Development 127, 1231-1241 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4289

Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina

Eric Birgbauer[1,*], Chad A. Cowan[2], David W. Sretavan[1,*] and Mark Henkemeyer[2]

[1]Departments of Ophthalmology and Physiology, Neuroscience and Biomedical Sciences Program, Beckman Vision Center, Box 0730, University of California San Francisco, San Francisco, CA 94143-0730, USA
[2]Center for Developmental Biology, and Kent Waldrep Foundation Center for Basic Neuroscience Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75235-9133, USA
*Authors for correspondence (e-mail: ericbirg@phy.ucsf.edu, dws@itsa.ucsf.edu)

Accepted 21 December 1999; published on WWW 21 February 2000

SUMMARY

Optic nerve formation requires precise retinal ganglion cell (RGC) axon pathfinding within the retina to the optic disc, the molecular basis of which is not well understood. At CNS targets, interactions between Eph receptor tyrosine kinases on RGC axons and ephrin ligands on target cells have been implicated in formation of topographic maps. However, studies in chick and mouse have shown that both Eph receptors and ephrins are also expressed within the retina itself, raising the possibility that this receptor-ligand family mediates aspects of retinal development. Here, we more fully document the presence of specific EphB receptors and B-ephrins in embryonic mouse retina and provide evidence that EphB receptors are involved in RGC axon pathfinding to the optic disc. We find that as RGC axons begin this pathfinding process, EphB receptors are uniformly expressed along the dorsal-ventral retinal axis. This is in contrast to the previously reported high ventral-low dorsal gradient of EphB receptors later in development when RGC axons map to CNS targets. We show that mice lacking both EphB2 and EphB3 receptor tyrosine kinases, but not each alone, exhibit increased frequency of RGC axon guidance errors to the optic disc. In these animals, major aspects of retinal development and cellular organization appear normal, as do the expression of other RGC guidance cues netrin, DCC, and L1. Unexpectedly, errors occur in dorsal but not ventral retina despite early uniform or later high ventral expression of EphB2 and EphB3. Furthermore, embryos lacking EphB3 and the kinase domain of EphB2 do not show increased errors, consistent with a guidance role for the EphB2 extracellular domain. Thus, while Eph kinase function is involved in RGC axon mapping in the brain, RGC axon pathfinding within the retina is partially mediated by EphB receptors acting in a kinase-independent manner.

Key words: Eph receptors, Ephrins, Axon guidance, Growth cones, Retinal ganglion cells, Tyrosine kinase, Visual system

INTRODUCTION

Among the earliest events in visual system development are RGC axon pathfinding within the retina to the optic disc and subsequent axon growth through the disc into the optic stalk to form the optic nerve. Guidance mechanisms underlying these pathfinding tasks are not completely understood. Studies demonstrate that at the optic disc, the axon guidance molecule netrin-1 controls, to a large extent, proper RGC axon growth through this exit point into the optic nerve (Deiner et al., 1997). However, since RGC axons in animals lacking netrin-1 or its receptor DCC are still able to find their way to the disc, guidance cues underlying pathfinding to the disc are distinct from those which mediate RGC axon growth through the disc and into the nerve. During pathfinding to the disc, RGC axons express Ig-CAMs (Bartsch et al., 1989; Paschke et al., 1992; Silver and Rutishauser, 1984), and anti-Ig-CAM antibody injections intraocularly in vivo, or applied onto retinal eye cup preparations in vitro, cause abnormal intra-retinal axon trajectories (Brittis et al., 1995; Ott et al., 1998). However, these perturbations do not affect all RGC axons, suggesting that additional guidance cues are involved in pathfinding to the optic disc.

Further along the visual pathway, the topographic mapping of RGC axons in the superior colliculus appears to depend on inhibitory interactions between Eph receptor tyrosine kinases on RGC axons and target derived ephrin ligands. Eph receptors belong to either the A or B subfamily which bind respectively to the A (GPI-linked) or B (transmembrane) subfamily of ephrin ligands (Brambilla et al., 1996; Eph Nomenclature Committee, 1997; Flanagan and Vanderhaeghen, 1998; Gale et al., 1996; Himanen et al., 1998). Experimental evidence indicates that the anterior-posterior orientation of the retinocollicular map is dependent on EphA receptors expressed in a nasal-temporal retinal gradient and an opposing anterior-posterior gradient of ephrin-A ligands in the target (Cheng et

al., 1995; Drescher et al., 1995; Frisén et al., 1998; Nakamoto et al., 1996). Although some EphB receptors are expressed in a dorsal-ventral gradient in the retina (Connor et al., 1998; Henkemeyer et al., 1996; Holash and Pasquale, 1995) and ephrin-B ligands are expressed as a medial-lateral gradient in the colliculus (Braisted et al., 1997; Connor et al., 1998), it is not known whether EphB receptors and B-ephrins mediate retinocollicular mapping along this axis.

EphB receptors have been shown to be required for the formation of specific commissures in the CNS. Targeted null mutation of *EphB2* (*Nuk*) resulted in a loss of the posterior tract of the anterior commissure (Henkemeyer et al., 1996), and an *EphB3* (*Sek4*) deletion resulted in partially penetrant defects in corpus callosum formation (Orioli et al., 1996). Elimination of both EphB2 and EphB3 receptors resulted in a stronger phenotype than a simple sum of individual phenotypes (Orioli et al., 1996), suggesting that EphB2 and EphB3 share overlapping or redundant functions. Furthermore, in this system, EphB2 was shown to act independently of its kinase domain, suggesting a non-cell-autonomous role of the extracellular domain by itself in pathfinding (Henkemeyer et al., 1996). This result, together with biochemical experiments demonstrating activation of ephrin-expressing cells following exposure to clustered Eph extracellular domains (Brückner et al., 1997; Holland et al., 1996), raises the possibility of bidirectional signaling during Eph-ephrin interactions in vivo (see also Mellitzer et al., 1999; Xu et al., 1999).

Since EphB receptors and B-ephrins are involved in CNS axon guidance, they likely also play a role in the development of retinal connections. Both A and B ephrins, in addition to being expressed in the superior colliculus, are present within the retina, suggesting that Eph-ephrin interactions mediate aspects of retinal development. A study using affinity reagents which bind Eph receptors and ephrin ligands has shown gradients of both A and B Eph and ephrin binding sites in the developing mouse retina (Marcus et al., 1996), although the individual receptors and ephrins, as well as the retinal cell types expressing them, were not identified. In chick, in situ hybridization studies have found several Eph receptors and ephrins in retina and showed that RGCs at different ages express multiple Ephs and ephrins (Braisted et al., 1997; Connor et al., 1998; Holash and Pasquale, 1995; Holash et al., 1997). The concurrent expression of both Eph receptors and ephrin ligands on RGCs (and presumably RGC axons; see Hornberger et al., 1999) raises the possibility that Eph-ephrin function is triggered in the retina as RGC axons interact with neighboring RGC axons.

In this study, we examine how axon guidance within the retina is affected following the loss of EphB receptor function. Similar to published studies in chick, embryonic mouse RGCs were found to express mRNAs encoding multiple Eph receptors (EphB1, B2, B3) and ephrins (ephrin-B1, B3). Targeted deletions of both *EphB2* and *EphB3* receptor genes, but not each alone, led to an increased incidence of RGC axon pathfinding errors during growth to the optic disc. Our data also indicate that guidance errors within the retina were not directly correlated with EphB tyrosine kinase activity. Instead, EphB receptors appeared to act in a kinase-independent manner to mediate RGC axon pathfinding within the retina to the optic disc, potentially by ephrin-B-expressing axons responding to the EphB extracellular domain.

MATERIALS AND METHODS

Animals

Mice with targeted deletions of *EphB2* (*Nuk*) and *EphB3* (*Sek4*) have been described previously (Henkemeyer et al., 1996; Orioli et al., 1996). The *EphB2$^{lacZ(kin-)}$* mice have the bacterial *lacZ* gene inserted into the kinase domain of *EphB2*, producing a fusion protein consisting of a truncated EphB2 protein, which lacks the kinase domain and cytoplasmic tail, linked to β-galactosidase (Henkemeyer et al., 1996). Embryos were removed from pregnant female mice by Cesarean section after anesthetizing the mother with intraperitoneal injections of 4.0 mg sodium pentobarbital. The morning of vaginal plug detection was counted as embryonic day 0 (E0).

