

New views on retinal axon development: a navigation guide

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ABSTRACT The eye is a peripheral outpost of the central nervous system (CNS) where the retinal ganglion cells (RGCs) reside. RGC axons navigate to their targets in a remarkably stereotyped and error-free manner and it is this process of directed growth that underlies the complex organization of the adult brain. The RGCs are the only retinal neurons to project into the brain and their peripheral location makes them an unusually accessible population of projection neurons for experiments involving *in vivo* gene transfer, anatomical tracing, transplantation and *in vitro* culture. In this paper, we review recent findings that have contributed to our understanding of some of the guidance decisions that axons make in the developing visual system. We look at two choice points in the pathway, the optic nerve head (onh) and the midline chiasm, and discuss evidence that supports the idea that key molecules in guiding axon growth at these junctures are netrin-1 (onh) and ephrin-B (chiasm). In the optic tectum where RGC axon terminals are arrayed in topographic order, we present experimental evidence to suggest that in the dorso-ventral dimension, the B-type ephrins and Eph receptors are of prime importance, possibly through attractive interactions. This complements the anterior-posterior topographic mapping known to be mediated through A-type ephrin/Eph repulsive interactions. An emerging theme is that guidance molecules such as ephrin-B and netrin-1 have complex patterns of restricted expression in the pathway and play multiple and changing roles in axon guidance.

KEY WORDS: retina, axon guidance, netrin, Eph receptor, ephrin

Introduction

Vision depends on the elaboration of appropriate functional connections between the retina and visual processing centres in the brain. During development, newly generated retinal ganglion cells (RGC) send axons that exit the eye through a circular region in the centre of the retina, the optic disc, and bundle together to form the optic nerves. RGC axons cross the ventral midline of the hypothalamus, at the optic chiasm, and extend dorsally in the optic tracts. In some vertebrate species, a proportion of RGC axons do not cross the midline and remain on the ipsilateral side of the brain. As a result, the optic tracts carry visual information from both eyes, a feature essential for binocular vision and depth perception.

Once they reach their principal target structure, the tectum (superior colliculi in Mammals), RGC axons stop and form synaptic connections with their target cells. Visual projections are organized such that two adjacent RGCs in the retina are connected to two adjacent points in the target field. This topographic arrangement allows the formation of a continuous image of the visual field onto the surface of the target structure.

Which are the molecules and mechanisms that guide migrating RGC axons during the establishment of this stereotyped pattern

of projection? The selection of the appropriate pathway by RGC growth cones occurs stepwise in response to a succession of different guidance cues expressed in their local environment. The past decades have seen significant advances in the identification of these signals, and the present review focuses on two families of molecules, the netrin and ephrin/Eph families, for which important functions have been attributed. We present here evidence from *in vivo* experiments that netrin and ephrin/Eph molecules control RGC axon navigation at various choice points along the visual pathway, and discuss recent advances in the understanding of the signal transduction mechanisms underlying retinal growth cone guidance. We confine our discussion mainly to work done in the *Xenopus* visual system but it should be noted that much important experimental work has been done in other experimental systems such as zebrafish, mouse and chick (Fricke *et al.*, 2001, Hutson and Chien, 2002, McLaughlin *et al.*, 2003a, McLaughlin *et al.*, 2003b).

Abbreviations used in this paper: CNS, central nervous system; onh, optic nerve head; RGC, retinal ganglion cell.

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Netrin-1 guides visual axons out of the eye

One of the first pathfinding tasks of visual axons is to exit the eye through the optic disk, at the retina-optic nerve junction (Fig. 1). RGC axons initially extend along the vitreal surface of the neural retina and follow a centripetal route towards the centre of the retina. Once they reach the optic disk, growth cones make a 90° turn to dive deep in the retina and exit the eye through the optic nerve head. Within the retina, RGC axons grow in close contact with Müller glial cell endfeet and with the vitreal basal lamina, both of which have been shown to express cell adhesion and extracellular matrix molecules, such as NCAM, L1 and chondroitin-sulfate proteoglycans that might stimulate axon growth and provide guidance signal to RGC axons (for review see Stuermer and Bastmeyer, 2000). It remains unclear, however, whether additional signals such as chemoattractants contribute to direct the navigation of RGC axons towards the optic disk. The search for candidate molecules involved in intraretinal pathfinding led to a focus on netrin-1, a laminin-related secreted protein, produced by glial cells at the optic disk and optic nerve head in fish (Lauderdale *et al.*, 1997, Strahle *et al.*, 1997), frogs (de la Torre *et al.*, 1997) and rodents (Deiner *et al.*, 1997), see Fig. 1. Netrin-1 was first reported to function as a long-range chemoattractive cue for commissural axons in the spinal cord (Kennedy *et al.*, 1994, Serafini *et al.*, 1994, Shirasaki *et al.*, 1996). In the retina, however, it is unlikely that netrin-1 acts at long-range to attract RGC axons, since no alteration of the optic disk-directed growth of RGC axons was observed in netrin-1 deficient mice (Deiner *et al.*, 1997). In this mutant, however, although RGC axons can reach the optic disk, they fail to enter the optic nerve head and instead grow aberrantly in other regions of the retina. This failure of many RGC axons to

