Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina

Abbie M. Jensen*† and Valerie A. Wallace†

MRC Developmental Neurobiology Programme, Laboratory for Molecular Cell Biology, University College London, Gower Street, London WC1E 6BT, UK

*Present address: Institute of Neuroscience, 1254 University of Oregon, Eugene, OR 97403-1254, USA
†Both authors contributed equally to this publication

SUMMARY

We show that Sonic hedgehog and patched are expressed in adjacent domains in the developing mouse retina. Treatment of cultures of perinatal mouse retinal cells with the amino-terminal fragment of Sonic hedgehog protein results in an increase in the proportion of cells that incorporate bromodeoxuridine, in total cell numbers, and in rod photoreceptors, amacrine cells and Müller glial cells, suggesting that Sonic hedgehog promotes the proliferation of retinal precursor cells. These findings suggest that hedgehog and patched are part of a conserved signalling pathway in retinal development in mammals and insects.

Key words: retina, Sonic hedgehog, patched, proliferation, Müller glia, ganglion cells, mouse

INTRODUCTION

The mammalian neural retina is a layered structure consisting of seven distinct cell types (Ramón y Cajal, 1892), six of which develop from proliferating multipotential precursor cells (Turner et al., 1990), while the other (astrocytes) migrates into the retina from the optic nerve head (Watanahe and Raff, 1988; Ling and Stone, 1989). Clonal analyses have revealed that there are no clear lineage relationships between the various cell types and, therefore, it has been inferred that environmental cues largely dictate cell fate during retinal histogenesis (Turner and Cepko, 1987; Holt et al., 1988; Wett's and Fraser, 1988), although few relevant cues have been identified. As part of an attempt to identify such cues, we investigated the possible role of the hedgehog (hh) gene family.

The hedgehog (hh) genes encode a family of secreted proteins that are involved in patterning events during embryogenesis in both Drosophila and vertebrates (for reviews see Ingham, 1994; Johnson and Tabin, 1995). There has been remarkable conservation in the use of these signalling proteins in the development of some parts of flies and vertebrates: they are used in similar ways, for example, in the patterning of appendages in Drosophila (Basler and Struhl, 1994; Tabata and Kornberg, 1994) and limb buds in chicks (Riddle et al., 1993).

The hh gene product is also required for proper development of the Drosophila eye (Heberlein et al., 1993; Ma et al., 1993; Heberlein and Moses, 1995). Although, in vertebrates, Sonic hedgehog (Shh) has been shown to play a role in the development of a number of cell types, including floor plate (Echelard et al., 1993; Roelink et al., 1994), and motor (Roelink et al., 1994; Marti et al., 1995), midbrain (Hynes et al., 1995) and forebrain (Ericson et al., 1995) neurons, evidence for a role for hh proteins in vertebrate eye development has been notably lacking. We examined the expression of Sonic hedgehog (Shh) and the related genes (Echelard et al., 1993) Indian hedgehog (Ihh) and Desert hedgehog (Dhh) in the developing mammalian retina. We show that Shh is expressed in the developing mouse retina: Shh expression is observed in the neural retina from embryonic day 14.5 (E14.5) to adult; no Ihh or Dhh was consistently detected at any age. We also show that patched (ptc), a novel transmembrane protein (Hooper and Scott, 1989; Nakano et al, 1989), expressed by cells responding to hh signals (Hidalgo and Ingham, 1990; Capdevila et al., 1994), is expressed in the retina at the same time as Shh and in a complementary pattern.

In both flies and vertebrates, the hh genes encode precursor proteins that undergo autoproteolysis to generate two fragments (Porter et al., 1995; Johnson and Tabin, 1995). Studies in vivo and in vitro have demonstrated that the Nterminal fragment mediates all the biological activity of the hh protein (Fan et al., 1995; Porter et al., 1995; Roelink et al., 1995; Marti et al., 1995). We have examined the effects of a recombinant NH2-terminal Shh protein (SHH-N) on developing mouse retinal cells in vitro and show that it promotes the proliferation of retinal precursor cells. Our findings suggest that hh proteins play a part in retinal development in vertebrates, just as they do in flies.

