

Ganglion cell axon pathfinding in the retina and optic nerve

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Abstract

The eye is a highly specialized structure that gathers and converts light information into neuronal signals. These signals are relayed along axons of retinal ganglion cells (RGCs) to visual centers in the brain for processing. In this review, we discuss the pathfinding tasks RGC axons face during development and the molecular mechanisms known to be involved. The data at hand support the presence of multiple axon guidance mechanisms concentrically organized around the optic nerve head, each of which appears to involve both growth-promoting and growth-inhibitory guidance molecules. Together, these strategies ensure proper optic nerve formation and establish the anatomical pathway for faithful transmission of information between the retina and the brain.

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1. Introduction

The eye as a light-capturing organ is beautifully crafted so that information at each point in our visual space is processed at a specific location on the retina. Neuronal signals corresponding to visual information in the external world must then be conveyed via the optic nerve to visual processing and cognitive centers in the brain. During development, a series of inductive, morphogenetic, and differentiation events contribute to the formation of the light focusing and detection structures of the eye. However, in order for visual information to be relayed to the CNS, a series of axon pathfinding events must also take place to ensure that the axons of embryonic RGCs find their way out of the eye to connect with CNS targets. RGCs are the only neuronal cell type in the retina that sends an axon out of the retina to the CNS. The numbers of RGC axons that must navigate correctly within the eye is quite large. In mice, over 50,000 RGC axons exit each retina into the optic nerve, while in humans, over a million RGC axons must be guided accurately during embryonic development of the retina. Given the structural similarities amongst vertebrate eyes and the central role of vision in animal behavior, it seems likely that highly conserved and robust axon guidance mechanisms ex-

ist to support proper RGC axon pathfinding within the retina and formation of the optic nerve.

2. Retinal development and axon outgrowth

The vertebrate retina originates as an outpouching from the embryonic diencephalon and remains attached to the brain via the optic stalk. Further invagination of the ventral aspect of the optic vesicle and optic stalk forms a groove called the optic fissure. Tissue on the two opposing sides of the optic fissure subsequently fuses to give rise to the eye cup. The optic nerve head region is located at the posterior pole of the eye cup and serves as the exit into the optic stalk. The first terminally differentiated RGCs start to appear as the process of optic fissure closure is near completion. These early generated RGCs are located in the central region of the retina, a short distance away from the nascent optic nerve head. Later differentiated RGCs are added in progressively more peripheral retinal regions. Given this central to peripheral gradient in RGC differentiation, RGC axons find their way into the optic nerve at different times. In contrast to the axons of the earliest generated RGCs that reside in the immediate vicinity of the optic nerve head, the axons of later generated RGC axons face a longer distance to travel from their site of origin and must develop pathfinding strategies to seek the optic nerve head. In mammals, RGC neurogenesis is limited to a specific period during in utero development, while in lower vertebrates such as fish and frogs, new RGCs are constantly added as the eyes continue to grow in adult

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animals. Therefore, in fish and frogs, the problem of axon guidance to the optic nerve head is an ongoing one, and one that must be solved throughout the life of the organism.

We have organized the content flow of this review in large part after the sequence of pathfinding tasks that a newly formed RGC growth cone will confront in order to find its way within the retina, to reach the optic nerve head, and finally to exit into the optic nerve. In addition to identifying guidance molecules and receptors that mediate retinal pathfinding, we will highlight some of the principles of RGC axon guidance as well as identify pathfinding events that are currently only poorly understood. While the main focus of this review is axon guidance within the vertebrate retina, particularly in mammals, research advances in other systems and organisms that may provide insight into this process will also be discussed. We also bring into the discussion research that may have significance for understanding human disease and congenital disorders.

3. RGC axonogenesis

The process of RGC axon pathfinding begins immediately following the elaboration of an axon from terminally differentiated RGCs. The molecular mechanisms surrounding initial axonogenesis from RGCs is not well understood in mammals. Our current understanding of this process is based on studies in *Xenopus* that have implicated cadherins, a calcium-dependent family of adhesion molecules. Cadherins are expressed in the early embryonic retina and are known to act as homophilic adhesion molecules to support the growth of chick RGC axons in vitro [1,2] and on glial cells in culture [3]. Analysis of *N*-cadherin function in vivo by the introduction of dominant-negative *N*-cadherin into the RGCs of *Xenopus* embryos demonstrated that cadherins function beyond their ability to support axon growth. Retinas treated with dominant-negative cadherin constructs showed a disruption of axonogenesis, with 70% of RGCs failing to send out an axon [4]. The specific molecular mechanisms through which cadherins may be linked to axonogenesis remain to be fully defined. These findings nevertheless suggest that molecular substrates that support axon growth may in fact have a role mediating the earliest stages of axonogenesis.

4. Random outgrowth versus directed pathfinding

Once axonogenesis has begun, the task becomes one of ensuring that every RGC is successful in sending an axon into the optic nerve. Two general types of developmental mechanisms can be envisioned. One involves the elaboration of multiple axons in many different directions. The axon that ultimately finds its way into the optic nerve head is then maintained. In this scenario, no specific pathfinding mechanism are required and enough axons are elaborated from each RGC such that on average at least one axon from each

RGC finds its way into the optic nerve. Such a mechanism resembles in some respects findings in hippocampal neurons where a number of cell processes are first elaborated in multiple directions and ultimately one process extends longer to become the axon [5,6]. Support for a mechanism involving multiple axons comes from anatomical studies demonstrating that some embryonic RGCs in fact have multiple transient axonal processes within the retina in addition to a single axon that has extended towards CNS targets [7]. On the other hand, results examining RGC axon trajectories that originate from small foci of fluorescent labeling within the retina argue against a model of simultaneous random outgrowth of multiple axons. The trajectories of embryonic RGC axons all appear to be specifically directed to the optic nerve head and axons do not extend randomly from the RGCs within the labeled region (see Refs. [8,9]). Thus, while transient axons may exist for some embryonic RGCs, these may be involved in developmental processes other than ensuring axon growth into the optic nerve. The overall picture, based on anatomical analysis and results from mechanistic studies discussed below, indicates the presence of molecular mechanisms that specifically guide RGC axons within the retina.

