A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve

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Summary

Retinal ganglion cell (RGC) axons have been shown to stimulate the proliferation of astrocytes in the developing rodent optic nerve, but the signals that mediate this effect have not been identified. The following findings suggest that Sonic hedgehog (Shh) is one of the signals. (1) RGCs express both Shh mRNA and protein, whereas the optic nerve contains the protein but not the mRNA. (2) Astrocytes and their precursors in the developing optic nerve express the Hedgehog (Hh) receptor gene Patched (Ptc), suggesting that they are being signalled by an Hh protein. (3) Ptc expression in the nerve is greatly decreased by either nerve transection or by treatment with neutralizing anti-Shh antibodies, suggesting that it depends on axon-derived Shh. (4) Astrocyte proliferation in the developing nerve is reduced by treatment with anti-Shh antibodies, suggesting that Shh normally helps stimulate this proliferation.

Key words: Astrocyte, Sonic hedgehog, Patched, Proliferation, Retinal ganglion cells, Axons, Mouse

Introduction

There is increasing evidence that neurons and glial cells influence each other’s development. The optic nerve is an attractive place to study such neuron-glial interactions, as it is one of the simplest parts of the central nervous system (CNS), being composed mainly of retinal ganglion cell (RGC) axons and two types of glial cells – astrocytes and oligodendrocytes. The astrocytes develop from neuroepithelial cells that form the optic stalk, whereas the oligodendrocytes develop from precursor cells that migrate into the developing nerve from the brain (Small et al., 1987; Ono et al., 1997).

Previous studies have shown that axons in the optic nerve signal to the glial cells in a number of ways: they promote the survival of oligodendrocytes (Barres et al., 1993), the proliferation and/or survival of oligodendrocyte precursors (Barres and Raff, 1993), and the proliferation of astrocytes (Burne and Raff, 1997). The signalling molecules that mediate these interactions have not been determined. In this study we provide evidence that Shh is an axon-derived signal that promotes astrocyte proliferation in the nerve.

Hh proteins play multiple roles in animal development (reviewed by Hammerschmidt et al., 1997). They are produced as precursors that undergo autoproteolysis to generate two fragments, a C-terminal fragment and an N-terminal fragment (Shh-N) that has the signalling activity (Bumcrot et al., 1995; Lee et al., 1994; Porter et al., 1995). Following autoproteolysis, Shh-N becomes covalently modified at its C terminus by the addition of a cholesterol molecule, which restricts the range of action of the protein (Porter et al., 1996a,b). Additional lipid modifications at the N terminus of the Shh-N fragment have also been described (Pepinsky et al., 1998). The receptor for Hh is the transmembrane protein Patched (Ptc) (Marigo et al., 1996; Stone et al., 1996), which is thought to antagonize the activity of Smoothened (Smo), a second transmembrane protein, that is required to transmit the Hh signal into the target cell (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Paradoxically, Hh signalling upregulates Ptc transcription so that Ptc expression is highest in cells adjacent to Hh-expressing cells, and ectopic Hh expression in vertebrates induces ectopic Ptc expression (Concordet et al., 1996; Goodrich et al., 1996; Marigo et al., 1996). Ptc expression is, therefore, a useful marker to identify cells that are responding to Hh signals.

In Drosophila, hh is associated with retinal axons, and it stimulates both ptc expression in glia and neurogenesis in developing visual centres in the brain (Huang and Kunes, 1996, 1998; Huang et al., 1998). We now provide evidence that Ptc is expressed by astrocytes and their precursors in the developing rodent optic nerve as a result of signalling by axon-derived Shh and that the Shh helps stimulate astrocyte proliferation in the nerve.

