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Control of retinal ganglion cell axon growth: a new role for Sonic hedgehog

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SUMMARY

Retinal ganglion cell (RGC) axons grow towards the diencephalic ventral midline during embryogenesis guided by cues whose nature is largely unknown. We provide in vitro and in vivo evidence for a novel role of Sonic hedgehog (SHH) as a negative regulator of growth cone movement. SHH suppresses both the number and the length of neurites emerging from the chick retina but not from neural tube or dorsal root ganglia explants, without interfering with their rate of proliferation and differentiation. Similarly, retroviral-mediated ectopic expression of *Shh* along the chick visual pathway greatly interferes the growth of RGC axons. Upon SHH addition to grown neurites, the intracellular level of cAMP decreases, suggesting that the dampening of growth cone extension mediated by SHH may involve interaction with its receptor Patched which is expressed by RGC. Based on these findings, we propose that *Shh* expression at the chiasm border defines a constrained pathway within the ventral midline which serves to guide the progression of RGC axons.

Key words: Retinal ganglion cells, SHH, Chick

INTRODUCTION

The successful projection of retina ganglion cell (RGC) axons to either the contralateral or to both the contralateral and ipsilateral side of the brain is fundamental to establish the appropriate and functional type of vision characteristic of each vertebrate species (Ramon y Cajal, 1901). Static and dynamic observations of the RGC axon tips have suggested that the optic chiasm is a major choice point where axons are sorted (Bovolenta and Mason 1987; Godement et al., 1990). However, the patterning of the optic stalk and the ventral hypothalamus, and, therefore, the establishment of the cellular and molecular mechanisms that guide the entire RGC axon population across the midline in some species (i.e. chick) or that allow the segregation of crossed and uncrossed axons in other species (i.e. mouse), are still poorly understood.

It has been proposed that the position of axon tracts and commissures, including the optic chiasm, may depend on the boundaries formed among expression domains of regulatory proteins, comprehending transcription factors and morphogenetic genes, which in turn control effector guidance molecules (Wilson et al., 1997). Indeed, a number of these molecules are expressed in the ventral diencephalon (i.e. Pax2, Vax1, Nkx2.1, Nkx2.2, Dlx2, BF-2, Six3, Shh, etc.; Martí et al., 1995b, Macdonald et al., 1995; Torres et al., 1996; Bertuzzi et al., 1999; Bovolenta et al., 1998) some of which define an overlapping zone of expression that might contribute to chiasm development (Marcus et al., 1999). This idea is in part supported by the analysis of phenotypes derived by the genetic disruption of a few of these molecules in mice and fish. Thus, in mice lacking *Vax1* (Bertuzzi et al., 1999; Hallonet et al., 1999), RGC axon outgrowth is altered, axons fail to enter the hypothalamus and form whorls of fibres at the distal end of the optic nerve. These alterations are associated with a clear reduction of the expression of *netrin 1*, a diffusible molecule involved in the axon pathfinding of a number of different neurones, including the RGC (Tessier-Lavigne and Goodman, 1996; Deinier et al., 1997). *Vax1*-deficient mice further show modifications of the *Shh* expression pattern in the hypothalamus (Bertuzzi et al., 1999).

In *Pax2*-deficient mice the optic chiasm is altered and retinal ganglion cells axons never cross the midline, leaving the projections entirely ipsilateral. Interestingly, in these mice *Shh* expression is ectopically maintained along the entire ventral midline, whereas in wild-type mice *Shh* expression is downregulated from the chiasm as RGC axons are guided towards this region (Torres et al., 1996). Similar results were observed in *noi* zebrafish mutants, which have alteration in *pax2/5/8*-like gene (Macdonald et al., 1997). These observations raised the possibility that the continuous expression of *Shh* at the axial midline might be directly or indirectly responsible for the failure of RGC axons to cross the midline, disrupting the development of the optic chiasm.

To address this issue, we have performed a series of experiments that demonstrate that SHH directly discourages the growth of RGC axons, providing a novel role for SHH. Because *Shh* expression retracts to the posterior and dorsal borders of the chiasm as RGC fibres accumulate in this region,

we propose that in vivo SHH contribute to define a constrained pathway for RGC axons, favouring the tight fasciculation of the optic commissure.

MATERIALS AND METHODS

Chick embryos

White-Leghorn eggs were incubated at 38ºC in an atmosphere of 70% humidity; embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Retrovirus production and embryo infections

Chick embryo fibroblasts (CEFs) were prepared from Line 0 eggs (Poultry Production Unit, Institute for Animal Health, UK). CEFs were obtained from the torso of embryonic day (E) 10 chickens and cultured following standard procedures (Fekete and Cepko, 1993). CEF cultures were grown and transfected with either alkaline phosphatase-RCASBP(B) or Sonic hedgehog-RCASBP(A). Assembled viruses were harvested and concentrated at the titre of approximate 10^8 cfu/ml, as described (Fekete and Cepko, 1993). Concentrated Shh-RCAS or AP-RCAS viruses were injected in the midline ventral forebrain region of stage HH13-14 embryos. Five days post-infection, embryos were harvested, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and processed for DiI labelling.

DiI labelling

Glass micropipettes with tip diameters of 5-10 μm were filled with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes) (2.5 mg/ml in DMSO). In each application, 25-50 nl of DiI was deposited onto the optic nerve head, after lens removal. The tissue was placed then stored in PBS containing 0.5% paraformaldehyde in the dark at 37ºC for 5 to 7 days. The heads were then washed in PBS, the brains carefully removed, and the ventral forebrain region dissected and split open to obtain an 'open-book' configuration. The preparations were mounted on a glass slide, and viewed and photographed using epifluorescence optics (Leica). The preparations were then washed in PBS and processed for in situ hybridisation.

In situ hybridisation and immunostaining

Whole-mount in situ hybridisation was performed as described (Bovolenta et al., 1998). Chick Sonic hedgehog (*Shh*) and Patched (*Ptc1*) probes have been described (Riddle et al., 1993; Marigo et al., 1996a). The hybridised sense and antisense probes were detected with alkaline-phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim). Whole-mount hybridised ventral forebrain preparations and embryos were photographed using a Leica MZ APO microscope. *Ptc1* hybridised retinas were sectioned on a vibratome (50 μm), and sections were mounted, coverslipped and photographed using a Leica DMR compound microscope. Immunohistochemistry was performed on cryostat (20 μm) sections or on the fixed explants, following standard procedures. The monoclonal antibody against an unique β-tubulin (Tuj-1) was obtained from MEDPASS, the polyclonal rabbit antiserum to the phospho-histone H3 from Upstate Biotechnology, the PAX2 rabbit polyclonal antiserum was purchased from ZYMED Laboratories, while the rabbit polyclonal anti-cAMP was obtained from CHEMICON. Additionally, anti-cAMP polyclonal antiserum was also kindly provided from Dr T. Wiemelt and the anti-Islet1 guinea-pig antiserum from Prof. T. Jessell. Secondary antibodies were biotin-conjugated anti-mouse or anti-rabbit IgG followed by peroxidase-coupled streptavidin (Jackson Laboratories, West Grove, PA). Antigen localisation was detected by incubation in PBS containing 30 μg/ml DAB to which 0.003% hydrogen peroxide was added or using the AEC system (DAKO Corporation, Carpinteria, CA). Alexa-594 conjugated streptavidin (Molecular Probes) was used to localise cAMP.