exit the eye results in a smaller optic nerve and hypo-pigmented streaks at the surface of the retinal pigmented epithelium, probably formed by ectopic axons growing between pigmented cells (Deiner *et al.*, 1997). Thus netrin-1 appears to act at a short-range to attract RGC growth cones into the optic nerve head. This effect is mediated by signaling involving the netrin-1 receptor, 'deleted in colorectal cancer' (DCC) expressed on RGC axons, since netrin-1 and DCC knock out mice exhibit similar retinal abnormalities.

Consistent with the proposed role of netrin-1 as a short-range chemoattractant, recombinant netrin-1 causes attractive turning of *Xenopus* retinal growth cone *in vitro*, an effect that can be blocked with antibodies to DCC (de la Torre *et al.*, 1997). In addition, this response can be modulated by the level of cytosolic cyclic nucleotides (Ming *et al.*, 1997, Song and Poo, 1999). For instance, it was shown that, in the presence of a cAMP analogue that activates protein kinase A (PKA), netrin-1-mediated attraction of retinal growth cones is converted into a repulsive activity (de la Torre *et al.*, 1997). Thus, the behaviour of RGC growth cones in response to netrin-1 might be dependent on other concomitant signals that modulate the level of intracellular cAMP. This is the case, for example, for the extracellular matrix protein laminin-1 which is able to abrogate the netrin-1-induced cAMP rise in cultured RGC axons. As a result, laminin-1 changes netrin-1 attraction into repulsion through a mechanism that involves $\beta 1$ integrin receptor expressed on RGC axons (Höpker *et al.*, 1999).

In the developing retina, laminin-1 is abundant in the vitreal basal lamina along which RGC axons navigate. Thus laminin-1 and netrin-1 are co-expressed at the vitreal surface of the optic disk, whereas only netrin-1 but not laminin-1 is found deeper in the optic nerve head (Fig. 1B). The addition of a mimetic peptide of laminin to a *Xenopus* retinal cup grown *in vitro* induces the failure of some RGC axon fascicles to leave the eye through the optic disk (Höpker *et al.*, 1999). These results are consistent with the idea that the restricted co-expression of laminin-1 and netrin-1 at the entrance to the optic nerve head results in a repulsive signal that serves to 'push' the growth cone away from the retinal surface and grow deep into the attractive netrin-1-rich/laminin-1-poor optic nerve head (Fig. 1B).

Developmental changes in netrin-1 responsiveness in RGC axons

Netrin-1 is expressed in the optic disk/nerve head but also further along the visual pathway (see summary diagram in Fig. 4) where it governs RGC axon guidance, as shown by the abnormal course of visual axons within the ventral hypothalamus of netrin-1- and DCC-deficient mice (Deiner and Sretavan, 1999). Analysis of netrin-1 expression in the brain of *Xenopus* embryos revealed that netrin-1 expression domains correspond to regions devoid of visual afferents that flank the trajectory of RGC axons (Shewan *et al.*, 2002). This suggests that, although netrin-1 can attract RGC axons in the optic disk, it might function later on as a repellent and tell visual axons where not to go. Consistent with this idea, the *in vitro* response of RGC axons to a gradient of netrin-1 critically depends on the developmental stages at which axons are assayed. This was shown in a "whole pathway" explant preparation, where RGC axons are challenged with netrin-1 at various points in their journey along the visual pathway (Shewan *et al.*, 2002). Unlike young axons that have not yet exited the eye and show attraction

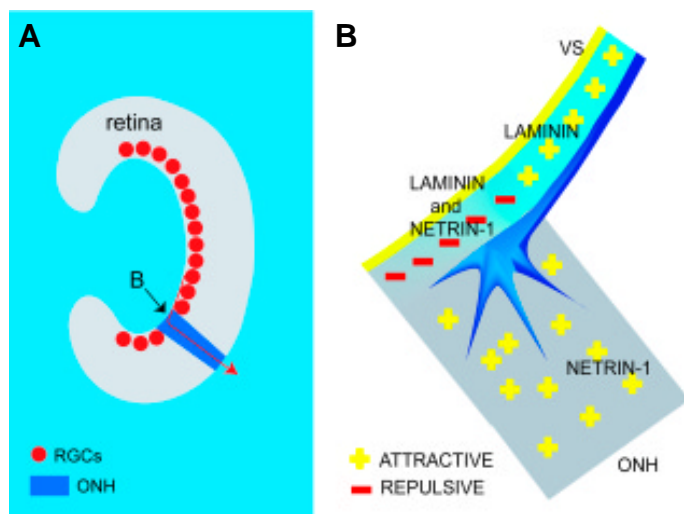


Fig. 1. Netrin-1 helps to guide the growth of axons out of the eye. (A) The arrangement of retinal ganglion cells (RGCs; red) along the vitreal surface of the retina (grey) where laminin is located. Axons grow across the vitreal surface and make a sharp bend at the entrance to the optic nerve head (ONH, dark blue; arrow at B) where netrin-1 is localized. **(B)** A growth cone entering the ONH where it encounters laminin and netrin-1 on the vitreal surface and netrin-1 only in the ONH. It is hypothesised that the laminin and netrin-1 signals combine to repel axons away from the surface and force axons into the netrin-1-rich/laminin-1-poor optic nerve head, and so out of the eye.

