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Review

Hedgehog signaling in vertebrate eye development: a growing puzzle

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Abstract. The vertebrate retina has been widely used as a model to study the development of the central nervous system. Its accessibility and relatively simple organization allow analysis of basic mechanisms such as cell proliferation, differentiation and death. For this reason, it could represent an ideal place to solve the puzzle of Hh signaling during neural development. However, the extensive wealth of data, sometimes apparently discordant, has made the retina one of the most complicated models for studying the role of the Hh cascade. Given the complexity of the field, a deep analysis of the data arising from different animal models is essential. In this review, we will compare and discuss all reported roles of Hh signaling in eye development to shed light on its multiple functions.

Key words. Hedgehog signaling; retina; neurogenesis; gliogenesis; neural patterning; proliferation; apoptosis; axon guidance.

Introduction

Hedgehog (Hh) signaling represents one of the best examples of conservation and versatility in developmental processes. In a 2001 review, Ingham and McMahon reported that Hh signaling plays a role in more than 20 different tissues, organs and/or cell types [1], and this list continues to grow rapidly. Also, the list of functions attributed to Hh signaling is quickly increasing. In the central nervous system (CNS), for example, besides the 'classical' functions of morphogen attributed to Hh signaling (i.e. dorso-ventral patterning of the neural tube), it is now clear that the cascade also regulates precursor proliferation and neuronal and glial cell specification [2]. Moreover, recent data suggest that Hh signaling is also involved in the control of programmed cell death and axon guidance [2].

The Hh family of signaling molecules has been identified by homology to the *Drosophila* segment polarity gene *hedgehog*. In vertebrates, there are three members of the *hedgehog* family: *Sonic* (*Shh*), *Indian* (*Ihh*) and *Desert* (*Dhh*) [1]. Hh proteins bind to Patched (Ptc or Ptch depending on species), a 12-transmembrane receptor protein coupled to Smoothened (Smo), a 7-transmembrane protein that transduces the signal. In the absence of Hh, Patched represses Smo. Hh binding to Patched overcomes the inhibition of Smo, which then transduces the signal intracellularly. Recent data suggest that this activity involves a relocalization of Smo from internal membranes to the cell surface [3]. Downstream of Smo, a multimolecular network, through interactions with microtubules, transduces the Hh signal to modify the activity of Gli proteins [4, 5]. These zinc-finger motif transcription factors, Gli1, Gli2 and Gli3, play critical roles in the mediation and interpretation of Hh signals through the activation and repression of Hh target genes.

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All these components of the cascade are expressed at various stages of retinal development (table 1, fig. 1). The analysis of phenotypes derived from an altered function of Hh signaling during retinogenesis leads to the conclusion that this cascade acts at many different steps of eye development: eye vesicle patterning, neuronal, glial and retinal pigment epithelium (RPE) differentiation, cell proliferation and survival, laminar organization, optic stalk development and axon guidance (table 2). All these activities will be reviewed in this article.

Eye vesicle patterning

In vertebrates, the eye field derives from a single morphogenetic field that later splits into two lateral optic primordia under the influence of the precordal plate [6]. This early event establishes the proximo-distal (P-D) axis of the optic vesicle: the distal-most region will invaginate, forming the optic cup and then the retina, while the proximal region will give rise to the optic stalk. Shh, secreted from the ventral midline, plays a primary role in this process. Genetic ablation of Shh in mouse leads to severe defects in the anterior neural tube and to cyclopia (i.e. the presence of an unseparated optic vesicle) [7]. Similarly, mutations in the human *Shh* gene cause holoprosencephaly, the fusion of cerebral hemispheres [8]. In zebrafish, the situation is more complex: the absence of Shh is not sufficient to induce cyclopia [9]. This could be due to a redundancy of Hh proteins. Indeed, besides *Shh*, two other members of the family are expressed in the zebrafish ventral midline: *tiggy-winkle hedgehog* (*twhh*) and *echidna hedgehog* (*ehh*) [10, 11]. These proteins might therefore compensate the absence of Shh.

Gain-of-function experiments led to the conclusion that Shh promotes proximal and represses distal fates by regulating the expression of *pax* genes (fig. 2A). In zebrafish and *Xenopus*, in fact, *Shh* overexpression promotes the expression of *Pax2*, a marker of the optic stalk, and represses the expression of *Pax6*, a retinal marker [10, 12, 13]. In addition, these two genes transcriptionally repress each other, forming a precise boundary between the retina and the optic stalk [14]. Recently, it was shown that Hh signaling is important to maintain the territories of the retina and the optic stalk even at later stages, when the P-D axis is already established [13, 15].

Just after the evagination of the two optic primordia from the lateral neural tube and the beginning of the P-D axis

Table 1. Hh cascade gene expression in eye development

Organism	Gene	Region/cell type	Stage	Reference
Zebrafish	*shh*	RGCs	from 28 hpf	27
		RPE	39–45 hpf	27, 23
	twhh	RGCs	from 28 hpf	27
		RPE	39–45 hpf	23
	Ptc-2	CMZ	55–80 hpf	23
Chick	*Ptc-1*	neuroepithelium, RGCs	E5	67
	Shh	RGCs	from E4	33
	Ptc-1	neuroepithelium	E4	33
		RPE	E3	17
Newt	*Shh*	neural retina*	adult	22
	Ptc-1	neural retina*, RPE*	adult	22
	Ptc-2	neural retina*, RPE*	adult	22
Xenopus	*Shh*	RGCs	from st 34	13
		neural retina*	adult	22
	bhh	central RPE	from st 34	13, 22
	chh	central RPE	from st 34	13, 22
	Ptc-1	peripheral RPE	from st 34	13
	Ptc-2	peripheral RPE	from st 34	13
	Gli1	peripheral RPE	from st 34	13
	Gli2	retinal stem cells + peripheral RPE	from st 34	13
	Gli3	retinal stem cells + peripheral RPE	from st 34	13
	Smo	retinal stem cells + peripheral RPE	from st 34	13
Mouse	*Shh*	RGCs	from E14.5	44
	Ihh	RPE, periocular mesenchyme		35, 36, 42
	Ptch	neuroepithelium	E14.5-E17	44
		Müller cells	from P7	44, 35
	Gli1	astrocytes optic stalk, optic disc cells	from E12	36, 42
		neuroepithelium	E18	35, 42