5. Establishing directionality

A major decision faced by a growth cone as it emerges from the parent cell body is to orient in the correct direction towards the optic nerve head. One proposed mechanism is the presence of inhibitory molecules that form a ring at the periphery of the retina, to prevent inappropriate axon growth in this direction. Immunostaining studies have reported a ring of chondroitin sulfate proteoglycans in peripheral retina adjacent to the youngest RGCs [10]. Chondroitin sulfate proteoglycans are highly negatively charged extracellular matrix molecules that have been shown to inhibit axon growth in vitro [11]. In a study in which chondroitin sulfate was removed enzymatically in retinal cup preparations in culture, RGCs showed abnormal axon trajectories away from the central retina [10]. These findings suggest a model in which inhibitory chondroitin sulfate localized next to newly born RGCs act as a fence to prevent axonal growth in the direction of peripheral retina and thus help guide axons centrally.

6. Slit 1 in a supporting role

In addition to inhibitory molecules limiting the direction of RGC axon growth, recent data suggest that specific growth-promoting mechanisms involving the Slit family of axon guidance molecules also exist to lead axons towards central retina. Slits are a well-studied family of guidance molecules originally discovered to play a critical role in the development of axon commissures in the *Drosophila*

nervous system [12]. Subsequent studies have demonstrated that mammalian Slit homologs also play important roles in axon guidance, olfactory development, and neuronal migration [13–16]. Slit molecules in the context of the visual system, have generally been described as inhibitory axon guidance molecules. For example, Slit molecules inhibit retinal growth cones [17–19] and Slit 1 and Slit 2 help to properly position RGC axons within the optic chiasm [20].

It is known, however, that the response of a growth cone to a specific axon guidance molecule can be switched from repulsion to attraction depending on the context of other signals impinging on the growth cone [21–23]. The same property appears to apply to RGC axon response to Slit as RGC axons in the retina have been reported to preferentially grow on retinal cells that express Slit. It has been suggested that by using this mechanism, RGC axons hop from cell to cell towards central retina [24]. Of note, this study also demonstrated that the retinal expression of Slit 1 is under the control of the homeobox gene *Irx4*. Furthermore, the disruption of proper Slit 1 expression leads to disorganized axon trajectories in the peripheral retina.

Taken together, published studies on RGC axon response to Slit molecules show that RGC axon responsiveness to Slit molecules differ depending whether the axon is interacting with Slit molecules within the retina or at the optic chiasm. In the molecular environment of the retina, axon growth is supported by Slit while the opposite is thought to occur in the chiasm. The molecular mechanisms governing this switch in responsiveness are of substantial interest.

7. Cell intrinsic mechanism for directionality

In addition to growth cone interactions with guidance molecules, another mechanism that can impart directionality is related to the intrinsic organization of cytoskeletal structures following terminal differentiation. This cell intrinsic means of governing initial axon polarity has not received much attention in studies of RGC axon guidance. In the developing grasshopper limb bud, a specific set of neurons gives rise to pioneer axons that head centrally towards the CNS. Prior to axonogenesis, these neurons have a characteristic arrangement of actin filaments, microtubules, and intracellular organelles such as the centrosomes and the Golgi apparatus. Furthermore, the pattern of these intracellular elements reliably predicts the polarity of the pioneer neuron, the neuron's site of axonogenesis, and ultimately the direction of initial axon outgrowth [25]. The molecular mechanism that defines axonal polarity has also been examined in a recent series of experiments on hippocampal neurons, where it was demonstrated that a protein complex including the molecules Par3, Par6, and a PI 3-Kinase enzyme can control the axon selection process [26]. It is not known at present whether intrinsic properties of newly formed RGCs also dictate the direction of initial axon outgrowth. If so, such a mechanism could act in concert with

cadherin and slit proteins in defining initial RGC axon orientation and growth within the retina.

8. Fasciculation

Once the polarity of outgrowth is established, RGC axons have to travel some distance from peripheral retina to the centrally located optic nerve head. Newly formed axons growing towards the optic nerve head encounter the axons of earlier generated RGCs and fasciculate with these axons to form small bundles or fascicles. These fascicles are particularly evident in the goldfish retina where bundles of axons can be seen traveling centrally from the edge of the retina to the optic nerve head (e.g. see [27]). Distinct axon bundles are typically not observed within the embryonic mammalian retina, presumably due to the large number of axons present. However, mammalian RGC axons are known to readily fasciculate with one another to form tight axon bundles *in vitro*.

Axon fasciculation is thought to be a convenient means for axons to follow their preceding counterparts along a defined pathway. A role for selective axon fasciculation in axon pathway formation was proposed in early studies of axon pathfinding in the developing grasshopper nervous system. Based on the observed ability of growth cones to preferentially fasciculate with one molecularly distinct pathway amongst many, the labeled pathway hypothesis proposed that molecular labels allowed axons to choose fasciculation partners which in turn guided axons to the appropriate targets. The fasciculation of later generated RGC axons with earlier generated axons that have already found their way to the optic nerve head plays a central role in axon pathfinding within the retina.