towards a source of netrin-1, RGC axons that have grown through the optic nerve head are insensitive to netrin-1. Moreover, after crossing the optic chiasm, RGC axons extending in the dorsal optic tract are strongly repelled by netrin-1. This later result supports the idea that netrin-1 functions as a repellent in the distal part of the visual pathway and helps to constrain the growth of RGC axons into the appropriate trajectory.

This developmental change in netrin-1 responsiveness can be in part explained by intrinsic changes in RGC growth cones, since RGC axons aged *in vitro* without having experienced the *in vivo* pathway also exhibit a repulsive response to netrin-1 (Shewan *et al.*, 2002). However, in cultures of retinal explants harvested at different developmental stages, growth cones either show an attractive or a repulsive behaviour but never exhibit a neutral response to netrin-1, as some axons do in the “whole pathway” preparation. Thus extrinsic factors present in the visual pathway might modulate the progressive maturational change that occurs in RGC growth cones response to netrin-1.

The age-related intrinsic change in netrin-1 responsiveness could, in theory, be mediated by a temporal change in netrin-1 receptor expression. In other systems, netrin-1-induced attraction requires DCC whereas repulsion is initiated by a receptor complex involving DCC and UNC-5 (Hong *et al.*, 1999). However, *in situ* hybridization in *Xenopus* retina failed to detect UNC-5 mRNA in RGC (Anderson and Holt, 2002) implying that UNC-5 does not mediate the repulsive responses to netrin-1 in the retina. Another potential receptor for netrin-1 is the membrane-associated adenosine A2b receptor, a G-protein-coupled receptor that induces cAMP production upon binding to netrin-1 (Corset *et al.*, 2000). A2b is proposed to be part of a receptor complex for netrin-1 that associates with DCC (Corset *et al.*, 2000). Interestingly, high A2b immunoreactivity is detected in young RGC growth cones and decreases in older growth cones, in a way that parallels a decrease of cytosolic cAMP in old RGC growth cone. The addition of both a cAMP analogue and an A2b specific agonist to cultures of old retinal axons can block the netrin-1-induced repulsion of older growth cones, whereas A2b antagonists used in cultures of young axons can convert netrin-1-mediated attraction to repulsion (Shewan *et al.*, 2002). Taken together, these results suggest that the age-dependant change in netrin-1 responsiveness could be induced by a change in A2b receptor expression in RGC axons. Further investigations will be necessary to understand how the down-regulation of A2b downstream led to growth cone repulsion and whether it acts directly by regulating the amount of cytosolic cAMP.

EphB and ephrin-B molecules are involved in intraretinal axon guidance

Eph receptors and ephrins represent another family of guidance molecule for which recent studies have provided evidence of important roles in developing visual projections. The Eph family of receptor tyrosine kinases comprises 15 identified members that interact with a second family of cell surface molecules, the ephrins. With a few exceptions, A-type Eph receptors interact with glycosylphosphatidylinositol anchored ephrins (ephrin-A) and B-type Eph receptors bind transmembrane ephrins (ephrin-B) (Nomenclature committee, 1997). Eph receptors and ephrins have the important property of being able to initiate bi-directional

