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Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development

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SUMMARY

The development of optic stalk neuroepithelial cells depends on Hedgehog (Hh) signaling, yet the source(s) of Hh protein in the optic stalk is unknown. We provide genetic evidence that sonic hedgehog (Shh) from retinal ganglion cells (RGCs) promotes the development of optic disc and stalk neuroepithelial cells. We demonstrate that RGCs express *Shh* soon after differentiation, and cells at the optic disc in close proximity to the Shh-expressing RGCs upregulate Hh target genes, which suggests they are responding to RGC-derived Shh signaling. Conditional ablation of *Shh* in RGCs caused a complete loss of optic disc astrocyte precursor cells, resulting in defective axon guidance in the retina, as well as conversion of the neuroepithelial cells in the optic stalk to pigmented cells. We further show that Shh signaling modulates the size of the Pax2$^+$ astrocyte precursor cell population at the optic disc in vitro. Together, these data provide a novel insight into the source of Hh that promotes neuroepithelial cell development in the mammalian optic disc and stalk.

Key words: Retinal ganglion cells, *Shh*, *Ihh*, *Pax2*, Optic disc/stalk, Neuroepithelial cells, Astrocyte precursor cells, Development, Mouse

INTRODUCTION

Two distinct inductive events govern vertebrate eye development: an initial morphogenetic process results in regional specification of the various eye structures, and cells in these defined anatomic domains then differentiate to acquire their respective fates and functions in the mature eye (Pei and Rhodin, 1970). In mice, the morphogenetic phase begins at about embryonic day 8.5 (E8.5) with the lateral outgrowth of the prosencephalon to form the optic vesicles. By mid-gestation, the optic vesicle contacts and induces the formation of a lens placode from the overlying surface ectoderm, and simultaneously invaginates to form the bilayered optic cup, which is connected to the diencephalon by the optic stalk. The invagination of the optic vesicle extends proximally to include the ventral portion of the distal optic stalk, creating a transient opening (optic fissure) through which the hyaloid vessels gain access into the retina. All the cells of the optic stalk express the homeobox transcription factor *Pax2*, and some of these cells protrude into the retina and persist into late embryogenesis as a cuff of cells that form an annulus around exiting RGC axons. These cells separate the axons from the retinal neuroepithelium (Otteson et al., 1998) and the potential subretinal space (Rhodes, 1982). Subsequent differentiation of neuroepithelial cells in the optic vesicle depends on its interaction with other neural and non-neural tissues. For example, neuroretinal differentiation requires FGF signaling (Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000; Zhao et al., 2001) from the surface ectoderm, whereas pigment epithelial specification depends on sustained expression of the microphthalmia-associated transcription factor *Mitf*, which is maintained by activin-like signaling from the extra-ocular mesenchyme (Fuhrmann et al., 2000). In addition, the induction of *Pax2* expression in neuroepithelial cells in the optic disc and stalk is necessary for their specification as glial cells (Nornes et al., 1990; Torres et al., 1996).

Optic stalk neuroepithelial cell development as astroglia requires their interaction with RGC axons (Juurlink and Fedoroff, 1980; Huxlin et al., 1992). Classical embryological studies demonstrate that RGC axon invasion of the optic stalk is associated with increased neuroepithelial cell proliferation, and survival and transformation into glial lineage precursor cells (Ulshafer and Clavert, 1979; Navascues et al., 1985). Moreover, the failure of axons to invade the optic stalk, as observed in ZRDCT-An mice with inherited optic nerve aplasia (Silver and Hughes, 1974; Silver et al., 1984) and ocular retardation mutant mice (Silver and Robb, 1979), as well as in *Pax6*$^{-/-}$ (Grindley et al., 1995) and *Math5*$^{-/-}$ (Brown et al., 2001) mutant mice, results in abortive neuroepithelial cell

development in the optic stalk. These studies emphasize the critical requirement of RGC axons in the induction and maintenance of gliogenesis in the optic stalk. The growth cones of RGCs have been shown to contain clusters of small axoplasmic vesicles (Kuwabara, 1975), which might contain factors that signal to cells in the optic disc and stalk, with which they make tight contacts en route to the brain (Horsburgh and Sefton, 1986). Although it is well established that RGC axons are necessary for the normal development of optic disc and stalk cells, the signals that mediate this RGC axon-to-neuroepithelial cell interaction are unknown.

We have investigated the role of Shh from RGCs in optic disc and stalk neuroepithelial cell development. The Hh gene family encodes secretory glycoproteins that are required for embryonic tissue patterning and organogenesis (Ingham and McMahon, 2001; McMahon et al., 2003). The three mammalian Hh genes, sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*) and desert hedgehog (*Dhh*), share the same signaling pathway components. The Hh receptor patched (*Ptch*) and one of the transcriptional adaptors of the pathway, *Gli*, are direct targets of Hh signaling, such that transcript levels of *Ptch* (Goodrich et al., 1996; Marigo and Tabin, 1996) and *Gli* (Marigo et al., 1996; Litingtung and Chiang, 2000; Bai et al., 2002) are upregulated in Hh responsive cells and vice versa. Hence, *Ptch* and *Gli* are established molecular readouts of Hh signal reception in several tissues.

Hh genes are important regulators of ocular morphogenesis and cellular diversification in several species examined. In the early somite stage mammalian embryo, *Shh* from the prechordal plate, and subsequently from the ventral forebrain neuroepithelium (Marti et al., 1995), patterns ventral forebrain structures including the hypothalamus and optic vesicles (Chiang et al., 1996; Rubenstein and Beachy, 1998). At later developmental stages, *Shh* and *Ihh* are expressed in an overlapping temporal fashion but in distinct spatial domains of the rodent eye (Levine et al., 1997; Wallace and Raff, 1999) (present study). Although a group of peri-ocular mesenchymal cells express *Ihh* at about E12, *Shh* is expressed in the emerging RGC layer (present study). Shh signaling from RGCs regulates the proliferation, differentiation and organization of retinal neuroblasts (Jensen and Wallace, 1997; Levine et al., 1997; Stenkamp et al., 2000; Zhang and Yang, 2001; Wang et al., 2002), and, in zebrafish, also drives neurogenesis across the retina (Neumann and Nuesslein-Volhard, 2000). *Dhh* is undetectable, by in situ hybridization, in or around tissues of the developing rodent eye.

Hh proteins are also axon-associated molecules in the visual systems of both invertebrate and vertebrate species (Kunes, 2000). In the fly, Hh transmitted along retinal axons induces neurogenesis and synaptic cartridge organization in the brain (Huang and Kunes, 1996; Huang and Kunes, 1998), whereas Shh from RGCs regulates astrocyte proliferation in the rodent optic nerve (Wallace and Raff, 1999). Recent biochemical analysis of adult hamster ocular and brain tissues provides further support for a possible anterograde transport of Shh in the mammalian visual system (Traiffort et al., 2001). At about E12 of mouse development, neuroepithelial cells in the optic stalk express *Ptch* and *Gli* in the absence of Hh mRNA expression (Wallace and Raff, 1999) (present study). The source of Hh in the optic nerve at this developmental stage is unclear. However, given that Hh proteins may be axonally transported, it is not inconceivable that Shh may be associated with the growth cones or axolema of RGCs and made accessible to neuroepithelial cells in the optic stalk. In addition, optic disc neuroepithelial cells express Hh target genes whereas differentiated RGCs express *Shh*, which suggests that RGC-derived Shh could signal to neuroepithelial cells at the optic disc. To investigate these two possibilities, we used a conditional gene ablation approach because *Shh*-knockout mice exhibit severe midline patterning defects and cyclopia (Chiang et al., 1996). By successfully disrupting the *Shh* allele in regions of the CNS, including retinal precursor cells, prior to RGC differentiation, we provide genetic evidence for a requirement of RGC-derived Shh signaling in the differentiation of optic disc and stalk neuroepithelial cells.

MATERIALS AND METHODS

Mice

The generation and characterization of *Thy1-Cre* mice has been reported elsewhere (Campsall et al., 2002). Cre recombinase gene expression in these mice is under the control of the regulatory elements of the murine *Thy-1.2* gene. $Shh^{conditional}$ (Shh^{c}) mice (Lewis et al., 2001) have exon 2 of the *Shh* allele flanked by loxP sites. As exon 2 of the *Shh* gene encodes about half of the N-terminal active Shh protein, Cre-mediated recombination at the Shh^{c} locus generates an Shh^{null} (Shh^{n}) allele. The breeding strategy to generate *Thy1-Cre;*$Shh^{n/c}$ (hereafter referred to as *ThyCre*$Shh^{n/c}$) embryos has been reported (Wang et al., 2002). Briefly, the *Thy1-Cre* mice were first crossed with heterozygous *Shh* mice and progeny that were heterozygous for both the *Cre* and *Shh* alleles were then crossed with $Shh^{c/c}$ mice to generate *ThyCre*$Shh^{n/c}$-mutant embryos. We obtained $Ihh^{-/-}$ embryos by crosses between heterozygous *Ihh* mice (St-Jacques et al., 1999). Genotyping was done by PCR as described previously (Campsall et al., 2002; Lewis et al., 2001; St-Jacques et al., 1999). All mice were maintained on a mixed genetic background, and mutant mice were analyzed in comparison to their wild-type littermates. Embryonic ages of mice were assessed from the day of observed vaginal plug that was designated as E0.

Histology, RNA in situ hybridization and immunohistochemistry

Tissues for histology, in situ hybridization and immunohistochemistry were dissected in PBS and fixed overnight in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After overnight protection in 30% sucrose/PBS, tissues were embedded in sucrose:OCT (Tissue-Tek) and stored at –80°C until the day of an experiment, when 10-14 μm cryosections were cut. In situ hybridization was performed according to Wallace and Raff (Wallace and Raff, 1999). Briefly, the sections were air-dried for at least 4 hours before overnight hybridization at 65°C in a moist chamber with a specific riboprobe (diluted 1:1000). Following the usual stringency washes and an alkaline phosphatase-conjugated anti-digoxigenin antibody treatment, staining in nitro blue tetrazolium/5-bromo-4-chloro-3-indoylphosphate revealed the blue color indicative of regions of specific in situ gene expression. Templates of full-length *Shh* (*Shhfl*), *Shh* exon 2 (*Shhexon2*), *Ptch*, *Gli*, *Pax2*, *Pax6*, Netrin 1 (*Ntn1*), *Mitf*, *Vax2*, *Pdgfra* and *Nkx2-1* were in vitro transcribed to generate the respective digoxigenin-labeled antisense riboprobes.