In this study, we examined the pattern of Eph and ephrin mRNA expression by in situ hybridization in embryos of both the CD-1 and C57/Bl6 background. No differences were observed in the spatial and temporal patterns of Eph and ephrin mRNA expression in these two strains. RGC pathfinding to the optic disc was analyzed mainly in mice of the CD1 strain, although some mice of the C57/Bl6 background were also used. The results based only on CD1 mice, including incidence and severity of pathfinding errors to the disc, were not different from those based on both mouse strains combined. Figs 6-8 and Table 1 include analysis of fifteen C57/Bl6 wild-type or heterozygote retinas and one C57/Bl6 *EphB2* mutant retina.

In situ hybridization

In situ hybridization was performed on 12-16 μm cryostat coronal retinal sections from E12-16 wild-type (C57/Bl6 or CD1) mouse embryos using digoxigenin-labeled antisense and sense control RNA probes (Braissant et al., 1996; Braissant and Wahli, 1998). If possible, embryos were fixed in 4% paraformaldehyde for 20-60 minutes before embedding and sectioning for better histological preservation. However, for some probes (EphB1, ephrin-B1 and ephrin-B3), successful in situ hybridization was possible only in fresh frozen unfixed tissue that was sectioned and briefly fixed before hybridization, resulting in a lower degree of histological preservation. In addition, Figs 1E, 2C, 5I are composites of two photos.

The probes used for individual Eph receptors and ephrin ligands are as follows: EphB1 – a 600 bp region of exon 3; EphB2 – a 3.1 kb full-length cDNA; EphB3 – 1.3 kb region from the 3′ end of the Sek4 cDNA, nucleotides 2290-4010, kindly provided by D. Wilkinson (Becker et al., 1994); ephrin-B1 – a 1 kb full-length cDNA; ephrin-B2 – a 1 kb full-length cDNA; and ephrin-B3 – a 300 bp region encoding the cytoplasmic domain.

Visualization of β-galactosidase activity in *EphB2$^{lacZ(kin-)}$* embryos

Visualization of β-galactosidase activity in the retinas was performed as described (Henkemeyer et al., 1996). After histochemical staining, the entire head including the retinas was sectioned coronally at 14-16 μm or the retinas were removed after marking orientation and prepared as whole mounts.

Quantitation of β-galactosidase reaction product was performed on images of representative coronal sections with a Macintosh computer using the public domain NIH Image program (developed by the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Sampling of the optic fiber layer (OFL) was performed using a circular area of a diameter equivalent to the thinnest region of the OFL to avoid sampling outside the OFL. The entire dorsal to ventral extent of the OFL, except the optic disc and the most peripheral ciliary margins of the retina, was sampled at set intervals and the mean pixel value of each spot recorded (see Fig. 3). The same process was done in the outer retina (outside the RGC layer) except that a larger spot, whose diameter was equivalent to about two-thirds the thickness of the outer retina, was used for sampling. Since a smaller spot was used for the OFL, more points

were collected than for the outer retina. Graphs were prepared with Igor software (Wavemetrics, OR) on an Apple Macintosh computer with the relative retinal position in the dorsal-ventral axis plotted on the x-axis.

Histology and immunostaining

For morphological analysis, wild-type (CD1 strain) and mutant embryos were fixed in glutaraldehyde-formaldehyde and embedded in Epon-Araldite as described by LaVail and Battelle (1975). 1 μm sections were cut coronally on a microtome and stained briefly with toluidine blue. Cell density in the RGC layer was counted in a 5000 μm^2 box using camera lucida from 9 wild-type and 11 double mutant, toluidine blue-stained, E16 coronal sections, approximately 200-300 μm dorsal or ventral from the optic disc. For immunolocalization, embryos were fixed in 4% paraformaldehyde and embedded in OCT (TissueTek). 12 μm cryostat sections were cut coronally for antibody incubation. DCC was detected with the monoclonal antibody 15041A (Pharmingen) and L1 was detected with a rabbit polyclonal serum (Chung et al., 1991). Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

DiI labeling and analysis of RGC axon trajectories within the retina

A modification of the method described by Deiner et al. (1997) was used for labeling RGC axons within the retina. Briefly, the cornea and lens were carefully removed from 4% paraformaldehyde-fixed mouse embryos, aged E13.5 to E17.5, and the excess liquid from the retinas removed by blotting with filter paper. A small crystal of DiI was gently pushed with a glass electrode into either dorsal or ventral retina at sites corresponding to approximately one-half to two-thirds of the distance from the optic disc to the retinal periphery. The specimens were then incubated for 4-6 hours at 37°C in 4% paraformaldehyde to allow DiI diffusion along the axons and then transferred to 4°C until analysis. The labeled retinas were removed, 3-4 small cuts were made at the periphery, and then the retinas were flat-mounted, RGC side up, on a slide with polyvinyl alcohol mounting medium (Elvanol, DuPont) and coverslipped.

Labeled retinas were examined under both epifluorescence and bright-field optics on a Nikon Microphot-SA microscope and images recorded with an Optronics color CCD camera (DEI-750). For fluorescence images, the microscope diaphragm was often closed down to exclude regions outside the area of interest to minimize light scattering from other parts, especially the DiI crystal itself. Some retinas were also examined and images acquired with a BioRad MRC600 or MRC1024 confocal microscope, including Fig. 4E,F,J,K. The confocal microscope and the standard fluorescence microscope did not differ in their ability to allow us to detect the presence or judge the severity of RGC axon pathfinding errors.

The trajectory of the DiI-labeled RGC axons was followed under epifluorescence from the labeling site to the optic disc, which was identified by bright-field optics. If all the labeled axons could be traced to the optic disc, it was counted as 'no errors'. A given retina was counted as having pathfinding errors if there were one or more labeled RGC axons that did not enter the optic disc but rather split away from the main bundle of axons and grew aberrantly away from the optic disc. The severity of the pathfinding errors was classified into two types. 'Type 1' was defined as a more severe phenotype consisting of several long aberrantly growing RGC axons having a total length of ≥1000 μm. 'Type 2' was defined as a milder phenotype consisting of only a few short aberrantly growing axons of total length less than 1000 μm (see text for details and examples). Fisher's two-sided exact T-test was used for statistical analysis.

During data analysis, we noticed that several retinas had a different type of labeled RGC axons which emanated radially from the optic disc and spread out into the region of the retina opposite to the DiI crystal. These were easily distinguished from pathfinding errors described above. These 'radial' labeled axons may reflect a certain degree of dye transfer at the optic disc or optic nerve where RGC axons are closely packed. Given that the incidence of 'radial' axons

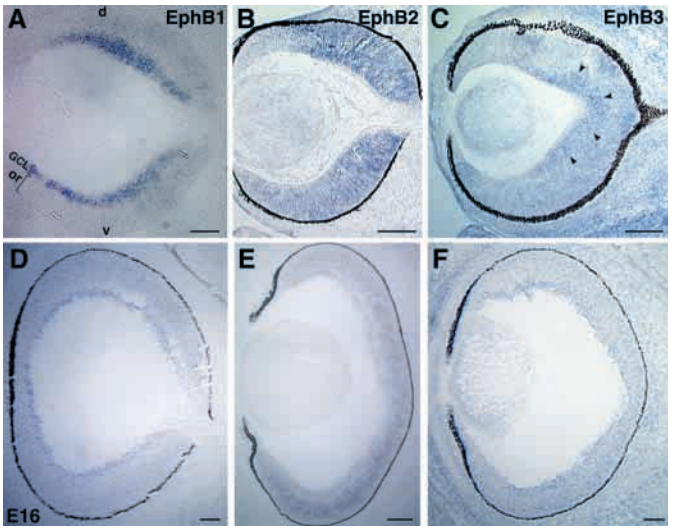

Fig. 1. Expression of EphB receptor mRNA. EphB1 (A,D), EphB2 (B,E) and EphB3 (C,F) mRNA expression in coronal sections of mouse retina. Top row is E13 (except A, E14.5) and bottom row is E16. EphB1, EphB2, and EphB3 are all expressed in the retinal ganglion cell layer (GCL), although EphB3 is only weakly expressed (C, arrowheads). EphB2 is also expressed in outer retina (or). EphB1 and EphB3 receptor mRNA appears uniformly expressed along the dorsal-ventral axis in the retina at both E13 and E16. EphB2 mRNA is uniformly distributed at E13, but by E16, a ventral-dorsal difference in expression is seen (see also Fig. 3). Dorsal retina (d) is up in each Fig. and ventral retina (v) is down. B,C,E are fixed tissue, while A,D,F are in unfixed tissue. The black structure in B-F is pigment in the retinal pigmented epithelium (RPE); the RPE is not visible in non-pigmented CD1 mouse embryos (A). Scale bars, 100 μm.

