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Topographic Mapping in Dorsoventral Axis of the *Xenopus* Retinotectal System Depends on Signaling through Ephrin-B Ligands

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Summary

Ephrin-B and EphB are distributed in matching dorsoventral gradients in the embryonic *Xenopus* visual system with retinal axons bearing high levels of ligand (dorsal) projecting to tectal regions with high receptor expression (ventral). In vitro stripe assays show that dorsal retinal axons prefer to grow on EphB receptor stripes supporting an attractive guidance mechanism. In vivo disruption of EphB/ephrin-B function by application of exogenous EphB or expression of dominant-negative ephrin-B ligand in dorsal retinal axons causes these axons to shift dorsally in the tectum, while misexpression of wild-type ephrin-B in ventral axons causes them to shift ventrally. These dorsoventral targeting errors are consistent with the hypothesis that an attractive mechanism that requires ephrin-B cytoplasmic domain is critical for retinotectal mapping in this axis.

Introduction

The development of appropriate connections between neurons and target cells in the brain is of fundamental importance in the visual system, where retinal ganglion cell (RGC) axons project a topographic representation of the visual world onto the brain. In the retinofugal projection of lower vertebrates, the temporo-nasal axis of the retina maps along the anterior-posterior axis of the tectum (the A-P axis), and the ventro-dorsal retinal axis to the dorso-ventral tectal axis (the D-V axis). Sperry (1963) proposed that orthogonal matching gradients of ligands and receptors establish this topographic projection. Recently it has become clear that gradients of the A class of Eph tyrosine kinase receptors and their ligands, the ephrins, are particularly important in the mapping of A-P axis (reviewed in Drescher et al., 1997; Flanagan and Vanderhaeghen, 1998; O'Leary and Wilkinson, 1999; Wilkinson, 2000). The map along the A-P axis is thought to depend on the repulsive activity of ephrin-A ligands on EphA-expressing axons. The molecular basis of D-V topography, however, remains unknown. In zebrafish, mutations in single genes affect the D-V retinotopic organization of the visual projection in the tectum (Baier et al., 1996; Trowe et al., 1996), but the identity of these genes is not known. Retinotectal mapping along the D-V axis is also affected in mouse mutant for the ephrin-A2 and ephrin-A5 ligands (Feldheim et al., 2000). However, these defects may be secondary to the loss of A-P labels. So far, the most promising candidates for receptors and ligands in the D-V axis of the type Sperry proposed are the B-type Eph receptors and their ligands.

In chick and mouse, EphB receptors and ephrin-Bs are expressed in opposite gradients across the retina, with high levels of EphB receptors in ventral retina and ephrin-B ligands abundant in dorsal retina (Holash and Pasquale, 1995; Marcus et al., 1996; Braisted et al., 1997; Holash et al., 1997; Connor et al., 1998; Birgbauer et al., 2000). In chick retina, the expression of both EphB receptors and ephrin-B ligands is under the control of the transcription factors cVax and Tbx5, respectively, which play a role in the early D-V patterning of the retina (Schulte et al., 1999; Koshiba-Takeuchi et al., 2000). Misexpression of both cVax2 and Tbx5 in chick retina causes topographic targeting errors of RGC axons along the D-V axis of the tectum (Schulte et al., 1999; Koshiba-Takeuchi et al., 2000), further suggesting a possible role of B-type Eph family molecules in D-V mapping.

Unlike the A-P axis, in the D-V axis, RGC axons expressing the highest level of EphB receptors project to the area of the tectum with the highest ligand expression. For example, in chick embryos, EphB2 is expressed on RGC axons from the ventral half of the retina (Holash and Pasquale, 1995); and a ligand for this receptor, ephrin-B1, is maximally expressed in the dorsal tectum (Braisted et al., 1997). This suggests that ephrin-Bs might be implicated in attractive rather than repulsive guidance (Braisted et al., 1997; O'Leary and Wilkinson, 1999). However, other results show that ephrin-Bs can act as repulsive guidance cues for EphB receptor-expressing neurons (Meima et al., 1997; Wang and Anderson, 1997; Kullander et al., 2001a; Yokoyama et al., 2001). This is consistent with the finding that in the *Xenopus* visual system expression of ephrin-B ligands in the optic chiasm repels EphB-expressing RGC axons causing them to project ipsilaterally (Nakagawa et al., 2000).

The possibility of "reverse" signaling through the ephrin ligands (i.e., upon activation by their cognate Eph receptor, ephrin ligands transduce a signal into the ephrin-expressing cell) has been shown for both ephrin-A (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001; Knoll et al., 2001) and ephrin-B molecules (Henkemeyer et al., 1996; Jones et al., 1998; Mellitzer et al., 1999; Xu et al., 1999; Adams et al., 2001). Genetic analyses show that ephrin-Bs might function as signaling receptors during axon pathfinding (Henkemeyer et al., 1996; Orioli et al., 1996; Birgbauer et al., 2000; Kullander et al., 2001b). In the eye of EphB2 and EphB3 mutant mouse, dorsal RGC axons that normally express low levels of EphB show defects in guidance to the optic disc (Birgbauer et al., 2000). This defect is partially rescued in EphB3 mutant mice by expressing a truncated EphB2 receptor that lacks tyrosine kinase activity (Birgbauer et al., 2000). It is therefore likely that the extracellular domain of EphB2, expressed on ventral retinal cells, provides a signal that directly guides ephrin-B-expressing RGC axons. Biochemical evidence

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demonstrating that the extracellular domain of EphB2 can trigger tyrosine phosphorylation of the ephrin-B cytoplasmic domain also supports the idea of reverse signaling (Holland et al., 1996; Bruckner et al., 1997). Recent in vitro data shows that the extracellular domain of EphB receptors applied to cultures of retinal explants can induce growth cone collapse and neurite retraction (Birgbauer et al., 2001). It is therefore possible that signaling through ephrin-B is involved in D-V axis formation in the retinotectal map.