While fasciculation is likely to be an important guidance mechanism particularly in pathways containing large numbers of axons, this mechanism depends fundamentally on accurate pathfinding by the very earliest axons that pioneer the pathway. The elaboration of the early axon tracts in the embryonic mouse brain including those related to formation of visual pathways has been examined [28,29]. Although recent studies have begun to uncover the presence of specific guidance molecules present very early in the region of the developing optic fissure (see later sections), how the earliest generated RGC axons find their way out of the eye is still a subject that is poorly understood.

9. Molecular basis of fasciculation

Studies of neuronal development in many different systems across species indicate that proteins of the Ig superfamily function critically in axon fasciculation. The Ig family consists of a large number of proteins each of which expresses a variable number of immunoglobulin repeats [30,31]. Many Ig family proteins exhibit homophilic bind-

ing, a feature that presumably allows axons to fasciculate with one another.

In the vertebrate retina, the two Ig superfamily members that have been most strongly implicated in axon fasciculation are L1 in rodents, and neurolin in the goldfish. In rats, antibody perturbation of L1 function results in RGC axons exhibiting a wandering phenotype instead of growing directly to the optic nerve head region [32]. However, large numbers of RGC axons still manage accurate pathfinding to the nerve head. Similarly, mice deficient in L1 have optic nerves that are roughly normal in size (D. Sretavan, unpublished observations), indicating that the vast majority of RGC axons still managed to accurately find the optic nerve after loss of L1 function. This suggests that in addition to L1, other Ig molecules or additional guidance mechanisms are also likely at work.

Experiments involving the injection of function blocking Fab fragments against neurolin into the eyes of goldfish resulted in the defasciculation of RGC axons and failure of “lost” axons to reach the optic nerve head. Many of the defasciculated axons turn around and travel towards the periphery of the retina, and some grow in circles [27,33]. Of note, since goldfish RGCs are continually added even in adult animals, neurolin function blocking was performed in adult goldfish. This result demonstrates the importance of Ig molecules in mediating RGC axon fasciculation beyond the early developmental period and into adulthood.

10. Other mechanisms of Ig protein and L1 function

It would be erroneous to have the impression that Ig molecules such as L1 function merely as a glue to mediate axon fasciculation. These molecules in fact have been demonstrated to have a wide range of functions, some of which have only recently been appreciated. For example, in addition to homophilic binding, heterophilic binding between L1 and integrin receptors have been reported [34,35]. Given that both L1 and integrin receptors are present on RGC axons [3,36–38], it is possible that L1–integrin interactions may function in RGC axon guidance *in vivo*.

Studies have also shown that L1 can modulate growth cone responses to inhibitory axon guidance molecules. In the corticospinal tract, L1 activation appears to switch growth cone responses to Sema3A from repulsion to attraction [21,39]. This finding suggests that L1 activation leads to signaling cascades within the growth cone that feed into and affect signaling downstream of other guidance receptors. Since L1 is expressed on RGC axons as they navigate within the optic nerve [40], optic chiasm [41], and in visual targets such as the superior colliculus [42], many RGC axon guidance events likely occur in the context of L1 signaling. Future investigations of growth cone signaling cascades activated in L1 signaling will likely contribute to a deeper understanding of the control of RGC axon pathfinding throughout the visual pathway.

11. Targeting the optic nerve head

Although the studies above demonstrate that RGC axon fasciculation mediated by Ig molecules is involved in RGC axon pathfinding to the optic nerve head, molecules that promote fasciculation by themselves are not enough to ensure that axons accurately target this exit point. Evidence indicates that inhibitory interactions mediated by the EphB and the ephrin-B families of transmembrane receptors and ligands are necessary for enhancing the accuracy of RGC axon targeting of the optic nerve head.

Eph and ephrin molecules are involved in a wide range of developmental events such as cell migration, blood vessel formation, and axon pathfinding [43–45]. Two subfamilies exist. Ephrin-A proteins are GPI-linked molecules that bind EphA receptor proteins, while ephrin-Bs are transmembrane molecules that bind to EphB proteins. EphB and ephrin-B molecules are notable in that interactions between members of these families can activate bi-directional signaling [46,47]. In conventional forward signaling, ephrin-B molecules bind and activate EphB molecules that act as receptor tyrosine kinases. In reverse signaling, EphB binding to transmembrane ephrin-B proteins result in the activation of ephrin-B as receptors, leading to phosphorylation and signaling cascades that involve PDZ-RGS [48] and NckB [49]. It is this reverse signaling mode that appears to mediate aspects of RGC axon pathfinding in the retina.

12. Graded expression of Eph and ephrins

The embryonic retina contains a number Eph and ephrin molecules that demonstrate two general characteristics in their expression pattern [9,50–53]. First, Eph and ephrins typically are not expressed in a uniform manner throughout the retina but usually exhibit graded patterns of expression. Second, each subfamily of Eph molecules is preferentially distributed in a particular retinal quadrant, while its corresponding ephrin is present in the highest levels in the opposing quadrant. For example, EphB receptor proteins are generally found in a high ventral to low dorsal gradient, while ephrin-B proteins are present in an opposite high dorsal to low ventral pattern. EphA and ephrin-A molecules demonstrate similar opposing gradients of expression along the nasal-temporal axis.