MATERIALS AND METHODS

Frozen sections

Eyes from embryonic and postnatal (Balb/c × C57Bl/6) F2 mice were dissected in phosphate-buffered saline (PBS), pierced with a 30 gauge needle and fixed in 4% paraformaldehyde in PBS.
Cell cultures

Neural retinas were dissected from perinatal (Balb/c × C57Bl/6) F2 mice. The vaginal plug date was taken as E0 and confirmed by comparison with developmental stages described by Thélier (1972). The neural retinas were incubated in 0.125% trypsin in Ca2+-, Mg2+-free PBS and triturated with a Pasteur pipette into a single-cell suspension in Minimum Eagle’s Medium (MEM) (ICN), containing 20% (v/v) fetal calf serum (FCS) (Gibco) and 0.04% DNase (Sigma). The cell suspension was centrifuged and the cells resuspended in MEM, containing 15% FCS and insulin (10 μg/ml) (MEM-FCS). Approximately 275,000 cells were centrifuged for 7 minutes at 800 g in a volume of 300 μl of MEM-FCS in an Eppendorf tube. The resulting pellet was left at room temperature for at least 1 hour before it was transferred onto a 13 mm polycarbonate filter (0.8 μm pore size; Nucleopore), floating in a 4-well multidish (Nunclon) in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium (with Glutamax) and Ham’s F12 (Gibco), supplemented with 1% FCS (heat inactivated; Gibco), insulin (10 μg/ml), transferrin (100 μg/ml), bovine serum albumin (BSA fraction V; 100 μg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), and gentamycin (25 μg/ml) (all from Sigma). Typically, two pellets were cultured on each filter in 400 μl of media. The cultures were maintained at 37°C in 5% CO2. For the double labelling, pellets were pulsed for 3 hours in culture medium containing 4 μM BrdU, cultured for 3 days in the presence or absence of SHH-N and pulsed with [3H]thymidine (0.5 μCi/ml, 94.0 Ci/mmol; Amersham) for the last 18 hours. For culture periods longer than 3 days, half of the medium was replaced with fresh medium every 3 or 4 days.

For monolayer cultures, cell suspensions (50,000 cells/75 μl) of E18 neural retinal cells were plated onto poly-D-lysine, laminin-coated 96-well tissue culture dishes in the supplemented culture media described above. Growth factors and SHH-N were added at the start of the culture period and [3H]thymidine (1 μCi/well) was added for the last 18 hours. After 3 days, the cells were harvested onto glass fibre filters and [3H]thymidine incorporation was assessed by scintillation counting.

Forskolin (Sigma) and Sp-5,6-DCI cBIMPS (Biolog) were dissolved in DMSO to 10 mM. The following growth factors were stored as concentrated stock solutions in 0.1% BSA/PBS: human bFGF and TGF-α (Preprotech); recombinant human BMP-2, BMP-4 and BMP-7 (a gift from Genetics Institute); platelet-derived human TGFβ1 (Genzyme Diagnostics) and recombinant human TGFβ2 (Boehringer Mannheim). The EGF receptor inhibitor, 4-(3-chloroanilino) quinazoline (a gift from Zeneca Pharmaceuticals) was added to cultures 30 minutes prior to addition of other growth factors. Purified baculovirus-derived SHH-N was a gift from H. Roelink and T. Jessell.