The present study investigates the function of B-type Eph receptors and their ligands in patterning the D-V axis of the visual projection in the vertebrate *Xenopus*. Opposing D-V gradients of EphB (high ventral to low dorsal) and ephrin-B (high dorsal to low ventral) are found in both the retina and the tectum. In an in vitro assay for axon growth, the extracellular domain of the EphB receptor modulates the elongation rate of ephrin-B-expressing retinal neurites, and a dominant-negative ephrin-B ligand, lacking a cytoplasmic domain, abolishes this effect when introduced into retinal cells. In an in vitro stripe assay, dorsal retinal axons showed an attractive guidance response to the EphB receptor. Finally, in vivo disruption of EphB/ephrin-B function induces targeting errors of visual axons along the D-V axis of the tectum that are consistent with a cell-autonomous function of ephrin-Bs in topographic mapping.

Results

EphB Receptors and Ephrin-B Ligands Are Expressed in a Complementary Pattern in *Xenopus* Retinal Cells and Axons

In *Xenopus*, the distribution of EphB receptors has been previously examined using the extracellular domain of the zebrafish ephrin-B2a fused to alkaline phosphatase (ephrin-B2-AP), thought to bind all B-type Eph receptors (Nakagawa et al., 2000). As development proceeds, the probe labeled a small region in the ventralmost part of the *Xenopus* retina. Figure 1A shows labeling at stage 40, when retinotectal projections begin to branch in the tectum. The zebrafish EphB4b ectodomain fused to alkaline phosphatase (EphB4-AP) used as an affinity probe shows that ephrin-B ligands distribute in a high-dorsal to low-ventral gradient in *Xenopus* retina from stage 28 (data not shown) to stage 40 (Figure 1B).

To examine the expression of ephrin-Bs and EphB receptors on axons and growth cones, we used AP-tag probes on cultured explants isolated from the most dorsal and ventral regions of the retina. Both axons and growth cones of ventral retinal neurons were found to bind ephrin-B2-AP (Figure 1C) but not EphB4-AP (Figure 1D), indicating that EphB receptors are expressed on the surface of growing retinal fibers. In contrast, axons extending from dorsal retinal explants expressed ephrin-B ligands as they were labeled with EphB4-AP (Figure 1F) but not ephrin-B2-AP (Figure 1E).

mRNA In Situ Reveals the Identity of Specific Types of EphB Receptors and Ephrin-B Ligands in the Developing *Xenopus* Retina

The AP-tag probes reveal the distribution of proteins, but do not distinguish which members of the Eph family

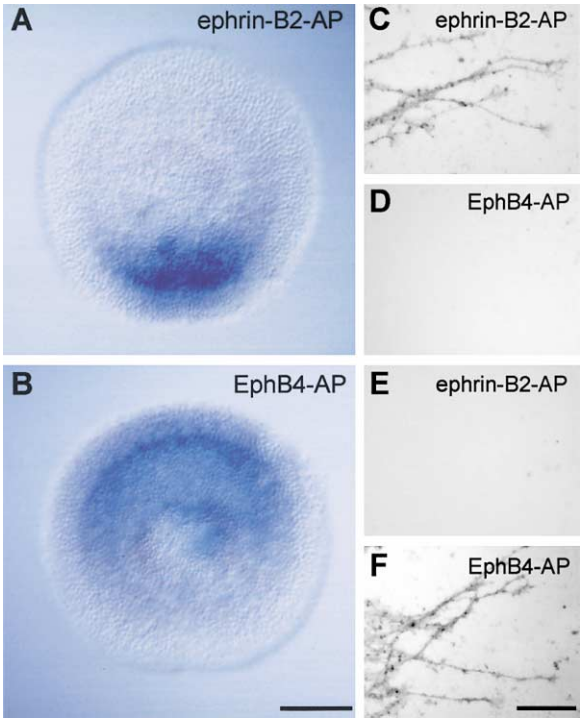

Figure 1. Localization of EphB Receptors and Ephrin-Bs in Developing Retina

Expression of EphB receptors and ephrin-Bs revealed by affinity probe with ephrin-B2-AP and EphB4-AP probes.

(A and B) Whole-mount dissected retina from stage 40 tadpoles, showing the complementary expression of EphB receptors and ephrin-Bs along the dorso-ventral axis of the retina.

(C–F) Retinal neurites from 24 hr explant cultures. Virtually all neurites from ventral retina (C and D) express EphB receptors, whereas those from dorsal retina (E and F) express ephrin-B ligands. In both cases, labeling is present in the growth cone and along the axon shaft. Scale bars: 50 μm, (A) and (B); 100 μm, (C)–(F)

of receptors and ligands are expressed in the *Xenopus* retina. This issue was addressed by in situ hybridization on stage 39 embryos. At this stage, RGC axons have just begun to innervate the optic tectum. *Xenopus* cDNAs for four B-class Eph receptors have been cloned, including EphB1 (Xek) (Jones et al., 1995; Scales et al., 1995; Smith et al., 1997), EphB2 (Tanaka et al., 1998), EphB3 (TCK) (Scales et al., 1995), and EphB4 (G51) (Brandli and Kirschner, 1995; Helbling et al., 1999). All four EphB receptors are found in the developing retina. EphB1 mRNA is detected in the inner plexiform layer (IPL), in which RGC dendrites and amacrine cell axons run, and outer plexiform layer (OPL) (Figure 2A). As reported previously (Jones et al., 1995), it appears uniformly distributed along the D-V axis of the retina. EphB2 mRNA is expressed in all retinal layers with the highest level in the IPL (Figure 2B) but is much stronger in ventral than dorsal retina (Figure 2B). Expression of EphB3 and EphB4 mRNAs is very low and is restricted to the IPL (Figures 2C and 2D). In cross-sections through the optic nerve head of the retina, however, it is possible to detect mRNAs for all EphB receptors. This indicates that transcripts are expressed in cells lining the optic nerve head,