13. Loss of targeting accuracy in EphB mutants

The dorsal-ventral gradient of Eph/ephrin expression helps axons belonging to RGCs in dorsal retina to find their way accurately into the optic nerve head region. Mutant mouse embryos lacking EphB proteins exhibit RGC axon guidance errors in which axons defasciculate from their neighbors within about 100 μm of the optic nerve head

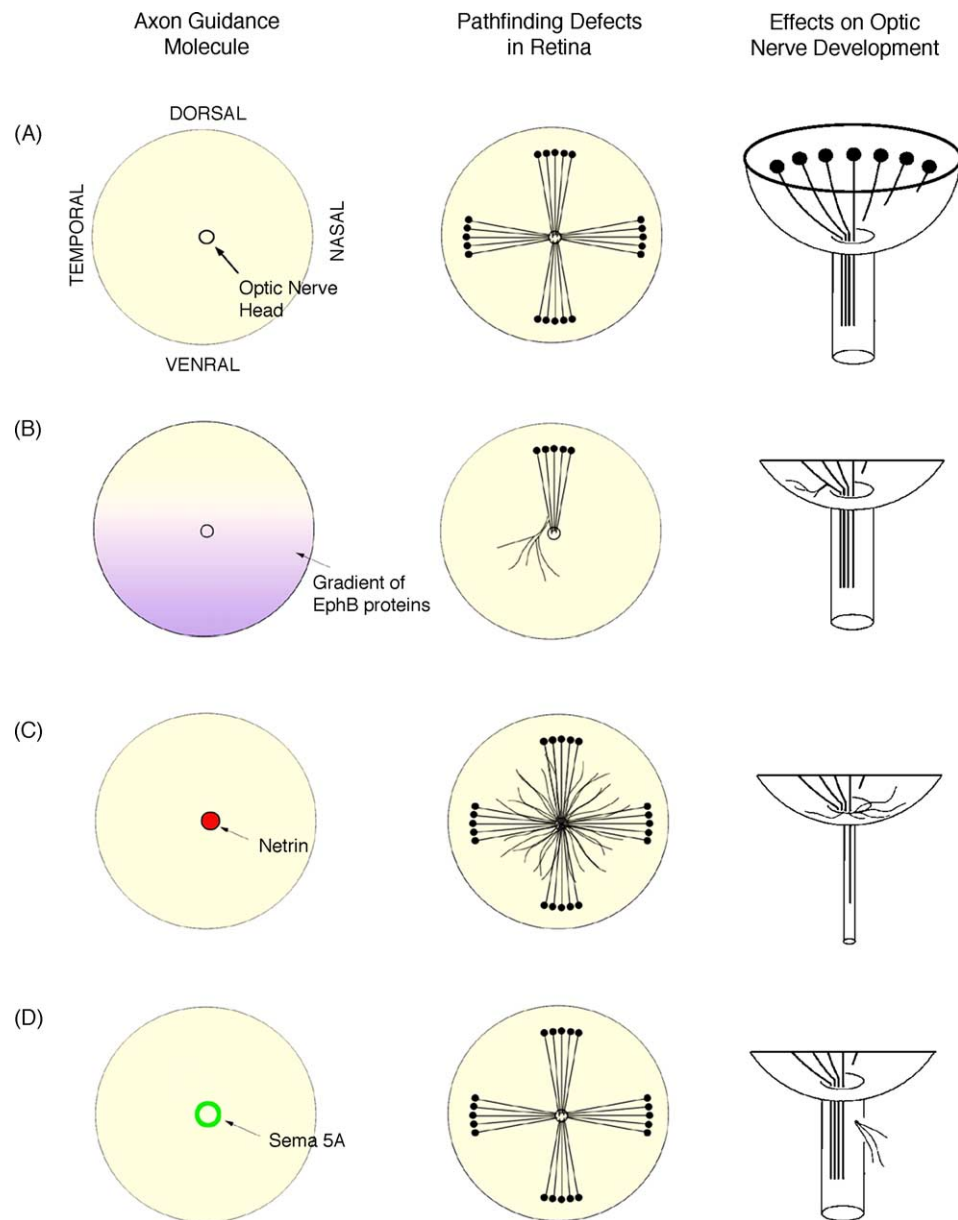


Fig. 1. Schematic diagram illustrating the pattern of expression of individual guidance molecules within the developing mouse retina and the resulting RGC axon guidance defects after loss of function perturbations. In each row, the diagram on the left shows the pattern of expression of an individual axon guidance molecule. The center diagram shows the axon guidance defects observed in the retina after functional perturbation. The diagram on the right shows the resulting size of the optic nerve and RGC axon guidance defects. (A) Wild type, (B) EphB, (C) Netrin-1, (D) Sema5A.

[9]. Defasciculated axons bypass the optic nerve head and instead grow along either side of this exit point, and extend inappropriately into the opposite side of the retina (Fig. 1). Analysis of mice lacking individual or combinations of EphB2 and EphB3 proteins revealed that the lack of a single EphB gene did not result in this mis-targeting phenotype. Instead, optic nerve head targeting were present only in animals lacking both EphB2 and EphB3 function. This requirement for the simultaneous loss of function of more than one set of EphB molecules likely reflects the functional redundancy of this family of guidance molecules within the retina.

A notable finding in EphB mutant animals is that only RGC axons originating from the dorsal quadrant of the retina show pathfinding defects. Since the overall levels of EphB proteins are in a high ventral to low dorsal gradient, the specific localization of pathfinding errors to only dorsal RGC axons suggests a non-cell autonomous function for EphB molecules in axon targeting of the optic nerve head. Furthermore, dorsal RGC axon pathfinding errors are rescued in mutant mice expressing only a truncated EphB2 protein without the intracellular kinase catalytic region, indicating that the extracellular domain of EphB2 plays a direct role in axon pathfinding. In vitro retinal growth cone assays show in

fact that the extracellular portion of EphB proteins acts as an inhibitory guidance molecule and triggers growth cone collapse [54]. The results in mutant animals and in cell culture are consistent with a model in which dorsal axons approaching the optic nerve head grow into an increasing gradient of inhibition and in doing so, maintain tight fasciculation to ensure precise targeting of the exit point. In addition, stray axons are normally prevented from growing into the opposite side of the retina because of the presence of high levels of EphB expression.