* Expression data obtained by reverse transcriptase polymerase chain reaction. st stage.

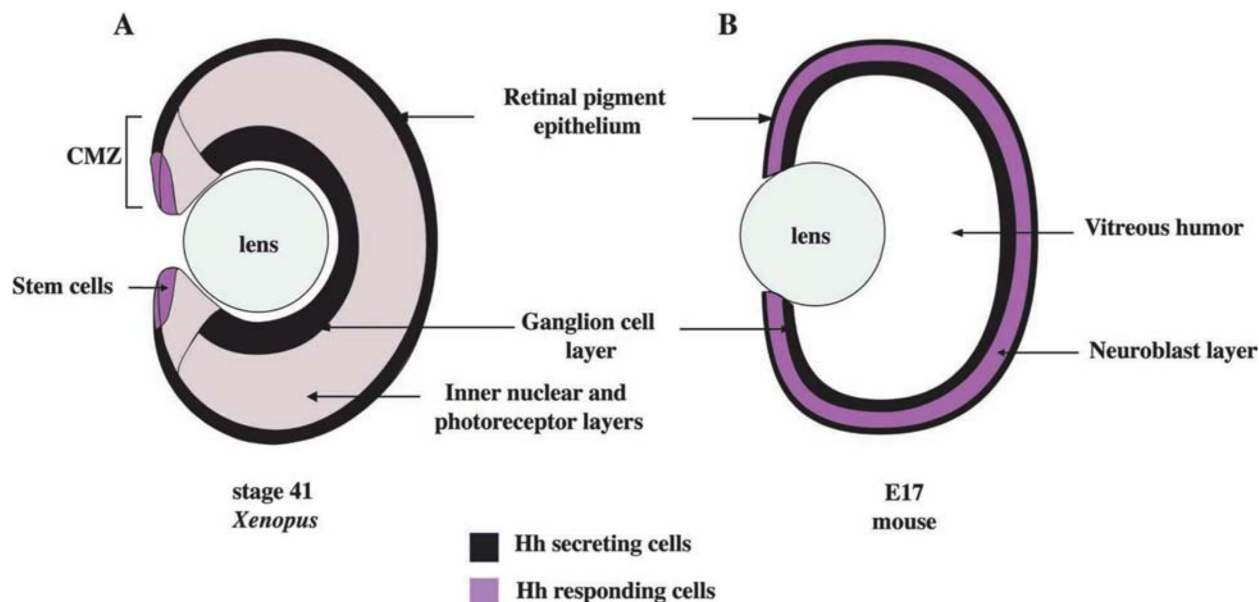

Figure 1. Examples of Hedgehog gene expression in vertebrate retina. Hedgehog proteins are expressed in vertebrate retinas by postmitotic RGCs and RPE cells. Downstream components of the cascade (i. e. *Patched*, *Smo* and *Gli* genes) are mainly expressed by undifferentiated precursors or proliferating retinoblasts. (*A*) Example of a stage 41 *Xenopus* retina. At this stage, the retina is made mainly of differentiated neurons, except in the CMZ, which contains stem cells, proliferating retinoblasts and differentiating neurons. Shh is expressed in the RGC layer, while the two other members of the family, *Bhh* and *Chh*, are expressed in the central part of the RPE. Stem cells, situated at the edge of the CMZ, strongly express *Smo*, *Gli2* and *Gli3* genes, being potential responding cells to RGC and RPE derived Hh signals. (*B*) Example of E17 mouse retina. Also in this case, the source of Shh is represented by the RGCs. RPE and surrounding mesenchymal cells (not shown) express Ihh. At this stage the signal is received by cells expressing Patched and Gli1 in the still proliferating neuroblast layer. **CMZ**, ciliary marginal zone; **RPE**, retinal pigment epithelium; **RGCs**, retinal ganglion cells.

Table 2. Reported roles for Hh signaling during eye development

Role	Organism	Experimental Condition	Loss of Function Phenotype	Gain of Function Phenotype	Reference
Optic vesicle separation/ P-D axis establishment	zebrafish	smu mutant; cyclopamine treatment	loss of proximal territories		62
		Shh overexpression		expansion of the proximal retina	12, 10
	Xenopus	cyclopamine treatment	loss of proximal territories		13
		Shh overexpression		expansion of the proximal retina	13
	mouse	Shh mutant	cyclopia		7
	Human	Shh mutation	holoprosencephaly		8
D-V axis establishment	chick	retroviral Shh overexpression		expansion of the ventral retina	17
		antibodies against Shh	expansion of the dorsal retina		17
	Xenopus	Shh overexpression		expansion of the ventral retina	18
Retinal neurogenesis	zebrafish	cyclopamine treatment	arrest of retinal differentiation		27
		antisense oligonucleotides	reduced photoreceptor differentiation		23, 32
		Shh mutant	reduced photoreceptor differentiation		38