Materials and Methods

Frozen sections and in situ hybridization

C57BL/6J mice were time mated, and the day of the vaginal plug was defined as embryonic day 0. Tissues for in situ hybridization were...
fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and cryoprotected in 30% sucrose in PBS. Prior to sectioning, the tissues were equilibrated in a 50:50 mixture of OCT compound and 30% sucrose, embedded in the same mixture, frozen and cut on a cryostat at 15 μm. The sections were transferred onto Vectabond- (Vector laboratories) coated slides, air dried for 2-6 hours at room temperature, and stored desiccated at −20°C. In situ hybridization was performed as previously described by Jensen and Wallace (1997), except that the signal was enhanced by performing the colour reaction in the presence of 10% polyvinyl alcohol (BDH) (as described by DeBlock and Debrouwer, 1993). The following templates (all subcloned into pBluescript) were used to generate digoxigenin-labelled antisense RNA probes, as previously described (Jensen and Wallace, 1997): murine Shh, a 2.6 kb full length cDNA, was transcribed using T7 RNA polymerase from an EcoRI linearized template; murine Ihh, a 1.8 kb cDNA, was transcribed using T7 polymerase from an XbaI linearized template; murine Ptc, an 841 bp fragment, was transcribed with T3 polymerase from a BamHI linearized template; rat GFAP, a 1.2 kb fragment, was transcribed using SP6 polymerase from an EcoRI linearized template; mouse PDGFRα was transcribed using T7 polymerase from a HindIII linearized probe.

Optic nerve transection, intracranial injections and cell culture

Postnatal day 2 (P2) mice were anaesthetized by cooling on ice, an incision was made in the left eyelid, and the eyeball was gently retracted. After severing the optic nerve with microscissors, the eye was removed, and the incision was closed with wound adhesive. The animals were killed 2 days after enucleation, and both optic nerves (cut and uncut) were dissected and processed for in situ hybridization.

Hybridoma cells secreting anti-Shh antibodies (5E1; Ericson et al., 1996) or anti-Thy-1.1 antibodies (OX-7; Mason and Williams, 1980) (both mouse IgG1) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 20% heat-inactivated fetal calf serum (FCS), in Dulbecco’s modified Eagle’s medium containing HEPES buffer (MEM-HEPES; ICN), and resuspended at a final concentration of 2 × 10^6 cells/ml. P1 mice were anaesthetized by cooling on ice, and the cells (2 × 10^5 cells in 1 μl) were injected into the subarachnoid space on both sides of the brain. After 3 days, the retina and optic nerves were dissected and processed for in situ hybridization or immunocytochemistry (see below).

For Shh stimulation of optic nerve astrocytes, optic nerves from P1 mice were trypsinized and triturated to obtain a single cell suspension. Cells were resuspended in a 1:1 mixture of DMEM:F12 supplemented with N-acetyl cysteine (60 μg/ml), 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). Approximately 4000 cells in 15 μl were placed on poly-D-lysine- (PDL) coated glass coverslips in 24-well plates for 15 minutes at 37°C to allow them to attach, and then 0.5 ml of the culture medium was added to each well. Three days later the medium was replaced with fresh medium, and the cultures were treated with growth factors (myristoylated Shh-N (a gift from Biogen), bFGF (Gibco) or FCS) for 2 days. To label dividing cells, the cultures were pulsed with bromodeoxyuridine (BrdU; Sigma) for the last 8 hours, and the cells on the coverslips were processed for immunocytochemistry for GFAP and BrdU as described below.

Western blotting and immunocytochemistry

Lysates of embryonic mouse tissues, perinatal mouse retinas, or postnatal rat optic nerves were prepared, electroblotted as described previously (Jensen and Wallace, 1997), and the blots were probed with affinity-purified anti-Shh antibodies (Ab80; Bumcrot et al., 1995, a