BrdU labelling

To label cells in S-phase of the cell division cycle, BrdU (1 mM stock in MEM; Boehringer Mannheim) was added to the culture media to a final concentration of 10 μM for the final 18 hours of culture. At the end of the culture period, pellets were washed in Ca2+-, Mg2+-free PBS, incubated in 0.125% trypsin in Ca2+-, Mg2+-free PBS and dissociated into a single-cell suspension in MEM containing 20% FCS and 0.04% DNase. The cells were centrifuged and resuspended in MEM with insulin (5 μg/ml) and the number of cells in each pellet was determined by counting a sample from the cell suspension prior to centrifugation. Approximately 100,000 cells were plated in 15-30 μl of MEM and insulin (5 μg/ml) onto poly-D-lysine-coated coverslips and incubated for 1 hour at room temperature to allow the cells to adhere. The cells were fixed in 70% ethanol for 10 minutes at −20°C, rehydrated in PBS, incubated in 2 M HCl for 15 minutes at room temperature to denature the DNA, washed in PBS, neutralised in 0.1 M Na2 B4 O7 (pH 8.5) for 5 minutes and washed in PBS. They were incubated in 20% goat serum in 50 mM tris buffer (pH 7.4), 10 mM lysisine, 145 mM NaCl and 1% BSA (TBSL; blocking solution) prior to incubation for 45 minutes in the Bu-20a monoclonal anti-
BrdU antibody (Magaud et al., 1988; culture supernatant diluted 1:1 in blocking solution). Cells were washed in PBS and incubated for 30 minutes in fluorescein-conjugated goat anti-mouse immunoglobulin (IgG+IgM; Jackson, diluted 1:100 in blocking solution). After washing in PBS, the coverslips were mounted onto glass slides in Citifluor (Citifluor, Ltd.) and examined in a Zeiss Axioskop fluorescence microscope. For autoradiography of [3 H]thymidine-labelled cells after BrdU staining, the coverslips were postfixed with 70% ethanol, air dried, coated in emulsion (K5, Ilford) and developed in D19 (Kodak) after 4 days. The labelled cells were counted blind. Experiments were repeated at least once, with the exception of the SHH-N dose response in pellets, and yielded similar results.

**Immunohistochemistry**

Cell suspensions were plated onto coverslips as described above. The adherent cells were fixed in 70% ethanol for 10 minutes at −20°C, washed in PBS and incubated in blocking solution. They were then incubated for 45 minutes at room temperature in the following primary antibodies diluted in blocking solution: the B630 monoclonal anti-rhodopsin antibody (Rohlich et al. 1989; purified antibody at 30 μg/ml) to identify rod photoreceptors; the HPC-1 monoclonal antibody (Barnstable et al., 1985; concentrated hybridoma supernatant diluted 1:20) to identify amacrine cells; a rabbit antiserum against CRALBP (Deleeruw et al., 1990; diluted 1:1250) to identify Müller cells and a rabbit antiserum against GFAP (Pruss, 1979; diluted 1:1000) to identify astrocytes. After washing in PBS, the cells were incubated for 45 minutes in appropriate fluorescein-conjugated secondary antibodies diluted 1:100 in blocking solution. The secondary antibodies were goat anti-mouse (IgG+IgM; Jackson ImmunoResearch) to recognize mouse antibodies and goat anti-rabbit (Accurate) to recognize rabbit antibodies. The cells were washed in PBS and then incubated with propidium iodide (4 μg/ml; Sigma) and RNAse (100 μg/ml; Sigma) in PBS at 37°C for 10 minutes to visualize cell nuclei. Finally, the cells were washed in PBS, mounted in Citifluor and examined as described above. The labelled cells were counted blind. Sections for anti-Shh staining were fixed with acid alcohol, rehydrated in PBS and incubated overnight in anti-Shh antiserum (1:300 H4 antiserum, a gift from S. Morton and T. Jessell) or normal rabbit serum. The signal was amplified by successive 1 hour incubations with the following: biotinylated anti-rabbit (Amersham), goat-anti-biotin (Sigma), biotinylated donkey anti-goat (Amersham) and streptavidin-FITC (Amersham). Sections were photographed using a Zeiss Axioskop microscope.

**RESULTS**

**Sonic hedgehog and patched are expressed in the developing mouse retina**

Sections of perinatal mouse retinas were examined for hh gene expression by in situ hybridization with digoxigenin (DIG)-labelled antisense RNA probes. Shh is expressed in the retina at all ages examined: embryonic day 14.5 (E14.5), E17, postnatal day 7 (P7), P14 and adult (Fig. 1 and data not shown). At E14.5, expression of Shh is confined to the inner half of the central retina, a region containing the first postmitotic neurons (Fig. 1). By E17, the Shh signal is clearly localized to cells in the developing ganglion cell layer, which contains both ganglion cells and amacrine cells. From P7 onwards, it is localized to both the ganglion cell layer and to a subset of cells in the inner half of the inner nuclear layer, possibly amacrine cells (Fig. 1, P7 and P14). This pattern of Shh expression is maintained in the adult (data not shown). Ihh expression was detected at P7 but, inconsistently, in cells of the inner nuclear layer and the outer nuclear layer, which contains photoreceptors (data not shown). Hybridization with either Dhh antisense or Ihh sense probes did not give a signal at any of the ages examined (data not shown).