Anti-neurofilament-associated protein immunohistochemistry was performed essentially according to Jensen and Wallace (Jensen and Wallace, 1997), with a monoclonal antibody-3A10 (Developmental Studies Hybridoma Bank). For collagen type IV immunoreactivity, sections were fixed in -20°C acetone, treated with 0.3% H_2O_2, and blocked in 20% sheep serum in 0.5% Triton X-100 before incubation for at least 1 hour at room temperature with polyclonal anti-collagen

type IV antibody (1:3000 Biogenesis). Using diaminobenzidine (DAB) as a substrate, conjugated antibodies were detected with the Vectorstain ABC Elite avidin/biotin/peroxidase kit (Vector Laboratories, Burlingame, California).

Retinal Explant Culture

Optic cups of E12 and E14 C57BL/6 embryos were dissected in MEM-HEPES (ICN) and cultured on 13 mm polycarbonate filters (pore size: 0.8 μm; Nucleopore) in serum-free conditions as described previously (Wang et al., 2002). The culture medium was composed of 1:1 DMEM/F12, insulin (10 μg/ml), transferrin (100 mg/ml), BSA Fraction V (100 mg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml) and gentamycin (25 μg/ml). Except for untreated controls, the eyecups were cultured in the presence of a recombinant myristoylated N-terminal active fragment of Shh (Shh-N) at 2 μg/ml, an anti-Hh antibody (5E1) at 30 μg/ml (Ericson et al., 1996) or an isotype-matched antibody (1E6) at 30 μg/ml. After 48 hours in culture, tissues were processed for in situ hybridization, and serial sections cut through the entire eyecup and analyzed for *Pax2* expression. All stained sections were examined on a Zeiss Axioplan microscope and digital images were captured with the Axio Vision 2.05 (Zeiss) camera and processed with Adobe Photoshop, version 7.

RESULTS

Temporal and spatial expression patterns of *Shh* and Hh target genes during ocular morphogenesis

To obtain an indication of the probable source(s) and roles of Hh signaling in the developing optic stalk we analyzed, by in situ hybridization, the temporal and spatial expression of *Shh*, *Ptch* and *Gli* in the developing optic primodia prior to, and during, RGC differentiation. At about E8.5, *Shh* expression in the prechordal plate resolves the single visual field into two separate optic primodia (Marti et al., 1995). By E9.0, *Shh* is expressed in the basal forebrain neuroepithelium (Fig. 1A), in response to which *Ptch* is upregulated in the ventral forebrain neuroepithelium and the surrounding cephalic mesenchyme close to the *Shh*-expressing cells at the midline (Fig. 1E). However, by E11 *Ptch* expression is graded in the ventral diencephalon (Fig. 1F). The anterior hypothalamic neuroepithelial cells closest to the midline source of Shh (Fig. 1B) express higher levels of *Ptch* compared with cells in more distal regions of the optic stalk (Fig. 1; compare B and F). This expression pattern of *Ptch* is consistent with the presence of a graded Shh activity from the ventral midline (Fig. 1B). Interestingly, from E12 onwards, Hh target gene expression is rather uniform along the entire length of the optic stalk and nerve (Fig. 1G,H). It is noteworthy that the period from E12 to E14 is associated with rapid glial cell development in the optic stalk (Kuwabara, 1975). The dynamic expression patterns of *Shh* and its target genes in the developing optic primodia, in conjunction with the demonstration that Shh is an axon-associated molecule in the visual system of perinatal and adult mammals (Wallace and Raff, 1999; Triaffort et al., 2001), suggest Shh from early-born retinal neurons (Fig. 1C,D) may be transported into the optic stalk to promote neuroepithelial cell development.

Neuroepithelial cells that originate from the optic stalk form a cuff around RGC axons at the optic disc. At a later developmental stage, these cells differentiate and migrate into the retina as retinal astrocytes (we will refer to these cells as optic disc astrocyte precursor cells). Prior to E12, cells in the region of the prospective optic disc, and at the lips of the optic fissure, express *Pax2* and *Ntn1* (Dressler et al., 1990; Nornes et al., 1990; Deiner et al., 1997; Otteson et al., 1998). However, following closure of the optic fissure, these genes are downregulated in the ventral optic cup, but persist in optic disc and stalk neuroepithelial cells (Fig. 1K,L). Thus, optic disc astrocyte precursor cells can be identified by the expression of genes, such as *Pax2* and *Ntn1*, that are not expressed by adjacent retinal neuroblasts. We also demonstrate that the optic disc astrocyte precursor cells are early targets of Hh signaling. RGC differentiation begins and spreads from the central to the peripheral retina. Soon after differentiation, RGCs express *Shh* (Fig. 1C,D,I), and the highest levels of Hh target gene expression in the retina at E12 are in optic disc astrocyte precursor cells that are adjacent to the Shh-expressing RGCs (Fig. 1J). The high Hh target gene expression by optic disc astrocyte precursor cells compared with adjacent retinal neuroblasts probably indicates that these cells are among the first cells to respond to RGC-derived Shh signal; the rest of the retina catches up in terms of *Gli* intensity at later developmental stages (Fig. 1N; data not shown). Although we did not perform co-localization experiments, examination of serial sections through the optic discs of E12 to E14 embryos for *Gli*, *Pax2* and *Ntn1* transcripts suggests the same cells are expressing these genes at the optic disc (Fig. 1J-L; data not shown). The differentiation program of optic disc astrocyte precursor cells involves the downregulation of Hh responsiveness, as evidenced by decreased *Gli* expression in $Pax2^{+}$/ $Pdgfr\alpha^{+}$ retinal astrocyte precursor cells at the disc and in those migrating into the retina (Fig. 1N-P; data not shown). Again, it is of interest to note that the period from E12 to E14 is coincident with rapid RGC differentiation and axon routing into the optic stalk, and that this window corresponds to the period of maximal response of optic disc astrocyte precursor cells to Hh signaling.

Ihh signaling is not required for *Gli* and *Ptch* expression in the retina and optic nerve

Another mammalian hedgehog homolog, *Ihh*, is expressed by a group of mesenchymal cells located outside the retinal pigment epithelium (Wallace and Raff, 1999) (data not shown), raising the possibility that *Ihh* could signal to cells in the retina, optic disc and stalk. To delineate the relative roles of the two Hh genes in ocular tissue patterning, we examined eye development and Hh target gene expression in ocular tissues of $Ihh^{-/-}$-mutant mice at various developmental stages. The eye sizes of *Ihh* mutants were comparable to their wild-type littermates (Fig. 2B,E). However, in contrast to wild type, *Gli* and *Ptch* expression were markedly downregulated in a layer of peri-ocular mesenchymal cells surrounding the retinal pigment epithelium (Fig. 2; arrowheads in A,D; data not shown). However, *Gli* expression in the neuroretina and optic nerve of $Ihh^{-/-}$ mice was not different from wild-type littermates (Fig. 2A,B,D,E). Astrocyte precursor cells at the optic disc and in the optic nerve also developed normally in $Ihh^{-/-}$ mice, as indicated by normal *Pax2* expression (Fig. 2F). The loss of *Gli* expression in the peri-ocular mesenchyme of $Ihh^{-/-}$ mice (Fig. 2D) indicates that RGC-derived Shh does not signal in the peri-ocular tissue. Likewise, Ihh signaling from the peri-ocular mesenchyme does not induce *Gli* expression in