Table 2 (continued)

Role	Organism	Experimental Condition	Loss of Function Phenotype	Gain of Function Phenotype	Reference
Retinal neurogenesis	chick	antibodies against Shh	increase in RGC differentiation		33
		retroviral Shh overexpression		decrease of RGC differentiation	33
	rat	recombinant Shh addition to retinal cultures		increased photo-receptor differentiation	37
RPE differentiation	*Xenopus*	cyclopamine treatment	reduced RPE differentiation		13
Müller cell differentiattion	Mouse	recombinant Shh addition to retinal cultures		increase in Müller cell differentiation	44
	Xenopus	Hh proteins overexpression		increase in Müller cell differentiation	M. A. A. et al., unpublished
Optic stalk differentiation	mouse	conditional ablation of Shh gene in the CNS	failure of astrocyte differentiation in the optic stalk		42
Cell proliferation	zebrafish	cyclopamine treatment	microphtalmia		27
	chick	antibodies against Shh	microphtalmia		17
	mouse	antibodies against Shh	reduced astrocyte proliferation in the optic nerve		36
		recombinant Shh addition to retinal cultures		increase in brdU incorporation	44
Cell death	zebrafish	cyclopamine treatment	increase in apoptosis		27
	chick	antibodies against Shh	increase in apoptosis		17
Laminar organization	mouse	conditional ablation of *Shh* gene in the CNS	disrupted retinal lamination		35
Retinal axon guidance	zebrafish	Shh mutant	absence of midline crossing of RGC axons		59
	chick	retroviral *Shh* over-expression in the optic chiasm		absence of midline crossing of RGC axons	67
	mouse	conditional ablation of Shh gene in the CNS	intraretinal navigation defects		42

formation, the optic vesicles are also patterned along a dorso-ventral (D-V) axis. This event will subsequently be of capital importance in retinal axon guidance and in the establishment of retinotectal topographic projections. Studies in chick, mouse and *Xenopus* suggest that Shh is also involved in the establishment of the eye D-V axis [16–18] (fig. 2B). Similarly to what happens in the neural tube, the optic vesicles receive two antagonistic signals: a source of Shh emanating from the ventral midline and a source of BMP4 in the dorsal optic vesicle itself. These molecules act in a coordinated manner to pattern the eye along the D-V axis, repressing each other [19]. It is likely that this mutual repression is achieved by the target genes *Vax2*, activated by *Shh* [18], and *Tbx5*, activated by BMP4 [18, 20]. These genes, in fact, are expressed in the ventral and in the dorsal halves of the optic vesicles, respectively, and their misexpression can ventralize or dorsalize the eye [20, 21]. This mutual repression between *Tbx* and *Vax* genes along the D-V axis of the eye vesicle resembles the repression between *Pax2* and *Pax6* along the P-D axis. Hh signaling thus contributes to both P-D and D-V axis establishment, activating *Vax2* and *Pax2*. It is likely that these genes are activated in parallel pathways, as *Vax2* is expressed in Pax2 mutants and vice versa [15]. Nonetheless, Hh signaling is not the only pathway controlling *Vax* gene expression. *Vax2* expression, for example, can be induced in the absence of Hh signals in zebrafish [15], as well as in *Xenopus*, when the

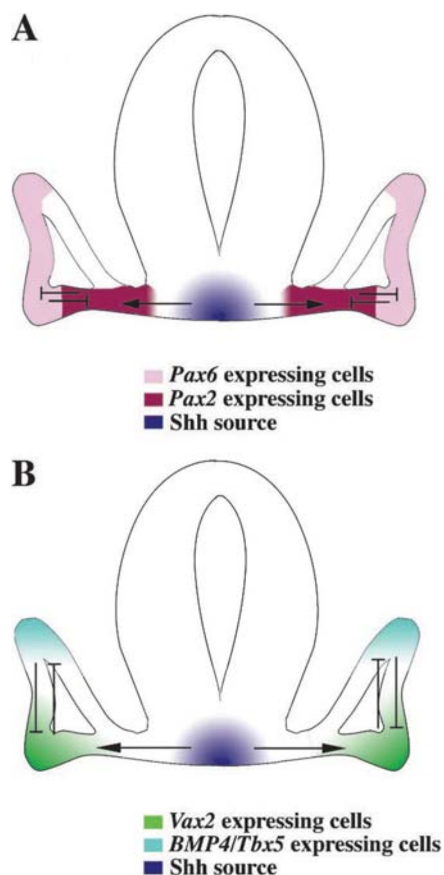

Figure 2. Functions of Hedgehog signaling in eye vesicle patterning. Ventral derived Shh is involved in both P-D and D-V axis establishment in the protruding optic vesicles. (*A*) Shh specifies proximal territories (i.e. optic stalk) by promoting the expression of *Pax2*. This latter gene transcriptionally represses *Pax6* expression. *Pax6* is one of the eye master regulator genes and specifies distal territories (i.e. neural and pigmented retina). *Pax6* and *Pax2* mutually repress each other, forming a precise boundary between the retina and the optic stalk. (*B*) Shh, together with other factors such as FGFs and retinoic acid (RA), contributes to the activation of *Vax2*, which specifies the ventral part of the retina. BMP4, expressed in the dorsal part of the retina, inhibits the ventralizing effect of Hh signaling through the activation of Tbx5 expression. Antagonist effects between Hh and BMP signals take place also in the establishment of the D-V axis in the neural tube.

Hh cascade is blocked by the alkaloid cyclopamine [13]. Fibroblast growth factor (FGF) is thought to be another activator of *Vax* genes: indeed, blocking the FGF receptor causes a complete loss of *Vax2* expression [15].