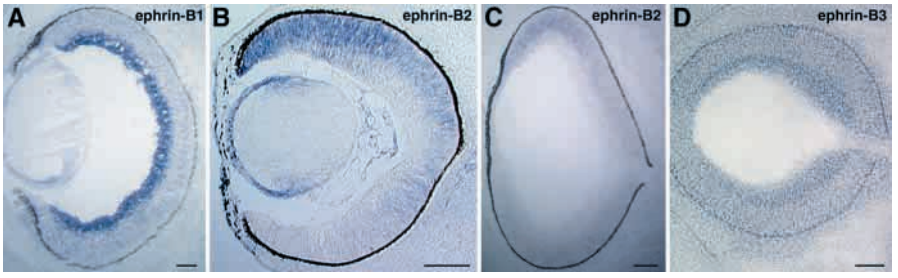

Fig. 2. Expression of ephrin-B ligand mRNA in developing retina. Ephrin-B1 (A) is expressed in the RGC layer of both dorsal and ventral retina. Ephrin-B2 (B,C) is not seen in the RGC layer but is found exclusively in the outer retina where it is expressed predominantly in dorsal compared to ventral retina at both E13 (B) and E16 (C). Ephrin-B3 (D) is expressed in the RGC layer uniformly along the dorsal-ventral axis. In situ hybridization was done on coronal sections of E16 (A,C), E13 (B), or E14.5 (D) retinas, either fixed before sectioning (B,C) or unfixed (A,D). As in Fig. 1, dorsal retina is up. Scale bars, 100 μm.

was not different in wild type compared with *EphB2* or *EphB3* single mutants or *EphB2 EphB3* double null mutants, these 'radial' axons were not considered for further analysis.

RESULTS

In mouse, retinal ganglion cell (RGC) neurogenesis occurs from embryonic day E11.5 though E18, starting first in central retina and then spreading more peripherally (Dräger, 1985). Each RGC, soon after its last mitosis, extends an axon which grows straight from the cell body to the optic disc at the center of the retina where it exits the retina to enter the optic nerve. We examined the expression and function of EphB receptors and ligands during early retinal development from roughly E13 to E16, a time when large numbers of RGC axons navigate to the optic disc.

Embryonic mouse RGCs express multiple EphB receptors

The expression of EphB1, EphB2, and EphB3 receptors in the embryonic mouse retina was examined by in situ hybridization. At these early stages of embryonic development, only two cell layers are readily apparent, the ganglion cell layer (GCL) containing the cell bodies of RGCs and the non-RGC layer, which we refer to as the 'outer retina', which contains primarily undifferentiated cells which will later organize into specific retinal layers.

At E13-14, soon after the first RGCs have been generated and their axons begun to navigate to the optic disc, cells of the RGC layer expressed all three EphB receptors examined, EphB1, B2 and B3 (Fig. 1A-C), although EphB3 expression appeared to be relatively weak. While EphB1 and EphB3 appeared to be restricted to RGCs (Fig. 1A,C), EphB2 was also seen in the outer retina (Fig. 1B). (The weak expression of EphB3 makes it difficult to be certain there was no outer retina expression.) During this time, the mRNA expression pattern of these three EphB receptors was roughly uniform in both dorsal and ventral retina, with no indication of a gradient.

As retinal development proceeded, some Eph receptors maintained a uniform pattern of expression in the retina, while others became differentially expressed along the dorsal-ventral retinal axis. At E16, EphB1 (Fig. 1D) and EphB3 (Fig. 1F) were still expressed uniformly and appeared to be restricted to RGCs. At this age, EphB2 has begun to exhibit a ventral-dorsal difference of mRNA expression (Fig. 1E). This gradient was most prominent in the outer retina and does not appear strongly in the RGC layer. Thus, the previously reported gradient of EphB2 (Connor et al., 1998; Henkemeyer et al., 1996) appears to develop from a uniform expression pattern in early retina.

Table 1. Incidence of observed RGC axon pathfinding errors

Genotype	*n*	Total incidence	Type 1	Type 2
Dorsal retina:				
Wild type	38	2 (5%)	0 (0%)	2
heterozygous	18	3 (17%)	1 (6%)	2
EphB2$^{-/-}$	15	0	0	0
EphB3$^{-/-}$	52	10 (19%)	1 (2%)	9
EphB2$^{-/-}$ *EphB3*$^{-/-}$	48	16 (33%)	10 (21%)	6
EphB2$^{lacZ(kin-)}$ *EphB3*$^{-/-}$	46	18 (39%)	2 (4%)	16
Ventral retina:				
Wild type	25	2 (8%)	1 (4%)	1
heterozygous	1	0	–	–
EphB2$^{-/-}$	0	–	–	–
EphB3$^{-/-}$	37	4 (11%)	0 (0%)	4
EphB2$^{-/-}$ *EphB3*$^{-/-}$	44	6 (14%)	1 (2%)	5

The number of E15-E17.5 retinas examined (*n*) after DiI tracing of RGC axons from dorsal retina or ventral retina for each genotype. The column labeled total incidence shows the number and percentage of retinas of each genotype having any aberrantly growing RGC axons. These are further divided into the incidence of the more severe type 1 errors (Type 1) and the milder type 2 errors (Type 2) in the last two columns. Heterozygous animals include mice heterozygous for only EphB2 (*n*=4), only EphB3 (*n*=8), or heterozygous for both EphB2 and EphB3 (*n*=6). *EphB2*$^{lacZ(kin-)}$ *EphB3*$^{-/-}$ embryos are homozygous for both the kinase-deleted *EphB2*$^{lacZ(kin-)}$ allele and the *EphB3* allele.

Ephrin-B expression in the developing retina

The expression of B-ephrins was also determined by using in situ hybridization. The three known B-ephrins were found expressed in mouse retina (Fig. 2). Ephrin-B1 mRNA was found in the RGC layer of both dorsal and ventral mouse retina at E16 (Fig. 2A), but expression was not detected at E13. This uniform expression differs from the dorsal-ventral gradient of ephrin-B1 expression described in chick (Braisted et al., 1997; Holash et al., 1997) and may represent a species or developmental difference. Ephrin-B2 mRNA was not observed in the RGC layer, but appeared in the outer retina, including the peripheral margin (Fig. 2B,C). Unique among the EphB receptors and ephrins examined, ephrin-B2 was expressed in a high dorsal to low ventral pattern of expression not only at E16 (Fig. 2C), but also at E13 (Fig. 2B). Ephrin-B3 was expressed in the RGC layer and did not appear to be expressed in the outer retina (Fig. 2D). The expression of ephrin-B3 in the RGC layer at E14.5 (Fig. 2D) appeared to be uniform along the dorsal-ventral axis.

Thus, in mouse, at least three EphB receptors (B1, B2, B3) and two ephrin-B ligands (B1, B3) are expressed in cells of the RGC layer during retinal axon guidance to the optic disc. Cells in outer retina, which include radial glial cells that may be sources of guidance cues for RGC axons (Bauch et al., 1998; Stier and Schlosshauer, 1995), express EphB2 and ephrin-B2 mRNA. Expression in RGCs at these early ages during axon pathfinding to the disc is predominantly uniform along the dorsal-ventral axis, but some differences in this axis are seen in the outer retina. As has been previously reported, later in development when RGC axons begin to innervate their target, the superior colliculus, dorsal-ventral gradients become more apparent (Braisted et al., 1997; Connor et al., 1998; Henkemeyer et al., 1996; Holash and Pasquale, 1995; Marcus et al., 1996).

Development of an EphB2 protein gradient along the dorsal-ventral axis

To confirm the initial uniform pattern of EphB receptors along the dorsal-ventral axis, we examined the expression and localization of EphB2 protein in retinas of mouse embryos in which the bacterial *lacZ* gene has been substituted for the kinase domain of the *EphB2* gene. This results in the expression, under its own promoter and enhancer sequences,

of a membrane-anchored, truncated EphB2-β-gal fusion protein that lacks its tyrosine kinase domain and carboxyl-terminal tail (Henkemeyer et al., 1996). Previous studies of this *EphB2$^{lacZ(kin-)}$* mutation have documented the presence of a high ventral to low dorsal gradient of EphB2 protein expression within the mouse retina at birth (P0; Henkemeyer et al., 1996).