The mechanisms underlying accurate targeting of axons from the nasal and temporal quadrants to the optic nerve head are unknown at this time. Candidates include the EphA and ephrin-A molecules as these molecules show opposing gradients of expression along the nasal-temporal axis [50].

14. Mechanisms other than EphB

The axon guidance defects present in the EphB2 and EphB3 mutant mice are relatively mild in the sense that the vast majority of RGC axons are still capable of finding their way to the nerve head and exit. To determine whether the concomitant loss of additional EphB molecules in the retina result in a more severe phenotype in axons from dorsal retina, we have examined RGC axon pathfinding in mutant mice simultaneously lacking EphB1, EphB2, and EphB3 molecules. Results show that the additional loss of EphB1 molecules did not result in a more severe phenotype. While RGC axon pathfinding errors were observed in these mutants (Fig. 2), the vast majority of RGC axons remained

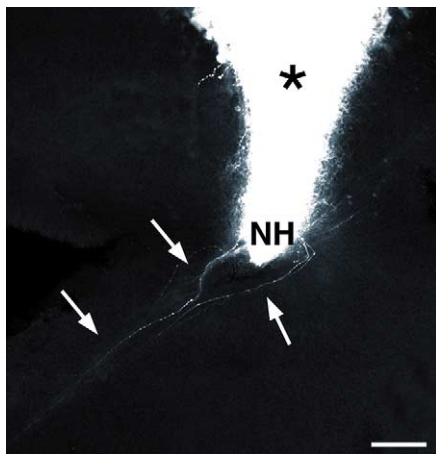


Fig. 2. Image showing axon guidance errors in the retina of an E14 mouse embryo lacking all three EphB1, EphB2, and EphB3 proteins. RGC axons in the dorsal retina were labeled using a small crystal of DiI placed in dorsal peripheral retina. The majority of labeled axons (asterisk) find their way accurately to the region of the optic nerve head (NH). A few RGC axons (arrows) have grown around either side of the nerve head and grow inappropriately within the ventral region of the retina. The severity of RGC axon guidance defects in animals lacking EphB1, EphB2, and EphB3 were similar to animals lacking just EphB2, and EphB3. Scale bar: 100 μ m.

unperturbed and correctly exited into the optic nerve. This observation suggests that in addition to EphB1, EphB2, and EphB3, other pathfinding molecules are likely involved in ensuring the accuracy of axon guidance from dorsal retina to the optic nerve head. These may include other members of the EphB family or other families of axon guidance molecules.

15. Pairing fasciculation with inhibition

The studies implicating both Ig molecules as well as members of the Eph/ephrin family in accurate axon targeting of the optic nerve head highlight the pairing of a RGC axon growth-promoting mechanism together with an inhibitory one as a pathfinding strategy in the developing retina. Fasciculation is a simple means of ensuring that a population of axons grows along the same route to a target. As a pathfinding mechanism, fasciculation works best when there are few axons, but may break down in the presence of many thousands of axons. For example, as RGC axons near the optic nerve head, the region is densely packed with L1 expressing RGC axons. Fasciculation on the basis of L1 mediated binding alone could lead axons away from the optic nerve head. To counter this possibility, the retina appears to deploy an inhibitory mechanism via Eph and ephrin molecules to minimize axon straying and maintain accurate targeting of the exit point.

The pairing of growth-promoting and growth-inhibitory axon guidance mechanisms appears to also be used in the peripheral regions of the retina. There, an inhibitory mechanism based on chondroitin sulfate proteoglycan and a growth-promoting mechanism based on RGC axon growth on Slit expressing cells assist each other in directing newly generated axons towards the central region of the retina. The strategy of pairing growth inhibition with growth promotion may in fact be generalized to other regions of the nervous system. It would be of interest to determine whether specific pairings of guidance molecules are maintained in neural development, and whether specific pairings are deployed to mediate particular pathfinding tasks.

16. Segmental defects in human retinal development

The dorsal specificity of RGC axon guidance defects in EphB2 and EphB3 mutants is intriguing given the occurrence of superior optic nerve hypoplasia (SSONH) in humans [55,56]. Patients with SSONH have a specific defect in the superior (dorsal) quadrant of the retina characterized by a decrease in the thickness of the optic fiber layer [57] and loss of inferior visual fields. Since central vision is well preserved in these patients, visual acuity is good and the inferior field loss is often identified only incidentally at eye exams. Subsequent clinical investigation then leads to a diagnosis of SSONH. Since the optic fiber layer

represents the axons of RGCs, a reduced fiber layer thickness in dorsal retina could result from a number of causes including selective RGC death in dorsal retina, abnormal axonogenesis, or axon pathfinding defects. Studies have not been performed to determine whether the expression of the EphB or ephrin-B families of guidance molecules may be affected in this patient population. Nevertheless, SSONH illustrates the quadrantic nature of eye development and underscores the fact that developmental abnormalities can lead to specific segmental defects in the adult eye.

17. Maternal diabetes, insulin receptors, and eye development

While the pathogenesis of SSONH remains unclear, a relationship between SSONH and maternal diabetes is well established [55,56]. A large percentage of patients with SSONH are born to mothers with Type 1 diabetes and a recent study reported that approximately 9% of offsprings from Type 1 diabetic mothers have SSONH [58]. It is unclear how maternal diabetes contributes to developmental defects in the retina. Maternal diabetes is well known to result in a higher incidence of congenital malformations in human patients [59]. Such developmental abnormalities are thought to result either from altered glucose levels during fetal development or involve secondary derangements in other metabolic pathways. If so, SSONH indicates that basic metabolic pathways may in fact have subtle levels of control over specific developmental events including axon guidance that when perturbed, results in spatially restricted developmental abnormalities.