Explant cultures

Tissue explants were dissected and collected in ice-cold Hanks' balanced salt solution (HBSS) (Gibco, BRL). Retinas were dissected from embryonic day (E)5 chickens, free from the associated pigmented epithelium, lens and vitreous and 250 μm wide strips of retinas were prepared. Dorsal neural tube and floor plate explants were dissected from E4 chick embryos and freed from surrounding non-neural tissues. Heparin-acrylic beads were soaked in either PBS or in recombinant purified human N-SHH (2.5 μg/ml; Biogen). Explants were embedded within three-dimensional collagen gels (Collaborative Biomedical Research) and cultured for 24-48 hours in 500 μl serum-free DMEM/F12 medium (Gibco, BRL), supplemented with N2 (Gibco, BRL) as previously described (Martí et al., 1995a). Collagen gel cultures were then harvested, fixed in 4% paraformaldehyde in PBS for 30 minutes and processed for immunostaining using standard procedures. In a second series of experiments, explants embedded in collagen gels were grown in the presence or absence of N-SHH (2.5 μg/ml) directly added to the culture medium. In a third set of experiments, retinal explants were plated vitreal side down on glass coverslips prepared with polylysine (20 μg/ml, Sigma) and laminin (10 μg/ml, Gibco, BRL) and cultured in the same DMEM/F12/N2 culture medium. Twelve to 24 hours after been plated, explants were treated with 2.5 μg/ml of recombinant N-SHH, Vehicle (Biogen) or anti-SHH blocking antibody, and direct effect over axon growth was scored using a Olimpus Camera coupled to a Zeiss Axiovert microscope. Video-time lapse analysis was performed using a COHU High performance CCD video camera and a VHS recorder (SVT S3050P, Sony) coupled to an inverted Olympus microscope, equipped with temperature and CO_2 control systems. Video recording was digitalised and processed using a MIRÓ DC30 plus card and the Adobe Premiere Program.

Analysis of neurite growth in collagen gel cultures

Neurite outgrowth from explants was quantified using the image analysis software (Q500 MC, Leica). The cultures were photographed under a stereo-microscope (Leica). Neurite outgrowth was quantitatively assessed on samples of scanned images representative of each experimental condition. An automatic analysis function was used to measure both the neurite number and length. The area covered by the explant was subtracted and the amount of neurite outgrowth was expressed as the number of pixels occupied by the neurites. To overcome the problem of explant size variability, the amount of fibre outgrowth was normalised for the explant size. All data were analysed by a one-way ANOVA with a PRISMA SOFTWARE for PC using the Tukey's test.

Analysis of neurite growth rate in culture

Neurite growth rate from retinal explants was quantified in 100 randomly chosen neurites from three different independent experiments. Neurites were individually followed during the time of culture and neurite length measured using an Argus-10 Image Processor (Hamamatsu) coupled to an inverted microscope. Relative axon length was expressed as means±s.e.m. Data were analysed by a two-way ANOVA using PRISMA SOFTWARE for PC.

Determination of cyclic AMP levels in growth cones

Retinal or DRG explants were established over laminin coated glass coverslips as described above. After 20 hours in cultured either N-SHH (2.5 μg/ml), vehicle or forskolin (20μM) were added to the culture medium. Explants were fixed 20 minutes after chemical addition and processed for immunocytochemistry with anti-cAMP antibodies as described above. In preliminary experiments, explants were fixed with either acrolein, following the procedure described by Wiemelt et al. (Wiemelt et al., 1997) or with 4% paraformaldehyde,

as suggested by CHEMICON manufacture procedures for anti-cAMP antiserum. Because no differences in staining intensities were observed with the two different procedures, additional experiments were performed in 4% paraformaldehyde-fixed tissue. Images of immunostained growth cones were analysed with confocal microscopy (Leica) and the immunofluorescent signal was quantified using the image analysis software Q500-MC (Leica). The labelling intensity normalised per growth cone area was determined using a grey scale in each case. The data obtained from three independent experiments performed in duplicate were statistically analysed with the PRISMA software for PC using the Student's *t*-test. The data reported in the results were obtained after the analysis of 25 (5,8,12), 16 (2,6,8) and 27 (6,9,12) retinal growth cones exposed to forskolin, N-SHH and control condition, respectively. For DRGs the total number of growth cones analysed are the following: 42 (15,16,11) for forskolin; 50 (11,18,21) for Shh and 34 (14,7,13) for controls. Data are expressed as mean±s.e.m. and significance was calculated using the unpaired *t*-test.

RESULTS

Shh is expressed in the dorsal and posterior borders of the chiasm

In the early chick embryo, Shh is expressed in the entire ventral midline of the CNS (Martí et al., 1995b). However, as the brain bends and develops, a clear gap in the expression of Shh can be observed, at the level of the optic recess from stage 19 onwards (Fig. 1A,B). This gap corresponds to the area where the PAX2-positive optic nerves enter the brain (Fig. 1C). To determine how this gap in expression relates to the formation of the optic chiasm, we have analysed in detail the topographical relationship between the expression of Shh and the ingrowth of RGC axons, visualised by labelling with the Tuj1 antibody. In early E3 embryos, where Shh is continuously expressed in the ventral midline (Fig. 1D,F,G), CD44/Tuj1-positive cells, crucial for the formation of the optic chiasm (Mason and Sretavan, 1997), appeared within the domain of Shh expression (Fig. 1E). At this stage, few RGC have differentiated in the retina (Prada et al., 1991; Fig. 1E). Later at E5, when Tuj1-positive RGC axons have invaded the midline (Fig. 1I-K) and PAX2-positive optic nerve cells have extended into the chiasm (Fig. 1C), Shh expression disappears from the region marked by CD44/Tuj1-cells (Fig. 1I,J) but is maintained in both the anterior and posterior dorsal edges, bordering the developing chiasm (Fig. 1C,H,K). These data, together with the analysis of the mouse and fish Pax2 mutants (Torres et al., 1996; Macdonald et al., 1997), suggest that Shh repression at the midline might be important to establish the correct navigation of visual axons, providing a scaffold for growth cone advance.