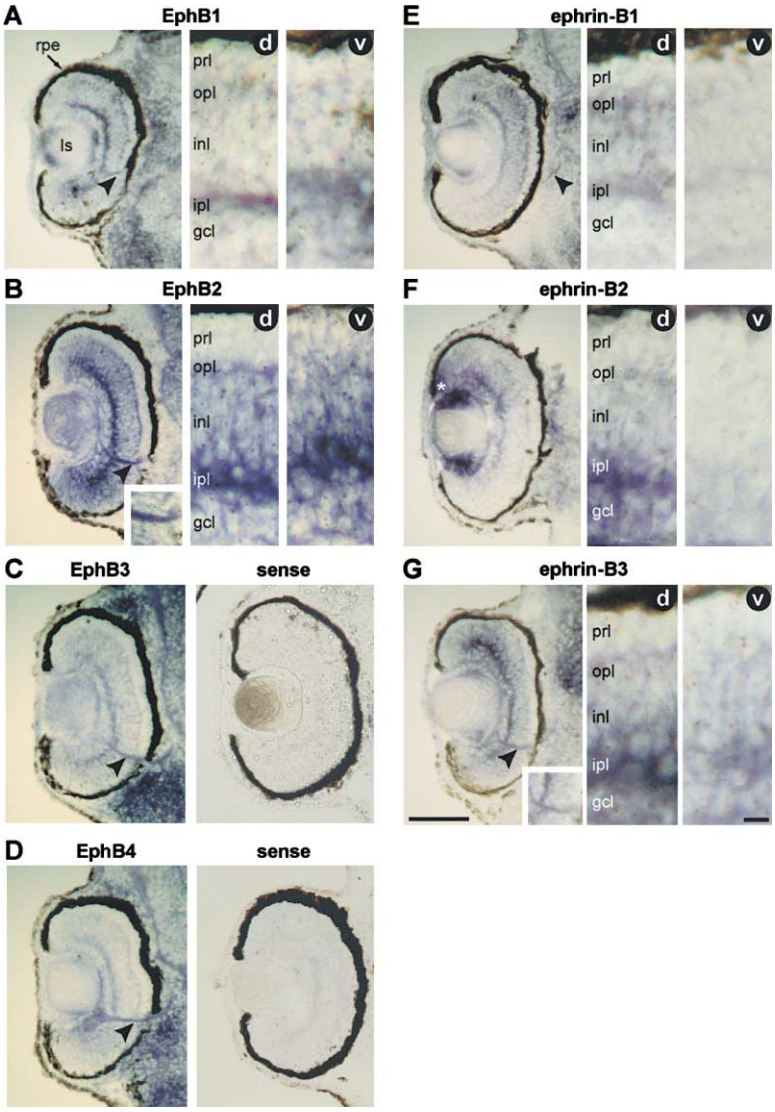

Figure 2. Expression of EphB Receptors and Ephrin-B mRNAs in the Developing Retina

Transverse sections through the retina of stage 39 embryos after RNA in situ hybridization with EphB1 (A), EphB2 (B), EphB3 (C), EphB4 (D), ephrin-B1 (E), ephrin-B2 (F), and ephrin-B3 (G) antisense riboprobes. In (C) and (D), the right panels show staining with EphB3 and EphB4 sense probes, respectively. In (A), (B), and (E)–(G), the middle and right panels are higher magnification views of dorsal (d) and ventral (v) regions of the retina. EphB2 mRNA (B) is expressed at higher level in ventral than dorsal retina. Ephrin-B2 (F) and ephrin-B3 (G) mRNAs are expressed in an opposite high-dorsal to low-ventral gradient. The arrowheads indicate staining in the optic nerve, and the asterisk shows the dorsal ciliary marginal zone. Abbreviations: rpe, retinal pigmented epithelium; ls, lens; prl, photoreceptor layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; and gcl, ganglion cell layer. Scale bars: 50 μm, (C), (D), and left panels in (A), (B), and (E)–(G); 10 μm, middle and right panels in (A), (B) and (E)–(G).

or alternatively, that axons exiting the eye transport the message (Figures 2A–2D).

Orthologs of the three ephrin-B ligands known in mammals and birds have been described in *Xenopus laevis*: ephrin-B1 (Xlerk) (Jones et al., 1997), ephrin-B2 (Smith et al., 1997), and ephrin-B3 (Helbling et al., 1999). All three are found in the *Xenopus* retina. Ephrin-B1 mRNA is expressed weakly in a high-dorsal to low-ventral gradient in IPL, OPL, and inner nuclear layer (INL) (Figure 2E). Ephrin-B2 mRNA is strong in the RGC layer and IPL, where it appears distributed in a high-dorsal to low-ventral gradient (Figure 2F). Expression is also evident in the dorsal but not ventral ciliary marginal zone. Finally, ephrin-B3 mRNA is expressed in most retinal layers and again distributed in a high-dorsal to low-ventral gradient (Figure 2G). An especially high level of expression was observed in the IPL. Ephrin-B1 and ephrin-B3 mRNAs, but not ephrin-B2 mRNA, are abundant in the optic nerve head and optic nerve (Figures 2E and 2G).