![Fig. 1. Shh, Ihh, Ptc and GFAP expression in the embryonic mouse eye and optic nerve. In situ hybridization for Ptc (A,E,H), Shh (B,F), GFAP (G), and Ihh (D) expression in horizontal sections of E12 (A,B,D,H) and E14 (E-G) eye and optic nerve. A diagram of the developing eye and optic nerve is shown in C; EL, eyelid; L, lens; ON, optic nerve; NR, neural retina; RPE, retinal pigment epithelium. Note that in (D) Ihh (arrowheads) is expressed in a single layer of cells outside the RPE (arrow) but not in cells of the optic stalk (within the dashed lines), whereas in H Ptc mRNA (blue dots) is present outside the RPE (arrow) and in the optic stalk (within the dashed lines) but not in the cells expressing Ihh (white arrowheads). The * in D and H indicates the lens. Scale bar, (A,B,E-G) 200 μm, and (D,H) 50 μm.](image)
kind gift from A. McMahon and D. Bumcrot). To assess DNA synthesis in optic nerve astrocytes and retinal cells in mice injected with hybridoma cells, two intraperitoneal injections of BrdU (16 mg/ml in MEM, 50 µl per injection) were given 2 hours apart. Two hours after the last injection, the neural retinas and optic nerves were dissociated into single cell suspensions with trypsin. The retinal cells were plated onto PDL-coated glass coverslips for 30 minutes, fixed with 70% ethanol, and immunostained with monoclonal anti-BrdU antibodies (Bu2a; Magaud et al., 1989), as described previously (Jensen and Wallace, 1997). Cells from optic nerves were cultured overnight on PDL-coated coverslips in DMEM, containing 0.5% FCS and supplemented as described above. The next day the cells were fixed and double-labelled by indirect immunofluorescence for GFAP and BrdU. For BrdU staining after in situ hybridization, sections were incubated in 2 N HCl for 20 minutes at 37°C to denature the DNA, washed in 0.1 M Tris pH 8.5, followed by PBS, and incubated with anti-BrdU antibody (Becton Dickinson; diluted 1:200) overnight at 4°C. The anti-BrdU antibody was visualized with fluorescein-conjugated goat anti-mouse IgG antibodies (Jackson Laboratories, diluted 1:100). For Pax-2 immunostaining after in situ hybridization, sections were incubated with rabbit anti-Pax-2 antiserum (Zymed Laboratories; diluted 1:400) overnight at 4°C. The Pax-2 antibodies were visualized with biotinylated goat anti-rabbit antibodies (Jackson Laboratories, diluted 1:100), followed by Streptavidin-FITC (Amersham, diluted 1:200). Cells and tissue sections were viewed with a Zeiss Axioplan fluorescence microscope.

RESULTS

Ptc is expressed by astrocyte lineage cells in the developing optic nerve

We showed previously that Shh is expressed in RGCs and Ptc is expressed in the adjacent neuroblast layer in the embryonic and postnatal mouse retina (Jensen and Wallace, 1997). To determine when these genes are first expressed in the retina we analyzed Ptc and Shh expression by in situ hybridization at embryonic day 12 (E12), prior to the period of maximal RGC production. Neither gene was expressed at detectable levels in the retina at this age (Fig. 1A,B), whereas by E14 Shh was expressed in the developing ganglion cell layer and Ptc was expressed in the adjacent neuroblast layer (Fig. 1F,E). Thus the onset of detectable Shh and Ptc expression in the neural retina occurs around the peak time of RGC production.

From E12 onwards, Ptc (Fig. 1A,E), but not Shh (Fig. 1B,F), was expressed in the optic nerve. As oligodendrocyte precursors do not enter the optic nerve until after E14 (Pringle et al., 1992), it seems likely that the Ptc+ cells in the nerve at E14 are cells of the astrocyte lineage, either astrocyte precursor cells and/or differentiated astrocytes. The cells in the nerve at this age, however, did not contain mRNA encoding the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 1G), suggesting that the Ptc+ cells are astrocyte precursors rather than differentiated astrocytes.