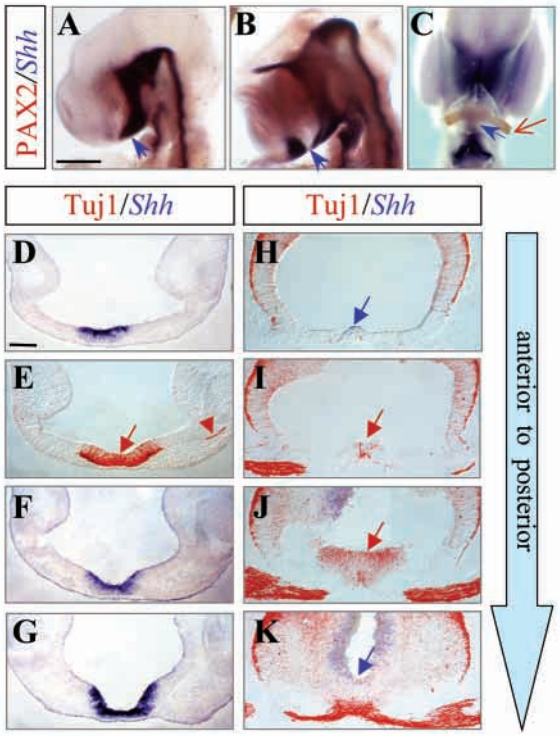

Fig. 1. Dynamic expression of *Shh* during chiasm formation in the chick. (A,B) Whole-mount in situ hybridisation in HH15 (A) and HH19 (B) embryos show that *Shh* mRNA is expressed in the entire ventral midline at early stages (arrow, A), but disappears from the chiasm midline as the visual pathway develops (arrow, B). (C) Ventral view of an E5 brain double labelled with a *Shh*-specific probe (blue staining) and an antiserum against PAX2 (brown staining). Note how *Shh* is expressed in the ventral diencephalic region, except in the midline where the optic nerves (red arrow) enter the chiasm. Note also how Shh expression border the posterior limit of the chiasm (blue arrow). Detailed analysis of chiasm development as determined by *Shh* (blue labelling) and Tuj1 labelling (red) of consecutive, frontal sections from E3 (D-G) and double labelling of frontal, consecutive sections from E5 (H-K) chick embryos. At E3, when very few RGC axons have entered the optic stalk (arrowhead in E), *Shh* expression extends throughout the entire midline (D,F,G) including the prospective chiasm region containing Tuj1-positive neurones (red arrow in E). At E5, Tuj1-positive RGC axons (red staining) invade the midline (I-K), and *Shh* mRNA expression now limits the borders of the RGC axon pathway, (blue arrows in H,K), but is downregulated from the position occupied by the Tuj1-positive chiasm 'guidepost' neurones (red arrow in I,J). Scale bar: 70 μm in A; 54 μm in B; 165 μm in C; 41 μm in D,E; 40 μm in F-H; 38 μm in I; 33 μm in J,K; 34 μm in L.

N-SHH suppresses neurite outgrowth from retinal explants

To address whether SHH could directly affect the growth of RGC axons, retina explants were co-cultured for 48 hours in standard three-dimensional collagen gels with heparin-acrylic beads impregnated with human N-SHH, placed at the approximate distance of 100 μm from the retinal tissue. Explants were prepared from E5 chick embryos, an age when many RGCs have already differentiated (Prada et al., 1991). Axon outgrowth from retinal explants was robust from all sides of the explants when the tissue was co-cultured with control beads (Fig. 2A). By contrast, the presence of N-SHH-impregnated beads greatly decreased the total outgrowth of retinal axons (Fig. 2B), without interfering significantly with the rate of RGC proliferation or differentiation (Fig. 2K,L). N-SHH-beads did not appear to reorient the growing fibres; however, the total number of neurites and their length was significantly reduced (Fig. 2J). This idea was confirmed by experiments in which N-SHH was directly added to the culture medium of the explants (compare Fig. 2C with 2D). Similar results were obtained when retinal explants were co-