In order to gain some insight into which cells in the retina may be responding to Shh signalling, we performed in situ hybridization with DIG-labelled antisense probes for Shh (left hand column) and ptc (right hand column) in E14.5, E17, P7 and P14 mouse retinas. Note that the retinal pigment epithelial cells (RPE) are naturally black, while the hybridization signal is blue. The * in E14.5 sections indicates the optic nerve head. NBL, neuroblast layer; PML, postmitotic layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

**Fig. 1.** In situ hybridization shows that Shh and ptc are expressed in the mouse retina. Photomicrographs of in situ hybridizations with DIG-labelled antisense probes for Shh (left hand column) and ptc (right hand column) in E14.5, E17, P7 and P14 mouse retinas. Note that the retinal pigment epithelial cells (RPE) are naturally black, while the hybridization signal is blue. The * in E14.5 sections indicates the optic nerve head. NBL, neuroblast layer; PML, postmitotic layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
signalling pathway in both flies and vertebrates (Concordet et al., 1996; Goodrich et al., 1996; Marigo et al., 1996). In the mouse retina, ptc message is detected at E14.5 and E17 in the neuroblast layer in a domain that is adjacent to cells that express Shh (Fig. 1). At P7 and P14, ptc message is localized to cells in the centre of the inner nuclear layer, a region containing Müller glia cell bodies (Fig. 1). This pattern of ptc expression is also maintained in the adult (data not shown). Hybridization with a sense probe for ptc did not give a signal at any of these ages (data not shown).

To confirm the presence of Shh protein in the retina, we performed western blotting using an affinity-purified anti-Shh antiserum, Ab80 (Bumcrot et al., 1995), which recognizes the 19 kDa NH2-terminal cleavage product of both Shh (Fig. 2A, lane 3, stomach; Fig. 2B, lane 3, lung) and Ihh (Fig. 2A, lane 2, kidney). The 19 kDa Shh band was detectable in retinal lysates from E16 to adult (Fig. 2A, lanes 4-7; Fig. 2B lane 7), demonstrating both that the protein is present and that it undergoes the expected proteolytic cleavage.

We also looked at the distribution of Shh in P1 and P14 retinal sections by immunocytochemistry, using H4, an anti-SHH-N antiserum. In the P1 retina, the Shh staining is localized to the ganglion cell layer, matching the Shh in situ hybridization signal (Fig. 2C). At P14, Shh staining is in the ganglion cell and inner nuclear layers, which is also consistent with the Shh in situ hybridization pattern (Figs 1, 2C). There is also staining in the inner (P1 and P14) and outer plexiform layers (P14) (Fig. 2C). The staining in the inner plexiform layer is consistent with Shh being in processes of ganglion and amacrine cells or from diffusion of the protein. The staining in the outerplexiform layer at P14 is likely to result from diffusion of Shh, as ganglion and amacrine cells do not extend processes into this layer. These staining patterns were also observed with another anti-SHH-N antiserum (not shown).

**Effect of SHH-N on proliferation in cultures of neural retinal cells**

To assess the role of Shh on retinal development, we added purified baculovirus-derived NH2-terminal Shh protein (SHH-N) to pellet cultures of neural retinal cells from perinatal mice. We used pellet cultures of retinal cells because, unlike dissociated cell (monolayer) cultures, proliferation, differentiation and, to some extent, lamination occur much like they do in vivo (Watanabe and Raff, 1990; Watanabe et al., 1996). Pellet cultures also have an advantage over explant cultures, in that all parts of the retina are intermixed in a pellet, whereas in explants there is spatial heterogeneity with respect to retinal maturity.