At postnatal day 0 (P0), Ptc and GFAP were expressed all along the length of the optic nerve, whereas oligodendrocyte precursors expressing the platelet-derived growth factor α receptor (PDGFαR) (Pringle et al., 1992) were confined to the chiasm end of the optic nerve (Fig. 2A-C). As GFAP is mainly present in astrocyte processes rather than in the astrocyte cell body, we could not use it as a marker to determine if some of the Ptc+ cells were astrocytes. Instead, we used the transcription factor Pax-2, which marks astrocyte lineage cells in the optic nerve (Macdonald et al., 1997; Mi and Barres, 1999)
shown). Thus the timing of declined in the first postnatal week and were rare by P14 (not shown) was detectable in the embryonic or postnatal optic nerve by in situ hybridization. At E12, Ihh mRNA (Fig. 1B and F) nor Dhh mRNA (not shown) was detectable in the embryonic or postnatal optic nerve by in situ hybridization. At E12, Ihh was expressed in a single layer of cells just outside the eye, adjacent to the retinal pigment epithelium (RPE), as well as along the optic stalk (Fig. 1D), while Ptc was expressed adjacent to these cells, both outside the RPE and within the optic stalk (Fig. 1H). As Ihh expression was no longer detectable around the eye or in the optic nerve by P0 (not shown), the most likely source of Hh protein in the postnatal optic nerve are the RGC axons, which might be expected to contain Shh, transported from the cell bodies of the RGCs. As shown in Fig. 3A, Shh was detected in lysates of postnatal rat optic nerve by western blotting using affinity-purified anti-Shh antibodies.

If the Shh protein present in the optic nerve is associated with RGC axons and Shh is responsible for the Ptc expression in the nerve, Ptc expression should greatly decrease when the nerve is cut and the axons degenerate. As shown in Fig. 4, we could not detect Ptc expression by in situ hybridization in the optic nerve 2 days after eye removal in P2 mice, while we could readily detect it in the uncut control nerve. Astrocytes were still present in the cut nerve, as GFAP expression was increased in the cut nerves compared to the uncut control nerves (data not shown).

To test directly whether Shh is responsible for Ptc expression in the postnatal optic nerve, we injected hybridoma cells that secrete a neutralizing monoclonal anti-Shh antibody (5E1; Ericson et al., 1996) into the brain of P1 mice and analyzed Ptc expression in the optic nerve 3 days later; as a control, we injected hybridoma cells that secrete a monoclonal anti-Thy-1 antibody (OX-7; Mason and Williams, 1980) of the same IgG subclass. As shown in Fig. 5, Ptc expression was undetectable by in situ hybridization in optic nerves of mice treated with the anti-Shh-secreting hybridoma cells (Fig. 5B), whereas in the nerves of mice treated with anti-Thy-1-secreting hybridoma cells Ptc expression was similar to that in un.injected littermates (Fig. 5A and not shown). In both cases, hybridoma cells expressing mouse IgG were detected around the optic chiasm of the injected animals (not shown).

Neutralizing anti-Shh antibodies reduce astrocyte proliferation in the developing optic nerve
To determine if Shh signalling influenced astrocyte development in the optic nerve, we examined GFAP expression in the nerves of mice injected with hybridoma cells, as described above. The level of GFAP expression was decreased in mice treated with anti-Shh-secreting hybridoma cells compared with mice treated with the anti-Thy-1-secreting hybridoma cells (Fig. 5C,D). This decrease could be explained by a decrease in (1) astrocyte proliferation, (2) astrocyte differentiation, (3) GFAP expression, or a combination of these mechanisms.

To determine the influence of Shh on astrocyte proliferation in the nerve, mice that had received anti-Shh-secreting hybridoma cells 3 days earlier were given 2 injections of BrdU 2 hours apart; the optic nerves were dissociated, and the proportion of GFAP+ cells that had incorporated BrdU was analyzed by indirect immunofluorescence. As shown in Fig. 6A, the proportion of BrdU+ astrocytes was decreased about 3-fold in anti-Shh-treated animals compared with either anti-Thy-1-treated or un.injected littermates, suggesting that Shh normally helps stimulate astrocyte proliferation in the developing nerve. As shown in Fig. 6B, BrdU incorporation in the retina was also reduced by the anti-Shh treatment, confirming that Hh signalling also helps stimulate precursor cell proliferation in the developing retina, as previously suggested by cell culture experiments (Jensen and Wallace, 1997).