Distribution of EphB Receptors and Transmembrane Ephrin-B Ligands in the Optic Tectum

To examine the expression of EphB receptors in the developing optic tectum, probes for EphB1, EphB2, EphB3, and EphB4 were used for in situ hybridization on cross-sections through the brain of stage 39 embryos. EphB1 transcripts are distributed in a high ventral to low dorsal gradient in the optic tectum (Figures 3A and 3B). The EphB1 gradient covers the tectal neuropil, the region where early RGC axons project (Holt and Harris, 1983). All other EphB receptors are evenly expressed with respect to the D-V axis of the tectum, with EphB2 transcripts being higher than EphB3 and EphB4 transcripts (data not shown).

In situ hybridization on sections from stage 39 *Xenopus* embryo brains revealed an opposing gradient of ephrin-Bs. Ephrin-B1 mRNA is in cells at the extreme dorsal aspect of the optic tectum (Figure 3C), a region normally devoid of visual afferents. Ephrin-B2 tran-

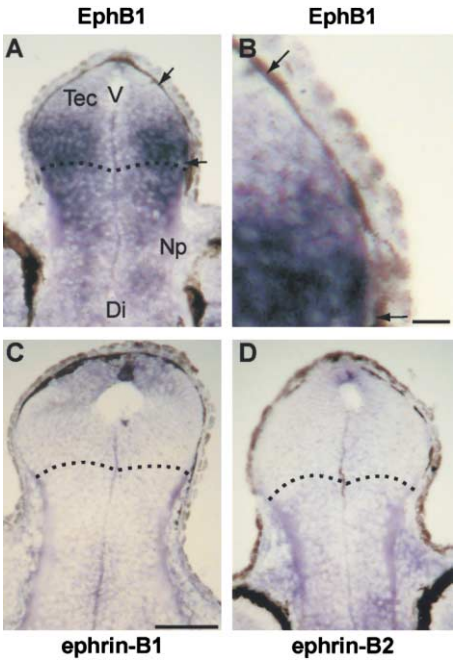

Figure 3. Expression of the EphB Receptor and Ephrin-B mRNAs in the Optic Tectum

Transverse sections through the midbrain of stage 39 embryos after RNA in situ hybridisation with EphB1 (A and B), ephrin-B1 (C), and ephrin-B2 (D) antisense riboprobes. EphB1 (A) mRNA shows a graded expression along the D-V axis of the tectum. (B) Higher magnification of the retinorecipient region, visualizing the high-ventral to low-dorsal gradient of EphB1 transcript. Arrowheads indicate the D-V borders of the region where RGC axons project. Dorsal RGC axons terminate in the tectal region with a high level of EphB1, whereas ventral RGC axons project to the tectal region with a low level of EphB1. Ephrin-B1 (C) mRNA is expressed in the dorsal midbrain, and ephrin-B2 (D) mRNA is restricted to cells at the dorsal midline. The dashed line indicates the approximate ventral border of the optic tectum. Abbreviations: Tec, tectum; Np, neuropil; Di, diencephalon; and V, ventricule. Scale bars: 50 μm, (A), (C), and (D); 10 μm, (B).

scripts are also restricted to visually uninnervated regions at the dorsal midline of the tectum (Figure 3D). Finally, expression of ephrin-B3 is high in the tectum but shows no D-V gradient (data not shown).

Disruption of Eph/Ephrin Interaction In Vivo Perturbs D-V Axis Termination of Optic Projections

To determine whether EphB/ephrin-B function is required for proper targeting of retinotectal projections, signaling was disrupted by applying exogenous soluble EphB2 ectodomain (EphB2-Fc) to the optic tectum in an exposed brain preparation. In these experiments, the brain was exposed at stage 35/36 when the first retinal axons have reached the middiencephalon in the contralateral side of the brain and run in a dorso-caudal direction to enter the optic tectum ~3 hr later (stage 37/38). At stage 40, the entire projection was visualized with the anterograde tracer HRP.

In control embryos, the whole HRP-filled projections look similar to those of nonexposed animals (data not shown): retinal axons cross the anterior tectal border of the tectum, where they widen and terminate (Figure 4A). When exposed to 16 μg/ml of EphB2-Fc, retinal axons pathfind correctly to the optic tectum. After entering their target, however, fibers terminate more dorsally than controls (Figure 4B). In fact, the EphB2-Fc-treated fibers appear to map to just the top half of the tectum, leaving the ventral tectum uninnervated. To quantify this effect, the D-V positions of the dorsalmost and the ventralmost optic terminals in the midbrain were measured using a coordinate system (Chien et al., 1995) (see Figure 7A and Experimental Procedures). Results are presented in Figure 4D. In control brains ($n = 10$), retinotectal fibers widen to span about 100 μm, covering a region between 53.1 ± 8.8 μm and 167.2 ± 17.7 μm below the dorsal midline. Optic projections exposed to 16 μg/ml of EphB2-Fc fusion protein ($n = 8$) widen to a lesser degree (about 70 μm). Treated axons also project significantly more dorsally than in controls and terminate in an area situated between 23.6 ± 7.0 μm and 93.2 ± 12.6 μm from the dorsal midline. Strikingly, the tectal region that normally receives afferents from the ventral half of the optic tract was found to be devoid of retinal terminals. A dose-response curve showed that 8 μg/ml EphB2-Fc ($n = 8$) was sufficient to induce a significant dorsal shift of the optic terminals in the tectum. Lower doses had no effect.