18. Insulin receptors and pathfinding

This discussion of maternal diabetes at first glance would appear to detour from our central theme of RGC axon guidance mechanisms. However, a recent study in *Drosophila* revealed a link between the development of retinal connectivity and insulin receptors [60]. In mutant fly embryos lacking a functional insulin receptor, photoreceptor cell axon pathfinding in visual targets such as the lamina and medulla is disorganized. It is as yet unknown whether insulin or an insulin growth factor is involved in photoreceptor cell axon pathfinding. In mice, insulin receptors have been reported to interact with IGF2 to promote embryonic growth [61]. Thus, insulin receptors may interact with ligands other than insulin itself to carry out axon guidance functions. Further studies will clarify the potential role of insulin itself in development of axon pathways in the fly visual system.

Do insulin receptors in mouse play an analogous role in RGC axon pathfinding? The developing nervous system including the retina is known to express a number of insulin and insulin growth factor-like (IGF) receptors ([62], reviewed in [63]). These receptors have been proposed to

mediate a spectrum of CNS developmental events ranging from metabolism, neurotransmitter uptake, synaptogenesis, and neurite outgrowth. It is not known at present whether insulin receptors mediate any aspect of RGC axon pathfinding within the retina or elsewhere in the mammalian visual system. The availability of multiple mouse lines with constitutive or conditional knockout of different insulin receptors [64] could contribute to analysis of this possibility.

19. Gate-keeping at the optic nerve head

Following the accurate targeting of RGC axons to the optic nerve head, one could imagine that RGC axons would then grow into the optic nerve in a straightforward fashion. However, this is not the case and a netrin-dependent mechanism of axon guidance is critical for the final exit from the retina into the nerve. Netrin-1 is an axon guidance molecule that was first described in spinal cord as an attractant of commissural axons from dorsal spinal cord to the ventral midline floor plate [65,66]. Axon response to netrin-1 is mediated by DCC, an Ig molecule member that serves as a netrin-1 receptor [67,68]. Both netrin-1 [69] and DCC deficient mice [70] show altered commissural axons trajectories towards the midline floor plate.

The requirement of netrin protein to guide RGC axons at the optic nerve head into the optic nerve is based on several lines of evidence [71]. Netrin protein is present in the embryonic eye at the time of optic fissure closure. It is expressed on the processes of neuroepithelial cells that surround and extend into the optic nerve head. RGC axons growing through the nerve head into the optic nerve express the netrin receptor molecule DCC and course in between the netrin positive neuroepithelial cell processes within the optic nerve head (Fig. 3A). The close relationship of RGC axons with cell processes carrying the netrin protein suggest that netrin served as a RGC axon guidance molecule, a possibility confirmed by in vitro studies demonstrating that recombinant netrin-1 protein is capable of promoting RGC axon outgrowth and does so by binding to DCC receptors on RGC axons. In vivo, genetic deletion of netrin-1 function in mice results in the failure of RGC axons to exit properly from the eye into the optic nerve and instead remain to grow abnormally into other regions of the retina. As a result, the optic nerve of netrin-1 mutant animals is reduced in size, exhibiting a condition known as optic nerve hypoplasia (ONH). Animals lacking DCC function have similar axon guidance defects at the optic nerve head as well as ONH, confirming that interactions between the ligand-receptor pair netrin-1 and DCC is critical for optic nerve formation.

Unlike the axon guidance defects observed in EphB2 and EphB3 mutant animals which affect only dorsal RGC axons and do not result in an apparent reduction in optic nerve size, the lack of netrin-1 mediated axon guidance affects axons from all retinal regions and leads to obvious ONH. ONH is

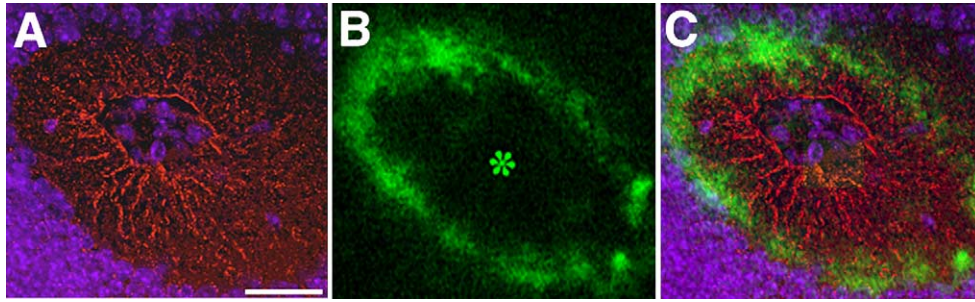


Fig. 3. Distribution of Netrin and Sema5A protein at the embryonic mouse optic nerve head. (A) Netrin protein decorates the processes of neuroepithelial cells at the optic nerve head. These processes form a reticular pattern through which RGC axons grow as they exit the retina into the optic nerve (from [71]). (B) Sema5A protein is localized to the perimeter of the embryonic optic nerve head (from [79]). The images shown in panels (A) and (B) are from different embryos. (C) The images shown in panels (A) and (B) are superimposed for illustration purposes to compare the location of Sema5A protein with respect to Netrin protein. Scale bar: 25 μm .

also found as a congenital malformation in human patients and has been reported as a major cause of childhood visual impairment (data from Blind Babies Foundation, 1995). In humans, the underlying etiology for ONH is not known and suggested mechanisms include a failure of RGC neurogenesis or abnormal development of CNS visual targets leading to retrograde loss of RGC and their axons. Given the finding of ONH in netrin-1 mutant mice, a third possibility is axon guidance defects preventing the proper exit of RGC axons from the retina into the optic nerve.