Neuronal and glial differentiation

Besides the Shh source derived from the ventral midline that is responsible for early eye vesicle patterning, there are at least two endogenous sources of Hh expression during retinal cell differentiation. Shh is secreted from postmitotic ganglion cells, while Indian or Desert Hedgehog homologue proteins, according to the species, are secreted from RPE cells [13, 22, 23, table 2].

Retinal ganglion cells

In vertebrates, retinal histogenesis proceeds from a single neuroepithelium of multipotent progenitors cells that gives rise to all cell types, following an established histogenetic order (reviewed in [24, 25]). Retinal ganglion cells (RGCs) are the first neurons to be born, followed by interneurons and photoreceptors and, lastly, by Müller glial cells. RGC differentiation begins roughly in the central part of the retina and proceeds towards the periphery along a central to peripheral pattern [26]. This wave of differentiation is reminiscent of the neurogenic wave that occurs during *Drosophila* eye differentiation. It has been shown that Hh signaling controls this process in a similar way in both *Drosophila* and zebrafish [27]. In the *Drosophila* eye imaginal disc, Hh protein, secreted by the newly differentiated ommatidia, stimulates the differentiation of the neighboring, but nonadjacent, immature ommatidia through the activation of *atonal* expression, a basic helix-loop-helix transcription factor (reviewed in [28]). *atonal* promotes the differentiation of the first cell type, the R8 photoreceptor that recruits the other cell types, completing the ommatidial differentiation. These newly born ommatidia will in turn secrete Hh, thereby propagating the wave of differentiation. In zebrafish, the retinal differentiation process is strikingly similar. The first patch of postmitotic neurons is found ventronasally, close to the optic stalk. Two waves of gene expression spread from these newly formed RGCs: a wave of *ath5* and a wave of *Shh*. *ath5* is an *atonal* homologue that transiently sweeps across the differentiating retinoblasts and then is maintained in the periphery of the retina, where cells keep differentiating [29, 30]. *Shh* expression is initiated first in differentiated RGCs and then extends as their differentiation proceeds [27]. Both *Shh* and *ath5* waves are necessary to the propagation of retinogenesis: blocking the Hh cascade with cyclopamine results in a severe arrest of retinal differentiation [27], and lakritz embryos that completely lack ath5 proteins do not develop RGCs [31]. Interactions between Hh signaling and *ath5* expression have been recently investigated by blocking Hh signals at different developmental stages in zebrafish embryos [32]. The authors suggest that extraretinal derived (probably from the ventral midline) Hh signals are involved in initiating *ath5* expression and retinal differentiation. On the other hand, the propagation of the *ath5* wave and the ongoing retinal differentiation would be dependent on intraretinal (i.e. RGC-derived) Hh proteins.
After retinal differentiation has occurred, Hh has an opposite role on RGCs: it inhibits the ultimate differentiation of retinal precursors, thereby controlling the number

of RGCs [33]. This process has been highlighted in chick where a dual role has been proposed for Hh signaling depending on the protein concentration: retinal progenitors that have not yet been reached by the differentiation wave receive low levels of Shh because they are still distant from the secreting cells. At this concentration, Shh would stimulate the differentiation of one fraction of these progenitors as RGCs, while the other cells would remain in the progenitor state to adopt later cell fates. Behind the wave of differentiation, Shh concentration becomes higher. This would inhibit the remaining progenitors from adopting the RGC fate, maintaining in this way a correct cell number in the RGC layer. Again, the similarities between vertebrate and *Drosophila* retinogenesis are remarkable. Before this dual role mechanism was characterized in chick, a work from Dominguez demonstrated that a similar mechanism also takes place in *Drosophila* [34]. In fact, *atonal* activation by Hh occurs at 5–7 ommatidial rows from the morphogenetical furrow (MF), the front of the differentiation wave. In contrast, at closer distances, *atonal* expression is inhibited by Hh. This response is crucial for the correct establishment of the ommatidial arrays of the *Drosophila's* eye. As is true in the neural tube, the fact that different retinal fates result from different Hh concentrations demonstrates that cells can read and interpret very precisely Hh protein levels.

Photoreceptors

The RGC wave of differentiation is followed by differentiation of the other retinal cell types. Does Hh signaling contribute to the differentiation of retinal cells other than RGCs? The matter has been studied in vivo and in vitro in different organisms. Besides the expression of *Shh* in the ganglion cell layer, Hh expression has been reported in the RPE in almost all vertebrates studied: rat, mouse, chick, *Xenopus*, newt and zebrafish [13, 22, 23, 35]. In mouse, Ihh expression has also been reported in the mesenchymal cells surrounding the eye [36]. This area of expression is situated on the opposite side of the retina from the ganglion cell layer. It is possible that cells situated in between these two sources of Hh, i.e. in the photoreceptor layer and in the inner nuclear layer of the retina, can sense these two Hh sources. In zebrafish it has been shown that Hh signaling also plays a role in photoreceptor genesis. Injection of antisense oligonucleotides against *Shh* and *twhh* reduces and delays photoreceptor differentiation [23]. Conversely, addition of Shh to rat retinal explants increases the proportion of photoreceptors [37], also suggesting a possible role for Hh in mammalian photoreceptor differentiation. The question has been further analyzed by Stenkamp and colleagues, who have undertaken a careful analysis of retinal gene expression in zebrafish *Shh* mutants (*syu*) [38]. No abnormalities were found in the expression of *neuroD*, *crx* and *rx2*. All these genes are implicated in photoreceptor differentiation [38 and references therein]. Instead, the expression of *rx1*, which is expressed in the photoreceptor layer and is thought to be involved in photoreceptor differentiation [39], was clearly reduced in *syu* mutants. This gene is thus a good candidate to mediate the effect of reduced Hh signaling in photoreceptors.