To test the specificity of this effect, purified Fc fragment alone (16 μg/ml, $n = 7$) was added to the bathing medium. In this case, retinal axons terminate in the appropriate region of the tectum (Figure 4D). Similarly, when the EphB2-Fc fusion protein was heat inactivated at 74°C before addition to the bathing medium (16 μg/ml, $n = 7$), no effect on retinal axon targeting could be detected (Figures 4C and 4D). This result demonstrates that the dorsal shift described above is specific to the active EphB2-Fc protein.

Eph/ephrin interactions have been shown to be involved in maintaining boundaries and tissue patterning (for review see Klein, 1999; Wilkinson, 2001). Thus one possibility is that exogenous EphB2-Fc perturbs the cellular organization of the tectum, and as a result, alters the distribution of topographic cues along the dorsoventral axis. However, the graded expression of EphB1 mRNA in the tectum of embryos treated with 16 μg/ml of EphB2-Fc appeared normal and did not differ from the pattern described in normal embryos (data not shown).

EphB Receptor Affects Neurite Outgrowth from Dorsal Retina but Not Ventral Retina in Cultures

We next examined whether EphB receptors could provide signals that modulate the outgrowth from *Xenopus* dorsal eye explants. We produced mouse L cells expressing *EphB4b* full-length gene (L-EphB4 cells). Cell surface expression of recombinant protein was confirmed by AP-tag staining (Figure 5B). No endogenous Eph receptors could be detected on control L cells using the same method (Figure 5A). Membrane particles were purified from the clones showing the highest level of recombinant protein expression and were used as growth substrates for retinal explants from stage 32 *Xenopus* embryos. Figures 5C and 5D illustrate the growth pattern of explants from the dorsal retina cultured for 40 hr on membranes from L-EphB4 cells and

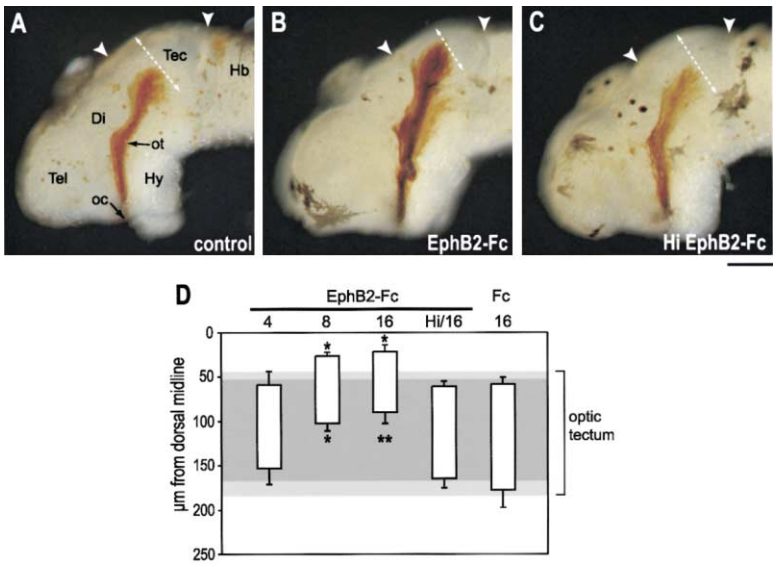

Figure 4. Exogenous EphB2-Fc Fusion Protein Causes RGC Axon Targeting Errors in the Tectum

Lateral view of whole-mount brains after labeling the visual projections with HRP. Control retinotectal projections (A) and visual projections in brains exposed to 16 μg/ml EphB2-Fc (B) or 16 μg/ml heat-inactivated EphB2-Fc (C). The anterior and posterior borders of the tectum are marked with arrowheads. The dashed line shows the D-V axis of the tectum and indicates the distance between the dorsal midline and the ventralmost retinal fibres. In brains exposed to EphB2-Fc fusion proteins (B), axons project more dorsally in the tectum than control axons. Projections treated with heat-inactivated EphB2/Fc appear normal (C). (D) Histogram showing the dorso-ventral position of visual projection in the optic tectum of brains treated with 4, 8, and 16 μg/ml EphB2-Fc or 16 μg/ml Fc fragment alone. Bars indicate the position of retinal projection along the D-V axis. The shaded area shows the average position of projection in control brains. Data are mean ± SEM. Asterisks show significant differences by a two-tailed t-test: *$p < 0.05$; and **$p < 0.01$. Abbreviations: Tec, tectum; Hb, hindbrain; Di, diencephalon; Hy, hypothalamus; Tel, telencephalon; oc, optic chiasm; ot, optic tract; Hi, heat inactivated. Scale bar: 100 μm.

parental L cells used as control. Axonal elongation was reduced in the presence of EphB4 receptor compared to control. Quantification of mean axon length confirmed that dorsal retinal neurites grew shorter on L-EphB4 cell membranes than on control membranes (Figure 5E). Explants from the ventral aspect of the retina, however, were not affected by the presence of EphB4 receptor and showed a similar extension rate on control and EphB4 membranes (Figure 5E). These results indicate that EphB4 selectively influences the outgrowth of ephrin-B-expressing dorsal retinal axons.