We found that at E12-14, EphB2 protein expression appeared uniform along the dorsal-ventral axis in both the OFL, which contains the RGC axons, and in the outer retina. At these ages, there was no apparent gradient of EphB2-β-gal expression either in coronal sections (Fig. 3A,B; E12 data not shown) or in retinal whole mounts (Fig. 3C). A high ventral to low dorsal pattern of expression emerged, beginning at E15, and appeared first in outer retina (Fig. 3D) and became quite apparent by E16 both in coronal section (Fig. 3E) and in retinal whole mount (Fig. 3F). In comparison, the RGC axons in the OFL did not begin to show a ventral-dorsal difference of EphB2 protein expression until later at E16 (Fig. 3E), a time when the outer retina already exhibited a strong ventral to dorsal difference in EphB2-βgal expression. Quantitation of β-galactosidase staining in the dorsal-ventral axis in both the OFL and outer retina (Fig. 3G-J) confirmed that a ventral-dorsal difference first appears in the outer retina (or) around late-E14 to E15 but does not appear in the OFL until around E16.

Axon pathfinding errors to the optic disc in retinas lacking EphB receptors

The presence of both EphB receptors and B-ephrins, as shown above, raises the possibility that they function within the developing retina. To investigate, we examined mice with targeted deletions in *EphB2* and *EphB3* to assay EphB function in RGC axon guidance within the retina. To determine the precision of RGC axon pathfinding to the optic disc, we applied the fluorescent dye DiI to selectively label and visualize the trajectories of a small group of RGC axons (Deiner et al., 1997). This type of micro-sampling rather than global labeling of all RGC axons was needed so that any deviations from normal pathfinding would not be obscured by axons from adjacent regions of the retina. We examined a total of 446 retinas from wild-type, heterozygous, and homozygous embryos with targeted disruptions of either *EphB2* or *EphB3* (single mutants), or both *EphB2* and *EphB3* (double mutants).

DiI placement in the retina normally labels a small population of RGC axons that have grown straight to the optic disc (Fig. 4A,B). In our analysis of EphB mutants, we observed aberrant RGC axons which split away from the main group of labeled axons to bypass and never reach the optic disc (Fig. 4C-H,J,K arrows). Some of these aberrant axons grew into the opposite side of the retina for a certain distance and then defasciculated from each other and grew as individual axons (Fig. 4C,E,F,J). By simple visual inspection, it was quite apparent that the severity of aberrant axons differed between retinas and could be grouped into two types. Some retinas had more severely aberrant axons, existing as one large bundle or as several relatively long axons (examples seen in Fig. 4C-F,J), while others were less severe, showing only one or two short aberrantly growing RGC axons (as seen in Fig. 4G,H,K).

Retinal development in *EphB2 EphB3* double mutant mice

In addition to their roles in axon guidance, Eph receptors have been shown to be involved in segmentation, cell migration, and angiogenesis (Adams et al., 1999; Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997; Wang et al., 1998; Xu et al., 1995, 1996, 1999). Therefore, to determine whether the observed aberrant RGC axons may be secondarily due to gross primary defects in retinal development, we examined retinal differentiation and morphology in *EphB2 EphB3* double mutant mice. The overall size and shape of the eye and the position of the optic disc within the retinas of these embryos appeared normal (data not shown), indicating that the absence of EphB2 and EphB3 function did not appear to affect early eye formation or optic fissure closure. Examination of retinal cell density, cell size, as well as thickness and positions of both the RGC layer and the outer retina showed no apparent difference from wild-type animals (Fig. 5A,D), reflecting the presence of normal retinal cell formation and migration. In addition, cell density in the RGC layer (cells/5000 μm^2) was similar between dorsal retina of wild-type (53±4) and double mutant (58±7) animals as well as in ventral retina of wild-type (47±4) and double mutant (57±9) embryos.

To determine if loss of EphB2 and EphB3 may have perturbed pathfinding by affecting the expression of other RGC axon guidance molecules, we analyzed the expression of a number of known retinal axon receptors and cell adhesion molecules. Immunostaining with antibodies against IgCAM superfamily members L1 (Fig. 5B,E) and the netrin receptor DCC (Fig. 5C,F), both of which have been implicated in RGC axon guidance (Brittis et al., 1995; Deiner et al., 1997), produced no detectable difference in the expression of these markers on RGC axons in the double mutants compared to wild type. In addition, in *EphB2 EphB3* double null mutants, netrin was expressed around the cells that line the optic disc, but not elsewhere in the retina, in a pattern indistinguishable from normal animals (data not shown). Lastly, to investigate whether retinal patterning is affected, we examined the expression of ephrin-B2 mRNA in the retinas of *EphB2 EphB3* double mutants (Fig. 5G). The results showed a high dorsal-low ventral pattern of ephrin-B2 mRNA expression similar to that in wild-type retina (see Fig. 2B). Thus, in *EphB2 EphB3* double mutant mice, major aspects of retinal development appeared to have proceeded normally through E16. Although we cannot rule out more subtle effects on retinal development following the loss of Eph receptor function, the finding of misrouted RGC axons in *EphB2 EphB3* double null mutants very likely reflects a direct role of Eph receptors in RGC axon guidance within the retina.

Increased pathfinding errors requires the absence of both EphB2 and EphB3 receptors

The incidence of retinas with RGC axon pathfinding errors was quantified for wild-type and mutant embryos labeled at E15-17 (Fig. 6B; Table 1). The sampling of large numbers of embryos revealed that in approximately 5% of wild-type retinas a few labeled RGC axons did not grow to the optic disc. These aberrantly growing RGC axons in the OFL appeared to be different to previously reported ectopically

located axons in the retinal pigment epithelium (RPE) of wild-type rodent and chick retina (Guillery et al., 1985; Halfter, 1988). To quantify the severity of RGC axon pathfinding errors in the OFL, the total length of aberrant axons for each retina containing errors was determined, and a histogram distribution of total aberrant length is presented in Fig. 6A. Retinas with total aberrant axon length of ≥1000μm were classified as containing type 1 errors, while retinas containing total aberrant axon length of less than 1000μm in length were classified as type 2 errors. Fig. 4C-F,J shows representative examples of type 1 errors, with total aberrant axon length ranging from 1225 μm (4D) to 4100 μm (4E). Examples of type 2 errors include Fig. 4G,H,K.

The incidence of pathfinding errors was highest in *EphB2 EphB3* double null mutants (33%; Fig. 6B), and many of these guidance errors were the more severe type 1 errors (Fig. 6C; Table 1). The results from *EphB2* and *EphB3* single mutants were quite different (Fig. 6B,C). *EphB2* single mutants were similar to wild-type embryos, as were heterozygous animals (Fig. 6B; Table 1). *EphB3* single mutants had a slightly higher total incidence of errors but did not have a significant difference in the more severe type 1 errors (Fig. 6C; Table 1). *EphB2 EphB3* double null mutants had a significantly higher incidence of the more severe type 1 errors (21%) than wild type, heterozygotes, and single homozygotes ($P<0.05$, Fisher's exact test, 2-sided; Fig. 6C).

In addition, we examined E13.5-14.5 retinas during the early period of RGC axon guidance to the optic disc. We found pathfinding errors in 7/46 (15%) retinas of *EphB2 EphB3* double null mutants compared to 1/19 (5%) of heterozygotes or 1/17 (6%) of *EphB3* single mutants. In examining the severity of these errors, 2 (4%) of *EphB2 EphB3* double mutant retinas showed the more severe type 1 errors; type 1 errors were not detected in the *EphB3* mutant or heterozygous retinas examined. Thus, EphB receptor function appears to be necessary for proper RGC axon pathfinding to the optic disc beginning at early stages of embryonic retinal development and not just at the later stages of E15-17.