We first tested the effect of SHH-N on the incorporation of bromodeoxyuridine (BrdU) into DNA. Retinal cell suspensions were centrifuged into pellets and cultured on floating polycarbonate filters. SHH-N was added to the culture medium at the start of the culture period and BrdU (10 µM) was added for the last 18 hours. After 3 days, the pellets were dissociated in trypsin and labelled with a monoclonal anti-BrdU antibody, and the proportion of labelled cells was determined. SHH-N increased the proportion of BrdU-labelled cells in a dose-dependent manner (Fig. 3A) and did so at all ages tested, including E16, E18 and P1 (Fig. 3B). These findings suggest that SHH-N acts as a mitogen and/or survival factor for proliferating cells in these retinal cultures.

To determine whether the SHH-N effect was on proliferation or survival of dividing cells in these pellets, we marked
the dividing cells at the start of the culture with BrdU (3 hour pulse). We then cultured the cells as pellets in the presence or absence of SHH-N for 3 days, pulsed with $[^3]$H-thymidine for the last 18 hours and then determined the proportion of BrdU+ cells that had incorporated $[^3]$H-thymidine. If SHH-N was affecting only the survival of the proliferating population, the proportion of double-labelled cells should not increase; if SHH-N was acting as a mitogen, the proportion of double-labelled cells should increase. As shown in Fig. 3C, treatment with SHH-N (190 nM) increased the proportion of double-labelled cells. Although this result does not rule out the possibility that SHH-N promotes cell survival, it does demonstrate that it acts as a mitogen for retinal cells.

Elevation of intracellular cyclic AMP inhibits SHH-N induced proliferation

An increase in the concentration of intracellular cyclic AMP (cAMP) has been shown to inhibit the effects of Shh on cell proliferation and cell fate determination in various developing tissues in both vertebrates and flies (for review see Kalderon, 1995). To determine if this is also the case for neural retinal cells, we tested the effect of forskolin, a potent activator of adenylate cyclases. We cultured E18 retinal pellets for 3 days in the presence of forskolin (50 μM), SHH-N (190 nM) or both, and pulsed them with BrdU for the last 18 hours. As shown in Fig. 4, forskolin completely blocked the SHH-N-induced proliferation in retinal cell pellets. The membrane-permeable, phosphodiesterase-resistant, cyclic AMP analogue Sp-5,6-DCI-cBIMPS (100 μM) had a similar, but smaller, effect (data not shown). Forskolin also inhibited SHH-N-induced proliferation in E16 retinal pellets cultured for 3 days (data not shown), and it had a small, but significant, inhibitory effect on basal proliferation in both E18 (Fig. 4) and E16 (data not shown) retinal pellets.

**SHH-N is mitogenic for neural retinal precursor cells**

The perinatal neural retina consists of proliferating precursor cells, postmitotic neurons and photoreceptors, and glial cells (which are known to maintain mitogenic potential). The results shown in Fig. 3 suggest that SHH-N promotes the proliferation of precursor cells, glial cells, or both. To test whether the SHH-N effects were on glial cells, we cultured E18 retinal cell
pellets for 7 days, with or without SHH-N, and added BrdU for the last 18 hours. We then dissociated the pellets and labelled the cells with anti-BrdU antibody and antibodies to identify the two classes of macroglial cells, i.e. anti-GFAP antibodies (Pruss, 1979) to label astrocytes and anti-CRALBP antibodies (Deleeuw et al., 1990) to label Müller cells (Deleeuw et al., 1990). In the presence or absence of SHH-N, astrocytes were too rare to quantify. The proportion of Müller cells that incorporated BrdU in the presence of SHH-N was not significantly different from control, i.e. 15.1±0.7% with SHH-N versus 15.5±0.9% without (n=4 pellets for each condition). Since SHH-N did not increase the proportion of Müller cells that incorporated BrdU and astrocytes were rare in the culture, it seemed likely that SHH-N promoted the proliferation of retinal precursor cells rather than glial cells.