The EphB-Induced Reduction in Axon Length Is Mediated by Ephrin-B Cytoplasmic Domain

To show that this effect is specifically mediated by ephrin-Bs, cDNA encoding a truncated form of the zebrafish ephrin-B2a ligand (ephrin-B2$^{\Delta C}$) was introduced into retinal precursors. This mutant ligand lacks the cytoplasmic domain. Therefore, it should be unable to transduce reverse signaling. The binding promiscuity between Eph receptors and ligands means that overexpressed ephrin-B2$^{\Delta C}$ may outcompete endogenous ephrin-Bs for binding to Eph receptors at the surface of adjacent cells and thus act as a dominant negative. Ephrin-B2$^{\Delta C}$ cDNA was injected together with myc-tagged green fluorescent protein (GFP-myc) into the two antero-dorsal blastomeres of four-cell stage *Xenopus* embryos. Explants from the dorsal retina of GFP-expressing eyes were then cultured on membrane substrates from L-EphB4 cells or control cells. Expressing neurites were immunostained with an anti-myc antibody, and the mean neurite length in each experimental condition was measured. The results (summarized in Figure 5F) indicate that wild-type fibers expressing GFP-myc alone were 21.2% shorter on EphB4 membranes than on control membranes. In contrast, retinal cells that received ephrin-B2$^{\Delta C}$ cDNA did not respond to the presence of EphB receptor and exhibited a similar elongation rate on control and EphB4 membranes. Dorsal retinal axons expressing the full-length ephrin-B2a (ephrin-B2^{WT}) cDNA grew shorter on an EphB4 substrate and showed a higher rate of growth reduction (31.0%) than control axons. These results demonstrate that the reduced growth response induced by EphB receptor requires ephrin-B cytoplasmic tail, and that ephrin-B2$^{\Delta C}$ is capable of inhibiting the function of endogenous transmembrane ephrins in growing retinal axons.

EphB1 Extracellular Domain Acts as an Attractive Guidance Cue for Dorsal Retinal Axons in a Stripe Assay

To test how the differential expression of the EphB receptor along the D-V tectal axis affects growth cone guidance, we examined the behavior of retinal axons in a stripe assay with the EphB1 receptor extracellular domain (EphB1-Fc). Stripes of artificially clustered EphB1-Fc were prepared on a polylysine and laminin-coated glass coverslip, onto which retinal explants from the dorsal part of the eye were cultured. As shown in Figures 5G, 5H, and 5K, 66.5% ± 5.8% of the explants examined showed an attractive response to stripes of EphB1-Fc (only 1.4% ± 1.4% of the explants showed a repulsive response and 32.1% ± 5.8% were apparently insensitive to EphB1-Fc). In the absence of clustered EphB1-Fc, however, the majority of retinal explants did not respond to the stripe substrate (attraction, 9.2% ± 6.7%; repulsion, 3.0% ± 3.0%; no response, 87.8% ± 5.1%; Figures 5I and 5K). A certain degree of unspecific guidance in this assay was previously described by Drescher and colleagues (Hornberger et al., 1999) and might be explained by mechanical forces. Retinal axons exhibited a similar behavior to control axons when EphB1-Fc was heat inactivated (attraction, 22.9% ± 10.4%; repulsion, 3.1% ± 3.1%; no response, 74.0% ± 7.3%) or when the clustering antibody was used alone (attraction, 19.2% ± 5.9%; repulsion:, 5.5% ± 5.6%; no

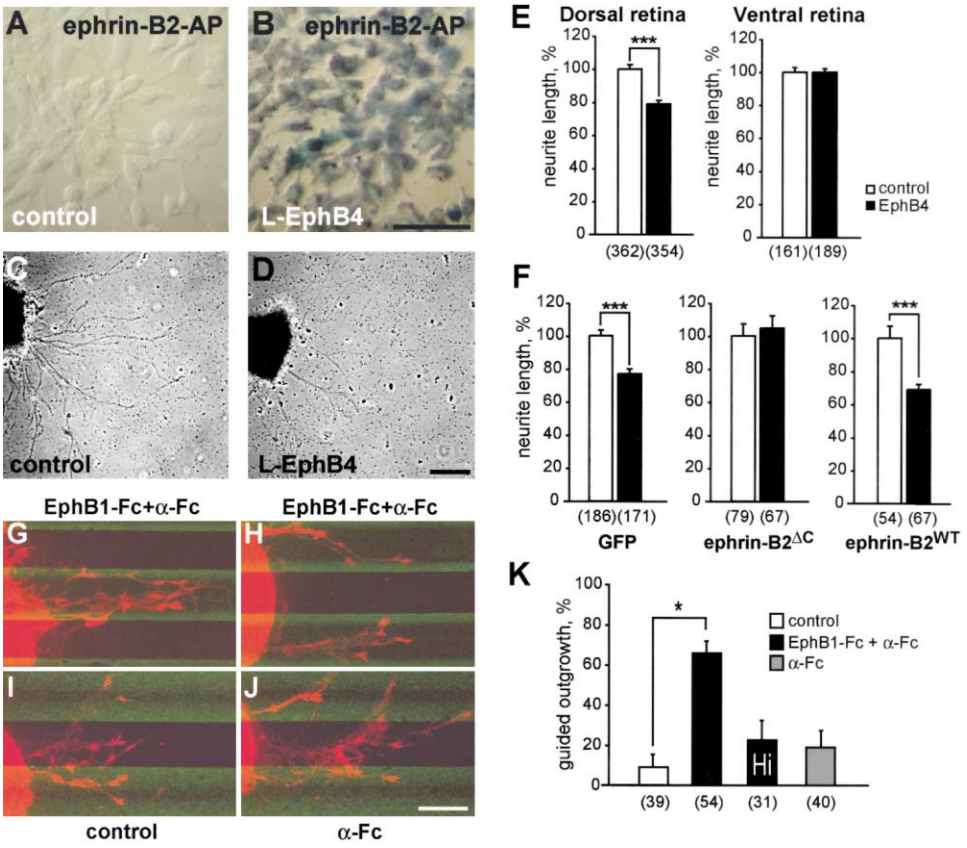

Figure 5. EphB Receptors Regulate Dorsal Retinal Axon Growth through Ephrin-B Cytoplasmic Domain

(A and B) Photomicrographs of control L cells and L cells stably expressing the EphB4 receptor, as indicated by the binding of ephrin-B2-AP probe.