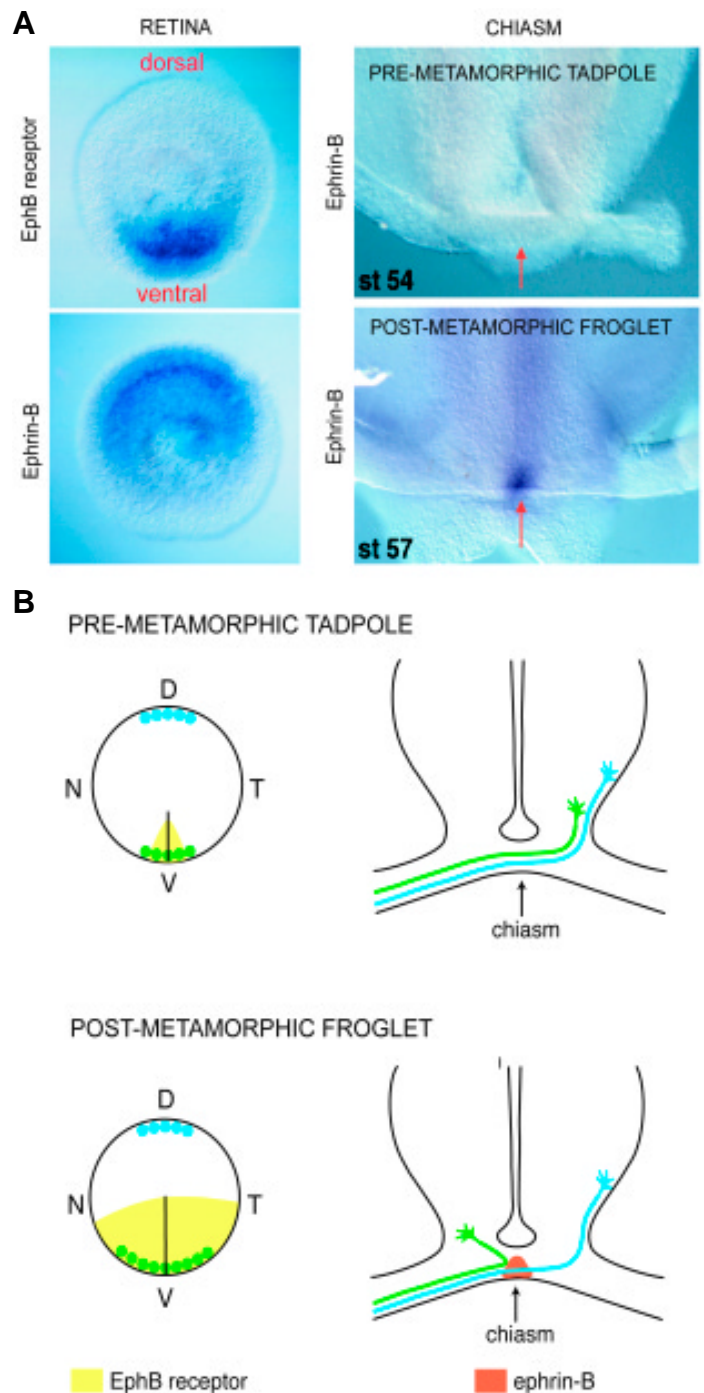


Fig. 2. Ephrin-B mediates divergent axon choice at the optic chiasm. (A) Shows the distribution of ephrin-B and EphB receptor in the embryonic retina (left two panels) and the chiasm of pre- and post- and metamorphic *Xenopus* (right two panels). Ephrin-B is expressed in a high-dorsal to low-ventral gradient in the retina while the EphB receptor is expressed in an opposing high-ventral to low-dorsal gradient. Ephrin-B is not expressed at the chiasm until metamorphosis which coincides with the initiation of the ipsilateral projection. A subpopulation of ventral EphB-expressing cells project ipsilaterally at metamorphosis. Photomicrographs adapted from Nakagawa *et al.*, 2000 and Mann *et al.*, 2002. **(B)** Model of repulsive axon guidance at the chiasm. See text for details. Diagram adapted from Mann and Holt, 2001.

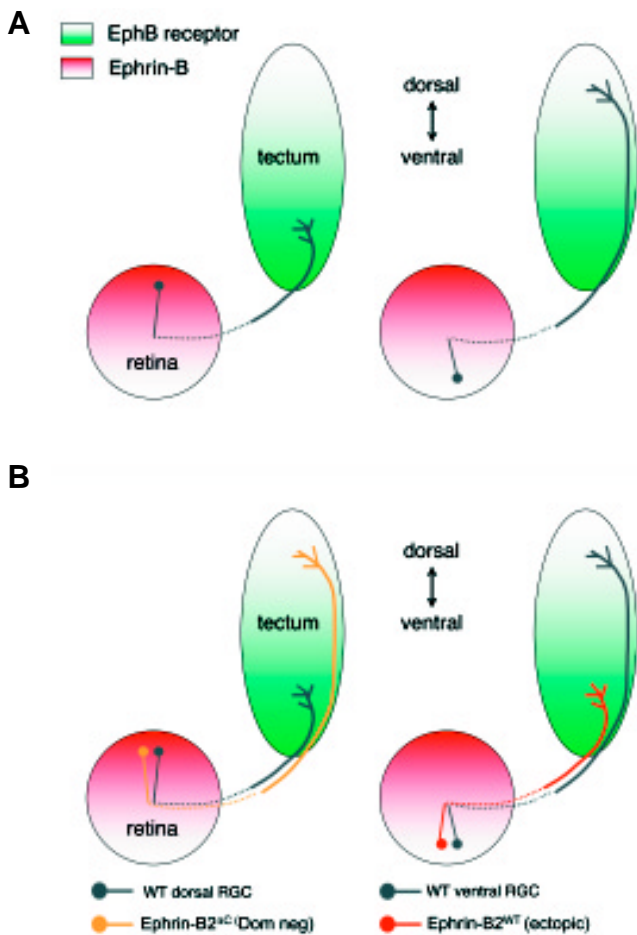


Fig. 3. Topographic mapping along the dorso-ventral axis involves attractive ephrin/EphB interactions. (A) The normal distribution of B-type ephrins and receptors in the retina and tectum. Regions of high ephrin-B expression (dorsal retina) project to regions of high receptor expression (ventral tectum) indicative of matching, attractive interactions. (B) Inhibition of EphB receptor function in dorsal retina causes axons to map significantly further dorsally (like ventral cells) and ectopic expression of EphB in ventral RGCs causes them to map ventrally (like dorsal cells) instead of dorsally. Summary of data from Mann *et al.*, 2002.