Dorsal RGC axons are more severely affected than ventral RGC axons

Given the emergence of a ventral-dorsal gradient of EphB2 receptors during retinal development, we examined whether there is a difference in pathfinding errors between RGC axons originating from dorsal retina compared to ventral retina (Fig. 7). Unexpectedly, in double mutants, there were significantly more pathfinding errors in axons originating from dorsal retina (33%) compared to ventral retina (14%; $P<0.05$; see Fig. 7A; Table 1). This difference was even greater when comparing the incidence of more severe type 1 errors from *EphB2 EphB3* dorsal retina (21%) versus ventral retina (2%; $P<0.05$; Fig. 7B; Table 1). A dorsal-ventral difference in the incidence of pathfinding errors (type 1 or 2) was not observed in wild-type or

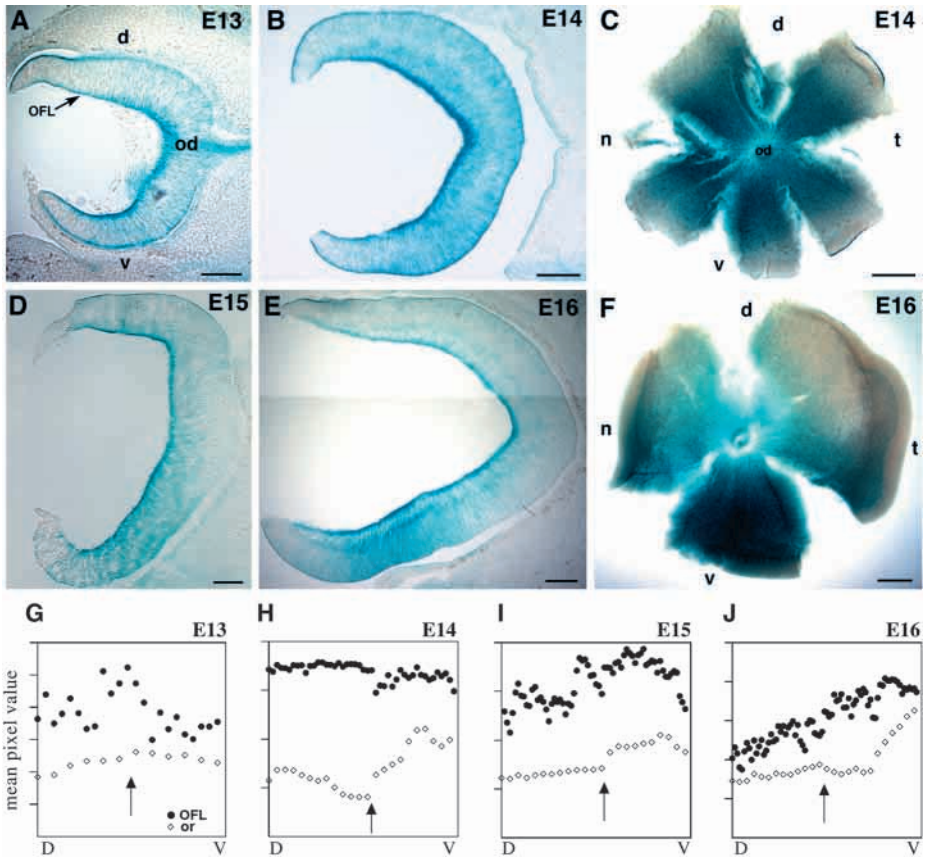

Fig. 3. EphB2-β-gal protein expression in E13-E16 retinas. At E13 (A) and E14 (B), EphB2-β-gal staining is strong in the OFL and weaker in the outer retina in both the dorsal (**d**) and ventral (**v**) regions of the retina in coronal sections. As seen in retina wholemounts at E14 (C), EphB2-β-gal is uniformly expressed throughout the retina with no apparent gradient. By E15 (D), a gradient of EphB2-β-gal expression has begun to appear in outer retina but is not yet present within the OFL. At E16 in coronal section (E), a dorsal-ventral difference of EphB2-β-gal staining is seen in the outer retina and also has begun to appear in the OFL. (E is a composite of two images) Because of the presence of a strong high ventral to low dorsal pattern of EphB2-β-gal expression at E16 in the thicker outer retina, the pattern of expression in the much thinner OFL is masked, and the retina when examined as a wholemount exhibits a strong ventral to dorsal gradient (F). Dorsal (d) is up and ventral (v) is down for all images. od, optic disc. In C, F: n, nasal; t, temporal. Scale bars, A,B,D,E: 100 μm; C,F: 200 μm. EphB2-β-gal protein expression was quantified during E13-E16 retinal development (G-J), both in the OFL (•) and the outer retina (◊). At E13 (G) and E14 (H), no general dorsal-ventral gradient is seen, although sometimes a difference is seen in the outer retina at late E14 (H). By E15 (I), a shallow gradient is seen in the outer retina (◊), but expression within the OFL (•) is quite uniform. By E16 (J), a ventral to dorsal gradient has begun to appear in both the OFL and the outer retina. The mean pixel value (y axis) is in arbitrary units for each retina, and therefore comparisons are only valid within a retina. Arrows indicate the location of the optic disc.

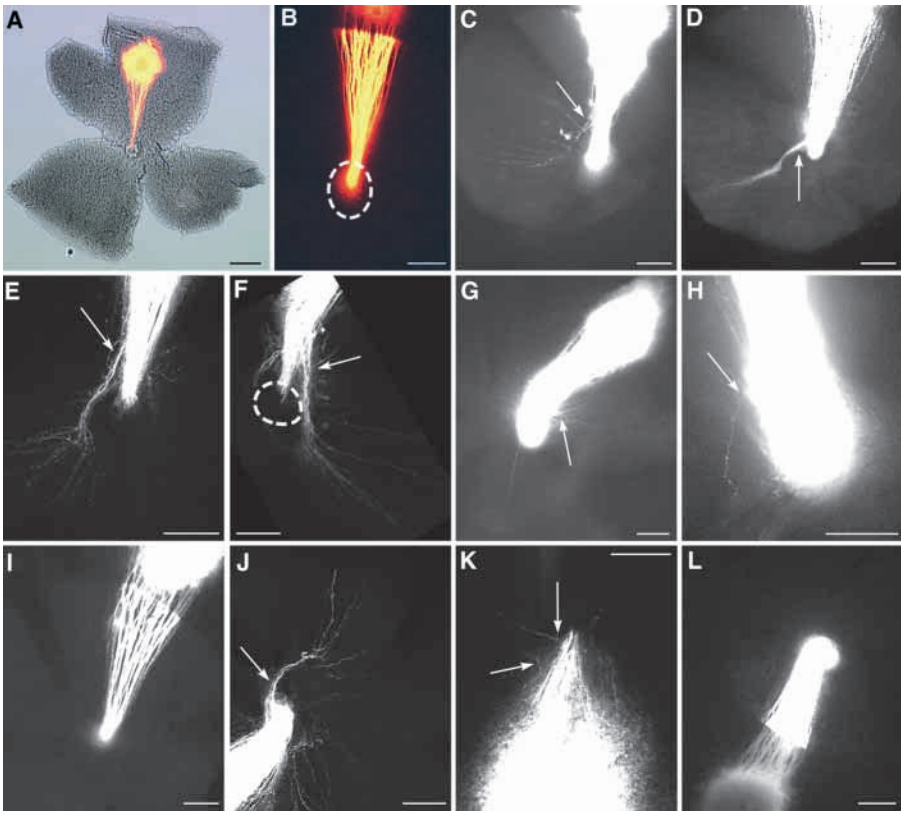

Fig. 4. DiI labeling of RGC axons and examples of aberrant axons in the vicinity of the optic disc in *EphB2 EphB3* mutant mouse retinas. (A) In wild-type animals, a small crystal of DiI placed into peripheral retina labels a 'cone' of RGC axons (red) extending from the labeling site to the optic disc. (B) Higher magnification of DiI labeled RGC axons in the vicinity of the optic disc (dashed circle) shows that normally all labeled RGC axons reach the optic disc. (C-L) Examples of RGC axon trajectories following DiI labeling in dorsal (C-I) or ventral retina (J-L). C-F and J show examples of pathfinding errors in *EphB2 EphB3* double null mutant retinas in which a small fascicle consisting of several long aberrantly growing RGC axons (arrows) split off from the main group of axons. (The dashed circle in F indicates position of the optic disc.) G,H and K show examples of milder errors in *EphB2 EphB3* double null mutant (G,K) or *EphB3* single mutant (H) retinas consisting of one or two labeled aberrantly growing RGC axons. I,L show retinas from *EphB2 EphB3* double null mutants in which no aberrantly growing RGC axons were detected. To avoid the bright fluorescence of the DiI crystal, the microscope diaphragm was stopped down for photography; portions of the diaphragm edge are seen in B,C,D,I,L. In all panels, dorsal retina is up. Scale bars, (A) 200 μm; (B-L) 100 μm.

heterozygous animals. This dorsal specificity is surprising considering that, during the developmental stages examined, EphB3 is expressed uniformly (Fig. 1) and EphB2 is first expressed uniformly and then is expressed predominantly in ventral retina (Figs 1 and 3). This preferential defect in dorsal axon pathfinding indicates that the loss of EphB2 protein does not affect axons which express the highest levels of EphB2 and that the loss of EphB3 protein may not affect all EphB3-expressing axons equally.