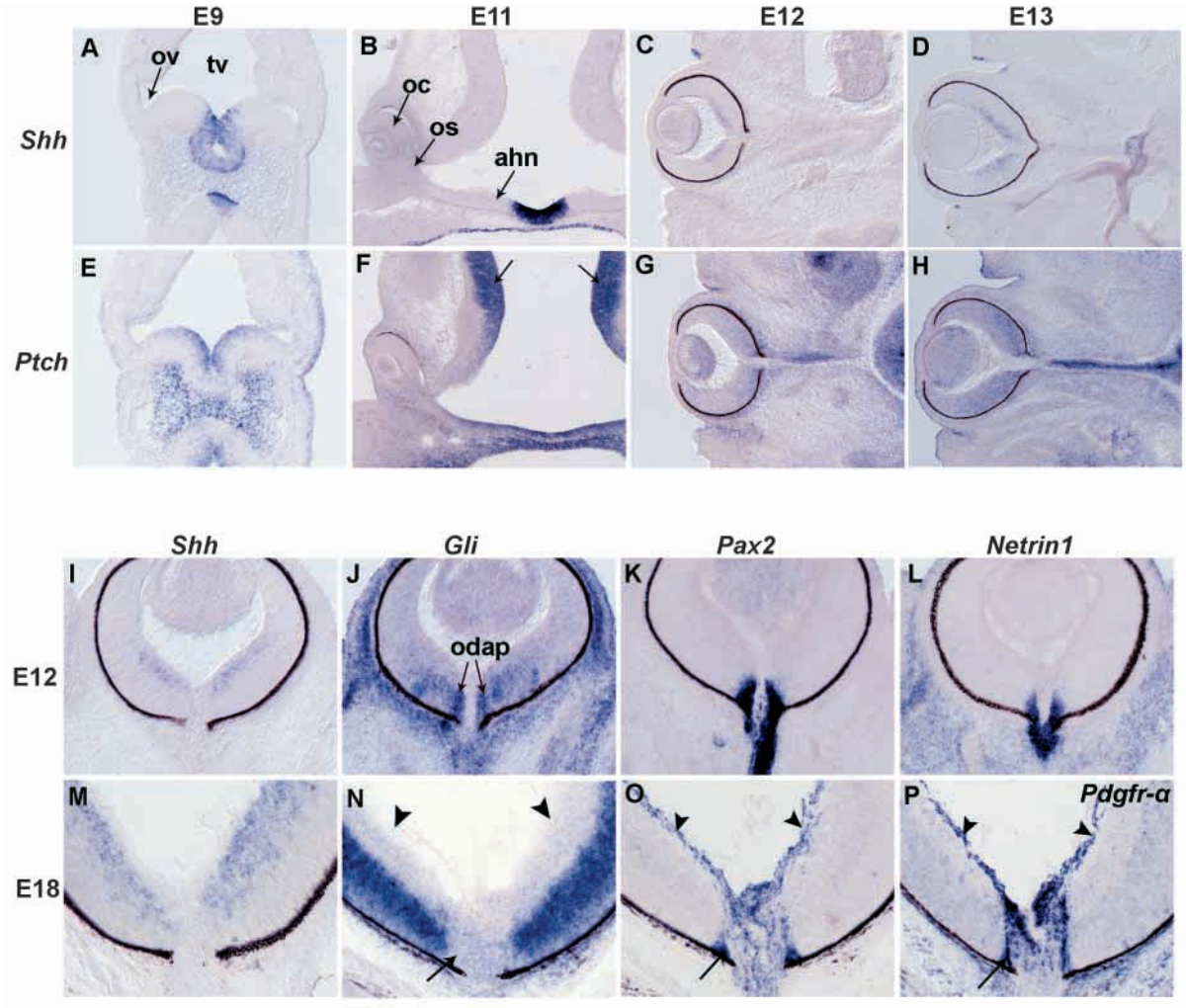

Fig. 1. Expression analysis suggests that Shh from RGCs signals to cells in the retinal neuroblast, optic disc and optic stalk. (A,E) Sections through the optic vesicle of an E9 embryo hybridized with *Shh* (A) and *Ptch* (E) riboprobes demonstrate the upregulation of *Ptch* expression in the neuroepitheliun and cephalic mesenchyme adjacent to the *Shh*-expressing cells. (B,F) Frontal sections through the developing eye of an E11 embryo reveals a graded *Ptch* expression (F) in the ventral forebrain, with high levels in the anterior hypothalamic neuroepithelium (ahn) and low levels in the optic stalk (os), consistent with an established morphogen gradient of *Shh* (B) from the ventral midline. *Ptch* (arrows in F) expression in the diencephalic neuroepithelium is due to *Shh* from the zona limitans intrathalamica (not shown). The period from E12 to E14 is when most neuroepithelial cells of the optic stalk transform into astrocyte progenitor cells (Kuwabara, 1975), and this period is coincident with the rapid RGC differentiation and expression of *Shh* (C,D), as well as uniform *Ptch* (G,H) expression in the optic nerve. (I-P) The response of optic disc astrocyte precursor cells (odap) to RGC-derived Shh signaling. As RGCs differentiate in the central retina and express *Shh* (I), optic disc astrocyte precursor cells and retinal neuroblasts in close proximity to the *Shh*-expressing cells respond by upregulating the Hh target gene, *Gli* (J). It is likely that the *Gli*-expressing cells at the disc are the same cells that express *Pax2* (K) and netrin 1 (*Ntn1*; L). Although RGCs continue to express *Shh* (M) into late embryogenesis and the underlying neuroblasts respond to this by expressing *Gli* (N), the *Pax2*- (O) and *Pdgfra*- (P) expressing retinal astrocyte precursor cells migrating into the retina (arrowheads in N,O,P), and those at the optic disc (arrows in N,O,P), downregulate their Hh responsiveness. ov, optic vesicle; tv, telencephalic vesicle; oc, optic cup; os, optic stalk; ahn, anterior hypothalamic neuroepithelium; odap, optic disc astocyte precursor cells.

the retinal neuroblasts of *ThyCreShh*$^{n/c}$ mice with conditional ablation of *Shh* in RGCs (Fig. 2C). Taken together, these findings suggest that Ihh signaling is received by nearby mesenchymal cells, and is not required for Hh target gene expression in the retina, optic disc and nerve.

Optic nerves of *ThyCreShh*$^{n/c}$ embryos are 'hypoplastic', hypocellular and pigmented

Our expression analyses suggest that Shh from RGCs may signal to optic disc and stalk neuroepithelial cells at the peak of their transformation into glial progenitors. To directly address this question, we used a conditional gene ablation approach to disrupt *Shh* in RGCs (see Materials and Methods). The *Thy1-Cre* line 703 mice used for this study expressed Cre recombinase in the developing CNS, including the optic cup, prior to RGC differentiation (Campsall et al., 2002). *ThyCreShh*$^{n/c}$ embryos were recovered at the expected Mendelian ratio. A total of 46 *ThyCreShh*$^{n/c}$-mutant embryos were generated for this study, of which 10 (21.7%) were excluded from the analysis because the eyes were closer to the

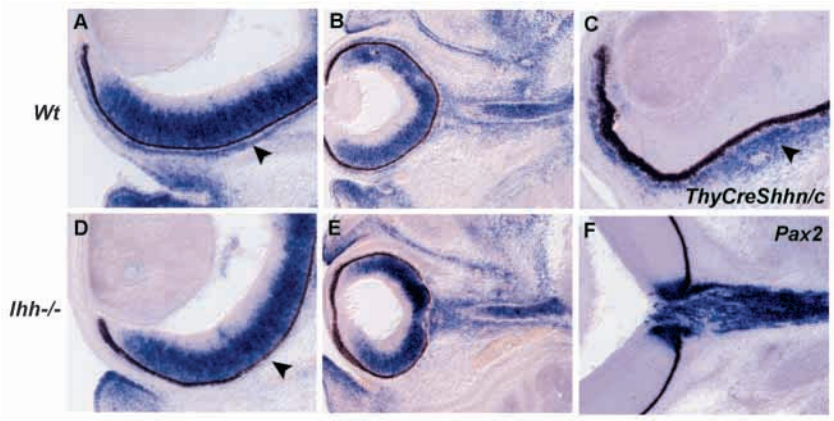

Fig. 2. Ihh signaling is not required for Hh target gene expression in the retina and optic nerve. (A-E) *Gli* and (F) *Pax2* transcripts detected by in situ hybridization in sections through the developing eye and optic nerve of E14 wild-type (A,B) and *Ihh*$^{-/-}$ (D-F) embryos, and E15 *ThyCreShh*$^{n/c}$ embryos (C). (A,B,D-F) Horizontal sections. (C) Frontal section. Compared with wild type (A,B), *Gli* expression is specifically lost in the peri-ocular mesenchyme of *Ihh*$^{-/-}$ mice (D,E) and in the retinal neuroblast layer of *ThyCreShh*$^{n/c}$ embryos (C). Arrowheads (A,C,D) indicate *Gli*-expressing cells adjacent to the retinal pigment epithelium. Astrocyte precursor cells in the optic nerve and at the optic disc develop normally in *Ihh*$^{-/-}$-mutant embryos, as indicated by normal *Pax2* expression (F).

midline and the neuroretina expanded into the optic stalk, suggestive of a possible loss of midline Shh signaling. The 36 (78.3%) *ThyCreShh*$^{n/c}$ mice included in this study developed grossly normal bodies and well separated bilateral eyes (Fig. 3D), but displayed a consistent craniofacial phenotype that was evident as early as E13. This phenotype consisted of microcephaly, hypoplastic craniofacial structures, micropthalmia and failure of eyelid closure throughout gestation (Fig. 3; compare A and D), which is probably caused by inactivation of the *Shh* allele in other regions of the CNS and ectoderm as well. A strong indication of efficient Cre-mediated disruption of *Shh* in RGCs of *ThyCreShh*$^{n/c}$ embryos was the loss of wild-type *Shh* transcripts in RGCs, as indicated by *Shhexon2* RNA in situ hybridization (Wang et al., 2002) (data not shown), as well as the marked downregulation of *Ptch* and *Gli* expression in retinal neuroblasts in contrast with wild-type littermates (Fig. 3B,C,E,F). The trace *Gli* and *Ptch* transcripts in the distal part of the retina of *ThyCreShh*$^{n/c}$ mice (Fig. 3E,F; arrows) are caused by the response of retinal neuroblasts to Shh signaling from a few RGCs in this area that escaped Cre-mediated recombination of the *Shh* allele. Shh from these cells will have no effect on the development of optic disc and stalk neuroepithelial cells because these are late generated RGCs.

The developing optic stalk and nerve of *ThyCreShh*$^{n/c}$ embryos was characterized in comparison to wild-type littermates. Ocular derivatives of the optic cup, surface ectoderm and neural crest cells, including the cornea, ciliary body, lens, neural retina and retinal pigment epithelium, appear to develop in their normal anatomic positions (Fig. 8; compare C and F). A striking finding was the complete loss of glial progenitor cells in the optic nerves of *ThyCreShh*$^{n/c}$ embryos. In contrast to wild type E17 optic nerves, which contained Ntn1^{+}/Pax2^{+} glial cells and RGC axons, as indicated by neurofilament staining (Fig. 4A-C), the optic nerves of *ThyCreShh*$^{n/c}$ embryos were thin, contained few cells (hypocellular) and were surrounded by a thick layer of pigmented cells (Fig. 4F-J). In all the *ThyCreShh*$^{n/c}$ embryos analyzed, the pigmented cells were continuous with the retinal pigment epithelium, but extended variable distances towards the ventral diencephalon (Fig. 5H). In conditions where pigmented cells did not populate the entire optic nerve, cells located closer to the diencephalon expressed *Ntn1* and *Pax2* (Fig. 4F,G; Fig. 5H; arrows).