signaling, where a signal is propagated in both the Eph receptor-bearing cell ('forward signaling') and in the ligand-expressing cell ('reverse signaling') (for review see (Kullander and Klein, 2002)).

Eph receptors and ephrins are known to be the largest class of molecules that exhibit polarized expression patterns in the retina. In general, A-type receptors and ligands are expressed in opposite nasal-temporal gradients and B-type receptors and ligands show complementary distributions across the dorsal-ventral axis (see Fig. 4). This applies to fish (Brennan *et al.*, 1997), amphibians (Mann *et al.*, 2002), birds (Connor *et al.*, 1998, Holash *et al.*, 1997) and mammals (Birgbauer *et al.*, 2000, Marcus *et al.*, 1996) and marsupials (Vidovic and Marotte, 2003, Vidovic *et al.*, 1999). The presence of both Eph receptors and their ephrin ligands suggest that these molecules might function to regulate intraretinal axon growth. Evidence for such a role has come from the study of mice deficient for two genes encoding the EphB2 and EphB3 receptors

(Birgbauer *et al.*, 2000). During RGC axon pathfinding to the disk, both receptors are initially distributed uniformly through the retina and later become differentially expressed along the dorso-ventral axis of the retina, with higher expression levels detected in the ventral part of the retina. Labelling of RGCs using the lipophilic dye, Dil, was used to show that RGC axons orient and grow normally towards the optic disk in the EphB2/EphB3 double mutant, but as they approach the centre of the retina, a small proportion of axons bypass the optic disk and extend abnormally into the opposite side of the retina. Several lines of evidence suggest that EphB molecules act in this system as guidance cues through a "reverse signaling" mechanism rather than functioning as traditional signaling receptors. First, the placing of Dil crystals into either the dorsal or the ventral retina shows that, although EphB2 and EphB3 are predominantly expressed in ventral retina, RGC axons from dorsal retina are the most severely affected. Second, partial rescue of the observed phenotype was obtained in mice which express a mutant EphB2 which lacks a cytoplasmic domain, suggesting that EphB2 functions in this system in a kinase-independent manner. These data led to propose a model in which ephrin-B-expressing RGC axons from the dorsal retina extending towards the optic disk encounter increasing amounts of EphB2 and EphB3 receptors that prevent dorsal axons from growing into the opposite side of the eye (Birgbauer *et al.*, 2000, Birgbauer *et al.*, 2001).

However, in mice deficient for EphB2 and EphB3, as well as mice lacking netrin-1, abnormal RGC axon behaviours are often only seen close to the optic disk suggesting that the mechanisms that direct the centripetal growth of RGC axons are mediated by other factors. Interestingly, two other Eph receptors, EphB5 and EphA7, distribute in centropolar gradients in chick retina, suggesting a possible role in directing the convergent growth of RGC axon the centre of the retina (Sefton *et al.*, 1997). However, experimental proof of such a function is still lacking.

EphB and ephrin-B control axon divergence at the optic chiasm

The optic chiasm is an important choice point along the visual pathway where RGC axons have to decide whether or not to cross the ventral midline of the brain. The degree of decussation of RGC axons at the optic chiasm depends on the type of animal. Fish and birds have no binocular vision and only crossed projections. In most mammals, all RGCs located in the temporal retina send axons ipsilaterally, whereas in species with less binocular vision uncrossed projections arise from a subpopulation of RGCs in the ventrotemporal (VT) region of the retina.

A key issue is to identify the nature of the molecular mechanisms that control the routing of RGC axons at the optic chiasm. Early studies in mouse have shown that the optic chiasm is the source of guidance molecules for crossed and uncrossed axons (for review see (Mason and Sretavan, 1997), but the first identification of the nature of the molecular actors that regulate axon divergence at the chiasm come from studies in the amphibians. During embryonic development in *Xenopus laevis*, all RGC axons from the two optic nerves cross each other at the optic chiasm. It is only during metamorphosis that the first ipsilateral projections start to develop from the VT retina, in order to subserve the acquisition of binocular vision in young froglets. The search for candidate molecules specifically expressed in the binocular part of the retina