20. Optic nerve hypoplasia versus aplasia

Although the lack of netrin-1 function clearly results in optic nerve hypoplasia, it does not lead to optic nerve aplasia, which is the total absence of axons in the optic nerve. The preservation of some RGC axon growth from the retina into the optic nerve suggests that additional axon guidance mechanisms may be at work and can act on a subset of RGC axons. One hypothesis is that early generated RGC axons that lie immediately adjacent to the optic nerve head region may not require netrin-1 for growth out of the eye through the nerve head into the optic nerve. A growth-promoting guidance molecule such as netrin-1, however, might be necessary for later arriving axons to enter behind their counterparts. Experiments in which the residual optic nerve axons in netrin-1 mutant embryos were retrograde labeled revealed that this was not the case. The retrograde filled RGCs that represent RGCs whose axons were able to leave the retina, were located throughout the retina including the peripheral regions (Fig. 4). This indicated that some late arriving axons at the optic nerve head were in fact able to find their way into the optic nerve despite the fact that many RGC axons ahead of them had abnormal trajectories. It is unclear whether this reflects a stochastic process or the existence of specific guidance mechanisms mediating the pathfinding behavior of a defined subset of RGC axons.

21. Septo-optic dysplasia (De Morsier's syndrome)

The absence of netrin-1 activity during neuronal development results not only in optic nerve hypoplasia, but also affects formation of other regions of the CNS. Notable

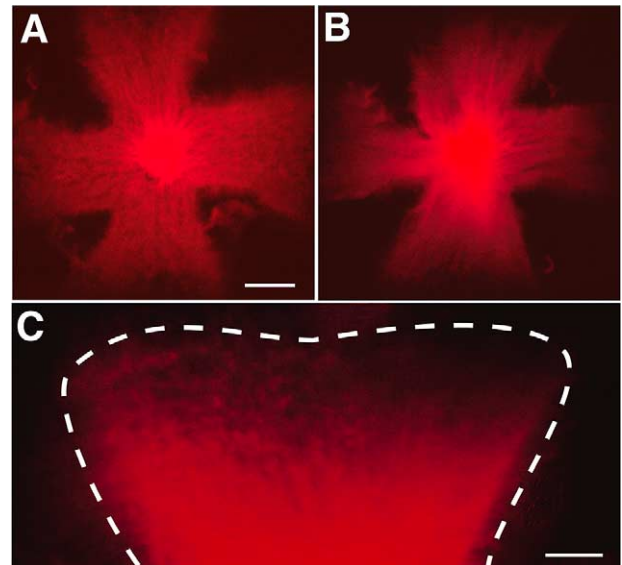


Fig. 4. Retinal wholemounts in an E14 Netrin-1 mutant mouse embryo and its wild-type littermate. DiI was deposited into the optic nerves to retrogradely label RGCs that have successfully exited the retina. (A) Retinal wholemount from a wild-type embryo showing the presence of retrogradely labeled axons and RGCs in both central and peripheral regions of all four quadrants of the retina. (B) Retinal wholemount from a littermate Netrin-1 mutant embryo showing that, as in wild-type littermates, retrogradely labeled axons and RGCs are also present in both central and peripheral regions of all four quadrants of the retina. (C) A higher magnification view of retrogradely labeled axons and RGCs in the peripheral retina of a Netrin mutant embryo. The dotted white line depicts the outline of the retinal tissue. This data demonstrate that in the absence of Netrin-1, a subset of RGCs do manage to send their axons out of the eye through an as yet unidentified mechanism. Furthermore, this subset of RGCs is dispersed throughout the retina, indicating that they likely do not only represent the earliest generated RGCs in the retina. Scale bar: 100 μm (A); 25 μm (C).

amongst these include abnormal corpus callosum development [72] and defects in the cellular organization of cell groups within the developing hypothalamus [73]. The concurrent findings of optic nerve hypoplasia, cortical midline abnormalities, and hypothalamic defects in the netrin-1 and DCC mutant mice resemble the similar constellation of findings in human patients affected by Septo-optic dysplasia (SOD), also known as De Morsier's syndrome [74,75].

SOD is relatively rare and is considered to be sporadic in occurrence, making this entity difficult to investigate genetically. There are, however, a few reports of sibling pairs in the literature. In a study involving one sibling pair, the homeobox transcription factor HESX1 was found to contain a missense mutation within the homeobox region that results in a loss of DNA binding. Mutant mice lacking HESX1 function exhibit developmental abnormalities in the corpus callosum, the eye, and pituitary dysplasia, suggesting that the HESX1 dysfunction in human patients is one cause of SOD. The targets genes of HESX1 transcriptional activity are not known but potentially could include genes encoding proteins that affect axon guidance and cell migration in the affected regions of the brain such as netrin-1 and its receptor DCC.

We are in the process of testing the hypothesis that alterations in the genes encoding netrin-1 or DCC might underlie SOD in humans. In parallel, we wish to also better understand the frequency of HESX1 mutations in SOD patients. DNA samples from 50 patients with either optic nerve hypoplasia alone or SOD have been analyzed for mutations in HESX1. Two cases revealed a heterozygous missense mutation within the homeodomain of HESX1, consistent with previous reports of heterozygous point mutations in the HESX1 homeodomain in patients with a range of pituitary disorders including SOD [76,77]. These previous studies demonstrated that some of the mutations affect HESX1 DNA binding, and mutant HESX1 protein can act in a dominant-negative fashion to affect DNA binding by wild-type HESX1 protein. Analysis of netrin-1 and DCC genes is only partially complete. To date, no gross genomic rearrangements or specific mutations in netrin-1 or DCC have been identified. The data available in the literature, together with our preliminary work, indicate that a substantial percentage of SOD patients have defects in genes other than HESX1. The functions of these unknown genes must ultimately impinge on RGC axon guidance and optic nerve development.