To obtain further insight as to the possible cause of the optic nerve phenotype of *ThyCreShh*$^{n/c}$ embryos, we compared optic vesicle development at early stages in wild-type and *ThyCreShh*$^{n/c}$ mice. Initially, the optic vesicles of both wild-type and *ThyCreShh*$^{n/c}$ mice were patterned normally, each forming two separate eyes with a distinct distal optic cup and a proximal optic stalk (Fig. 5; compare A-C with E-G). The development of the optic primodia of *ThyCreShh*$^{n/c}$ mice subsequently lagged behind those of wild-type littermates, such that, from E12 onwards, *ThyCreShh*$^{n/c}$ embryos displayed smaller eyes (Fig. 3; compare B,C with E,F). At E12, wild-type optic nerves contained *Ntn1*- and *Pax2*-expressing astrocyte precursor cells (Fig. 5D; data not shown), whereas *ThyCreShh*$^{n/c}$-mutant optic nerves were occupied with

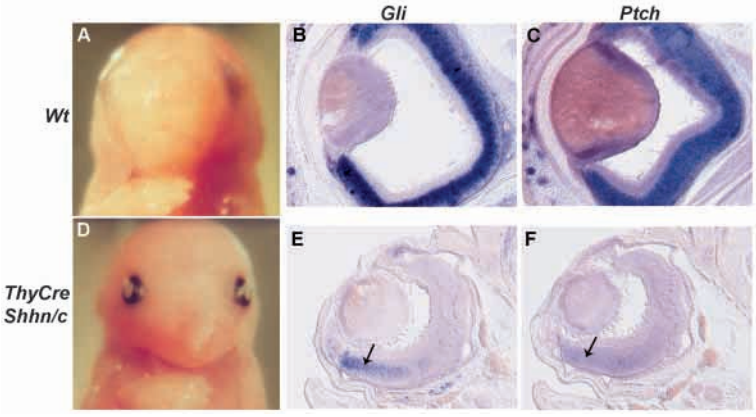

Fig. 3. Craniofacial phenotype and Hh target gene expression in the retina of wild-type and *ThyCreShh*$^{n/c}$ mice. (A,D) Photographs of E17 embryos showing that *ThyCreShh*$^{n/c}$ embryos (D) develop well separated bilateral eyes but, compared with wild type (A), have hypoplastic craniofacial structures and failed eyelid closure. (B,C,E,F) Expression of Hh target genes in retinal neuroblasts of E17 wild-type (B,C) and *ThyCreShh*$^{n/c}$ embryos (E,F) reveals a marked downregulation of *Gli* (E) and *Ptch* (F) expression in the retina of *ThyCreShh*$^{n/c}$ embryos in comparison with wild-type littermates (B,C). Arrows (E,F) point to retinal neuroblasts responding to Shh signaling from a few RGCs that escaped Cre-mediated recombination of the *Shh* allele in this part of the retina.

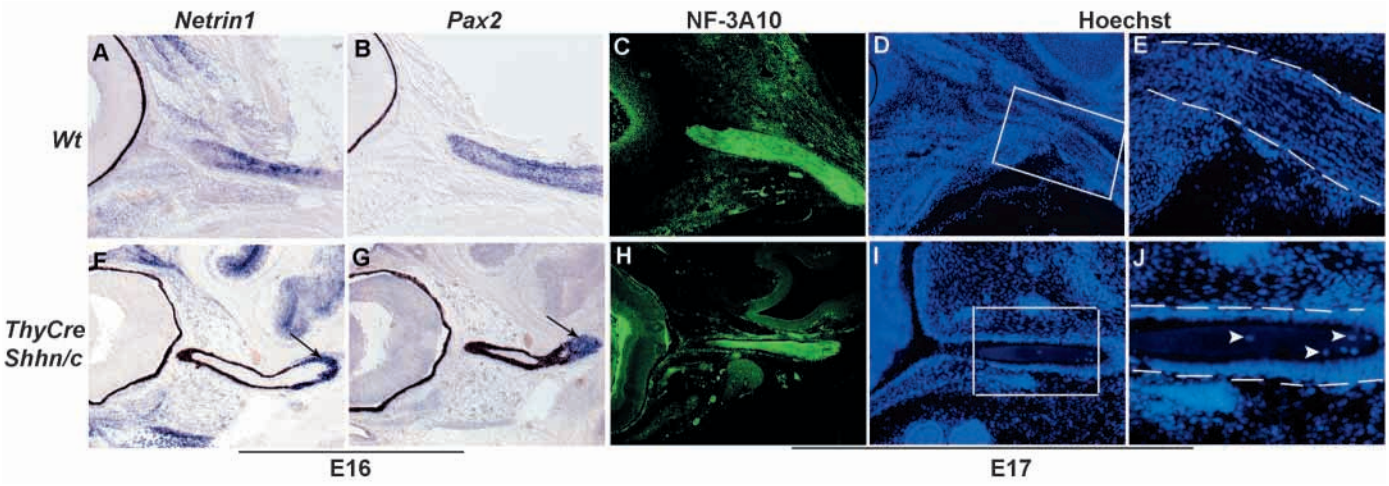

Fig. 4. The optic nerves of *ThyCreShh*$^{n/c}$ embryos are hypocellular and covered by a thick layer of melanotic cells. (A,B,F,G) RNA in situ hybridization for *Ntn1* (A,F) and *Pax2* (B,G) expression in sections through the eye and optic nerve of E16 wild-type (A,B) and *ThyCreShh*$^{n/c}$ (F,G) embryos. Note the expression of *Ntn1* and *Pax2* (arrows in F,G) in only proximal optic stalks of *ThyCreShh*$^{n/c}$ mice. Although RGC axons invaded the optic nerves of *ThyCreShh*$^{n/c}$ mice, as evidenced by anti-neurofilament-3A10 immunostaining (compare C and H), neuroepithelial cells in the nerve failed to differentiate as glial cells, resulting in the hypocellularity of the optic nerves (compare D,E with I,J). (E,J) High magnifications of the boxed areas in D and I. Dashed lines (E,J) outline the optic nerves, and arrowheads (I) indicate nuclei of some cells in optic nerves of *ThyCreShh*$^{n/c}$ mice.

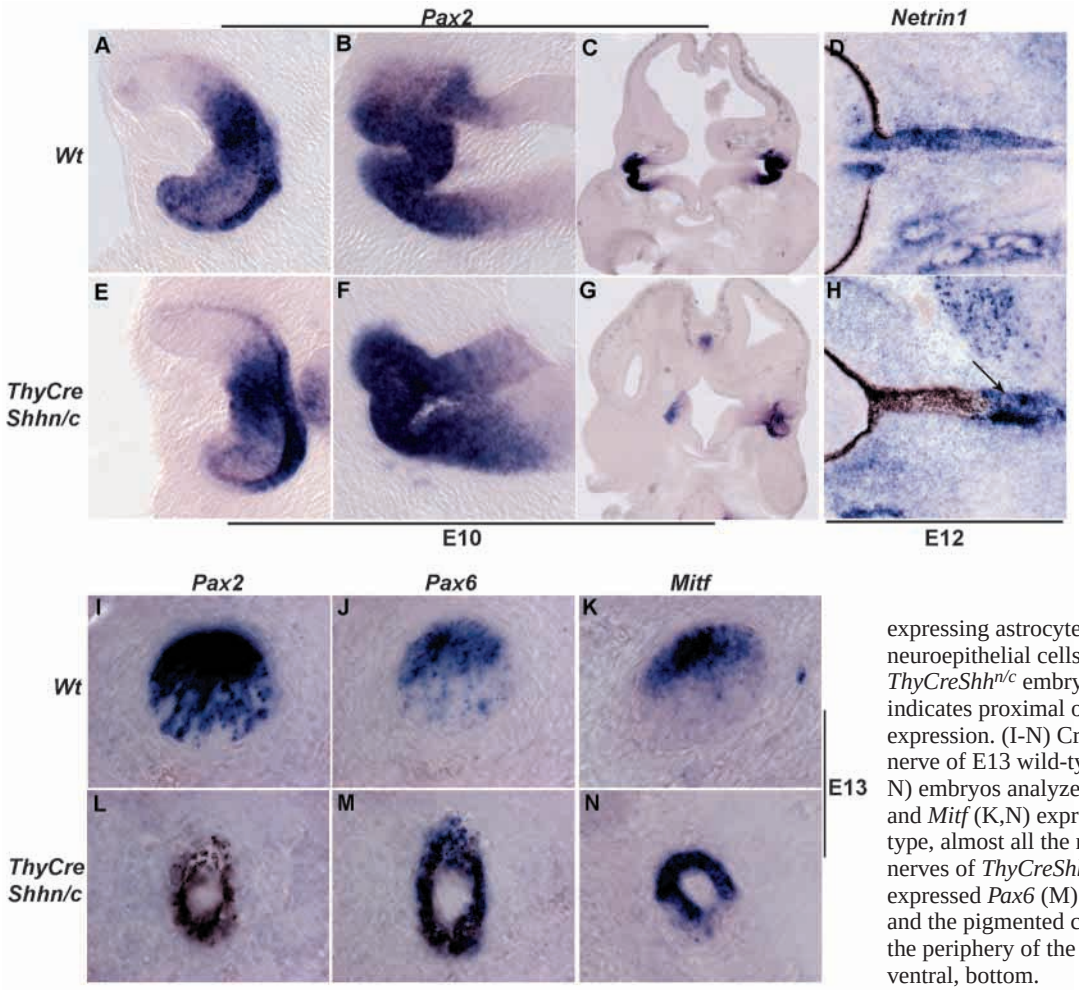

Fig. 5. Normal optic vesicle patterning and abnormal gene expression in the optic nerves of *ThyCreShh*$^{n/c}$ mice. (A-C,E-G) *Pax2* expression detected by in situ hybridization in sections through the developing optic vesicle of E10 wild-type (A-C) and *ThyCreShh*$^{n/c}$ (E-G) embryos. Pax2 expression and optic vesicle morphology appear normal in *ThyCreShh*$^{n/c}$ when compared to wild-type littermates. Note that wild-type (C) and *ThyCreShh*$^{n/c}$ (G) embryo sections were not exactly through the same frontal plane. By E12, wild-type (D) optic nerves contained *Ntn1*-expressing astrocyte lineage cells, but the neuroepithelial cells in the optic stalk of *ThyCreShh*$^{n/c}$ embryos (H) were pigmented. Arrow indicates proximal optic stack with normal Ntn1 expression. (I-N) Cross sections through the optic nerve of E13 wild-type (I-K) and *ThyCreShh*$^{n/c}$ (L-N) embryos analyzed for *Pax2* (I,L), *Pax6* (J,M) and *Mitf* (K,N) expression. Compared with wild-type, almost all the neuroepithelial cells in the optic nerves of *ThyCreShh*$^{n/c}$ mice were pigmented and expressed *Pax6* (M) and *Mitf* (N), but not *Pax2* (L), and the pigmented cells were already separating to the periphery of the axons. (I-N) dorsal, top; ventral, bottom.