led to a focus on Eph receptors. High levels of B-type Eph receptors are expressed in the VT region of *Xenopus* retina through development and the corresponding ephrin-B ligand is detected at the optic chiasm of metamorphosing animals but not in premetamorphic embryos (Nakagawa *et al.*, 2000); see Fig. 2). In addition, precocious expression of ephrin-B ligand at the chiasm of premetamorphic embryos is sufficient to misroute some axons from the VT retina ipsilaterally (Nakagawa *et al.*, 2000). Taken together, these results suggest that ephrin-B ligand forms a repulsive 'barrier' at the chiasm that repels the subpopulation of EphB-expressing fibers into the ipsilateral tract (Fig. 2). Does the same ephrin-B-based mechanism work in other vertebrates? This idea is supported by the fact that in chick embryos, which lack permanent ipsilateral projections, EphB binding sites (ie. ephrin-B) cannot be detected at the ventral midline of the diencephalon (Nakagawa *et al.*, 2000). On the other hand, one ephrin ligand, ephrin-B2, is specifically expressed in radial glia cells at the chiasm of mouse embryos in a pattern that correlates with the temporal development of ipsilateral projections in mice (Williams *et al.*, 2003). A recent study provides evidence that ephrin-B2 is necessary for the development of ipsilateral projections, since inhibition of ephrin-B2 activity by soluble EphB4-Fc completely abolished the formation of ipsilateral projections in a semi-intact preparation of the visual pathway (Williams *et al.*, 2003).

Another important question is to understand how ipsilaterally projecting RGCs are specified to respond to ephrin-B repulsive activity at the optic chiasm. This issue is complicated by the fact that in mouse only a small proportion (about 3%) of RGCs in the VT retina send axons ipsilaterally. Among the putative receptors for ephrin-B2, EphB1 was the most likely candidate, since its expression is restricted to some RGCs in mouse VT retina, unlike EphB2 and EphB3 which show a widespread expression (Williams *et al.*, 2003). Anterograde and retrograde labelling of visual projections in EphB1 null mutant revealed a strong reduction in the number of ipsilaterally projecting RGC axons that instead project aberrantly to the contralateral side of the brain (Williams *et al.*, 2003). Recently the zinc-finger transcription factor, *Zic2*, was found to be expressed exclusively in RGCs with uncrossed projections and the extent of *Zic2* expression in different vertebrates correlates with the degree of binocularity (Herrera *et al.*, 2003). Gain and loss of function experiments in mouse demonstrate that expression of *Zic2* is required for axons to be repelled at the chiasm and to project ipsilaterally. These findings raise the possibility that *Zic2* could directly regulate the expression of EphB1 in subsets of growing RGC axons.

Topographic mapping of visual projection depends on Eph family molecules

Topographic mapping of RGC axons in their target occurs along two orthogonal axes: the nasal-temporal axis of the retina maps along the posterior-anterior axis of the optic tectum and the dorsal-ventral retinal axis along the ventral-dorsal tectal axis (lateral-medial (L-M) in mammals; see summary diagrams in Figs. 3,4). The search for candidate molecules involved in setting-up topographic mapping of visual projections has first involved the A-type subclass of Eph and ephrins, which are expressed in opposite gradients along the A-P axis of the retina and tectum, respectively. A large amount of evidence from both *in vitro* and *in vivo* experiments

have led to a model in which the graded repulsion of EphA-expressing RGC axons and ephrin-A in the target prevent temporal axons from terminating in the ligand-rich posterior part of the tectum (for review see (Drescher *et al.*, 1997, McLaughlin *et al.*, 2003a).

The mechanisms involved in D-V patterning have been discovered more recently. Due to the polarized expression of B-type Eph molecules in the D-V axis of developing retina in many vertebrate species, EphB and ephrin-B were proposed to be choice candidates for such a function. In chick and mouse, gradients of ephrin-B1 ligand were detected in the tectum and superior colliculi respectively (Braisted *et al.*, 1997, Hindges *et al.*, 2002). Unlike in the A-P axis, ephrin-B is expressed in the dorsal tectum (medial SC) where ventral RGC expressing high levels of EphB receptors terminate, suggesting that attractive rather than repulsive interactions between Eph and ephrin are involved in mapping along this axis. In mouse, RGC axons enter their target structure, form interstitial branches at appropriate A-P positions that extend along the latero-medial (L-M) axis towards their appropriate topographic target region. In double mutants for EphB2 and EphB3 receptors, injection of Dil into the VT retina of postnatal animal revealed ectopic termination of RGC afferents in regions lateral to the appropriate target zone along the L-M axis. In contrast, dorsal axons terminate in their appropriate region (Hindges *et al.*, 2002). The same aberrant termination zone of ventral RGCs axons were