Eph function in RGC axon guidance independent of the kinase domain

Eph receptors have cytoplasmic protein tyrosine kinase domains and are therefore thought to function by transducing

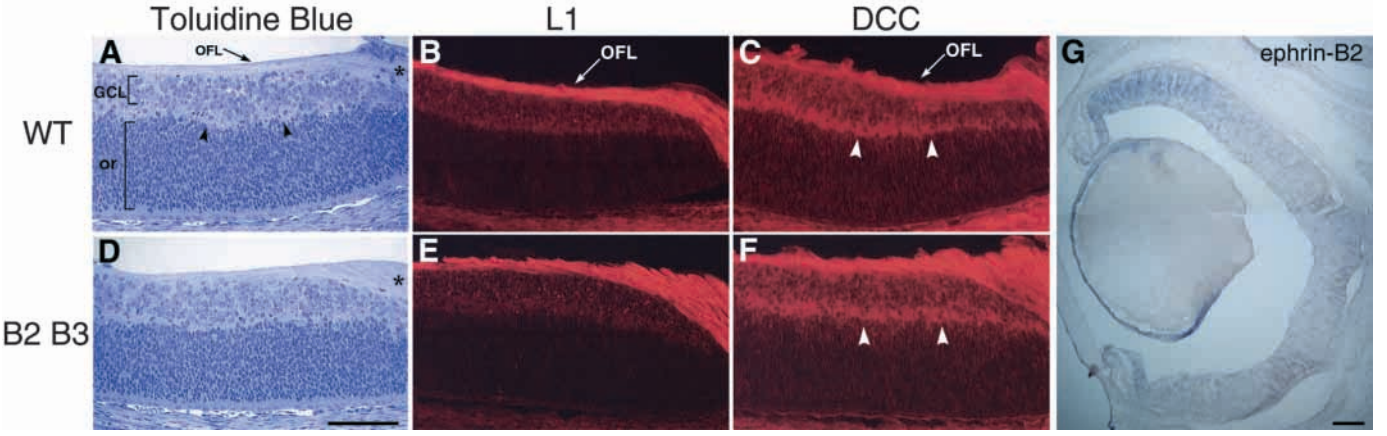

Fig. 5. Morphology and immunostaining of wild-type (A-C) and *EphB2 EphB3* double null mutant (D-F,G) retinas in coronal section. Toluidine blue stained 1 μm plastic sections (A,D) show no apparent histological difference between wild-type retina (A) and *EphB2 EphB3* double null mutant retina (D). Antibodies against L1 (B,E) and DCC (C,F) label RGC axons of the OFL strongly in both wild-type (B-C) and mutant (E-F) retina. In addition, RGC cell bodies in the ganglion cell layer are labeled weakly. DCC is also present in the RGC dendrites in the developing IPL (arrowheads in C,F and A). All sections in A-F show a region of E16 dorsal retina at the level of the optic disc; RGC axons exiting the retina at the optic disc can be seen on the right of each panel (indicated by * in A and D). (G) In situ hybridization of E16 *EphB2 EphB3* double null mutant retinas shows a normal dorsal-ventral distribution of ephrin-B2 mRNA in coronal section (dorsal is up). OFL, optic fiber layer; GCL, retinal ganglion cell layer; or, outer retina. Scale bars, (A-F) 100 μm; (G) 100 μm.

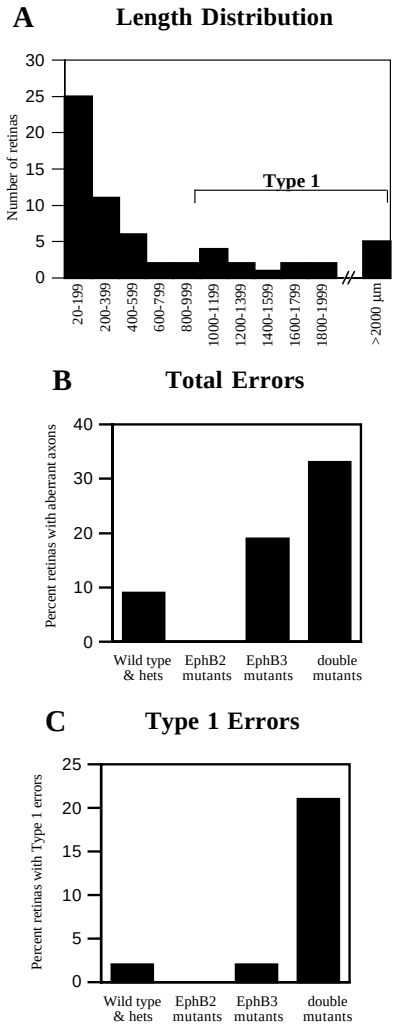

Fig. 6. Incidence of retinas showing RGC axon pathfinding errors in E15-17.5 embryos. (A) Histogram showing distribution of total length of aberrant axons in retinas with pathfinding errors from all genotypes. Retinas were grouped by total aberrant axon length into 200 µm intervals as shown on the x-axis. There were 5 retinas with total length >2000 µm, ranging up to 6000 µm, which have been displayed as one bar. Retinas with total aberrant axon length ≥1000 µm, as indicated by the bracket, were classified as showing type 1 errors. (B) The total incidence of retinas (expressed as a percentage of retinas examined) showing any RGC axon pathfinding defects (both type 1 and type 2 errors) from dorsal DiI labeling for wild-type and heterozygous animals, *EphB2* single mutants, *EphB3* single mutants, or *EphB2 EphB3* double null mutants. There were zero retinas with labeled pathfinding errors from *EphB2* retinas. (C) The incidence, expressed as a percentage of retinas examined, that had the more severe type 1 errors consisting of several labeled aberrant RGC axons.

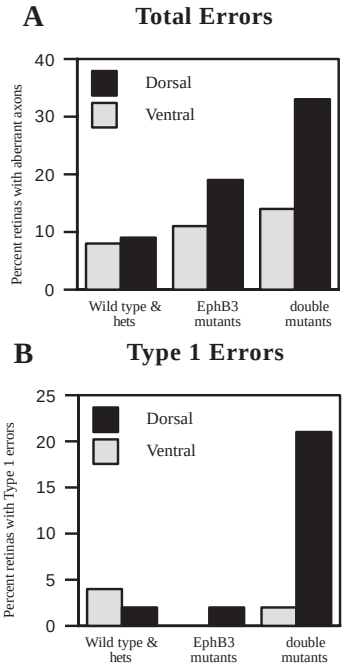

Fig. 7. Incidence of RGC axon guidance defects from dorsal versus ventral retina. (A) The total incidence of retinas in E15-17.5 embryos that show any RGC axon pathfinding errors from dorsal DiI labeling (black bars) or from ventral DiI labeling (stippled bars) for the various genotypes. (No ventral labeling was done for *EphB2* single mutants.) (B) Incidence of the more severe type 1 errors in dorsal versus ventral retina.

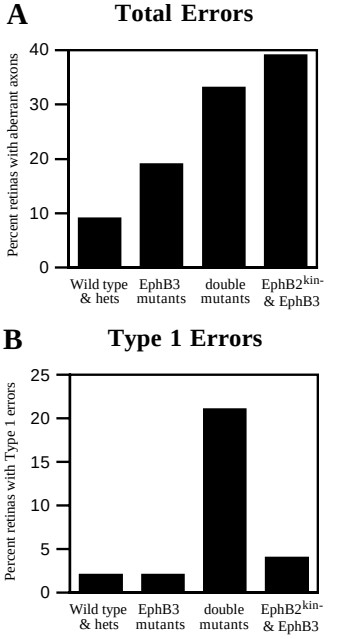

Fig. 8. Incidence of pathfinding errors from dorsal retina in E15-17.5 embryos. (A) The total incidence of any RGC axon pathfinding errors from wild-type and heterozygous animals, *EphB3* single mutants, *EphB2 EphB3* double null mutants (labeled 'double mutants'), and *EphB2*$^{lacZ(kin-)}$ *EphB3* double mutants (labeled '*EphB2*$^{kin-}$ & *EphB3*'). (B) Incidence of the more severe type 1 errors.

signals through phosphorylation. However, recent experiments have highlighted the potential for Eph molecules to function not through kinase action but by 'reverse' signaling through B-ephrins (Brückner et al., 1997; Henkemeyer et al., 1996; Holland et al., 1996). The requirement for EphB2 kinase activity in RGC axon guidance to the optic disc was investigated in mutant embryos containing the *EphB2*$^{lacZ(kin-)}$

allele which lacks the kinase domain and cytoplasmic tail of EphB2. We found that *EphB2*$^{lacZ(kin-)}$ *EphB3* double homozygotes exhibited much milder defects in RGC axon guidance to the optic disc compared to the *EphB2 EphB3* double null homozygotes (Fig. 8; Table 1). While there was an increased incidence of the less severe type 2 errors in *EphB2*$^{lacZ(kin-)}$ *EphB3* double mutants (Table 1, Fig. 8A), the incidence of the more severe type 1 errors (4%) was significantly less in this mutant combination when compared to *EphB2 EphB3* double null mutants (21%, $P<0.05$; Fig. 8B). This incidence of type 1 errors in *EphB2*$^{lacZ(kin-)}$ *EphB3* double mutants was similar to *EphB3* single mutants (2%, $P=0.6$). Thus, EphB2 protein lacking its tyrosine kinase domain can function in aspects of RGC axon guidance to the optic disc, demonstrating a potential role of its extracellular domain in axon guidance.