pigmented cells (Fig. 5H) interspersed with RGC axons. A day later, the pigmented cells appeared to have moved to the periphery of the optic nerve (Fig. 5L-N), which is consistent with the idea that pigmented cells and axons are repellant (Silver and Sapiro, 1981). This observation raised the question of a possible abnormal transcriptional control of neuroepithelial cell development in the optic stalk. We addressed this issue by examining a number of transcription factors shown to be necessary for the normal differentiation of the optic stalk and retinal pigment epithelium. *Pax2* and *Pax6*, for example, undergo dynamic regulation in the optic stalk and nerve, with the gradual repression of *Pax6* by *Pax2* (Schwarz et al., 2000) such that by E14 all the cells in the optic nerve express *Pax2* and not *Pax6*. Sagittal sections of E13 wild-type brains revealed high levels of *Pax2* and low levels of *Pax6* expression in the optic nerve (Fig. 5I,J). Also at this developmental stage, *Mitf* was expressed only in the dorsal regions of the distal optic nerve in wild-type mice (Fig. 5K). By contrast, the distal two thirds of the optic nerves of *ThyCreShh*$^{n/c}$ embryos contained no *Pax2*-expressing glial cells. Instead, almost all the neuroepithelial cells in the optic stalk were pigmented, and expressed *Pax6* and *Mitf* (Fig. 5L-N), which indicates abnormal transcriptional regulation of the differentiation program of optic stalk neuroepithelial cells in these mutant mice.

ThyCreShh$^{n/c}$ embryos have RGC axon guidance defects in the retina

We showed previously that RGC development is induced across the retina of *ThyCreShh*$^{n/c}$ mice, and that it may even be increased (Wang et al., 2002) (Y.P.W., unpublished). Hence, the optic nerve 'hypoplasia' of *ThyCreShh*$^{n/c}$ mice suggested that not all the axons exited the retina. We therefore stained retinal sections of perinatal *ThyCreShh*$^{n/c}$ mice with an anti-neurofilament-3A10 antibody, which revealed a remarkable axon guidance defect in the retina that was very similar to that observed in *Ntn1*$^{-/-}$ mutant mice (Fig. 6C-F) (Deiner et al., 1997). Compared with wild-type mice (Fig. 6A,B), RGC axons of *ThyCreShh*$^{n/c}$ embryos were misrouted to sub-retinal spaces in several regions of the retina and at the optic disc (Fig. 6C-F). Of the axons that reached the disc, some failed to exit into the optic nerve, and instead coiled in the sub-retinal space (Fig. 6; white asterisks in C and D). The similarity of the retinal phenotype of *ThyCreShh*$^{n/c}$ embryos to that of *Ntn1*$^{-/-}$ mutants, and the observation that optic disc astrocyte precursor cells are early targets of Hh signaling, suggested that netrin signaling was disrupted in optic disc astrocyte precursor cells. We therefore examined the development of the optic disc astrocyte precursor cells in *ThyCreShh*$^{n/c}$ embryos. We were unable to detect any *Ntn1/Pax2*-expressing cells at the optic discs of E12 *ThyCreShh*$^{n/c}$ embryos by in situ hybridization analysis (Fig. 7A,D; data not shown). From E15 onwards, the optic disc astrocyte precursor cells express *Pdgfra* as they migrate into the retina over RGC axons, such that they are easily detected in sections at, or within the vicinity of, the optic nerve head (Fig. 7B,C). In contrast to wild-type littermates (Fig. 7B,C), three *ThyCreShh*$^{n/c}$ embryos examined at this developmental stage consistently demonstrated absence of *Pax2*, *Pdgfra* and *Ntn1* expression in the optic disc region (Fig. 7E,F; data not shown).

We needed to rule out confounding factors that might also contribute to the eye phenotype of *ThyCreShh*$^{n/c}$ embryos. First, Shh is a mitogen for retinal neuroblasts (Jensen and Wallace 1997; Levine et al., 1997; Stenkamp et al., 2000) and perinatal optic nerve astrocytes (Wallace and Raff, 1999). Thus the absence of retinal astrocyte precursor cells at the optic nerve head of *ThyCreShh*$^{n/c}$ embryos could have resulted from failure to stimulate the proliferation of these cells in the absence of RGC-derived Shh signaling. We addressed this issue by examining the retinas of cyclin D1$^{-/-}$ mice that are highly defective in cellular proliferation in the retina (Fantl et al., 1995; Sicinski et al., 1995). Although cyclin D1$^{-/-}$ mice at E18 displayed thinner retina than wild-type littermates, *Pax2* and *Pdgfra* were expressed in the optic disc and retina, and there was no RGC axon misrouting (Fig.

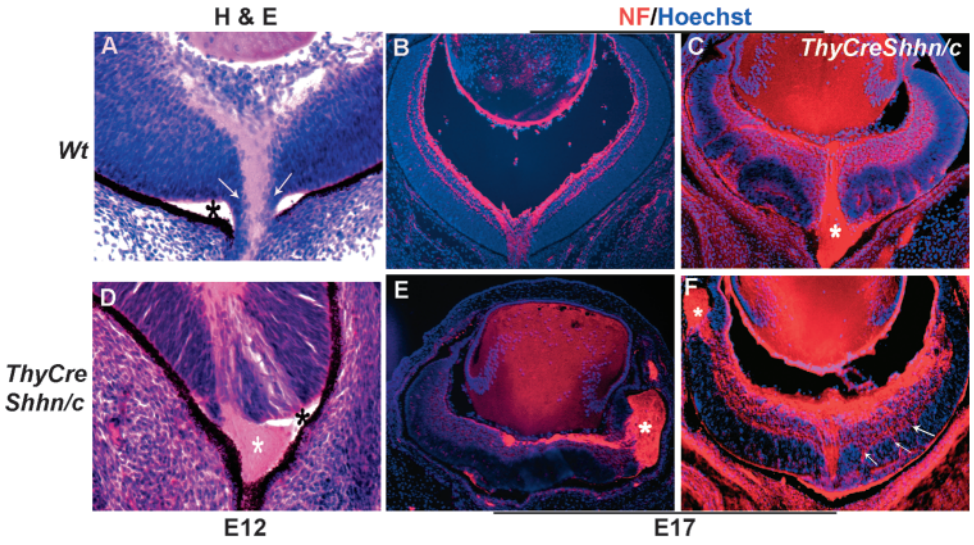

Fig. 6. RGC axon guidance defects in *ThyCreShh*$^{n/c}$ mice. (A,D) Hematoxylin and Eosin staining of sections through the optic disc of E12 wild-type (A) and *ThyCreShh*$^{n/c}$ (D) embryos. Note that in the wild type, the eosinophilic RGC axons are separated from the potential subretinal space (black asterisk in A) by the optic disc astrocyte precursor cells (white arrows in A), and that these cells are missing in *ThyCreShh*$^{n/c}$ mutants (D) thereby exposing the subretinal space (black asterisk in D) to invasion by RGC axons (white asterisk in D). Anti-neurofilament-associated protein immunostaining (B,C,E,F) of E17 retinal sections of wild-type (B) and *ThyCreShh*$^{n/c}$ embryos (C,E,F) reveals the retinal axon guidance defects of *ThyCreShh*$^{n/c}$ embryos. Compared with wild type (B), *ThyCreShh*$^{n/c}$ embryos display axon coiling in the subretinal space at the optic disc (white asterisks in C and D), and axon misrouting into the peripheral retina (open white asterisks in E,F). Note the severe axon misguidance in (F) that creates what looks like multiple optic discs (white arrows in F).

7G-J; data not shown). Second, the failure of RGC axons to exit the retina of *ThyCreShh*$^{n/c}$ mice could have resulted from abnormal formation or faulty closure of the optic fissure, as observed in the inherited optic nerve aplastic strain, ZRDCT-An (Silver et al., 1984), *Chx10*^{or} (Smith et al., 2002) and *Bst*/+ (Rice et al., 1997) mutant mice. To determine whether the eye phenotype of *ThyCreShh*$^{n/c}$ mice was caused by abnormalities in optic fissure closure, we examined sagittal sections through the eye at E13, a time at which the optic fissure should normally be closed. In both wild-type and *ThyCreShh*$^{n/c}$ embryos, the optic fissures were completely closed, and the expression of *Vax2*, a homeobox transcription factor that is important for this process (Barbieri et al., 2002), was localized to the ventral retina (Fig. 8A,D). In addition, the expression of *Pax6* and other neuroretinal genes in the retina of *ThyCreShh*$^{n/c}$ embryos was comparable to wild-type littermates (Fig. 8B,E; data not shown). The extension of the choroid fissure into the distal optic stalk is also necessary for the hyaloid vessels to enter the optic cup and establish the vascular tunic of the lens (Smith et al., 2002). Collagen type IV immunostaining for retinal blood vessels of wild-type and *ThyCreShh*$^{n/c}$ embryos revealed normal hyaloid vasculature in the posterior chamber of the eye (Fig. 8C,F), again suggestive of normal formation and closure of the optic fissure in *ThyCreShh*$^{n/c}$ mice.

The ocular phenotype of *ThyCreShh*$^{n/c}$ mice is not a consequence of loss of midline Shh early during eye development

Cre-recombinase activity in line 703 *Thy1-Cre* mice used for this study was heterogeneous and not restricted to the retina, as some of the *ThyCreShh*$^{n/c}$ embryos we recovered had holoprosencephaly, which indicates that midline *Shh* expression was affected in these mice (these mice were not included in our analysis). Given the importance of midline-derived Shh signaling in proximal-distal patterning of the optic vesicle, we were concerned that the eye phenotype we observed could have resulted from loss of Shh at the midline. To address this issue, we first examined the expression of *Shhexon2*, *Ptch* and *Gli* in the developing hypothalamus by in situ hybridization. These transcripts were detectable in *ThyCreShh*$^{n/c}$ embryos at comparable levels to their wild-type littermates (Fig. 9A,D; data not shown). We next examined some markers of the diencephalon such as *Nkx2-1* (*Titf1* – Mouse Genome Informatics) and *Ntn1*, whose expression may be regulated by Shh signaling (Sussel et al., 1999; Pabst et al., 2000; Hynes et al., 2000). Analysis of *Nkx2-1* and *Ntn1* expression in E13, E16 and E17 *ThyCreShh*$^{n/c}$ embryos showed comparable expression patterns to wild-type littermates (Fig. 9B,C,E,F; data not shown), which indicates the hypothalamus was present in *ThyCreShh*$^{n/c}$ mice, and its ventral molecular identity was preserved as well. Based on the above findings, and the presence of bilateral eyes in *ThyCreShh*$^{n/c}$ embryos, it is unlikely the eye and optic nerve defects are due to loss of *Shh* expression at the ventral midline.