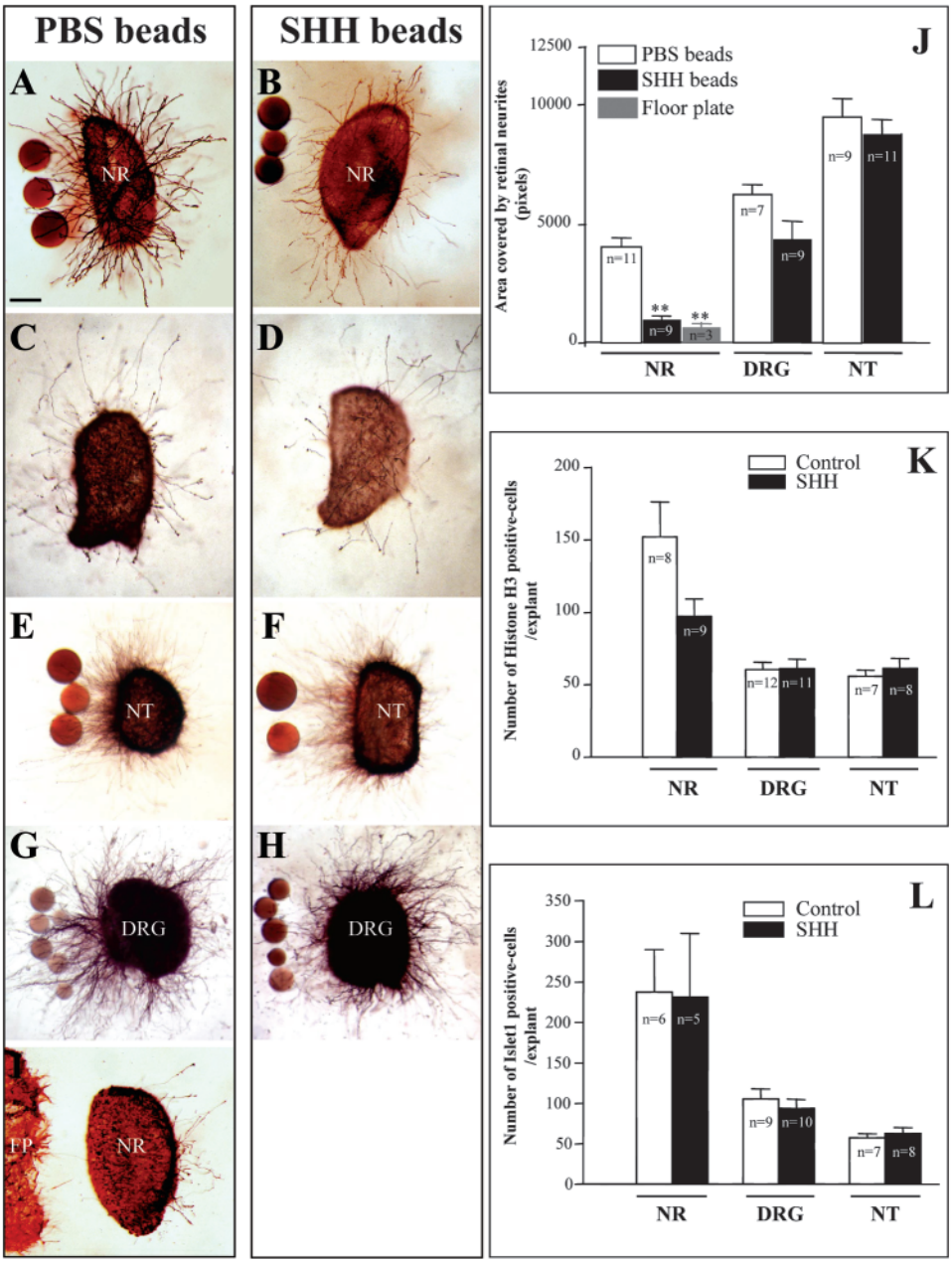

Fig. 2. N-SHH specifically suppress the growth of RGC axons. E5 chick neural retina (NR) (A-D,I), dorsal neural tube explants (NT) (E,F) and E12 dorsal root ganglia (DRG) (G,H) were cultured in collagen gel matrix 100 μm from control (A,E,G), N-SHH-soaked beads (B,F,H) or floor plate explants (I). In presence of N-SHH-soaked beads, both the length and the number of neurites from retinal explants (B) were decreased when compared with the growth in the presence of control beads (A). Similarly, addition of soluble N-SHH directly to the culture medium reduced RGC axon growth (D) when compared with controls (C). Similar results were obtained when retinal explants were co-cultured in the presence of floor plate (FP) explants (I). Neurite outgrowth from dorsal neural tube explants (E,F) and dorsal root ganglia (G,H) was unaffected by the presence of N-SHH. (J) Quantification of neurite outgrowth (both neurite lengths and numbers) from (n) explants. Neurite outgrowth is expressed as the number of pixels occupied by neurites normalised for the explant size. (K) Quantification of cell proliferation in the explants, as assessed by staining with an antiserum the mitotic marker phospho-histone H3. (L) Quantification of cell differentiation in the explants, as determined by counting the number of Islet1-positive cells in the explants. Scale bars: 50 μm in A-H; 55 μm in I.

cultured in the presence of the floor plate tissue. Indeed, as previously shown in the mouse (Wang et al., 1996), the floor plate tissue, a rich source of SHH (Martí et al., 1995b), greatly reduces the outgrowth of RGC axons (Fig. 2I,J). The floor plate totally suppressed the growth of neurites from the facing side of the retina explants and further appeared to orient away the direction of the extension of the fibres emerging from the remaining sides of the explants. These differences are not surprising, however, as the floor plate is a rich source of other diffusible molecules, as Netrins and Slits (Tessier-Lavigne and Goodman 1996; Brose et al., 1999). The latter, which is clear repellent for RGC axons (Erskine et al., 2000; Ringstedt et al., 2000), is likely to be the additional responsible for the strong inhibitory activity of the floor plate.

To determine whether SHH activity might be due to a nonspecific suppressing effect of this molecule over axon growth, two other sources of axons were co-cultured in collagen gels with SHH-releasing beads. Dorsal neural tube explants appeared as a good choice because at least a sub-population of dorsal neurones (the commissural neurones) normally project towards a SHH-rich intermediate target, the floor plate (Bovolenta and Dodd, 1990). The overall outgrowth from dorsal neural tube explants were not significantly affected by the presence of SHH when compared with control culture (Fig. 2E,F,J), either when the total extent of growth was considered or when the outgrowth from the side of the explant facing the beads was measured. Similar results were also obtained when dorsal root ganglia (DRG) were used as an alternative source of axons (Fig. 2G,H,J). In both type of tissues, N-SHH addition did not appear to affect either cell proliferation or differentiation (Fig. 2K,L).

N-SHH induces growth cone arrest

We next asked whether SHH effect was the result of its direct activity on RGC growth cones. SHH-responding cells express Patched (*Ptc1*), a transmembrane receptor protein required for propagation of SHH signalling (Marigo et al., 1996b; Stone et al., 1996; Ingham 1998). If SHH was acting directly on the growth cone, RGC should express the *Ptc1* gene. In situ hybridisation analysis of E5 retinal tissue shows that *Ptc1* mRNA is expressed, albeit at low levels, in the retinal neuroepithelium including in the RGC layer (Fig. 3A,B). Similar localisation of *Ptc1* has been reported for the mouse retina (Wallace and Raff, 1999). As Patched localised to the RGC, we next asked how the presence of SHH modified the behaviour of already established neurites. Thus, retina explants were plated over dishes coated with laminin, an extracellular matrix molecule which allows the profuse outgrowth of neurites from a wide variety of neural tissue sources. Twelve hours after plating, a large number of fibres had extended a considerable distance from the explants. At this time the behaviour of several randomly chosen neurites was observed by means of digital images, captured at fixed intervals during the next two hours (Fig. 3C,D), after which, either N-SHH (2.5 μg/ml) or its vehicle were included into the culture medium. Addition of the vehicle did not significantly alter the behaviour of the neurites, which continued to extend at a steady pace (Fig. 3C). By contrast, many of the fibres growing in the presence of N-SHH showed a clear reduction in the shaft length and a rounding up of the growth cone, as early as 15 minutes after SHH addition. Neurite retraction persisted for the following hour, thereafter growth cone slowly reassumed their extension (Fig. 3D). Accurate measure of the neurite length in 100 randomly selected neurites clearly demonstrated that N-SHH induced a significant and transient decrease of the neurite length as compared with control cultures (Fig. 3E). Time lapse video recording of individual growth cones further confirmed and extended this observation (Fig. 4A). While the addition of the vehicle or of N-SHH pre-incubated with anti-SHH blocking antibody did not alter neurite behaviour, the presence of N-SHH alone induced a slowing down of the growth cone movements that was followed by a partial retraction of the axon shaft, as already shown in Fig. 3. Full but reversible and re-inducible growth cone collapse was observed only in a minority of the cases. All the collapsed growth cones shared the characteristic of belonging to newly initiated neurites, just emerging from the explant edge (Fig. 4A). N-SHH activity on growth cone movement appeared again neuronal type specific, as DRG growth cone behaviour was not affected by N-SHH addition, as analysed in similar time lapse video microscopy experiments (data not shown).