DISCUSSION

Embryonic mouse RGCs express multiple EphB receptors and ephrin-B ligands during a primary period of RGC axon pathfinding to the optic disc. During this period (E12-16), EphB receptors are distributed uniformly or in a high ventral to low dorsal expression pattern while ephrin-B ligands are either distributed uniformly or in the opposite high dorsal to low ventral manner. Analysis of *EphB2 EphB3* null embryos showed an increased incidence of RGC axon pathfinding errors to the optic disc. Surprisingly, although EphB2 is more strongly expressed in ventral retina, the concurrent elimination of EphB2 and EphB3 affected dorsal RGC axons and not ventral axons. In addition, analysis of mutant animals expressing a truncated EphB2 receptor lacking its kinase domain indicated that the EphB2 extracellular domain is important for dorsal RGC pathfinding to the disc. Our data, together with previous studies of RGC axon mapping in targets, demonstrate that Eph molecules mediate RGC axon pathfinding at multiple sites along the visual pathway. However, unlike RGC axon guidance in the superior colliculus which is proposed to involve Eph tyrosine kinase activity, RGC axon guidance within the retina to the optic disc is mediated in part by a kinase-independent function of EphB2.

Severity of axon guidance defects in EphB mutants

The *EphB2 EphB3* double null mutants showed a stronger phenotype than either single mutant alone, especially with regard to the more severe type 1 errors. However, there were also many cases of *EphB2 EphB3* double null mutants in which no aberrantly growing RGC axons were seen following micro-labeling at a single retinal site (Table 1; Fig. 4I,L). Since our DiI labeling technique only sampled a subset of the RGC axons in each retina, it is possible that aberrant RGC axons were also present, but not labeled, in these retinas. Nevertheless, aberrantly growing axons made up only a small portion of all RGC axons and overall the majority of the RGC axons grew correctly to the optic disc and entered the optic nerve. This observation suggests overlapping function with other guidance molecules, possibly other EphB receptors. EphB1 is a good candidate since it is strongly expressed in RGCs during this period of retinal development (Fig. 1A,D).

In *EphB2 EphB3* double null mutants, the location of RGC pathfinding errors near the optic disc is similar to the RGC axon guidance defects seen in netrin-1- and DCC-deficient embryos (Deiner et al., 1997). However, the *netrin-1* and *DCC* mutant phenotypes were much more severe, with the majority of RGC axons affected and resulting in optic nerve hypoplasia, a finding which was not present in *EphB2 EphB3* mutants. Interestingly, in neither netrin-1-, DCC-, nor EphB2 Eph3-deficient embryos were RGC axon pathfinding defects found further from the optic disc in peripheral or mid-retina. Thus, the retina may consist of at least two guidance regions, one near the optic disc in which netrin-1/DCC and EphB2, EphB3 interactions are important and another more peripheral region(s) in which other guidance mechanisms are used.

Difference in dorsal versus ventral retina

Given the expression patterns of EphB2 and EphB3, the finding that deletion of these two receptors preferentially affected RGC axon guidance from dorsal but not ventral retina was unexpected. EphB2 mRNA and protein are expressed uniformly in dorsal and ventral retina at E12-14 and begin to appear more strongly in ventral compared to dorsal retina at E15-16. Thus the affected RGC axons are not those that express the highest levels of EphB2. EphB3 mRNA is present uniformly in dorsal and ventral retina throughout the period studied. Although we have not examined EphB3 protein expression, we note that EphB2 protein generally reflects the pattern of EphB2 mRNA expression, suggesting that EphB3 protein is likely uniformly expressed in the dorsal-ventral axis and that the affected dorsal axons may not express the highest levels of EphB3 receptor.

This dorsal effect may reflect developmental differences between dorsal and ventral retina. For example, in fish retina the IgCAM molecule neurolin is expressed on RGC axons in all regions of the retina (Paschke et al., 1992), but antibody perturbation of its function affects RGC axon trajectories in dorsal but not ventral retina (Ott et al., 1998). In humans, patients with superior segmental optic nerve hypoplasia have abnormalities in superior (dorsal) but not inferior (ventral) RGC axons (Kim et al., 1989). These examples along with the present findings of specific dorsal retinal defects in EphB mutant mice underscores differences in developmental mechanisms between dorsal and ventral retina.

Receptor tyrosine kinase function and RGC axon pathfinding

A role of Eph receptor molecules independent of their kinase domain for pathfinding within the retina is in contrast to the proposed kinase-dependent function of Eph receptors in mediating RGC axon mapping onto CNS targets. However, since retinotopic mapping at the target has thus far only been demonstrated with EphA receptors, and our results here involve EphB receptors, it is formally possible that a difference exists such that EphA receptor function depends on kinase activity while EphB function is kinase independent. However, another possibility is that Eph receptors mediate RGC axon pathfinding at multiple sites along the visual pathway by different mechanisms. Thus, Eph function in the retina itself may be kinase independent, but Eph kinase activity may be essential later during RGC axon topographic mapping within

CNS targets. It is possible to differentiate between these two models by examining the role of EphA receptors in intra-retinal axon pathfinding and the involvement of the kinase domain of EphB receptors in RGC axon mapping within the superior colliculus.

Possible function of EphB extracellular domains

The results from analysis of *EphB2$^{lacZ(kin-)}$ EphB3* double mutants indicate that the occurrence of the severe type 1 errors appears to be due to the loss of the extracellular domain and not the intracellular kinase domain of EphB2. This finding, along with the more severe dorsal effect even though EphB2 and EphB3 expression is not specifically dorsal, is difficult to explain using the conventional model of ephrins binding to Eph receptors on axon growth cones and triggering intracellular signaling through Eph phosphorylation. Rather, it indicates that the EphB2 extracellular domain may act independently in pathfinding, possibly by 'reverse' signaling through ephrin-B molecules. Indeed, there is biochemical evidence that EphB2 can trigger phosphorylation of ephrin-B molecules (Brückner et al., 1997; Holland et al., 1996) as well as in vivo evidence suggesting reverse signaling in axon pathfinding of the anterior commissure (Henkemeyer et al., 1996) and cell sorting in the hindbrain (Mellitzer et al., 1999; Xu et al., 1999).

Some aspects of the pathfinding errors in the retina could be explained by a reverse signaling model. For instance, dorsal RGC axons that express ephrin-B1 and ephrin-B3 may respond to an increasing level of EphB2 protein as they approach the disc. If ephrin-expressing axons normally fasciculate in response to EphB extracellular domains, elimination of EphB2 and EphB3 could result in a RGC axon 'defasciculation' phenotype near the optic disc. However, a simple reverse signaling model through ephrin-B1 or B3 is not sufficient since only dorsal axons are affected even though ephrin-B1 and B3 are found in both dorsal and ventral retina. In conclusion, our results together with published studies demonstrate that Eph-ephrin interactions are important in RGC axon pathfinding at multiple locations in the visual system and that RGC axon pathfinding to the optic disc involves EphB molecules acting in a kinase-independent manner.