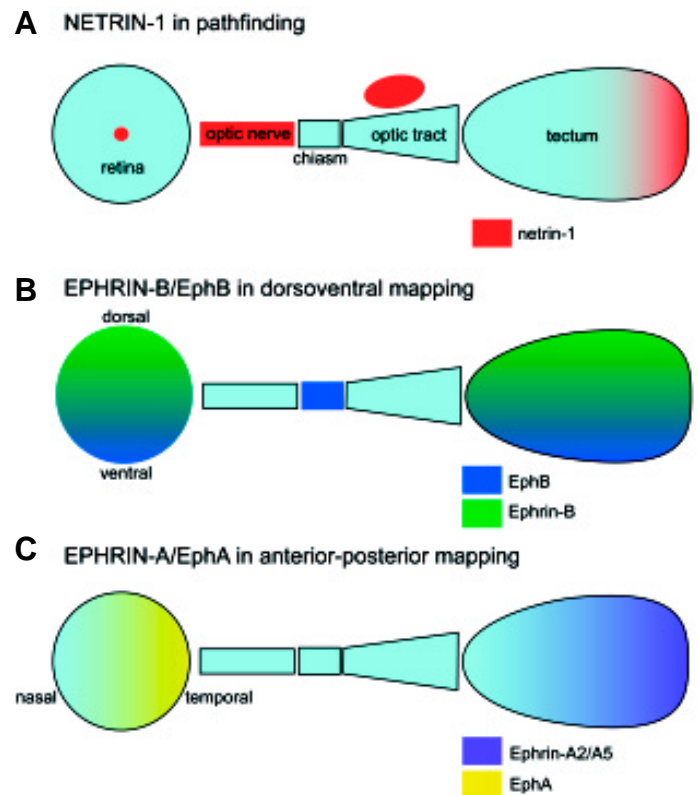


Fig. 4. Summary of distribution and role of netrin-1, A- and B-type ephrins and receptors in the developing visual pathway. Eye: dorsoventral axis is top to bottom, nasotemporal axis left to right. Tectum: mediolateral (mammals) and dorsoventral (frogs) is top to bottom, anterior-posterior axis is left to right.

observed in mutants for kinase-inactivated EphB2, suggesting that forward rather than reverse EphB signaling is most probably involved in L-M targeting. Consistent with the hypothesis of an attractant effect between B-type Eph and ephrin, these results indicate that signaling through EphB receptors is required for RGC axons to form branches that extend dorsally in the SC toward region of high ephrin-B expression.

In lower vertebrates such as frogs, B-type Eph receptors and ephrins have also been shown to play an important role in D-V patterning. In the tectum of *Xenopus* embryos, a D-V gradient of EphB1 can be detected, with a maximum expression level in the ventral part of tectum where ephrin-B expressing dorsal RGC axons terminate (Mann *et al.*, 2002). EphB and ephrin-B function was disrupted *in vivo* by applying the extracellular domain of the EphB2 receptor to the optic pathway in an "exposed brain preparation". This treatment causes targeting errors of retinal axons which terminate in a more dorsal position in the tectum, leaving the ventral tectum (normally innervated by ephrin-B-expressing RGCs) completely devoid of retinal afferents (Mann *et al.*, 2002). Taken together, these findings suggest that ephrin-B "reverse signaling" in dorsal RGC axons might be important for the recognition of D-V positional cues. To address this idea directly, a dominant negative ephrin-B ligand that lacks a cytoplasmic domain was expressed by targeted lipofection into dorsal retinal precursors. Analysis of D-V topography shows that transfected axons project significantly more dorsally in the tectum (Fig. 3). In contrast, misexpression of full-length ephrin-B ligand in ventral RGCs causes them to terminate more ventrally in their target (Fig. 3) (Mann *et al.*, 2002). These results demonstrate that the cytoplasmic tail of ephrin-Bs is important for appropriate D-V mapping and support the idea of an attractive mechanism by which EphB1 receptor in the tectum provides topographic guidance cues for ephrin-B-expressing RGC axons.

Mechanisms of Eph and ephrin bifunctional activity

The studies discussed above have contributed significantly to our understanding of Eph receptors and ephrin *in vivo* functions during RGC axon development. However, the mechanisms by which these molecules control growth cone behaviour remain poorly understood. *In vivo* gain and loss of function experiments suggest that Eph/ephrin activation leads to growth cone responses that can be attributed to both repulsion (in retina and optic chiasm) and attraction (in tectum and superior colliculi). Similarly, dual function of B-type Eph molecules has been observed *in vitro*: dorsal retinal neurons adhere and grow preferentially on EphB substrate (Holash *et al.*, 1997, Mann *et al.*, 2002) but are repelled by soluble EphB added to the culture medium (Birgbauer *et al.*, 2001, Mann *et al.*, 2003).