SHH-mediated growth cone arrest depends on the levels of cyclic AMP

The response of the growth cone to extracellular guidance cues appears to be mediated by the intracellular levels of cAMP, with low levels of the cyclic nucleotide favouring growth

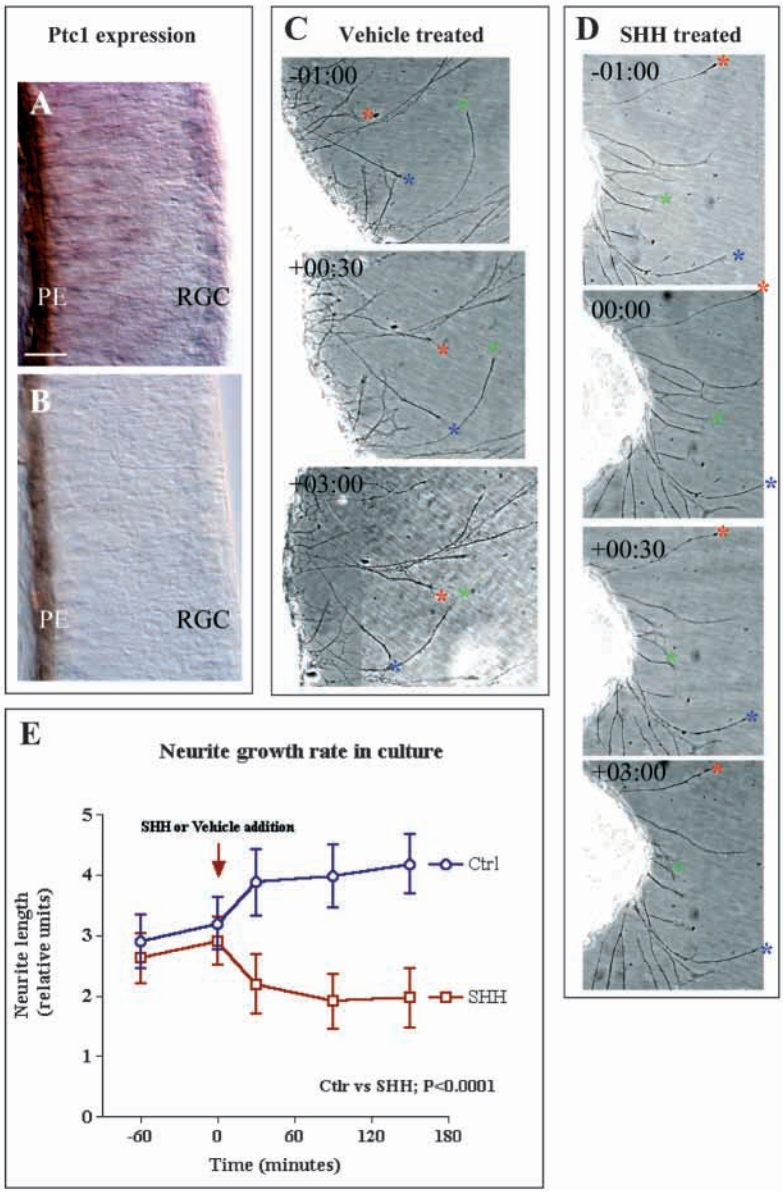

Fig. 3. *Ptc1* expression and SHH activity at the growth cone. (A) In situ hybridisation of E5 chick retina with antisense probe shows low levels of *Ptc1* mRNA in the retinal neuroepithelium and moderate in the retinal ganglion cell layer (RGC). PE, pigment epithelium. (B) Hybridisation with the sense probe is shown for comparison. (C-E) E5 chick retina explants were cultured on laminin substrate for 12 hours and the behaviour of neurites was observed by means of digital images, captured at fixed intervals during the next 2 hours. Colour-coded asterisks identify individual neurites. (C) Control explant treated with vehicle where neurites continued to extend at a steady pace. (D) Explant treated with purified N-SHH (2.5 μg/ml) already shows neurite retraction 15 minutes after N-SHH addition. Neurite retraction persisted for the following hour, thereafter growth cone slowly reassumed their extension. (E) Statistical analysis of neurite growth rate in cultured explants treated with vehicle (blue) or with N-SHH (red) shows a clear reduction in the total neurite length after N-SHH treatment, neurite extension is slowly reassumed 2 hours after the treatment. Scale bar: 24 μm in A,B; 100 μm in C,D.

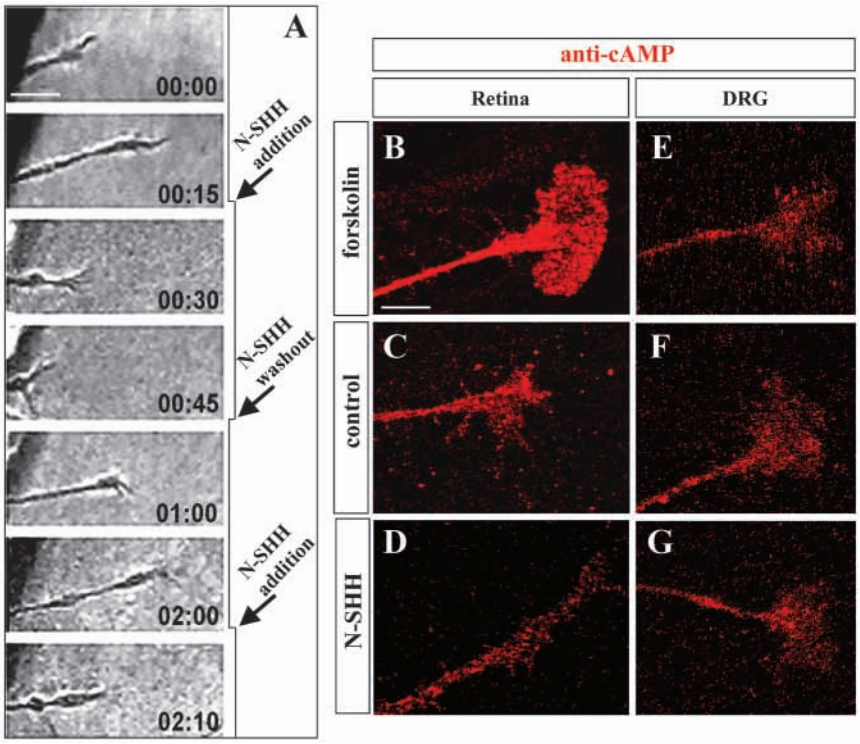

Fig. 4. N-SHH induces the retraction on newly initiated neurites and lowers the cAMP levels in the growth cone. E5 chick retina (A-D) or E12 DRG (E-G) explants were cultured on laminin substrate for 20 hours. (A) The behaviour of neurites was observed with time lapse video recording of individual growth cones. Addition of N-SHH to the culture medium induced a slowing down of the growth cone movement which was followed by a partial retraction of the axon shaft. Growth cone collapse is reversible and re-inducible as observed after N-SHH washout (45 minutes later), and N-SHH re-addition (2 hours later). (B-D) Retinal and DRG (E-G) growth cones immunostained with anti-cAMP antibody. Note the intense staining of RGC growth cone after incubation with forskolin (B) and medium intensity in control conditions (C). Note how 20 minutes exposure to N-SHH greatly reduces the levels of cAMP in retinal (D) but not DRG (G) growth cone, which presents an intensity similar to those observed in control (F) or after forskolin addition (E). Scale bar: 12 μm in A; 6 μm in B-G.