If SHH-N stimulates the proliferation of precursor cells, then it should increase both the total number of cells in the pellets and the number of various types of differentiated cells that develop in the cultures. As shown in Fig. 5, SHH-N, in a dose-dependent manner, increased the total number of cells in E18 pellet cultures after 7 days, as well as the numbers of rod photoreceptors (identified with an anti-rhodopsin monoclonal antibody, B630 (Rohlich et al., 1989)), amacrine cells (identified with the HPC-1 monoclonal antibody (Barnstable et al., 1985)) and Müller cells (CRALBP+).

In the developing neural plate, SHH-N has been shown to have a concentration-dependent effect on cell fate determination (Marti et al., 1995; Roelink et al., 1995). As can be deduced from Fig. 5, there was no strict effect of SHH-N on cell fate determination for the three retinal cell types examined, with the possible exception of Müller cells. At 190 nM SHH-N, the highest concentration tested, all cell types (rod cells, amacrine cells and Müller cells) increased in total number. SHH-N increased the number of Müller cells to a greater extent, however, than amacrine cells or rod cells. In addition to increasing total Müller cell numbers, SHH-N also increased the overall proportion of Müller cells (but not of amacrine cells or rod cells) that developed in E18 retinal pellets after 7 days: Müller cells were approximately 10% of the cells in the absence of SHH-N and 18% in the presence of 190 nM SHH-N.

**SHH-N does not act indirectly by increasing the levels of bFGF or TGFα**

We next compared the ability of SHH-N to stimulate proliferation in cultures of dissociated embryonic neural retina cells (monolayers) with the ability of previously identified retinal cell mitogens to do so, i.e. TGFα and bFGF (Anchan et al., 1991; Lillien and Cepko, 1992). We dissociated E18 neural retinal cells and cultured them on poly-D-lysine and laminin-coated 96-well tissue culture dishes (50,000 cells/well) in the presence or absence of the signalling proteins for 3 days (plateau concentrations of TGFα and bFGF were predetermined). The cultures were pulsed with [3H]thymidine (1 μCi/well) for the last 18 hours. As shown in Fig. 6A, whereas both TGFα and bFGF were strongly mitogenic, SHH-N was only weakly so, and only at the highest dose tested (190 nM SHH-N) (Fig. 6A). The ability of SHH-N to stimulate proliferation potently in pellets but only weakly in monolayers suggests that its mitogenic effect on retinal cells may depend on cell-cell interactions that occur in pellets but not in dissociated cell cultures.

In some developing systems, hh family members have been shown to act, in part at least, by inducing the production of other signalling molecules, especially members of the TGFβ superfamily (for review Roelink, 1996). The mitogenic effect of SHH-N in pellet cultures required more than 18 hours exposure, which is consistent with the possibility that SHH-N is inducing secondary signalling molecules (data not shown). It is unlikely that the mitogenic effect of SHH-N is mediated by either TGFα or bFGF, as these factors did not stimulate proliferation in pellets (Fig. 6B). We also tested BMP-2, BMP-4, BMP-7, TGFβ1 and TGFβ2, and found that they did not stimulate proliferation in pellets over a dose-response range of 0.1 ng/ml to 10 ng/ml (Fig. 6B and data not shown), suggesting that the SHH-N effect is not mediated by any of these molecules.

Although TGFα failed to increase BrdU incorporation in pellets, the possibility remained that SHH-N increased the number of EGF receptors on retinal cells thus enabling the cells to respond to saturating amounts of TGFα (or EGF) made in the pellets (Lillien, 1995). To investigate this possibility, we first tested the ability of a previously characterized inhibitor of the EGF receptor tyrosine kinase (Ward et al., 1994) to block TGFα-induced proliferation in monolayer cultures of E18 retinal cells. As shown in Fig. 6C, the inhibitor (100 nM) effectively blocked [3H]thymidine incorporation across a range of TGFα concentrations.

We next tested the ability of the inhibitor to block the SHH-N-induced BrdU incorporation in E18 retinal pellets. As shown in Fig. 6D, the EGF receptor inhibitor (100 nM) had no effect on SHH-N-induced BrdU incorporation. Nor did it reduce the basal level of BrdU incorporation, suggesting that EGF receptor activation is not required for the basal levels of proliferation that we observe in retinal pellet cultures.