Shh does not stimulate astrocyte proliferation in culture
To determine whether Shh was sufficient to stimulate astrocyte proliferation in vitro, optic nerve cells were isolated from P1 mice and cultured in serum-free medium for 4 days and then treated with recombinant myristoylated Shh-N or bFGF for an additional 2 days. The cultures were pulsed with BrdU for the last 8 hours, and the proportion of GFAP+ cells that had incorporated BrdU was determined. Whereas treatment with bFGF, which has been shown to be mitogenic for astrocytes (Burne and Raff, 1997; Mi and Barres, 1999), increased the proportion of BrdU+ astrocytes in the cultures, treatment with Shh-N did not (Fig. 7). The same dose of the same batch of myristoylated Shh-N did stimulate BrdU incorporation in
cultures of cerebellar granule neurons (not shown), indicating that the Shh-N protein was active. Thus Shh-N is not sufficient to drive proliferation of astrocytes in vitro, suggesting that its effect on astrocyte proliferation in vivo requires cell-cell interactions that do not operate in our dissociated cultures.

**DISCUSSION**

Several lines of evidence suggest that RGC axons stimulate the proliferation of astrocyte lineage cells in the developing rodent optic nerve. First, the proliferation of GFAP+ astrocytes is reduced after optic nerve transection (Burne and Raff, 1997). Second, astrocyte proliferation and the total number of astrocytes are increased in the developing optic nerve of Bcl-2 transgenic mice that contain 80% more RGC axons than normal (as the result of decreased normal RGC death during development), suggesting that axons are normally limiting for the proliferation of astrocyte lineage cells (Burne et al., 1996). Third, when axons are prevented from entering the optic stalk, as in the ocular retardation mutant mouse (Silver and Robb, 1979) or after optic nerve transection in the chick (Ulshafer and Clavert, 1979), glial cells, including astrocytes, fail to develop, suggesting that axons are required for the survival and/or proliferation of astrocyte lineage cells in the nerve. RGC axons have also been shown to stimulate glial cell proliferation in the optic tectum of chickens (DeLong and Sidman, 1962). In the present study we provide evidence that Shh is one of the axonal signals that helps promote astrocyte proliferation in the rodent optic nerve.

We can detect Shh protein but not mRNA in the optic nerve, whereas we can detect Shh protein in the retina and Shh mRNA in RGCs (Jensen and Wallace, 1997). These findings suggest that Shh is present in the optic nerve and that it is most likely associated with RGC axons in the nerve.

Four lines of evidence suggest that the targets of Shh-signalling in the optic nerve are mainly cells of the astrocyte lineage, rather than cells of the oligodendrocyte lineage. First, Ptc expression, a marker for Hh-responding cells, is present in the embryonic nerve before oligodendrocyte precursors have migrated into the nerve. Second, most cells in the newborn nerve that express Pax-2, a reported marker for astrocyte lineage cells (Burne et al., 1996), are Ptc+. Third, in the newborn optic nerve, where most of the dividing cells are astrocytes (Skoff et al., 1976a,b), all of the cells that become labelled following an injection of BrdU are Ptc+. Fourth, Ptc expression disappears after the second postnatal week, when astrocyte proliferation falls off and oligodendrocyte development is maximal (Skoff et al., 1976a,b; Barres et al., 1992).

Three lines of evidence suggest that RGC-axon-derived Shh helps stimulate astrocyte proliferation in the developing optic nerve. (1) Both Ptc expression (this study) and proliferation (Skoff et al., 1976a,b) in astrocyte lineage cells decreases at around the same time in the developing optic nerve. (2) Both Ptc expression (this study) and proliferation (Burne and Raff, 1997) in astrocyte lineage cells shut down prematurely when the developing nerve is transected, suggesting that they both depend on intact axons. (3) An injection of hybridoma cells secreting neutralizing anti-Shh antibodies results in the loss of Ptc expression and reduces BrdU incorporation in astrocytes, suggesting that both Ptc expression and normal astrocyte proliferation depend on Shh. This treatment also reduces the level of GFAP mRNA in the nerve, which may simply reflect the decrease in astrocyte proliferation, although it could also reflect a decrease in GFAP expression in astrocytes.