We would like to thank David Wilkinson for kindly providing the EphB3 in situ probe and Peter Bacchetti of the UCSF Division of Biostatistics for help with the statistical analysis. We appreciate the help of Jun Velarde and Doug Yasumura in plastic sectioning. We thank Chris Severin for assistance in animal genotyping. E. B. was supported by a NIH NRSA Postdoctoral Fellowship, D. W. S. by NIH EY10688, the That Man May See Foundation, and NIH EY02162 to the Dept. of Ophthalmology, and M. H. by the March of Dimes Birth Defects Foundation, the Muscular Dystrophy Association, and NIH NS36671. D. W. S. is the recipient of a Jules and Doris Stein Professorship from Research to Prevent Blindness.

REFERENCES

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W. and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev.* **13**, 295-306.

Bartsch, U., Kirchhoff, F. and Schachner, M. (1989). Immunohistological localization of the adhesion molecules L1, N-CAM, and MAG in the developing and adult optic nerve of mice. *J. Comp. Neurol.* **284**, 451-462.

Bauch, H., Stier, H. and Schlosshauer, B. (1998). Axonal versus dendritic outgrowth is differentially affected by radial glia in discrete layers of the retina. *J. Neurosci.* **18**, 1774-1785.

Becker, N., Seitanidou, T., Murphy, P., Mattei, M. G., Topilko, P., Nieto, M. A., Wilkinson, D. G., Charnay, P. and Gilardi-Hebenstreit, P. (1994). Several receptor tyrosine kinase genes of the Eph family are segmentally expressed in the developing hindbrain. *Mech. Dev.* **47**, 3-17.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M. and Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* **137**, 354-366.

Braissant, O. and Wahli, W. (1998). A simplified in situ hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. *Biochemica (Boehringer Mannheim)* **1998**, 10-16.

Braisted, J. E., McLaughlin, T., Wang, H. U., Friedman, G. C., Anderson, D. J. and O'Leary, D. D. M. (1997). Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. *Dev. Biol.* **191**, 14-28.

Brambilla, R., Bruckner, K., Orioli, D., Bergemann, A. D., Flanagan, J. G. and Klein, R. (1996). Similarities and differences in the way transmembrane-type ligands interact with the Elk subclass of Eph receptors. *Mol. Cell. Neurosci.* **8**, 199-209.

Brittis, P. A., Lemmon, V., Rutishauser, U. and Silver, J. (1995). Unique changes of ganglion cell growth cone behavior following cell adhesion molecule perturbations: a time-lapse study of the living retina. *Mol. Cell. Neurosci.* **6**, 433-449.

Brückner, K., Pasquale, E. B. and Klein, R. (1997). Tyrosine phosphorylation of transmembrane ligands for Eph receptors. *Science* **275**, 1640-1643.

Cheng, H. J., Nakamoto, M., Bergemann, A. D. and Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82**, 371-381.

Chung, W. W., Lagenaur, C. F., Yan, Y. M. and Lund, J. S. (1991). Developmental expression of neural cell adhesion molecules in the mouse neocortex and olfactory bulb. *J. Comp. Neurol.* **314**, 290-305.

Connor, R. J., Menzel, P. and Pasquale, E. B. (1998). Expression and tyrosine phosphorylation of Eph receptors suggest multiple mechanisms in patterning of the visual system. *Dev. Biol.* **193**, 21-35.

Deiner, M. S., Kennedy, T. E., Fazeli, A., Serafini, T., Tessier-Lavigne, M. and Sretavan, D. W. (1997). Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. *Neuron* **19**, 575-589.

Dräger, U. C. (1985). Birth dates of retinal ganglion cells giving rise to the crossed and uncrossed optic projections in the mouse. *Proc. R. Soc. Lond. B Biol. Sci.* **224**, 57-77.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M. and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* **82**, 359-370.

Eph Nomenclature Committee. (1997). Unified nomenclature for Eph family receptors and their ligands, the ephrins. *Cell* **90**, 403-404.

Flanagan, J. G. and Vanderhaeghen, P. (1998). The ephrins and Eph receptors in neural development. *Annu. Rev. Neurosci.* **21**, 309-345.

Frisén, J., Yates, P. A., McLaughlin, T., Friedman, G. C., O'Leary, D. D. M. and Barbacid, M. (1998). Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. *Neuron* **20**, 235-243.

Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G. et al. (1996). Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* **17**, 9-19.

Guillery, R. W., Lysakowski, A. and Price, S. (1985). On the distribution and probable origin of axonal bundles in the pigment epithelium of the eyecup. *Brain Res.* **349**, 293-295.

Halfter, W. (1988). Aberrant optic axons in the retinal pigment epithelium during chick and quail visual pathway development. *J. Comp. Neurol.* **268**, 161-170.

Henkemeyer, M., Orioli, D., Henderson, J. T., Saxton, T. M., Roder, J.,

Pawson, T. and Klein, R. (1996). Nuk controls pathfinding of commissural axons in the mammalian central nervous system. *Cell* **86**, 35-46.

Himanen, J. P., Henkemeyer, M. and Nikolov, D. B. (1998). Crystal structure of the ligand-binding domain of the receptor tyrosine kinase EphB2. *Nature* **396**, 486-491.

Holash, J. A. and Pasquale, E. B. (1995). Polarized expression of the receptor protein tyrosine kinase Cek5 in the developing avian visual system. *Dev. Biol.* **172**, 683-693.

Holash, J. A., Soans, C., Chong, L. D., Shao, H., Dixit, V. M. and Pasquale, E. B. (1997). Reciprocal expression of the Eph receptor Cek5 and its ligand(s) in the early retina. *Dev. Biol.* **182**, 256-269.

Holland, S. J., Gale, N. W., Mbamalu, G., Yancopoulos, G. D., Henkemeyer, M. and Pawson, T. (1996). Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* **383**, 722-725.

Hornberger, M. R., Dutting, D., Ciossek, T., Yamada, T., Handwerker, C., Lang, S., Weth, F., Huf, J., Wessel, R., Logan, C. et al. (1999). Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. *Neuron* **22**, 731-742.

Kim, R. Y., Hoyt, W. F., Lessell, S. and Narahara, M. H. (1989). Superior segmental optic hypoplasia: a sign of maternal diabetes. *Arch. Ophthalmol.* **107**, 1312-1315.

Krull, C. E., Lansford, R., Gale, N. W., Collazo, A., Marcelle, C., Yancopoulos, G. D., Fraser, S. E. and Bronner-Fraser, M. (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr. Biol.* **7**, 571-580.

LaVail, M. M. and Battelle, B. A. (1975). Influence of eye pigmentation and light deprivation on inherited retinal dystrophy in the rat. *Exp. Eye Res.* **21**, 167-192.

Marcus, R. C., Gale, N. W., Morrison, M. E., Mason, C. A. and Yancopoulos, G. D. (1996). Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina. *Dev. Biol.* **180**, 786-789.

Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

Nakamoto, M., Cheng, H. J., Friedman, G. C., McLaughlin, T., Hansen, M. J., Yoon, C. H., O'Leary, D. D. and Flanagan, J. G. (1996). Topographically specific effects of ELF-1 on retinal axon guidance in vitro and retinal axon mapping in vivo. *Cell* **86**, 755-766.

Orioli, D., Henkemeyer, M., Lemke, G., Klein, R. and Pawson, T. (1996). Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J.* **15**, 6035-6049.

Ott, H., Bastmeyer, M. and Stuermer, C. A. (1998). Neurolin, the goldfish homolog of DM-GRASP, is involved in retinal axon pathfinding to the optic disk. *J. Neurosci.* **18**, 3363-3372.

Paschke, K. A., Lottspeich, F. and Stuermer, C. A. (1992). Neurolin, a cell surface glycoprotein on growing retinal axons in the goldfish visual system, is reexpressed during retinal axonal regeneration. *J. Cell Biol.* **117**, 863-875.

Silver, J. and Rutishauser, U. (1984). Guidance of optic axons in vivo by a preformed adhesive pathway on neuroepithelial endfeet. *Dev. Biol.* **106**, 485-499.

Smith, A., Robinson, V., Patel, K. and Wilkinson, D. G. (1997). The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. *Curr. Biol.* **7**, 561-570.

Stier, H. and Schlosshauer, B. (1995). Axonal guidance in the chicken retina. *Development* **121**, 1443-1454.

Wang, H. U. and Anderson, D. J. (1997). Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* **18**, 383-396.

Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**, 741-753.

Xu, Q., Alldus, G., Holder, N. and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the *Xenopus* and zebrafish hindbrain. *Development* **121**, 4005-4016.

Xu, Q., Alldus, G., Macdonald, R., Wilkinson, D. G. and Holder, N. (1996). Function of the Eph-related kinase rtk1 in patterning of the zebrafish forebrain. *Nature* **381**, 319-322.

Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-271.