Retinal pigment epithelium

Besides being important for photoreceptor differentiation, Hh signaling in the RPE is essential for differentiation of the RPE itself. Studies in chick showed that Shh is required for ventral RPE formation, since there is a lack of pigmentation in the ventral eye when embryos are treated with anti-Shh antibodies [17]. As this treatment also reduces the expression of *otx2*, and this latter gene is involved in the control of RPE versus neural retinal fate, the authors speculated that the loss of pigmentation is due to a conversion of RPE to neural retina caused by the lack of *otx2*. We have shown that in *Xenopus*, Hh signaling is essential for all RPE cell differentiation. Blocking the Hh cascade in developing embryos with cyclopamine has a severe consequence on RPE formation, causing a reduction in pigmentation and the reduction or loss of expression of RPE differentiation markers. Moreover, our expression data in tadpole retina suggest that Hh signals secreted from the mature central RPE are received by young RPE precursors in the periphery of the retina that express downstream components of the cascade. As they grow and become more centrally located, these precursors lose the ability to receive Hh signals (i.e. they stop expressing downstream components) and eventually, in a third phase, they will fully differentiate and secrete Hh proteins. This is a classical case of Hh action, in which the tissue sending the signal is postmitotic and fully differentiated, while receiving cells are still in the precursor state. We think that in *Xenopus*, RPE defects are due to an arrest in the process of RPE differentiation rather than to a switch of cell fate. This idea is supported by the fact that RPE cells in which Hh signaling is blocked do not display any marker of neural retina. It would be of interest to investigate whether RPE progenitors respond differently to various doses of Hh source, as is true in chick RGCs and *Drosophila* photoreceptor differentiation.

Glial cells

So far, we have described the role of Hh signaling in the differentiation of neural and pigmented retina. A wealth of data suggest that Hh signaling also controls glial specification throughout the nervous system. The most docu-

mented case is the differentiation of oligodendrocytes, the myelin-forming glial cells in the CNS. Oligodendrocytes arise from specific regions mostly situated in the ventral region of the neural tube [40]. In vitro studies revealed that oligodendrocytes can be induced in culture by adding Shh. Further, inhibiting Shh signaling prior to the appearance of the oligodendrocytes blocks their emergence [41]. Also in this case, Shh appears to act on precursors, contributing to the initial commitment to the oligodendrocyte fate (reviewed in [40]). Neuron-to-glia interactions controlled by the Hh cascade are not limited to oligodendrocyte specification in the neural tube. The cerebellum represents another good example. Purkinje cells form the main source of Hh signaling in this case. Purkinje cells are the first to differentiate, and Shh produced by these cells stimulates the differentiation of Bergmann cells, an astrocyte-like radial glial cell type [5]. In the mouse retina, it has been shown that Hh signals from RGCs influence the differentiation of another major class of glial cells, astrocytes. This induction occurs through an axon-to-astrocyte signaling in the optic nerve that stimulates astrocyte proliferation [36]. It is likely that RGC-axons secrete Shh protein: this signal would be received by astrocyte precursors in the developing optic nerve since they express the Hh receptor *Ptch* [36]. Blocking of Shh activity using neutralizing antibodies reduces astrocyte proliferation in the optic nerve. Recently, it has been demonstrated that RGC derived Shh is also required for glial cell specification in the optic stalk and disc [42]. Mice carrying a conditional *Shh* mutation in retinal precursors display a failure of optic stalk differentiation and a transformation of optic stalk neuroepithelial cells into pigmented cells. This effect could be due to a loss of Pax2 activity, as *Pax2* mutant mice display the same phenotype, characterized by a pigmented optic nerve [43].

Müller cells represent the major glial cell type in the neural retina. Like the other retinal cell types, these cells are derived from multipotent precursors that divide asymmetrically. At each division, precursors give rise to a postmitotic progenitor that differentiates and a new dividing precursor. Müller cells are the last cell type to be born in the developing retina. They play an important trophic role, are responsible for reuptake of neurotransmitters and are essential for maintenance of a correct lamination of cell layers [35]. Treatment of cultures of perinatal mouse retinal cells with the amino-terminal fragment of Shh results in an increase in the proportion of Müller glial cells [44]. Moreover, a role for Hh signaling on Müller cell differentiation is supported by in vivo experiments in *Xenopus*. We have observed that overexpression of Hh genes in *Xenopus* retina leads to a dramatic increase in Müller glial cells [M. A. A., M. P., unpublished data]. It is not completely clear, however, whether this increase is due to an expansion of glial precursor proliferation, or to a direct role on Müller cell specification. We are currently addressing this question.