(C and D) Typical examples of 40 hr retinal explant cultures on membrane carpet from L-EphB4 and control cells. Explants harvested from the dorsal side of the retina extend shorter neurites in the presence than in the absence of EphB4 receptor.

(E) Quantitation of mean neurite length in cultures of dorsal and ventral eye explants on membrane substrates from L-EphB4 and control cells.

(F) Histogram showing the mean length of axons from dorsal retinal explants isolated from embryos injected with ephrin-B2^{WT}, ephrin-B2$^{\Delta C}$, or GFP-myc alone. In (E) and (F), data are presented in percentage and normalized to 100% for values obtained on control membranes.

(G–J) Photomicrograph of dorsal retinal axons growing on alternative stripes prepared with clustered EphB1-Fc (EphB1-Fc + α-Fc), clustering antibody alone (α-Fc) or PBS (control). Axons are labeled with Alexa594-conjugated phalloidin (red) and stripes are visualized with an Alexa488-conjugated antibody (green). Dorsal retinal axons show an attractive response to clustered EphB1-Fc, whereas they show no preference for control stripes.

(K) Quantitative analysis of axon guidance in stripe assay. Bars show the average percentage of explants showing a clear attractive response to stripes prepared with PBS (control), clustered EphB1-Fc (EphB1-Fc + α-Fc), and clustering antibody alone (α-Fc). Statistical analysis using a Mann-Whitney U test indicate that the percentage of explants showing an attractive response to EphB1-Fc is statistically different from the percentage of explants in which axons show a preference for stripes coated with the clustering antibody ($p < 0.05$) and heat inactivated EphB1-Fc ($p < 0.05$). Hi, heat inactivated. Bars indicate mean ± SEM. Asterisks show significant differences from control: *$p < 0.05$; ***$p < 0.001$; n = total number of neuritis, (E) and (F); n = total number of explants (K). Scale bar: 100 μm, (A)–(D); 50 μm, (G)–(J).

response, 75.3% ± 6.0%) (Figures 5J and 5K). These results indicate that dorsal retinal axons showed an attractive response to the EphB1 extracellular domain.

Expression of Truncated Ephrin-B Ligand in Dorsal Retina Leads to In Vivo Targeting Errors of RGC Axons in the Optic Tectum

Are axonally expressed ephrin-Bs involved in D-V retinotectal mapping? We compared the D-V positioning of the terminals of retinal fibers expressing wild-type (ephrin-B2^{WT}) and dominant-negative (ephrin-B2$^{\Delta C}$) ligands in vivo using the lipofection technique (Holt et al., 1990). cDNAs for these constructs were colipofected with GFP-myc at stage 18–19 into the lateral part of the eye field that gives rise to the dorsal retina. This approach has the advantage that it specifically modifies the expression of surface ligands on RGCs without affecting the molecular environment along the optic pathway or optic tectum. RGC axons transfected with the wild-type and the dominant-negative ephrin-B2 ligands exhibited no gross defects in their trajectory along the optic pathway and successfully innervated their target (Figures 6B and 6C).

To analyze topography, embryos were allowed to survive for 2 days after lipofection and were then fixed at stage 40. At this stage, the dorso-ventral order of visual projections is well established (Holt and Harris, 1983; Chien et al., 1995). Individual transfected axons were detected with an anti-myc antibody, and the D-V posi-