inhibition or repulsion (Song and Poo, 1999). An increase in the activity of the cAMP-dependent protein kinase (PKA), one of the principal mediators of cAMP effects, inhibits the response of cells to Hedgehog signalling (Jiang and Struhl, 1995; Epstein et al., 1996; Hammerschmidt et al., 1996). We therefore postulated that SHH activity on growth cone motility could be mediated by a decrease in the levels of cAMP in the growth cone. To test this hypothesis, we used an antibody that binds to free cAMP in the cytosol (Wiemelt et al., 1997; Hopker et al., 1999). As measured by immunofluorescence intensity, the levels of cAMP could be modulated in RGC growth cones extending over a laminin-coated dish. Addition of forskolin, a specific activator of adenylate cyclase, induced a significant increase (mean of grey scale intensity/normalised per growth cone area ± s.e.m: 268±27; *n* (number of growth cones analysed)=25; 143% intensity of controls; $P<0.01$) of cAMP when compared with control (188±15, *n*=27; 100%), while addition of N-SHH to the culture medium led to a significant and reproducible decrease (129±16, *n*=16; 68%; $P<0.01$) in the staining intensity when compared with controls (Fig. 4B-D). Moreover, addition of forskolin to the culture medium appeared to revert the N-SHH mediated inhibition of RGC growth cone movements as analysed by time-lapse video microscopy (data not shown). By contrast, similar experiments showed that N-SHH non-responding DRG growth cones (control values: 232±19; *n*=34) presented unchanged levels of cAMP independently of the presence of N-SHH (201±10; *n*=50) or forskolin (233±20; *n*=42) in the culture medium (Fig. 4E-G). These observations support the idea that SHH effects on RGC axon are mediated by modulation of PKA.

SHH alters RGC axon pathway in vivo

To confirm and extend all the above observations, we have asked whether RCAS-mediated ectopic expression of *Shh* along the visual pathway could interfere with RGC axon navigation. Chick embryos of HH13-14 were infected in the ventral forebrain and analysed at E5-6, when many RGC axons have already passed through the chiasm, entering the lateral optic tract (Rager, 1980). The pathway taken by RGC axons was visualised by anterograde tracing with the lipophylic dye DiI, injected into the right eye of each embryo. In control or RCAS-AP infected embryos, DiI-labelled axons grew through the optic disc into the optic nerve. At the chiasm, the majority of axons crossed the midline to invade the contralateral optic tract (Fig. 5A,B), while an extremely reduced number of axons transiently projected into the ipsilateral optic tract, as previously reported (O'Leary et al., 1983). RCAS-*Shh* infected embryos presented several alterations as compared to this normal pathway. The alterations were dependent on the degree of viral infection in each embryo (Fig. 5C-G). In most cases (78%; *n*=32), when *Shh* expression was abundant in the entire ventral forebrain, including the optic nerve (Fig. 5C), axons advanced very little along the nerve and they barely reached the chiasm region (Fig. 5D). In 15% of treated embryos, viral infection and therefore *Shh* ectopic expression was confined to the midline hypothalamic region (Fig. 5E,G). In these animals, axons grew along the nerve and some of them were able to cross the midline and project contralaterally (Fig. 5F), whereas a consistent proportion of axons was misrouted into the ipsilateral optic tract (Fig. 5H), resembling the pathway taken by RGC axons in *Pax2/noi* mice/fish mutants (Torres et al., 1996; Macdonald et al., 1997).

Early ectopic expression of *Shh* in zebrafish embryos induces the expansion of the *pax2*-positive proximal domain of the eye vesicle, the optic stalk, at the expenses of its *pax6*-positive distal domain, the retina (Macdonald et al., 1995). Therefore the outgrowth failures of RGC axons could be the result of a not properly patterned eye or simply could

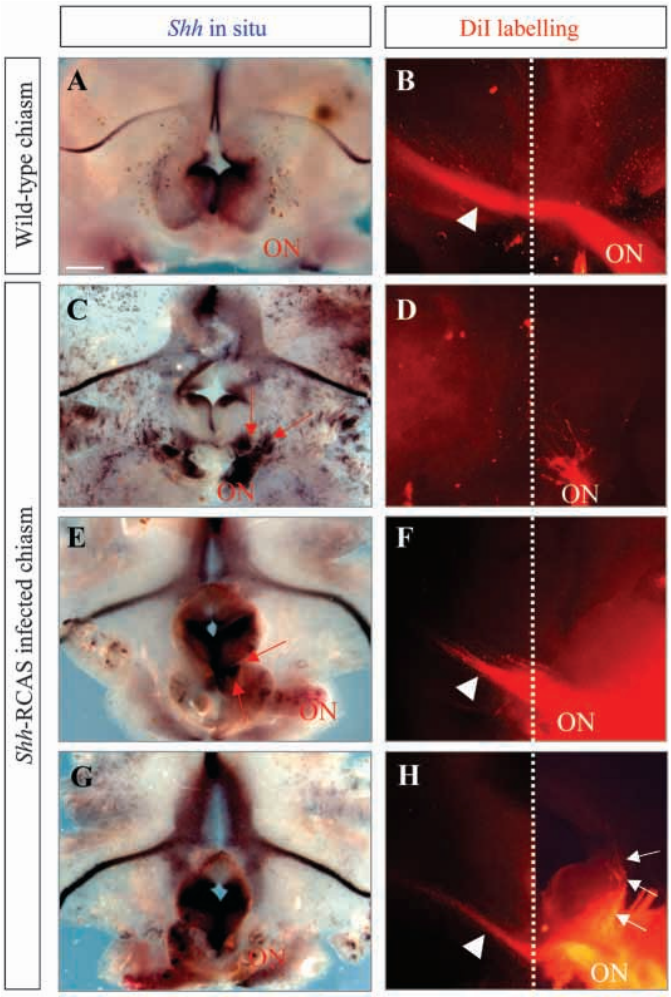

Fig. 5. RCAS-mediated ectopic expression of *Shh* alters RGC axon pathway in vivo. HH13-14 chick embryos were infected in the ventral forebrain and analysed at E5-E6 when many RGC axons have already passed through the chiasm. DiI crystals were placed into the right eye of each embryo to visualise RGC axon pathway. In control or RCAS-AP infected embryos, *Shh* expression is normal (A) and DiI-labelled axons grew through the optic disc into the optic nerve (ON) and crossed the midline (broken white line) to invade the contralateral optic tract (arrowhead in B). RCAS-*Shh* infected embryos presented several alterations as compared to this normal pathway. (C) In most cases (78%, *n*=32) *Shh* expression was ectopically extended into the hypothalamic region (red arrows) and into the optic nerve (ON). (D) In these animals, DiI labelling shows that axons advanced very little along the nerve (ON) and they barely reached the chiasm region. (E,G) In 15% of treated embryos, viral infection and therefore *Shh* ectopic expression was confined to the midline hypothalamic region. (F,H) In these animals, few axons grew across the midline (broken white line) into the contralateral optic tract (arrowheads in F,H) and a consistent small proportion of axons was misrouted into the ipsilateral optic tract (arrows in H). Scale bar: 79 μm in A,C,E,G; 37 μm in B,D,F,H.