While Shh has been shown to have effects mainly on cell fate choice and the patterning of developing tissues (reviewed by Hammerschmidt et al., 1997), there are several examples in vertebrate development where it has been shown to stimulate cell proliferation. Overexpression of Shh, for example, increases proliferation in lung, muscle and skin (Bellusci et al., 1997; Duprez et al., 1998; Fan et al., 1997; Oro et al., 1997), and Shh-N increases BrdU incorporation in sclerotome cells and myoblasts in vitro (Duprez et al., 1998; Fan et al., 1995). Shh has also been shown to be a mitogen for granule neuron precursor cells in the developing cerebellum (Wallace, 1999; Wechsler-Reya and Scott, 1999). We showed previously that Shh-N is mitogenic for mouse neural retina precursor cells in pellet cultures (Jensen and Wallace, 1997), and our present finding that anti-Shh antibodies reduce neural retina cell proliferation in vivo suggests that Shh normally acts to stimulate precursor cell proliferation in the developing retina. Taken together, our previous and present findings suggest that RGC-derived Shh normally promotes cell proliferation in both the developing neural retina and optic nerve.

Our results suggest that Shh protein is transported to the RGC axon from the soma. If the protein simply diffused in the extracellular fluid from the neural retina into the nerve, one might expect that it would induce a gradient of Ptc expression along the nerve, with the highest levels of expression closest to the eye. We find, however, that the level of Ptc expression is uniform along the developing nerve. It is also unlikely that the Shh in the nerve comes via retrograde transport from the target cells that the RGCs innervate: although Shh is expressed in adult RGCs (Jensen and Wallace, 1997), we cannot detect Shh expression in the superior colliculus of adult mice (unpublished observations). How then does Shh get from the RGC soma to the axon in a form that can signal to astrocyte lineage cells in the nerve? The simplest possibility is that it is inserted into the plasma membrane of the soma and diffuses along the surface of the axon to the nerve. We have been unable to immunolocalize Shh on the surface of RGC axons, however, perhaps because the protein is present at amounts too low to detect in this way. Another possibility is that Shh is transported within vesicles in the axon and is released by exocytosis, either at the nerve terminals, followed by retrograde transport along the axonal surface back to the nerve, or in the nerve itself. Our results do not distinguish between these possibilities, but the previous finding that an intraocular injection of colchicine inhibits mitogenic signalling between axons and astrocytes in the optic nerve (Burne and Raff, 1997) raises the possibility that microtubule-dependent processes may be involved in the transport of Shh to the nerve.

We showed previously that RGCs express Shh in the adult retina (Jensen and Wallace, 1997). Ptc expression, however, is not detectable in the optic nerve after P14, suggesting that astrocytes in the nerve are no longer responding to an Hh signal. One possible explanation for this paradox is that Shh may not be transported down the axons after P14. Other
possibilities are that myelination prevents astrocytes from gaining access to Shh protein or that astrocytes become unresponsive to Shh as they mature.

Shh is probably not the only axonal signal involved in promoting astrocyte proliferation. Another candidate is basic fibroblast growth factor (bFGF), which is made by RGCs (de Iongh and McAvoy, 1992), is mitogenic for optic nerve astrocytes in culture (Burne and Raff, 1997; Mi and Barres, 1999), and might bind to FGF-receptor-3 on astrocytes (Miyake et al., 1996). RGCs also produce GGF2 (Shi et al., 1998), which has also been shown to be mitogenic for astrocyte precursors (Mi and Barres, 1999). By contrast, recombinant Shh-N is not mitogenic for optic nerve astrocytes cultured under the same conditions (Burne and Raff, 1997; Mi and Barres, 1999; and this study), suggesting that Shh signalling to astrocytes may depend on cell-cell interactions that fail to occur in dissociated cell culture. The addition of purified RGCs to cultures of astrocyte precursor cells also did not stimulate their proliferation (Mi and Barres, 1999). RGC-derived Shh signals may have been blocked in these co-cultures, however, because the culture medium contained CPT-cAMP, a cell permeable analogue of cAMP, which promotes RGC survival; increasing intracellular cAMP levels results in activation of protein kinase A, which has been shown to block Shh signalling (reviewed in Goodrich and Scott, 1998).

