müller cells (C. Neophytou, unpublished observations), is just as effective as serum at inhibiting rod development in dissociated-cell cultures. Third, a monolayer of purified Müller cells markedly inhibits rod development when new-born retinal cells are cultured on top of it in serum-free medium. Fourth, in pellet

cultures serum only weakly stimulates Müller cell proliferation and it only weakly inhibits rod development.

Evidence that Müller-cell-derived LIF inhibits rod development

How do Müller cells block rod differentiation? We find that culture medium conditioned by Müller cell monolayers (MCM) is just as effective at blocking rod development as the monolayers themselves, suggesting that the inhibition is mediated by a soluble factor. Five lines of evidence suggest that LIF or a closely related cytokine is the relevant factor. (1) Others have shown that LIF (Ezzeddine et al., 1997) and CNTF (Ezzeddine et al., 1997; Kirsch et al., 1996) can block rod development in cultures of rat retinal cells, although it has also been reported that CNTF promotes rod development in cultures of chick retina (Fuhrmann et al., 1995). (2) We show here that both LIF and CNTF, but not the related cytokine IL-6, are potent inhibitors of rod differentiation in cultures of newborn mouse retinal cells, mimicking the effect of serum, Müller cells and MCM. Moreover, while serum (which has only a weak stimulatory effect on Müller cell proliferation in pellet cultures) has only a weak inhibitory effect on rod development in pellet cultures, both LIF and CNTF have a powerful inhibitory effect on rod development in such cultures. (3) A mutant form of human LIF, hLIF05, which antagonises the action of those cytokines that depend on the LIF β receptor (reviewed by Ip and Yancopoulos (1996), including LIF (Hudson et al., 1996), CNTF, oncostatin M and CT-1 (A. B. Vernallis, K. Hudson and J. Heath, unpublished), blocks the inhibitory effect of MCM. This finding is consistent with the possibility that one or more of these four cytokines mediates the inhibitory effect of MCM. (4) Although we can detect CNTF in cultured Müller cells by both immunofluorescence staining and metabolic labelling (C. Neophytou, unpublished observations), CNTF does not have a secretory signal sequence and is not normally secreted by cells in vitro (Stockli et al., 1989). Moreover, we have not been able to remove the rod inhibitory activity from MCM by immunoprecipitation with the rabbit anti-CNTF antiserum K10 (Stockli et al., 1991), and the same antiserum does not neutralise the action of MCM when added to retinal cell cultures (C. Neophytou, unpublished observations). Taken together, these findings make it unlikely that CNTF mediates the inhibitory effect of the MCM. (5) Unlike CNTF, LIF does have a secretory signal sequence and is normally secreted by cells that make it (for an example see Richards et al., 1996). Although we could not detect LIF in cultured retinal cells by immunocytochemistry, probably because it is expressed at very low levels, rabbit antibodies raised against recombinant mouse LIF, which specifically block signalling by mouse LIF, almost completely block the inhibitory effect of MCM. This finding suggests that most, if not all, of the inhibitory activity in the MCM is due to LIF. It remains to be determined whether LIF (and CNTF) act directly on rod precursor cells, or indirectly via another cell type.

Inhibition at a committed pre-rod stage of development

It has recently been suggested that CNTF and LIF inhibit rod development in cultures of rat neural retinal cells by respecifying cells fated to become rods so that they become bipolar cells instead (Ezzeddine et al., 1997). This interpretation was

partly based on the finding that the CNTF treatment that led to a decrease in rhodopsin⁺ rods also led to a reciprocal increase in cells expressing several bipolar cell markers, although, interestingly, the increase in bipolar-like cells was apparently not seen in mouse cultures. As resistance to the CNTF effect on rod development occurred around the time rod precursor cells began to express rhodopsin, it was also concluded that commitment to terminal rod differentiation may coincide with the initiation of rhodopsin expression.

Our findings in cultures of mouse neural retinal cells lead us to different conclusions. We conclude that serum, Müller cells, LIF and CNTF all inhibit rod development mainly by arresting differentiation at a committed, postmitotic, pre-rod stage of development, and that commitment to a rod fate can occur before cells express rhodopsin. The following results support these conclusions. First, in our cultures, either dissociated-cell cultures or pellet cultures, even though LIF and CNTF greatly inhibit rod cell development, they do not detectably increase the number of cells that stain with either the 115A10 monoclonal antibody (Onoda and Fujita, 1987) or a monoclonal antibody against protein kinase C (Zhang and Yeh, 1991), both of which mainly recognise bipolar cells. Although we cannot exclude the possibility that LIF and CNTF divert a small number of precursor cells to become bipolar cells instead of rods, this cannot be their main effect on rod development in our cultures. Second, when serum, MCM, LIF or CNTF inhibit the development of rhodopsin⁺ rods in our cultures, the number of cells with a nuclear morphology that is highly characteristic of rhodopsin⁺ rods in culture is the same as in untreated cultures. Such cells do not incorporate BrdU and do not stain with antibodies against non-rod types of retinal cells. It seems very likely that these cells, even when rhodopsin⁻, are committed to a rod fate. Third, when the LIF β receptor antagonist hLIF05 is added to cultures after 5 days of treatment with either MCM or CNTF and the cultures are maintained for another 7 days, the final number of rhodopsin⁺ rods is almost as high as in cultures that received no treatment at all, suggesting that the inhibition by the MCM and CNTF is largely reversible, at least after 5 days in vitro. This finding makes it highly unlikely that the main effect of CNTF (or LIF) in our cultures is to respecify rod precursors to become some other cell type. A similar result is obtained when hLIF05 is added after 5 days to MCM-treated cultures of newborn rat neural retinal cells, making it unlikely that there is a fundamental difference between mouse and rat in the way LIF or CNTF influence rod development in culture. Taken together, our results suggest that these cytokines block rod differentiation at a postmitotic, pre-rod stage of development and that commitment to a rod fate can occur before cells express rhodopsin.

Relevance to in vivo development

An important question raised by our studies and those of others (Ezzeddine et al., 1997; Kirsch et al., 1996) is whether Müller cells and LIF (or related cytokines) normally exert an inhibitory influence on rod cell differentiation during retinal development in vivo. Could such an influence, for example, explain in part the long delay between the time a precursor cell stops dividing and the time it expresses rhodopsin, arrestin and recoverin? There is increasing evidence that inhibitory cell-cell interactions play an important part in regulating retinal cell neurogenesis, but many of these interactions seem to be

mediated by the Notch signalling system (Austin et al., 1995; Dorsky et al., 1995, 1997). One observation suggests that Notch signalling may normally regulate rod development, at least in mice: when one of the two copies of the *hes1* gene, which encodes a transcription factor regulated by the Notch signalling pathway, is inactivated by targeted gene disruption, rhodopsin⁺ rods develop prematurely (Tomita et al., 1996).

By contrast, there is little evidence so far that LIF normally regulates rod development. Although LIF receptor β mRNA is expressed in the retina (Ip et al., 1993), it is unclear which cells express it. It has been found that more rods develop in retinal explants from LIF receptor β 'knockout' mice (Li et al., 1995; Ware et al., 1995) than in explants from wild-type mice (Ezzeddine et al., 1997), but these mutant mice die too early to know whether rod development in vivo is affected. In the future it will be important to determine whether LIF is normally expressed in the developing mammalian retina. If it is, it will be important to determine which retinal cells express it, which cells express the LIF β receptor and whether rod development is abnormal in LIF 'knockout' mice.

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