correspond to a general delay in the embryo development. To exclude both possibilities, tissue sections of control and infected embryos were immunostained with antibodies against PAX2 and PAX6 to check for proximo-distal patterning and with Tuj1 antibody to verify cell differentiation and fibre extension. Patterning of RCAS-*Shh* infected eyes appeared

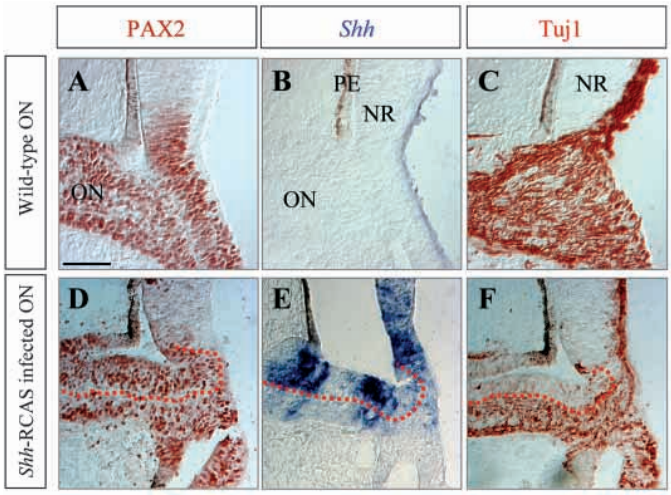

Fig. 6. Patterning and neural differentiation are not affected in the RCAS-*Shh* infected eyes. Images illustrate the expression of PAX2 (A,D), *Shh* (B,E) and Tuj (C,F) on consecutive sections of control (A-C) and infected (D-F) embryos. (A,D) PAX2 expression is normal in the optic nerve without extending into the *Shh* infected neural retina (E). Tuj1 immunostaining highlighted that the RGG axons avoided the portion of the optic disk that ectopically expressed *Shh* (compare C with F). NR, neural retina; PE, pigment epithelium. Scale bar: 67 μm.

normal as shown by PAX2 (Fig. 6A,D) and PAX6 (not shown) expression, even in patches of high *Shh* ectopic expression (Fig. 6E). Furthermore, neural differentiation in infected retinas appeared normal as shown by Tuj1 (Fig. 6C,F), although nerve fibres avoided crossing throughout patches of high *Shh* expression (Fig. 6E,F), confirming the data obtained with DiI labelling of RGC axons.

DISCUSSION

SHH plays a central role in vertebrate development. It specifies the identity of different cell types in the ventral neural tube, controls digits formation in the limb and regulates cell proliferation in particular CNS regions (Hammerschmidt et al., 1997). The present study shows a novel role for SHH, demonstrating that it guides the outgrowth of the RGC axons by directly signalling at the growth cone.

The position of the optic chiasm and, therefore, the location at which retinal RGC axons cross the chiasm appears to be greatly influenced by SHH. The formation of the chiasm occurs in steps. First, before the arrival of RGC axons, a group of Tuj1/CD44-positive neurones and a specialised set of midline radial glial cells differentiate in the ventral hypothalamus, playing a crucial role in positioning and further patterning of the chiasm. It has been proposed that the induction of both types of these ‘guidepost cells’ may require the activity of SHH (Mason and Sretavan, 1997). In the chick, SHH induces the differentiation of ventral forebrain neurones (Ericson et al., 1995), including possibly the early differentiating neurones of the chiasm. SHH is required, together with BMP7, for the determination of the diencephalic ventral midline (Dale et al., 1997), which includes the hypothalamic radial glia cells.

Therefore, the initial positioning of the chiasm might depend on SHH signalling.

In a second phase, when optic fibres approach the hypothalamus, *Shh* expression is downregulated precisely at the location occupied by the Tuj1-positive 'guidepost' neurones. Analysis of the *Pax2*-null mice, which present morphological alteration of the chiasm, associated with a persistent expression of *Shh* at the optic recess and aberrant projections of the RGC axons (Torres et al., 1996), suggests that *Shh* repression at the midline might be important to establish the correct navigation of the visual axons. We have provided several pieces of cell biological evidence to indicate that SHH interferes with the extension of retinal axons. SHH dampened neurite outgrowth from retina explants without orienting away the direction of fibre extension. This activity of SHH very much resembles that emanating from chiasm explants, which, possibly by retaining *Shh* expression at the borders, suppressed RGC neurite extension in co-culture experiments (Wang et al., 1996). Two recent reports have demonstrated that local graded levels of SHH signal can differentially modulate RGC differentiation without affecting cell proliferation (Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001a). However, in our experimental conditions, exogenously added N-SHH does not appear to modify differentiation or proliferation within the retinal explants, excluding the possibility that the SHH-induced decrease in neurite outgrowth is a consequence of a low number of differentiated RGC in the explants. Instead, this effect is directly mediated by SHH. RGC express the SHH receptor *Ptc1*. Addition of SHH to retinal neurites extending over a laminin substrate induces a rapid, reversible and re-inducible retraction of the fibres and the growth cones. This behaviour appears to be mediated by a SHH-induced decrease of the cAMP levels within the axon tips. The growth suppressing activity of SHH on RGC axons is further sustained by the behaviour of RGC projections after RCAS-mediated ectopic expression of *Shh* in ovo. The presence of *Shh* along the optic nerve or at the hypothalamic region clearly impaired RGC axon growth without affecting, as recently reported (Zhang and Yang, 2001a), at the stages in which the infections were performed, the proximodistal patterning of the eye (Macdonald et al., 1995; Zhang and Yang, 2001b). The growth of the axons was very poor along *Shh*-positive nerve, and the extend of outgrowth correlated with the level of retroviral infection. Furthermore, axons appeared to avoid regions of high *Shh* ectopic expression in the nerve.