Which mechanisms underlie the bifunctional action of Eph and ephrin? As shown previously for netrin-1, changes in cytosolic cyclic nucleotides might modulate Eph/ephrin signaling. For example, the repulsive response to soluble EphB relies on low levels of cGMP (Mann *et al.*, 2003). Another factor known to influence Eph/ephrin responsiveness is the degree of clustering. As both receptor and ligands are membrane-anchored molecules, soluble forms of Eph and ephrin require to be artificially oligomerized in order to activate their binding partner *in vitro* (Davis *et al.*, 1994). In endothelial cells, different oligomeric forms of ephrin-B1-Fc

induce distinct cell responses mediated by the recruitment of distinct downstream signaling complexes (Stein *et al.*, 1998). Moreover, in culture of retinal explants, clustered (multimeric) ephrin-B1-Fc fusion protein induces growth cone collapse whereas unclustered (dimeric) ephrin-B1-Fc does not. In addition, unclustered but not clustered EphB2-Fc stimulates retinal growth cone retraction (Mann *et al.*, 2003). How does this relate to the *in vivo* situation? The above data suggest that signals or adaptor proteins that control the degree oligomerisation might modulate whether Eph and ephrin act as attractants or repellents. Alternatively, the differences in the density of Eph/ephrin expression might be critical to determine growth cone response. This latter idea is supported by recent experiments where growing visual axons are confronted by ectopic ephrin-B1 expressed in the chick tectum: high ephrin-B1 expression attracts RGC axon interstitial branches, whereas lower expression causes branch repulsion (McLaughlin *et al.*, 2003b).

Another proposed mechanism that modulates repulsion versus adhesion involves extracellular proteolytic cleavage of ephrin ectodomain. Upon binding to cognate Eph receptors, ephrin ligands are cleaved from the cell surface by the Kuzbanian metalloprotease, and this processing is thought to be important for axon growth cones to break adhesive contacts with an ephrin-expressing cell (Hattori *et al.*, 2000). In principle, Eph/ephrin interactions occurring in the absence of metalloprotease activity would prevent growth cone retraction and result in sustained adhesive behaviour. This mechanism has been described for A-type Eph and ephrin and it is not known whether it occurs in the B-subclass of Eph and ephrin molecules, although proteolytically-released forms of ephrin-B ligands exist *in vitro* (Nicola *et al.*, 1996). Instead, in cell lines and primary neuronal cultures, the entire EphB-ephrin-B complex is removed from the cell surface by endocytosis (Mann *et al.*, 2003, Marston *et al.*, 2003, Zimmer *et al.*, 2003). In cultures of retinal cells, endocytosis occurs during 'reverse signaling': internalization of EphB receptor occurs in dorsal retinal growth cones and appears to be necessary for growth cone collapse, since an inhibitor of endocytosis blocks this response. In primary cultures of mouse hippocampal neurons, it has been shown that the intracellular domain of EphB receptors is important for endocytosis and subsequent growth cone collapse (Zimmer *et al.*, 2003). In addition, previous studies reported that ephrin-A5 mediates repulsion through activation of a full-length EphA7 and adhesion through a truncated EphA7 receptor that lacks a cytoplasmic domain (Holmberg *et al.*, 2000). Since truncated forms of EphB receptors have been detected in mouse neural system (Ciossek *et al.*, 1995), it would be interesting to determine their exact localization and examine how they could possibly determine EphB/ephrin-B activity.

Protein synthesis and degradation in visual axon navigation

Recent studies have revealed that several aspects of growth cone navigation, including the mechanisms by which external signals are transduced within the growth cone, might be regulated by rapid and local changes in protein levels. For example, in *Xenopus* retinal axons, netrin-1-induced growth cone turning can be blocked with specific translation inhibitors and proteasomal inhibitors (Campbell and Holt, 2001). EphB-induced collapse, however, does not depend on protein synthesis, but can be blocked with inhibitors of the proteasomal pathway which also

inhibit EphB internalisation into retinal growth cones (Mann *et al.*, 2003). In contrast, growth cone repulsion stimulated by ephrin-B is not affected by blocking protein synthesis or degradation (Mann *et al.*, 2003). The nature of the proteins involved in mediating growth cone steering is not yet known and efforts are now being made to identify the mRNAs present in the axons and growth cones.

In *Xenopus* embryos, *in situ* hybridization methods have revealed strong positive signals for mRNAs of various B-type Eph/ephrin molecules in the optic nerve suggesting that translation regulatory mechanisms might control the levels of EphB and ephrin-B protein expression at the growth cone (Mann *et al.*, 2002). Evidence for such a mechanism come from the study of spinal commissural neurons in which EphA2 mRNA is translated in the distal part of the axons arriving at the ventral midline (Brittis *et al.*, 2002). Controlled translation could be involved in regulating the localized increase of EphB1 in ipsilateral axons that approach the chiasm midline. In addition, mRNA translation could control the subcellular localisation of EphB receptors, a mechanism that would explain why interstitial branches but not main visual axons are sensitive to ephrin-B1 ligand in their target structure (Hindges *et al.*, 2002, McLaughlin *et al.*, 2003b).

Conclusion

Netrin and Eph/ephrin molecules belong to the growing list of guidance molecules that have been shown to contribute to the accurate development of visual projections. Although they play distinct roles, they share the ability to exert bi-functional activity on growing RGC axons, they are reused along the visual pathway and their role is conserved among vertebrate species.

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