Cell proliferation and survival

If a lack of Hh function is the cause of diseases such as holoprosencephaly or cyclopia, it is now also clear that the Hh cascade, when inappropriately regulated, drives tumorigenesis (reviewed in [45]). Indeed, *Ptch* was found to be mutated in basal cell carcinoma [46, 47]. Several studies have also described *Ptch* and *Smo* mutations in sporadic medulloblastomas and related primitive neuroectodermal tumors (reviewed in [48]). Involvement of Hh in tumorigenesis can be attributed to the fact that during normal development, Hh signaling plays a role in modulating the proliferation of progenitor cells and can act as a survival factor. In the last 2 years, several studies have begun to shed light on the molecular mechanisms of these processes. Again, the cerebellum represents a good model to study the effect of Hh signaling on progenitor cell proliferation. As already explained above, Purkinje neurons are the main source of Shh in the cerebellum. Hh cascade is active in the external granule layer (EGL) as revealed by the expression of *Ptch-1* and *Gli1* downstream genes. This layer is made of mitotic progenitors of granule neurons and undergoes, in mammals, a dramatic expansion during the early postnatal period and then disappears. Three elegant studies have shown that Shh is necessary and sufficient to promote EGL cell proliferation [5, 49, 50]. Implantation of hybridoma cell grafts that secrete anti-Shh neutralizing antibody led to mice with impaired cerebellar development and reduced BrdU incorporation. Conversely, adding ectopic Shh protein to granule precursor cultures increases their BrdU incorporation [49]. In the retina, similarly to what happens in the cerebellum, Hh proteins are secreted by postmitotic cells, and the signal is received by proliferating cells (figs 1 and 3). We have shown that *Gli2*, *Gli3* and *Smo* are expressed in retinal stem cells in the ciliary marginal zone (CMZ) of *Xenopus* tadpoles (fig. 1). Retinal progenitor cultures respond to ectopic Shh addition in a way similar to cerebellar cells, by increasing proliferation, as seen by BrdU incorporation data [37, 44].

Besides this in vitro data, there is evidence that Hh signaling controls retinal proliferation in vivo. Blocking the Hh cascade causes microphtalmia, a reduction of the eye size. This phenotype has been observed in zebrafish and in *Xenopus* by treating the embryos with cyclopamine [27; our unpublished observations]; in chick, by treating the embryos with anti-Shh neutralizing antibodies [17]; and in mice carrying a conditional mutation in the *Shh* gene [35]. These data fit perfectly with studies in other CNS compartments where it has been shown that Hh signaling is an important factor for the control of the size of

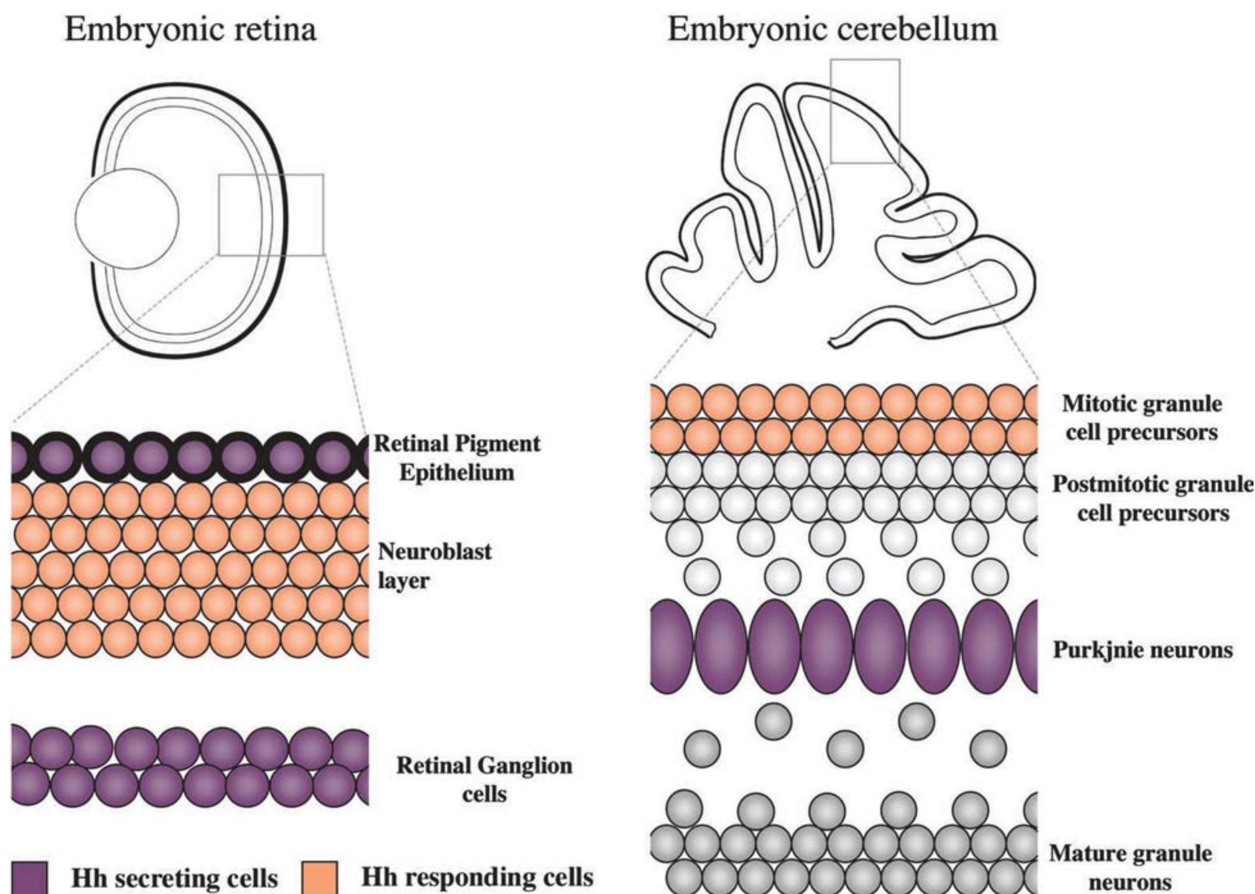

Figure 3. Hedgehog signaling: parallels between retina and cerebellum. As shown in figure 1, Hedgehog proteins are secreted from post-mitotic RGCs and RPE in embryonic mouse retina. Downstream components of the cascade are expressed by mitotic retinal precursors in the neuroblast layer. In mouse embryonic cerebellum, the source of Hh derives from postmitotic Purkinje cells, and the signal is received by mitotic granule cell precursors.