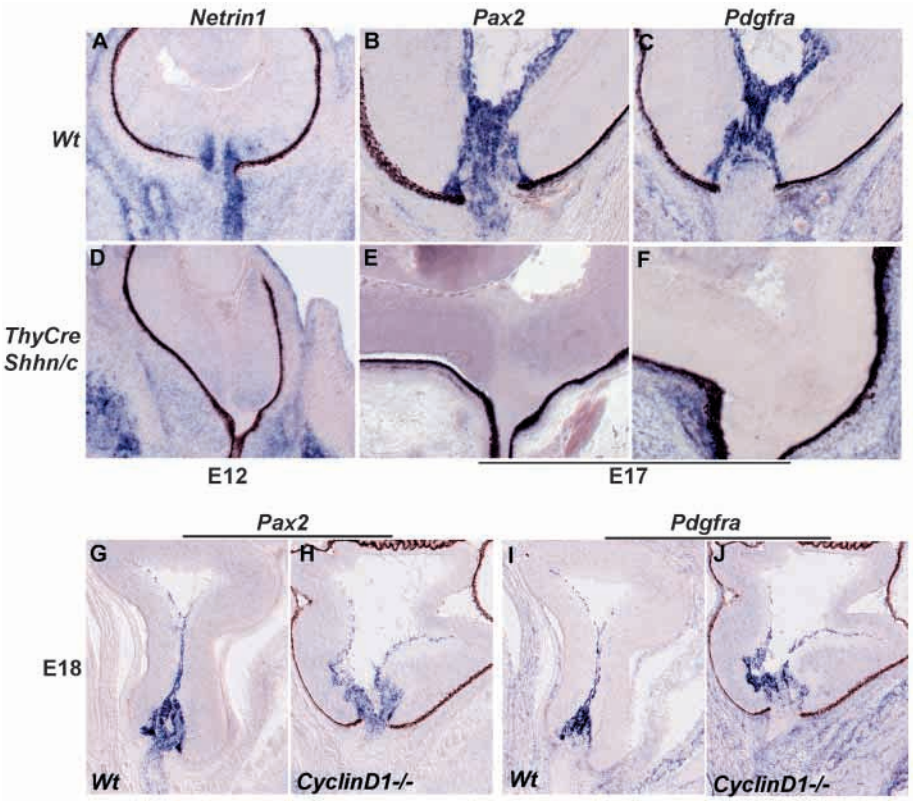

Fig. 7. Loss of optic disc astrocyte precursor cells in *ThyCreShh*$^{n/c}$ mice is not caused by a global defect in proliferation. *Ntn1* (A,D), *Pax2* (B,E) and *Pdgfra* (C,F) transcript expression detected by in situ hybridization in sections through the optic disc of wild-type (A-C) and *ThyCreShh*$^{n/c}$ (D-F) embryos, at E12 (A,D) and E17 (B,C,E,F). Compared with wild-type mice (A-C), there was no *Ntn1* (D), *Pax2* (E) or *Pdgfra* (F) expression in optic discs of *ThyCreShh*$^{n/c}$-mutant mice. *Pax2* (G,H) and *Pdgfra* (I,J) expression is comparable between E18 wild-type (G,I) and cyclin D1$^{-/-}$ (H,J) mice.

Shh modulates optic disc astrocyte precursor cell population in vitro

Our in vivo analysis is consistent with a model whereby Shh from RGCs acts both early and transiently to promote the development of astrocyte precursor cells at the optic disc. To test whether Hh signaling can modulate the size of the Pax2^{+} optic disc astrocyte precursor cell population, we cultured optic cups from E12 C57BL/6 embryos for 48 hours in the presence or absence of recombinant myristoylated N-terminal active fragment of Shh (Shh-N), an anti-Hh antibody-5E1 (Ericson et al., 1996) or a 5E1 isotype-matched antibody-1E6, and performed in situ hybridization on serial sections through the optic disc for *Pax2* expression. Compared with controls, Shh-N markedly increased, whereas 5E1 decreased, the size of the Pax2^{+} cell population at the optic disc (Fig. 10A-C). Treatment with 1E6 was similar to untreated controls (data not shown). The effect of anti-Hh antibody 5E1 was due to blockage of endogenous Shh produced by RGCs, which is present in

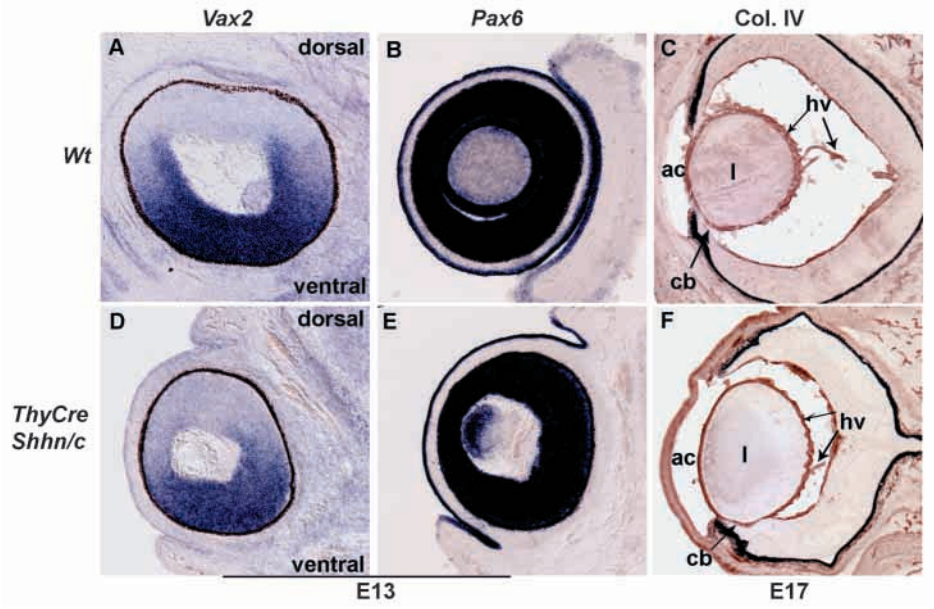

Fig. 8. Dorsal ventral patterning and optic fissure closure are normal in *ThyCreShh*$^{n/c}$ embryos. (A,B,D,E) Sagittal sections through the eyes of E13 wild-type (A,B) and *ThyCreShh*$^{n/c}$ (D,E) embryos hybridized with *Vax2* (A,D) and *Pax6* (B,E) riboprobes. Note the absence of coloboma (D,E), and the normal expression of *Vax2* (D) in the ventral retina, and *Pax6* (E) in the retina, lens, and corneal epithelium of *ThyCreShh*$^{n/c}$ embryos in comparison with wild-type littermates. (C,F) Collagen type IV immunostaining reveals the presence of normal vascular tunics in the posterior chamber of E17 *ThyCreShh*$^{n/c}$ eyes (compare hyaloid vessels in C and F). ac, anterior chamber; l, lens; hv, hyaloid vessels; cb, ciliary body.

the cultures during the first 24 hours (Wang et al., 2002). As the entire disc region of each explanted eyecup was covered in three sections irrespective of the treatment, the change in size of the Pax2$^+$ cells at the optic disc was quantitative. However, by E14 Shh-N treatment had no effect on the size of the Pax2$^+$ population, nor was it associated with an increase in *Gli* expression in optic disc astrocyte precursor cells after two days in culture (Fig. 10; compare E and F, and D and H). Thus the timing of Hh responsiveness of optic disc astrocytes is maintained in vitro and probably represents a cell intrinsic property of this population. However, anti-Hh treatment of E14 explants reduced the size of the Pax2$^+$ cell population (Fig. 10G), which indicates that the size of this population may be under the control of Hh signaling at least at early time points in these explants.

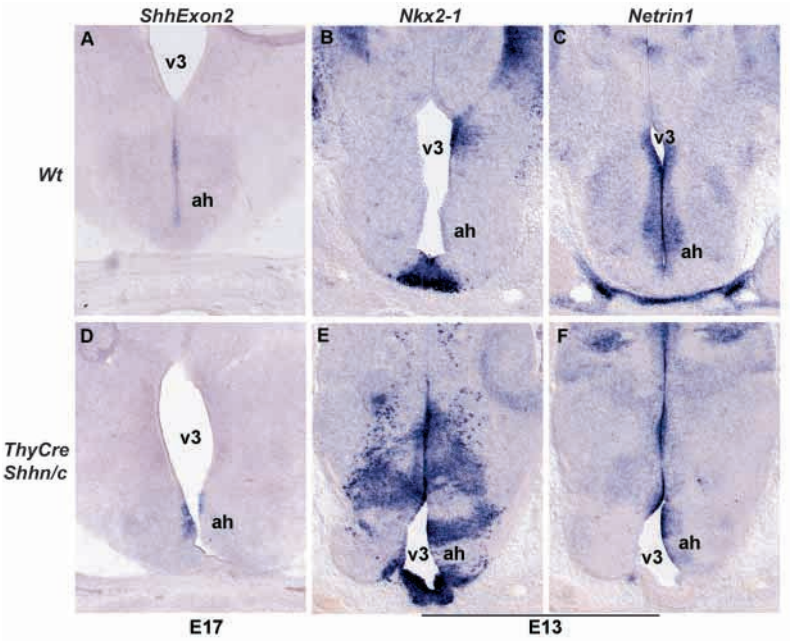

Fig. 9. Normal development and expression of ventral markers in the hypothalamus of *ThyCreShh*$^{n/c}$ embryos. (A-F) Frontal sections through the hypothalamus hybridized with *Shhexon2* (A,D), *Nkx2-1* (B,E) and *Ntn1* (C,F) riboprobes reveal comparable expression of functional *Shh* (A,D), *Nkx2-1* (B,E) and *Ntn1* (C,F) in wild-type (A-C) and *ThyCreShh*$^{n/c}$ (D-F) mice. ah, anterior hypothalamus; v3, third ventricle.