**DISCUSSION**

Although it seems likely that they evolved independently, the
retinas of *Drosophila* and vertebrates share some developmental mechanisms. The *eyeless* gene, for example, is required for eye development in *Drosophila* (Quiring et al., 1994) and its homologue *Pax-6* is required for eye development in vertebrates (for review see Macdonald and Wilson, 1996). Moreover, the Notch-Delta signalling system mediates lateral inhibition in both the fly (Cagan and Ready, 1989) and vertebrate retina (Austen et al., 1995; Dorsky et al., 1995).

Largely as a result of genetic analyses, much more is known about the extracellular and intracellular signalling events involved in retinal development in *Drosophila* than in vertebrates. *Hh*, for example, has been shown to be required for progression of the morphogenetic furrow in the eye imaginal disc (Heberlein et al., 1993; Ma et al., 1993 and reviewed in Heberlein and Moses, 1995) and *ptc* is also part of this signalling pathway controlling the movement of the furrow (Heberlein et al., 1995; Ma et al., 1993). In the present study, we show that *Shh* and *ptc* are expressed in the perinatal mouse neural retina and that purified SHH-N promotes the proliferation of neural retinal precursor cells in culture. These findings suggest that the *Shh* and *ptc* signalling pathway plays a part in neural retinal development in vertebrates, just as it does in flies.

**Shh and ptc expression**

*Shh* is expressed in the mouse retina from E14.5 to adult and is localized to the ganglion cell layer, which contains both ganglion and displaced amacrine cells. We have evidence that *Shh* is expressed in at least a subset of ganglion cells since we can detect the protein in the optic nerve head by immunocytochemistry and in the optic nerve by western blot analysis (unpublished observations). From P7 onwards, it is also expressed in a subset of cells in the inner nuclear layer, a region that contains amacrine, bipolar, horizontal and Müller cells. *ptc* is expressed in cells in the neuroblast layer at E14.5 and E17 and eventually becomes localized to cells in the middle of the inner nuclear layer. *Shh* and *ptc* expression are generally non-overlapping even though they are both expressed in cells of the inner nuclear layer at later stages of retinal development (P7 to adult). From P7 onward, *Shh* expression is localized to cells in the inner half of the inner nuclear layer, presumably amacrine cells, whereas *ptc* expression is localized to cells in the centre of the inner nuclear layer, presumably Müller glia.

**Sonic Hedgehog promotes retinal precursor cell proliferation**

SHH-N promotes the proliferation of neural retinal cells at all perinatal ages that we tested. The mitogenic effect of SHH-N on neural retinal cells is blocked by raising cAMP with forskolin, a potent activator of adenylate cyclases. This result is consistent with the observations that the ability of SHH-N to stimulate BrdU incorporation and *Pax-1* induction in the sclerotome and to induce dopaminergic neurons in the midbrain is also blocked by agents that raise cAMP (Fan et al., 1995; Hynes et al., 1995). The SHH-N effect is not on glial cell proliferation as there are too few astrocytes to quantify in cultures with or without SHH-N, and the proportion of Müller cells that incorporate BrdU is not increased in the presence of SHH-N. Our observation that there is an increase in both the total cell number and the number of neurons and glia in pellets treated with SHH-N is consistent with SHH-N acting as a mitogen for retinal precursor cells.

The concentration of SHH-N that promotes retinal cell proliferation is in the range that was previously found to induce the differentiation of mouse midbrain dopaminergic neurons (Hynes et al., 1995), but it is much higher than the concentration required either to induce the differentiation of chick motor neurons or floor plate (Roelink et al., 1995) or to induce the proliferation of mouse sclerotome cells (Fan et al., 1995). One possible explanation for these differences is that our assay and that of Hynes et al. (1995) requires several days, while the assays that demonstrate effects at much lower concentrations are assessed after only 24 hours.

Shh has been shown to induce the development of motor neurons in the spinal cord (Roelink et al., 1994, 1995), dopaminergic neurons in the midbrain (Hynes et al., 1995),

![Fig. 6. Mitogenic effect of SHH-N, TGFβ and bFGF.](image-url)
ventral neurons in the forebrain (Ericson et al., 1995) and sclerotome cells in the developing somite (Fan et al., 1995). We find no evidence that SHH-N induces the development of rod cells or amacrine cells, as the proportions of these cells do not increase in SHH-N-treated cultures. The proportion of Müller cells, however, does increase in SHH-N-treated cultures, suggesting that SHH-N promotes the induction or differentiation of Müller cells, or acts as a survival factor for these cells.