neural structures [51, 52 and references therein]. As the main Hh source, the notochord has a key role in regulating the growth of the brain vesicle: in chick, transient displacement of the notochord from the midbrain results in a reduced expansion of the brain vesicle. Implanting Shh secreting cells restored the expansion [53]. The authors found that this reduced growth is due both to a decrease in cell proliferation and to an increase in cell death. Indeed, the control of growth of all neural structures is not only a matter of cell proliferation: apoptosis is also known to be an important process in the control of the size and the shape of several structures. Craniofacial neural crest cells, for example, contribute to the formation of the skull, the meninges and the cranial sensory ganglia. Interestingly, the depletion of neural crest cells leads to phenotypes that are reminiscent of those associated with reduced Hh signaling [54]. Indeed, Ahlgren and Bronner-Fraser showed that the inhibition of Shh causes craniofacial neural crest death [55]. In the developing eye, interfering with Hh signaling is also associated with an increase in apoptosis: this has been reported in the zebrafish and chick retina [17, 27]. In zebrafish, cell death has been examined in *Shh* (syu) mutant embryos [29, 38]. It seems that, at early stages, cell survival is not dependent upon Hh signals, but after retinal differentiation Shh plays a role in retinal cell survival [27, 38].

Laminar organization

The vertebrate retina is a highly organized structure, made of three nuclear layers separated by two fiber layers and surrounded by a pigmented epithelium. In vitro studies have shown that neural-retinal explants can reaggregate in culture, forming spheres containing all cell types, arranged in layers as in the retina, but with an inverse organization (reviewed in [56]). These inside-out spheres are characterized by the presence of rosettes of photoreceptors and radially arranged Müller cells. Retinal rosettes can be observed in vivo in several pathological or experimental conditions, in the embryo when proliferation is blocked [57] or in retinoblastoma [58]. RPE and

Müller glial cells are known to play primary roles in maintaining retinal organization [56]. If neural-retinal explants are cocultured in the presence of RPE, or with the addition of Müller cell conditioned medium, retinal spheres assume a proper laminar organization [56]. Recently, it was shown that RGC-derived Shh plays a role in this process since, when added to mouse retinal explants, it restores normal lamination and avoids rosette formation [35]. Accordingly, treating explants with anti-Shh neutralizing antibodies greatly increases disorganization and photoreceptor rosetting [35]. Also, retinal disorganization and photoreceptor rosetting has been observed in mice carrying a conditional ablation of the *Shh* gene [35]. Do Hh signals other than those derived from RGCs play a role in maintaining retinal laminar organization? As RPE is known to be important for this event, RPE-derived Hh proteins could be good candidates. However, at least in mouse, this hypothesis is unlikely, as mice mutant for *Ihh* (the only member of the family to be expressed in the RPE) display no abnormalities in their retinas. In zebrafish, a fraction of *syu* mutant retinas fail to become laminated [38]. However, the authors interpret this event as delayed differentiation, with consequent persistence of retinal cells in the progenitor state, rather than direct action of Shh in the maintenance of retinal lamination.

Axon guidance

Recent studies have begun to shed light on the role of Hh signaling in axon growth and guidance, but the mechanisms underlying these processes are still unclear and poorly understood. The first evidence that the Hh cascade is also involved in the control of axon guidance derives from genetic screens in zebrafish in which a large number of mutant lines with abnormal retinal projections have been identified (reviewed in [59, 60]) In the developing zebrafish, as in lower vertebrates, RGC axons project contralaterally to the other side of the embryo. This means that RGCs extend their axons towards the midline and cross it at the optic chiasm; then they project in the opposite side of the brain to their targets. In mammals and birds, not all RGCs project their axons contralaterally: some axons turn sharply at the optic chiasm and project ipsilaterally. Several mutations affecting retinal midline crossing have been discovered to belong to the Hh cascade: *sonic you* (*syu*) [9] , *detour* (*dtr*) [61], *you-too* (*yot*) [61] and *slow-muscle-omitted* (*smu*) [62] are the loci coding for Shh, Gli1, Gli2 and Smo, respectively. Mutations in these genes share the same phenotype: midline crossing is disrupted and RGCs project ipsilaterally [59]. One possible hypothesis to explain this phenotype is that in Hh cascade mutants, the ventral midline is lacking or strongly reduced. This region is an important source of diffusible factors implicated in axon guidance; some of them, like netrins, are essential to attract axon growth cones towards the floor plate [63]. This region has been shown to attract axons that normally cross the midline and to repel uncrossed axons [64]. The developing retinal axon growth cone that navigates from the eye to the brain could be influenced by several Hh sources: the first consists of endogenous Shh proteins, which are transported anterogradely from the RGC soma. As discussed above, it seems that Hh proteins secreted by axons in the optic nerve are important in promoting astrocyte proliferation and optic stalk development, and some of the navigation defects, including axon misrouting to subretinal spaces and failure to exit, found in Hh mutants, could be due to an astrocyte deficiency. The second Hh source that can influence axon navigation derives from the retina. Besides the Shh gradient emanating from the RGCs themselves, Hh is also expressed in the RPE. It has been shown that RGC-derived Shh ablation causes intraretinal axon navigation defects [42], with several axons misrouted to subretinal spaces and failing to exit the retina through the optic disc. A similar phenotype has been observed in netrin1 mutant mice [65]. As Shh is required for the proper development of netrin-expressing optic disc cells, it is probable that intraretinal navigation defects are due to a loss in netrin signaling. Once out of the retina, the growth cone navigates towards the ventral midline. This zone, i.e. the floor plate with the underlying notochord, represents the most important Hh source in the whole embryo. Nevertheless, even if Shh expression is maintained all along the ventral midline, it is downregulated in the optic chiasm just when retinal axons are approaching [43]. If *Shh* expression is inappropriately expressed in the optic chiasm, as occurs in Pax2 mutant mice [43] or in Pax2.1/noi mutant zebrafish [66], axons do not cross the midline, as in syu mutants. Consistent with these data, Bovolenta and colleagues have shown that Shh repels RGC growth cones and inhibits their growth [67]. The authors demonstrated that Shh overexpression in the chiasm region of chick embryos interferes with the retinal axon pathway: as in *Pax2* mutants, axons were misrouted ipsilaterally. Together with Pax2 mutant mice and fish, these data suggest that RGC axons must find a Shh-free 'corridor' in order to cross the ventral midline. What are the mechanisms underlying this behavior? Addition of recombinant N-Shh to retinal explants, cultured on a laminin-coated dish, decreased cyclic AMP (cAMP) levels in growth cones. Since cAMP plays a strategic role in the developing growth cone, variations in cAMP levels can determine growth cone behavior in the presence of netrin-1. If cAMP levels diminish, axons are repelled from a netrin source instead of being attracted [68, 69]. Thus, ectopic expression of Shh could change the growth cone response to netrin by regulating cytosolic cAMP levels. A very recent study has shown that Hh signaling has an opposite effect on dorsal spinal cord commissural