22. Switching off netrin-dependent guidance

Having successfully navigated within the retina to reach the optic nerve head, the final task for RGC axons is to pass through the optic nerve head and into the optic nerve. This behavior raises an important question in the field of axon guidance. How do axons that have grown into an area con-

taining an attractive guidance molecule such as netrin, ultimately free themselves to continue onwards to other parts of the pathway? One mechanism that has been proposed from studies in *Xenopus* is based on the observation that both laminin and netrin are found at the developing optic nerve head. As in the mammalian retina, netrin-1 in *Xenopus* is likely an attractive guidance molecule involved in axon exit through the optic nerve head. In vitro, *Xenopus* RGC axons exposed to laminin switch their responsiveness to netrin from attraction to inhibition [78]. This response switching was proposed to enable RGC axons to leave the netrin rich environment of the optic nerve head.

Several lines of evidence suggest that this mechanism of “driving” axons beyond the optic nerve head out of the retina may not be entirely conserved in the mammalian retina. First, in vitro experiments demonstrate that embryonic mouse RGC axons extending on laminin in fact respond to netrin as a growth-promoting molecule [79]. Secondly, in mice, netrin-1 protein is present along the entire length of the developing optic nerve and into the ventral diencephalons but is absent from the midline [71]. As a result, a mechanism involving the switching of axon responsiveness to netrin from attraction to repulsion would likely function in the ventral diencephalon and not necessarily at the optic nerve head. Lastly, given the apparent co-localization of laminin and netrin at the optic nerve head, it is unclear how RGC axons would be able to enter the optic nerve head in the first place.

23. Staying within the optic nerve

While the optic nerve is the main thoroughfare for RGC axon growth and is presumably dominated by a growth-promoting environment, there is evidence that inhibitory guidance forces are necessary to properly channel RGC axons within the optic nerve. Recent data show that neuroepithelial cells at the optic nerve head and in the optic nerve express Sema5A, a transmembrane member of the semaphorin family of guidance molecules [80,81]. During the period of optic nerve development, Sema5A protein is present as a ring surrounding the RGC axons exiting the retina and coursing into the optic nerve (Fig. 2B). This cuff of Sema5A contrasts with the pattern of netrin protein expression in the same region, in which netrin is found on the radially aligned processes of the neuroepithelial cells that are intermingled with the exiting RGC axons (Fig. 2A and C). In vitro, recombinant Sema5A inhibits RGC axon growth and causes growth cone collapse. Furthermore, antibody perturbation of Sema5A function in living optic nerve preparations results in RGC axons escaping from the main body of the optic nerve (Fig. 1). Together, these data support a model in which Sema5A forms an inhibitory sheath surrounding the developing optic nerve and serves to prevent RGC axons straying from the normally tightly fasciculated optic nerve bundle.

24. Other players in the optic nerve

The axon guidance defects that arise after anti-Sema5A function blockade are relatively mild and do not affect large numbers of axons. A number of technical factors, such as antibody penetration or duration of exposure, could contribute to this. However, it is important to note that other guidance molecules are also known to be present within the optic nerve, and these may act in concert with Sema5A to maintain optic nerve integrity. For instance, axons traveling in the optic nerve continue to express L1, which promotes axon fasciculation and keeps the axons bundled together. In addition, netrin-1 expression extends from the optic nerve head along the length of the optic nerve. This netrin expression also likely helps to keep axons within the central region of the nerve. Finally, inhibitory slit proteins are also expressed along the length of the optic nerve [17,19]. The role of inhibitory slit “guardrails” in optic chiasm positioning has been proposed [20]. It is possible that Slit expression along the optic nerve length serves a similar ensheathing function as Sema5A, and prevents RGC axon defasciculation from the nerve.

25. Guidance and other retinal cells

It probably has not escaped the reader that this review has focused entirely on axon guidance mechanisms mediating the pathfinding of RGC axons without mentioning the dendrites or the axons of other cell types. While RGCs are the only projection neurons in the retina, and proper RGC axon pathfinding is thus critical for conveying visual information to brain targets, the development of RGC dendritic fields and the axonal and dendritic connections of other retinal interneurons are also essential for signal processing in the retina. Recent work in this area has led to the identification of the transmembrane immunoglobulin molecules *sdk-1* and *sdk-2* that help specify retinal connectivity through homophilic interactions [82]. The retina, given its laminar organization and the wealth of information concerning its histological and synaptic connections, offers a good system to study guidance mechanisms underlying the development of intrinsic circuitry.

26. Summary

In this review, we have described some of the progress that has been made in our understanding of the molecular basis for RGC axon pathfinding in the developing eye leading to the formation of the optic nerve. In mouse development, these mechanisms operate over a substantial period of time covering roughly the last third of gestation and ensure the accurate targeting of over 50,000 RGC axons to the optic nerve head and into the optic nerve. Although a number of axon guidance molecules essential for this process have now

been identified, there is much that is still unknown. The data at hand provisionally support a pathfinding strategy in which distinct guidance mechanisms operate in concentric zones surrounding the optic nerve head, each of which appears to involve the exposure of RGC axons to growth-promoting as well as growth-inhibitory guidance molecules. By deploying specific pairings of guidance molecules at strategic locations, the visual system ensures that large numbers of RGC axons all project correctly to a specific point. The sequence of axon pathfinding mechanisms used by the retina may potentially be of interest in guiding regenerating axons in the adult nervous system following injury and disease.

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