DISCUSSION

Three conclusions can be drawn from the present study: (1) Shh signaling is required for Hh target gene expression in the developing retina, whereas Ihh signaling is required for Hh target gene expression in the peri-ocular mesenchyme; (2) *Shh* expression in RGCs is required for glial lineage specification from optic stalk neuroepithelial cells; and (3) *Shh* expression in RGCs is required to induce the development of astrocyte precursor cells at the optic disc. The last two conclusions are based on the observation that conditional ablation of *Shh* in retinal neuroepithelial cells prior to RGC differentiation resulted in failure of optic disc astrocyte precursor cell development, and the conversion of optic stalk neuroepithelial cells into pigmented cells. In addition, modulating the levels of Shh signaling in retinal explants in vitro altered the size of the optic disc astrocyte precursor cell population. As *Ntn1* expression in optic disc astrocyte precursor cells is required for local axon guidance at the optic disc (Deiner et al., 1997), netrin signaling was perturbed in the conditional mutant mice, which resulted in severe intraretinal RGC axon guidance defects. These observations support a model whereby RGCs, the first neurons to differentiate in the retina, signal through Shh to

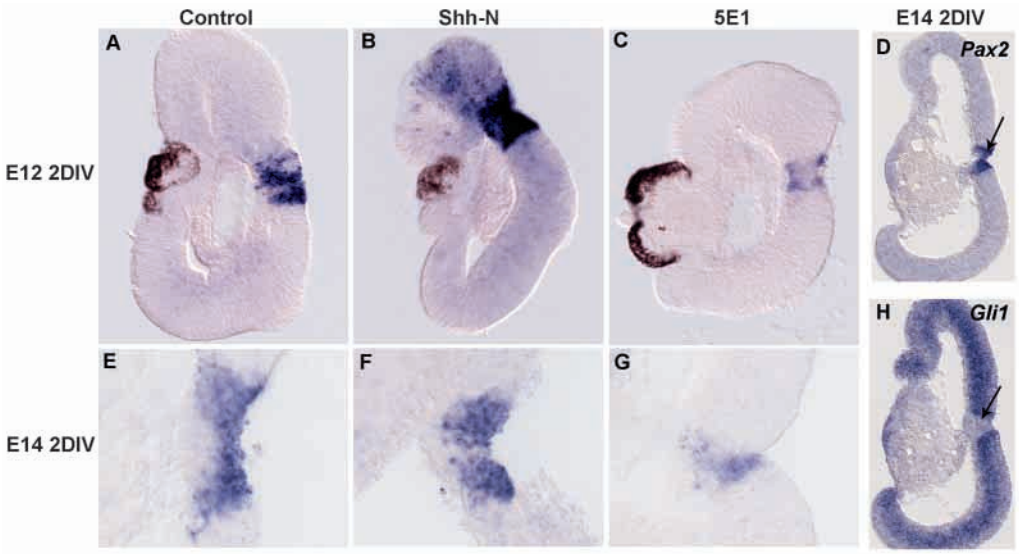

Fig. 10. Modulation of the Pax2-positive astrocyte precursor cell population at the optic disc by Shh signaling. Optic cups of E12 (A-C) and E14 (D-H) C57BL/6 embryos were cultured for 48 hours, untreated (A,E) or treated with recombinant Shh-N (B,D,F,H) or the anti-Shh antibody 5E1 (C,G), and sectioned for in situ hybridization for *Pax2* (A-G) and *Gli* (H) expression. Compared with control (A), the size of the Pax2-positive cell population at the optic disc was markedly increased by Shh-N treatment (B), whereas 5E1 treatment (C) resulted in an almost complete loss of these cells. (D,H) The kinetics of Hh responsiveness by optic disc cells is maintained in vitro, as the Pax2-positive cells (arrow in D) of the E14 Shh-N treated explants downregulate *Gli* (arrow in H) expression by the second day in culture. Consistent with this observation, the size of the Pax2-positive cell population at the optic disc did not differ much between controls (E) and Shh-N treated (F) cultures, but was much reduced in the 5E1 (G) treated explants at E14.

setup their axon guidance system and promote the development of glial support cells along their path.

RGC-derived Shh and peri-ocular mesenchymal Ihh signal in distinct domains of the developing eye

There is an overlap in the timing of expression of *Shh* in the neuroretina and *Ihh* in the peri-ocular mesenchyme, which raises the possibility that either Shh or Ihh, or both, could induce Hh target gene expression in the retina, optic stalk or peri-ocular mesenchyme. Indeed, there are several examples where more than one Hh member is responsible for tissue patterning and/or where one Hh family member compensates for the loss of another (Pathi et al., 2001; Zhang et al., 2001; Wijerde et al., 2002). As there is currently no blocking antibody available that discriminates between the two Hh proteins, we employed a genetic approach to delineate the relative roles of Shh and Ihh signaling in eye development. Evidence from our analysis conclusively demonstrates that Ihh signaling is not required for *Ptch* or *Gli* expression in the retina and optic nerve, and probably does not contribute to the development of these structures. However, Ihh appears to play a role in the development of peri-ocular structures, as $Ihh^{-/-}$ mice have ocular phenotypes suggestive of abnormal peri-ocular mesenchymal tissue development (G.D.D., unpublished). Further evidence for the lack of Ihh signaling in the retina and optic nerve was accrued from analysis of $ThyCreShh^{n/c}$ mice. In these conditional mutant mice, Ihh signaling could induce *Gli* expression in the peri-ocular mesenchyme, but not in the retina or optic nerve. Whatever limits the spread of Ihh into the retina or optic nerve, and RGC-derived Shh into the peri-ocular environment, is unclear at this time, but may be related to the tight junctions formed between retinal pigment epithelial cells.

Shh signaling from RGCs is required for Hh signal reception in the optic stalk

There is ample evidence that axon-derived factors promote glial cell development (Fields and Stevens-Graham, 2002). Indeed, several experiments conclusively demonstrate that glial cell numbers in the optic nerve are regulated by RGC axons. Optic nerve astrocyte proliferation was increased in *Bcl2* transgenic mice, which have more RGC axons than their wild-type littermates (Burne et al., 1996). Furthermore, when RGC axons of wild-type and Wallerian-degeneration-deficient mutant mice were severed behind the eye, astrocyte proliferation decreased, which suggests that factors from the RGC body are involved in the induction of astrocyte proliferation in the optic nerve. Subsequent intra-ocular colchicine and tetrodotoxin injection experiments revealed that astrocyte proliferation in the rodent optic nerve depends on fast axonal transport (Burne and Raff, 1997). However, the axonal factors that mediate astrocyte proliferation in the optic nerve have remained elusive. We showed previously that Shh protein, but not the mRNA was present in the perinatal optic nerve, and RGC axotomy and functional anti-Hh antibody blockage abrogated *Ptch* expression, as well as astrocyte proliferation, in the perinatal optic nerve, which is consistent with a model whereby Shh is transported in an anterograde fashion into the optic nerve (Wallace and Raff, 1999). We have also observed a marked downregulation of *Gli*, *Ptch* and cyclin D1 expression in postnatal optic nerves of mice with conditional disruption of *Shh* in parts of the retina (G.D.D., unpublished), which provides further evidence that Hh signaling in the perinatal optic nerve is dependent on *Shh* expression in the retina. Recently, a role for anterograde transport of Shh has been demonstrated in the visual system of the adult hamster (Triaffort et al., 2001), and in the fornix of the adult rat forebrain (Lai et al., 2003), suggesting that axon-associated Shh signaling occurs in other parts of the adult CNS as well. The failure of optic stalk neuroepithelial cells of $ThyCreShh^{n/c}$ mice to differentiate as glial progenitors at E12 is consistent with an even earlier requirement of Shh signaling from RGC axons in optic stalk neuroepithelial cell development. In the fly, in-growing retinal axons contain Hh that induces *patched*

expression in glial cells along their trajectory and in the brain, and also triggers neurogenesis in the visual ganglia (Huang and Kunes, 1996). Thus, anterograde axonal transport of Hh proteins may be a conserved phenomenon from flies to mammals.

Given that *ThyCreShh$^{n/c}$* mice develop smaller brains than their wild-type littermates, it could be argued that the optic nerve phenotype is caused by insufficient signaling from Shh at the midline, which results in proximal-distal defects in optic vesicle patterning. However, evidence from our analysis and other studies strongly disputes this possibility. *ThyCreShh$^{n/c}$* mice express *Shh* and Shh-dependent markers at the ventral diencephalon. In addition, it is unlikely that Shh diffusion from the midline can account for Hh target gene expression in the optic nerve by E12, and thereafter, as the furthest distance Shh seems to travel extracellularly is in the range of 20-30 cell diameters (Lewis et al., 2001, Wijerde et al., 2002), and from E12 onwards there is no graded *Ptch* or *Gli* expression in the optic stalk to suggest graded Shh signaling from the ventral midline. The ocular phenotypes associated with loss of midline Shh, such as cyclopia in *Shh$^{-/-}$* mice (Chiang et al., 1996), and the failure of the optic vesicle to transit from vesicle to cup stage, as in *BF1$^{-/-}$* mice (Huh et al., 1999), were never observed in *ThyCreShh$^{n/c}$* mice. As cholesterol modified Shh molecules are associated with the lipid raft machinery in the optic nerve (Traiffort et al., 2001), it is very likely that RGC-derived Shh is associated with their growth cones or axons in lipid rafts, and signals to cells in the optic disc and stalk. To directly address the possibility that cholesterol modification is essential for axonal transport of Shh proteins in the visual system may require, for example, the generation and analysis of conditional mice that express functional Shh without the cholesterol moiety in RGCs. Our prediction is that such mice will develop similar optic nerve phenotype as those observed in *ThyCreShh$^{n/c}$* mice.