axons: it attracts them towards the ventral midline, cooperating with netrin [70]. These neurons, born in the dorsal neural tube, extend their axons ventrally where they cross the midline. Why do dorsal commissural and RGC axons respond in opposite ways to the same stimulus? The first difference that we can observe is that RGC axons themselves express Hh proteins, while dorsal commissural axons do not. Is this difference sufficient to explain these different behaviors? In this review we have shown that Hh and BMP have often opposite signaling roles. Intriguingly, dorsal commissural axons that intrinsically express BMP4 are repelled by BMP signaling from the dorsal midline [71] and attracted towards a Hh source.

Controversies

We can observe from these data that Hh signals deeply influence the development of the visual system. However, some issues remain elusive. The most intriguing point is the observation that altering Hh signaling produces different outcomes according to the animal model used. For example, as described above, the downregulation of the Hh signal causes differentiation defects in either RGCs, or photoreceptors, or RPE, or astrocytes. These apparent discrepancies observed by researchers raise interesting questions: (i) Are there different roles for the Hh pathway in different species of vertebrates? (ii) How can different experimental conditions be compared? (iii) Do Hh functions change at different developmental stages? The first point is supported by the analysis of *Gli* gene functions in zebrafish and mouse [72]. In zebrafish, Gli1 acts as the main activator of the Hh cascade, while in mouse it has a dispensable role [*72*]. In contrast, Gli2 is thought to be the major mediator of Hh signaling in mouse. This suggests that *Gli* genes may also be differentially recruited in the retina of different species. Moreover, intrinsic differences in developmental processes could make molecules act differently, or more simply, could provide us a different readout of a given experiment. For example, the knock down of *shh* and *twhh* in zebrafish has been shown to impair photoreceptor (especially rod) development, while this has not been reported in other vertebrates. It is worth noting, however, that photoreceptor differentiation in teleost fish takes place in a peculiar mode compared with other vertebrates (reviewed by [73]). Indeed, during retinal development, mouse and *Xenopus* progenitors can give rise to all cell types [74, 75], while fish progenitors will not generate rod photoreceptors. The latter are subsequently added to the differentiated retina from specialized precursors.

Discrepancies could also not result from different roles of the Hh pathway in different species of vertebrates, but rather from different experimental conditions as cells are extremely sensitive to variations in Hh concentration. Therefore, it is likely that blocking the cascade by different methods (i.e. by pharmacological treatments, injecting antisense oligonucleotides or knocking down a single gene of the cascade) results in different phenotypes due to diverse degrees of inhibition. For example, the zebrafish shh mutants (syu) display a reduction in RGC formation [27], while knocking down shh in chick by antibody neutralization leads to an increase in RGC differentiation [33]. These opposite effects allowed to develop a model on the role of Hh signaling on RGC genesis based on dose-dependant action.

Last but not least, it is now clear that Hh function changes in the course of retinogenesis. Indeed, the Hh pathway seems to be recruited at several steps of eye development. One can therefore expect various phenotypes depending precisely when the pathway is altered.

Although the wealth of data provided by the large multiplicity of models and techniques seems to cloud our understanding of the role of the Hh pathway in eye development, it is actually helpful to fully analyze the pathway.

Conclusions

Hh signaling has a fundamental role during neural development, and it is clear that cells respond to Hh signals in several ways. They can differentiate, proliferate or die, and the choice among these outputs is finely regulated. Here we have reviewed Hh functions in one of the most important and versatile models for the study of the CNS, the vertebrate retina. Although we are still far from a comprehensive vision of the role of Hh signaling during retinal development, all the recent data reviewed in this paper highlight the sequential roles of the Hh pathway for the various developmental processes of retinogenesis. Insight into the molecular mechanisms of this pathway during eye development will contribute to a better understanding of its involvement in neoplastic transformation of cells arising from neuronal precursors. The rapidity with which our knowledge is increasing is encouraging, and new breakthroughs will surely help to solve this intriguing puzzle.

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