Our results raise the possibility that Ihh is involved in the development of the embryonic optic stalk. At E12 Ihh is expressed in cells located just outside the RPE and along the outer surface of the optic stalk (see Fig. 1D), whereas Ptc is expressed in cells directly adjacent to the Ihh-expressing cells, both within the optic stalk and outside the RPE (Fig. 1H). Although the
5E1 monoclonal anti-Shh antibody cross-reacts with Ihh (Ericson et al., 1996), it is unlikely that the reduction in Ptc expression and astrocyte proliferation that we observe in postnatal animals injected with 5E1 hybridoma cells results from the neutralization of Ihh, as we cannot detect Ihh expression by in situ hybridization anywhere in the eye or optic nerve of postnatal animals, despite two previous reports that Ihh mRNA is detectable by RT-PCR in the RPE (Levine et al., 1997; Takabatake et al., 1997). It is possible, however, that cells in the developing optic nerve receive two sequential Hh signals: an early Ihh signal from cells adjacent to the nerve and a later Shh signal from RGC axons.

A remarkable number of signalling mechanisms that operate in animal development have been evolutionarily conserved between flies and vertebrates. Shh signalling in the development of the visual system is now another example: hh in Drosophila is transported along retinal axons and stimulates both the proliferation of neural precursor cells and the expression of ptc in glial cells in the optic ganglion (Huang and Kunes, 1996), and Shh in rodents stimulates the proliferation of retinal precursor cells (Jensen and Wallace, 1997) and is apparently transported along retinal axons to influence the proliferation of astrocytes in the optic nerve (this study). Results in the fly visual system might help explain why Shh does not stimulate astrocyte proliferation in dissociated cell culture. hh signalling in lamina precursor cells induces the expression of EGF receptors in these cells, allowing the cells to respond to axon-derived Spitz, a second axon-derived signal that is required for the final differentiation of these neurons (Huang et al., 1998). Although glia in the lamina also respond to hh by expressing ptc, their migration and development requires a third, as yet unidentified, retinal-axon derived factor (Huang and Kunes, 1998). It is possible that other signals are also required for Shh to stimulate astrocyte proliferation in the rodent optic nerve and that these signals fail to operate in culture.

Shh may also mediate some forms of neuron-to-glia signalling in the adult retina and cerebellum, as RGCs and cerebellar Purkinje cells express Shh (Jensen and Wallace, 1997; Wallace, 1999; Wechsler-Reya and Scott, 1999) while Müller glia and Bergmann glia express Ptc (Traiffort et al., 1998 and unpublished observations). In all of these regions of the vertebrate CNS – optic nerve, retina and cerebellum – neurons seem to communicate with astrocyte-like glial cells via Shh, but, with the exception of the developing optic nerve, the functional significance of the signalling is entirely unknown.

RGC axons also signal to oligodendrocyte lineage cells in the developing optic nerve, but, as discussed earlier, the signalling mechanisms apparently do not involve Hh proteins. It seems that RGC axons can influence developing optic nerve glial cells by at least four distinct mechanisms. First, they promote the survival of newly formed oligodendrocytes by mechanisms that do not depend on either electrical activity (Barres and Raff, 1993) or axonal transport (Burne and Raff, 1997). Second, they promote the proliferation and/or survival of oligodendrocyte precursor cells by mechanisms that depend on electrical activity (Barres and Raff, 1993) but not on axonal transport (Burne and Raff, 1997). Third, they stimulate
astrocyte proliferation by mechanisms that depend on axonal transport but not on electrical activity (Burne and Raff, 1997). Fourth, RGCs may also control the timing of oligodendrocyte differentiation by stimulating Notch signalling in oligodendrocyte precursor cells (Wang et al., 1998). It remains a major challenge to determine the various molecular mechanisms that mediate neuron to glial cell communication.

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