In various developing Drosophila tissues (Heberlein et al., 1993) as well as in chick hindgut (Roberts et al., 1995) and limb bud (Lauffer et al., 1994), hh proteins act by stimulating the production of secondary signalling proteins, which are members of the TGF-β superfamily. Frequently, these are bone morphogenic proteins (BMPs), which are often expressed adjacent to sites of hh expression (Bitgood and McMahon, 1995). The possibility that SHH-N is inducing secondary signalling molecules in retinal cultures is supported by the observation that the SHH-N mitogenic effect in pellet cultures requires more than 18 hours exposure. We tested several candidate signalling molecules that might act to relay the SHH-N signal, including BMP-2, BMP-4, BMP-7, TGF-β1 and TGF-β2. None of these, however, are able to mimic the effect of SHH-N on retinal pellet cultures. SHH-N also does not act as a mitogen in pellet cultures by increasing the production or release of two other previously identified mitogens for retinal precursor cells, TGF-α and bFGF, as neither of these mimics the effect of SHH-N nor does it require EGF receptor signalling, as an inhibitor of the EGF receptor does not block the mitogenic effect of SHH-N.

Although we were not able to mimic the mitogenic effect of SHH-N with other signalling molecules, our observations are consistent with the possibility that the mitogenic effect of SHH-N in retinal pellet cultures depends on cell-cell interactions occurring within the pellets. In contrast to TGF-α and bFGF, for example, SHH-N does not increase proliferation in low density dissociated-cell cultures of neural retinal cells, which suggests that its mitogenic effect may depend on cell-cell interactions that do not occur in such cultures; it does increase cell numbers in monolayers cultured at very high density for 7 days (unpublished observation). The nature of these putative cell interactions and whether they are required to relay the SHH-N signal or increase responsiveness to SHH-N or some other signal remains to be determined. The effects of Shh on neural plate and sclerotome have all been demonstrated in explant cultures; it would be of interest to know if they can also be seen in dissociated cultures of these tissues.

Our finding that SHH-N potentiates the high basal level of proliferation in pellet cultures of neural retinal cells suggests that it is not saturating in these cultures, possibly because retinal ganglion cells (RGC), which are likely to be the major source of endogenous Shh, do not survive in these cultures (unpublished observation). It is possible, therefore, that Shh produced by RGCs stimulates proliferation of retinal precursor cells in vivo. The observation that ptc is expressed in the neuroblast layer adjacent to the cells that express Shh is consistent with this hypothesis. Neuroblasts could come into contact with a source of Shh through their processes, which extend into the ganglion cell layer (Polkey et al., 1989). Alternatively, Shh may diffuse away from its source in the ganglion cell layer to signal cells in the neuroblast layer. The possibility that RGC-derived signals control retinal precursor proliferation is, however, inconsistent with the observation that elimination of RGCs by neonatal optic nerve transection apparently does not affect retinal cell proliferation or survival (Beazley et al., 1987). RGCs may not, however, be the only source of Shh: there are Shh-expressing cells in the inner half of the inner nuclear layer (possibly amacrine cells) and these cells may continue to be a source of the protein after axotomy. Alternatively, there may be enough deposits of Shh protein remaining in the retina to promote proliferation after axotomy. Finally, these RGC-derived proliferative signals may only be important during embryonic retinal development.

The observation that Shh is expressed in the adult retina, long after the retina has completed its development, suggests that Shh has additional functions. It could influence retinal synapses or cell survival, glial cells in the optic nerve, or cells in target structures in the CNS. These last two possibilities are likely given the recent findings of Huang and Kunes (1996) who demonstrate that Shh transmitted along retinal axons is required for the development of the visual centre in the Drosophila brain.

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REFERENCES


Drosophila in mouse retina

Sonic hedgehog in mouse retina