Floor plate explants strongly inhibit neurite outgrowth of mouse (Wang et al., 1996) and chick retina. SHH, which is expressed at high level in the floor plate, most probably contributes to this activity. However, we were able to only marginally antagonise the floor plate inhibitory activity with antibodies that block SHH (data not shown). This poor reversion is not surprising, however, as additional inhibitory cues, including Slit2 (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000), may contribute to inhibitory activity of the floor plate.

Previous observations on the behaviour of RGC axons after experimental manipulation in chick can be interpreted as an active avoidance of RGC axons of regions with high levels of *Shh* expression. Implantation of *Shh*-expressing cells at the midline of the dorsal tectum results in the ventralisation of this region with a consequent alteration of the normal dorsoventral topographic organisation of the RGC projections. The authors, however, noted that, as an exception to the new organisation of the projections, fibres that should have projected to the dorsalmost portion of the tectum terminated in a more central position (Nomura and Fujisawa, 2000). In view of our results, SHH secreted by the dorsally grafted cells might be directly responsible for the deflection of the projections.

The guidance of RGC axons to their final target is achieved, as for many other neuronal cell types (Tessier-Lavigne and Goodman, 1996), by a combination of contact mediated and diffusible, attractive and repulsive cues expressed at critical points along the visual pathway. Thus, a combination of chondroitin sulphate proteoglycans, L1 and netrins contribute to the initial outgrowth of RGC axons along the vitreal surface of the retina towards the optic disk (Britts et al., 1992; Deiner et al., 1997; de la Torre et al., 1997). Once axons reach the hypothalamic region, secreted and contact-mediated inhibitory cues may be responsible for leading growth cones towards the chiasm midline (Sretavan and Reichardt, 1993; Wizenmann et al., 1993; Wang et al., 1996). Here, growth promoting molecules associated to the specialised radial glial cells further contribute to funnel axons into the optic tract (Wang et al., 1995). In vitro experiments have also shown that chiasm membrane contains contact-dependent cues able to selectively repel ipsilateral but not contralateral projecting fibres (Wizenmann et al., 1993; Wang et al., 1995). At least one class of the molecules responsible for this activity are Ephrin B proteins, as recently shown by elegant experiments in the metamorphosing frog (Nakagawa et al., 2000). Additionally, A-class Ephrins and Slits, expressed in both the eye and the ventral diencephalon, rostral to the RGC axon trajectory, might further provide general inhibitory cues to the growth cones, as, at least in vitro, they inhibit RGC axon outgrowth (Erskine et al., 2000; Niclou et al., 2000; Ringstedt et al., 2000; Marcus et al., 2000). However, the restriction of *Shh* expression at the chiasm border might be particularly significant for RGC axon guidance at the midline, considering that RGC are the only ventrally decussating fibres that do so in a *Shh*-free environment. Interestingly, in *Xenopus* embryos, RGC axons originating from a third ectopically transplanted eye tended to grow along the ipsilateral side of the CNS but crossed the midline when they encountered the chiasm region (Sharma, 1972; Harris, 1986). Possible interpretations of this behaviour could take in account that RGC growth cones fail to decussate in the otherwise *Shh*-rich ventral midline.

Diffusible guidance cues, such as BDNF or netrins, provide information to the neurones by binding to specific receptors on the surface of the growth cone, triggering a cascade of cytoplasmic events, including the rearrangement of cytoskeletal components, that eventually leads to favour or discourage growth cone advance. The signalling of the different cues converge into common second messengers: the cyclic nucleotides. Thus, high levels of cAMP in the neurones seem to favour growth cone advance, while low levels of cAMP are associated with the inhibition and retraction of the growth cone movements (reviewed by Song and Poo, 1999) (see also Fig. 7). We show here that addition of N-SHH to extended RGC neurites significantly lower the cytoplasmic levels of cAMP into the growth cones and that forskolin effectively antagonises N-SHH activity. The first step involved in the

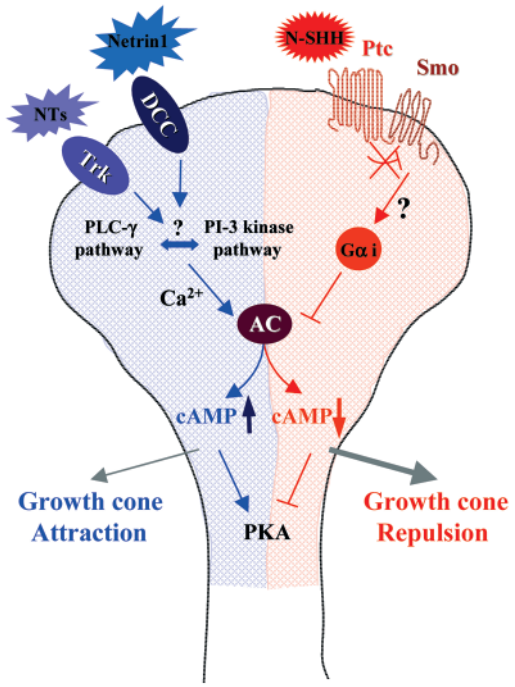

Fig. 7. Proposed model for SHH-mediated signalling leading to the suppression of RGC growth cone extension. On the left (blue background) is depicted a summary (Song and Poo, 1999) of the signalling pathway proposed for the activity of different diffusible growth cone guidance cues. On the right (red background) schematic representation of SHH signalling leading to impairment of growth cone movement (see Discussion).

transduction of SHH signalling requires the presence of its receptor Patched, an eleven-pass transmembrane protein which is also a downstream target of *Shh* activity (Marigo et al., 1996b). Under basal conditions, Ptc associates with and sequesters the activity of Smoothened (Smo), a G-protein associated receptor-like protein. In response to the binding of SHH, Ptc releases Smo, which then activates a Gαi subunit to inhibit cAMP production within the cell. Transcription of *Shh* downstream targets further requires the activity of the GLI family of zinc-finger transcription factors, which are in turn negatively regulated by the cAMP-dependent protein kinase (PKA; Jiang and Struhl, 1995; Epstein et al., 1996; Hammerschmidt et al., 1996; Ingham, 1998). Taking together this information, we propose that SHH, possibly upon binding to Patched, induces a Smo-mediated decrease of cAMP in the axons and growth cones. The cytoplasmic decrease of cAMP leads to a rapid and transcription-independent retraction of the neurites and to an inhibition of the growth cone advance (Fig. 7). Growth of neurites from dorsal neural tube or DRG explants is not affected by SHH. At the moment we do not know if the SHH activity on retina growth cones extends to other neuronal cell types. However, SHH inhibits the migration of neural crest cells (Testaz et al., 2001). These observations complement and reinforce the idea emerging from our studies that SHH, besides its well characterised effects as a patterning, mitogenic and trophic molecule (Hammerschmidt et al., 1997), has also the additional function of controlling cell movement, including in this novel role the guidance of the progression of the axon growth cone.

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