Regulation of *Pax2* expression and gliogenesis in the optic stalk requires RGC-derived Shh signaling

The homeobox transcription factor *Pax2* plays an important role in normal development of the proximal optic vesicle, where it is expressed early in embryogenesis (Favor et al., 1996; Torres et al., 1996; Otteson et al., 1998). It also appears that *Pax2* is required to suppress pigment cell formation in the optic disc and stalk, as *Pax2* expression is never observed in pigmented cells and *Pax2$^{-/-}$* mice develop a pigmented optic nerve (Torres et al., 1996). Indeed the pigmented optic nerve phenotype of *ThyCreShh$^{n/c}$* mice is histologically indistinguishable from that of *Pax2$^{-/-}$*-mutant mice (Torres et al., 1996). In an experiment where RGC axons were prevented from entering the optic stalk, the neuroepithelial cells became heavily pigmented with increased apoptosis (Ulshafer et al., 1979). This finding, together with the pigmented optic nerve phenotype of *Pax2$^{-/-}$* mice, suggests that factors from retinal axons may signal to optic stalk neuroepithelial cells to maintain *Pax2* expression, thereby preventing them from differentiating as pigmented cells. Hh signaling regulates *Pax2* expression (Macdonald et al., 1995; Ekker et al., 1996), but it is not known whether this effect is direct or indirect. Misexpression of *shh* or *twhh* in the zebrafish embryo results in the expansion of the *pax2* expression domain at the expense of *pax6* in the optic primodium, with a reduction in eye pigmentation (Macdonald et al., 1995; Ekker et al., 1996). Thus, the similarities in the optic nerve phenotypes of *ThyCreShh$^{n/c}$* and *Pax2$^{-/-}$* mice provides strong support for a direct link between RGC-derived Shh signaling in the maintenance of *Pax2* expression in the optic nerve.

Optic disc astrocyte precursor cell development depends on RGC-derived Shh signaling

Optic disc astrocyte precursor cells are first targets of Hh signaling in the retina, and our findings suggest that RGC-derived Shh signaling promotes their development. First we show that there is a temporal and spatial correlation in the pattern of *Shh* and *Gli* expression in the central retina, consistent with local Shh signaling from RGCs. Next we provide genetic evidence that optic disc astrocyte precursor cells fail to develop in mice with targeted disruption of *Shh* in RGCs. Finally, we demonstrate that Shh signaling modulates the size of the optic disc astrocyte precursor cell population in vitro. It is probable that the initial requirement for RGC-derived Shh signaling is to maintain *Pax2* expression in optic disc astrocyte precursors, which is consistent with our in vitro data where we show that functional blockage of RGC-derived Shh signaling results in a remarkable decrease in the size of the optic disc Pax2$^+$ cell population. Later, Shh signaling may also be associated with the induction of *Pdgfra* expression in optic disc astrocyte precursor cells, as the peak of *Gli* expression by the Pax2$^+$ optic disc cells coincides with their initial expression of *Pdgfra*, and Gli has been shown to directly activate the expression and phosphorylation of *Pdgfra* in cell lines (Xie et al., 2001). The maintenance of *Pdgfra* expression in more differentiated retinal astrocyte precursor cells is unlikely to be dependent upon sustained Shh signaling, as the expression of *Pdgfra* in astrocyte precursor cells at the disc is followed by the downregulation of *Gli* expression.

There is probably a cell intrinsic program that regulates the response of optic disc cells to Hh signaling. In contrast to astrocytes in the optic nerve that continue to respond to Hh signaling into the postnatal period (Wallace and Raff, 1999), the response of optic disc astrocyte precursor cells is transient, averaging 4-5 days. One possible explanation for this difference is that optic disc astrocyte precursors are responding to Shh released from the growth cones of RGCs, such that once RGC axons have finished invading the optic disc, Shh becomes limiting to optic disc cells. But this explanation is unlikely given that astrocytes in the optic nerve are able to receive axon-associated Hh signals right into the perinatal period (Wallace and Raff, 1999), and that the addition of recombinant Shh-N to retinal explants in vitro did not restore *Gli* expression in cells at the optic disc. Another explanation for the downregulation of Hh responsiveness in optic disc astrocyte precursor cells could be an alteration in the levels of their extra-cellular matrix components in this region of the retina, as extracellular matrix has been shown to have positive and negative influences on Hh signaling (Pons et al., 2001).

Regulation of gliogenesis by Shh signaling

Glial cell specification from uncommitted neuroepithelial cells requires Shh signaling (Pringle et al., 1996). For example, oligodendrocytes are generated in specific domains of the ventral neuroepithelium under the influence of Shh signaling (reviewed by Bongarzone, 2002). However, in the optic stalk,

which is part of the ventral neuroepithelium, Shh signaling rather induces specification of astroglial lineage cells from uncommitted neural stem cells. Upon induction by Shh in the ventral forebrain, oligodendrocyte precursor cells migrate into the perinatal optic nerve where they differentiate into mature oligodendrocytes. How neuroepithelial cells interpret Shh signals appears to depend on the history and spatial location of a given group of cells. For example, in the ventral spinal cord (Orentas et al., 1999; Lu et al., 2000), metencephalon (Davies and Miller, 2001) and telencephalon (Nery et al., 2001; Tekki-Kessaris et al., 2001), Shh signaling has been shown to regulate the development of oligodendrocytes by inducing the expression of oligodendrocyte fate specification genes such as *Olig1* and *Olig2* in neuroepithelial cells (Sussman et al., 2002). However, in the optic stalk, Shh signaling rather induces and maintains the expression of *Pax2* that is required for astrocyte, but not oligodendrocyte, lineage commitment.

Shh may also be involved in the development and maintenance of the integrity of glial cells in other regions of the adult CNS. For instance, the proliferation and organization of Müller glia in the developing and adult retina (Jensen and Wallace, 1997; Wang et al., 2002) require Shh signaling, whereas in the adult cerebellum, Bergman glia respond to Shh signaling from cerebellar Purkinje cells (Traiffort et al., 1998).

Misguidance of retinal axons in *ThyCreShh$^{n/c}$* embryos is due to the loss of *Ntn1*-expressing optic disc astrocyte precursor cells

Ntn1 from optic disc cells is required for the proper guidance of RGC axons into the optic stalk (Deiner et al., 1997; Shewan et al., 2002). The identical retinal axon guidance defects of *ThyCreShh$^{n/c}$*- and *Ntn1$^{-/-}$*-mutant mice suggests that Shh is required for the development of the Ntn1-positive cells, or for the induction of *Ntn1* expression in optic disc astrocyte precursor cells or both. However, the failure to observe any *Pax2* or *Pdgfra* expression at the optic disc in *ThyCreShh$^{n/c}$* mice implies these cells were unspecified in the mutant mice. It is likely that the loss of optic disc astrocyte precursors that ultimately results in a failure of *Ntn1* expression at the optic disc could have resulted in the axon guidance problems. However, it should also be noted that the loss of optic disc cells in *ThyCreShh$^{n/c}$* mice could lead to abnormal optic disc formation, as well as exposure of the potential subretinal space to exiting RGC axons, both of which could exacerbate the axon misguidance.

Unlike *Ntn1$^{-/-}$* mice, the eyes of *ThyCreShh$^{n/c}$* embryos are small and the retinal layers are disrupted (Wang et al., 2002), which could have caused the axon misrouting. However, two lines of evidence indicate that disorganized retinal morphology was not responsible for the axon misrouting. First, retinal rosettes were not observed until E17, but RGC axon coiling in subretinal spaces at the optic disc was evident as early as E12. Second, misguided RGC axons were not restricted to spaces in between retinal rosettes, as axons were seen misrouted into peripheral retina in the absence of lamination defects, and some misguided RGC axons were observed going through rosettes. The smaller eye size of *ThyCreShh$^{n/c}$* mutants is consistent with the role of Shh in ocular growth and proliferation. However, it is unlikely that the micropthalmia is responsible for the axon misrouting, as micropthalmia per se does not result in RGC axon misguidance in the retina. Conceivably, the misrouted axons in between the retinal pigment epithelium and the neuroretina could obstruct communication between these retinal layers thereby leading to the rosettes observed in *ThyCreShh$^{n/c}$*-mutant retina; however, evidence from our studies and others do not support this conclusion. We observed regions of normal retinal layering with misrouted axons in subretinal spaces and, moreover, the retinal rosettes we observed in *ThyCreShh$^{n/c}$*-mutant retina were largely not associated with misrouted axons in the subretinal space. Furthermore, the axon misrouting in *Ntn1$^{-/-}$*-mutant mice did not result in any retinal lamination abnormalities (Deiner et al., 1997).

As in *Ntn1$^{-/-}$*-mutant mice, RGC axons were still able to exit the retina of *ThyCreShh$^{n/c}$* mice. The earliest age at which RGC-derived Shh signaling appears to act on optic disc astrocyte precursor cells is E12, but *Ntn1* is expressed in the optic fissure and stalk prior to this age (Deiner et al., 1997). As RGC differentiation begins some hours earlier than E12, it is possible normal axon guidance could have occurred at the optic disc prior to the loss of *Ntn1*-expressing cells. Thus, some of the later generated RGC axons would then respond to guidance cues, such as N-CAM (Brittis et al., 1995), from the early axons and follow them into the optic nerve.

RGC-derived Shh signaling in eye development

Shh signaling from RGCs plays an important role in the development of the vertebrate visual system. RGC-derived Shh regulates the proliferation and differentiation of retinal neuroblasts, thereby controlling the number and organization of their synaptic connections (Jensen and Wallace, 1997; Levine et al., 1997; Stenkamp et al., 2000; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001). By promoting the organization and structural integrity of Müller glia in the retina, RGC-derived Shh signaling also helps maintain the normal layering of the retina (Wang et al., 2002). In addition, the proliferation of perinatal optic nerve astrocytes is regulated in part by RGC axon-associated Shh signaling (Wallace and Raff, 1999). We now provide evidence that Shh from early-born retinal neurons is required for optic disc and stalk neuroepithelial cell development.

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