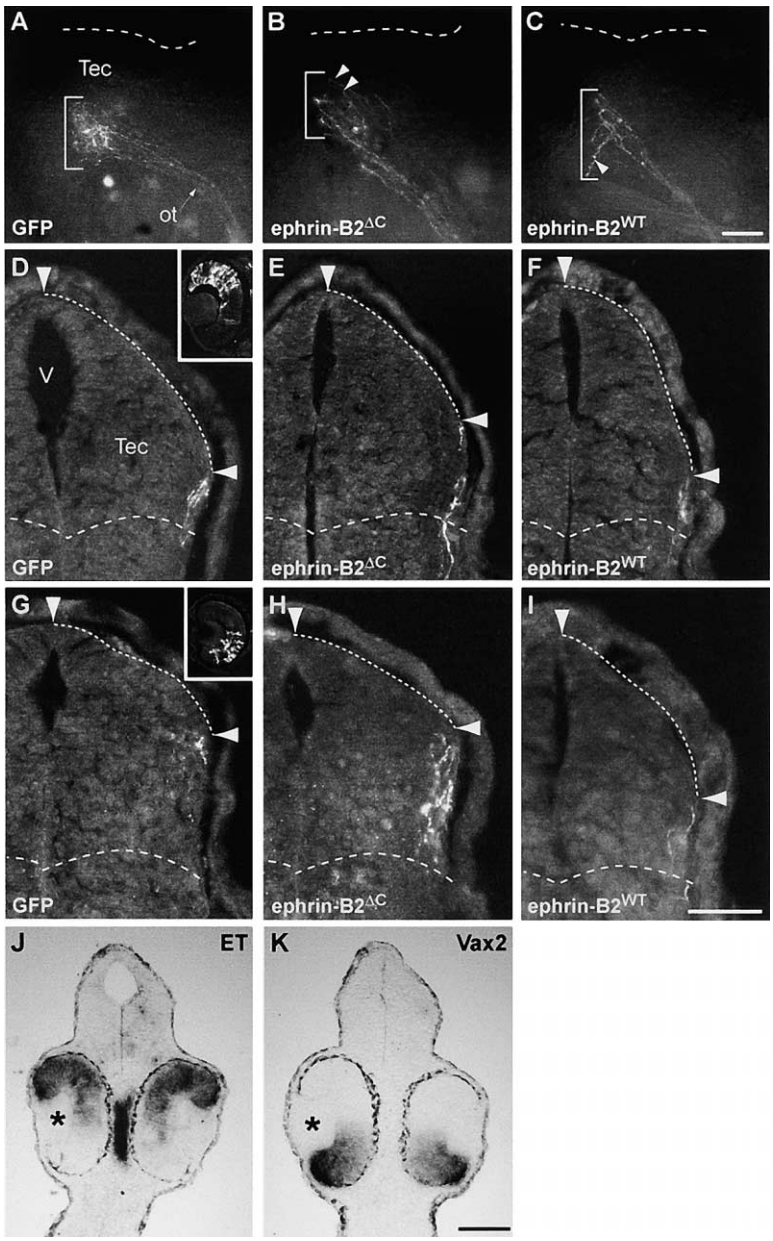

Figure 6. Expressing the Dominant-Negative Ephrin-B in Dorsal Retinal Cells and the Wild-Type Ephrin-B Ligand in Ventral Retinal Cells Affects Dorso-Ventral Topography

(A–C) Lateral view of whole-mount stage 40 tectum, showing projections from dorsal RGCs lipofected with GFP-myc alone or in combination with ephrin-B2$^{\Delta C}$ and ephrin-B2^{WT}. Caudal is left, dorsal is up, and the approximate location of the dorsal midline is indicated by the dashed lines.

(D–I) Cross section through the tectum of stage 40 embryos, showing projections from dorsal (D–F) and ventral (G–I) RGCs lipofected with GFP-myc alone or in combination with ephrin-B2$^{\Delta C}$ and ephrin-B2^{WT}. The dotted line between the two arrowheads indicates the distance from the dorsal midline to the branch tip, and the dashed line shows the approximate ventral border of the tectum. Inset in (D) and (G) shows transfected retinal cells located in the dorsal and ventral side of the eye respectively. Dorsal RGC axons transfected with ephrin-B2$^{\Delta C}$ (B and E) project more dorsally than control axons (A and D), whereas control and ephrin-B2$^{\Delta C}$-transfected ventral RGC fibers terminate in the same tectal region (G and H). After lipofection of ephrin-B2^{WT} (C and F), the majority of dorsal retinal projections terminate in their appropriate topographic location. Some axons, however, send branches that aberrantly grow ventrally in the midbrain. In contrast, ephrin-B2^{WT}-expressing ventral RGC axons terminate more ventrally than control axons (G and I).

(J and K) Expression of ET and Vax2 mRNAs in stage 32 embryos is not affected by unilateral injection of ephrin-B2$^{\Delta C}$ cDNA. Asterisks indicate the injected side. Abbreviations: Tec, tectum; V, ventricle; ot, optic tract. Scale bars: 30 μm, (A)–(C); 50 μm, (D)–(I); 100 μm, (J) and (K).

tion of each terminal in the tectum was determined on whole-mount brain preparations (see Figure 7A). To control the origin of the retinal fibers, we also analyzed the location of transfected cells in cross-sections through the eye (see Figure 7B). We included only specimens in which all labeled fibers came from RGCs whose location in the dorsal part of the retina could be confirmed.

Dorsal retinal axons lipofected with the dominant-negative ephrin-B2$^{\Delta C}$ projected significantly more dorsally in the tectum than control axons lipofected with GFP-myc (Figures 6A, 6B, 6D, and 6E). These ephrin-B2$^{\Delta C}$ dorsal retinal axons project about 30 μm dorsal to their normal position (+29.9 ± 6.0 μm in whole-mount brains, +28.6 ± 5.3 μm in sections; Figures 7C and 7D), roughly 30% of the dorso-ventral extent of the tectal neuropil. Qualitatively, this result is similar to the dorsal shift of retinal projections induced by disrupting EphB/ephrin-B interactions using exogenous EphB2-Fc. Quantitatively, however, expressing the dominant-negative ephrin-B2$^{\Delta C}$ construct in dorsal cells causes a smaller change in D-V position than applying EphB2-Fc to the optic pathway.

In contrast to RGC axons expressing ephrin-B2$^{\Delta C}$, axons transfected with the wild-type ephrin-B2^{WT} showed a tendency to project slightly more ventrally than control fibers (Figures 6A, 6C, 6D, and 6F), about 10 μm below their appropriate termination zone (−9.2 ± 5.0 μm in whole-mount brains, −11.5 ± 5.3 μm in sections; Figures 7E and 7F). However, this change is not statistically significant.

To confirm that introducing ephrin-B2$^{\Delta C}$ in retinal precursors does not change the identity of transfected cells, we examined the expression pattern of markers for D-V asymmetry in the retinas of embryos injected with

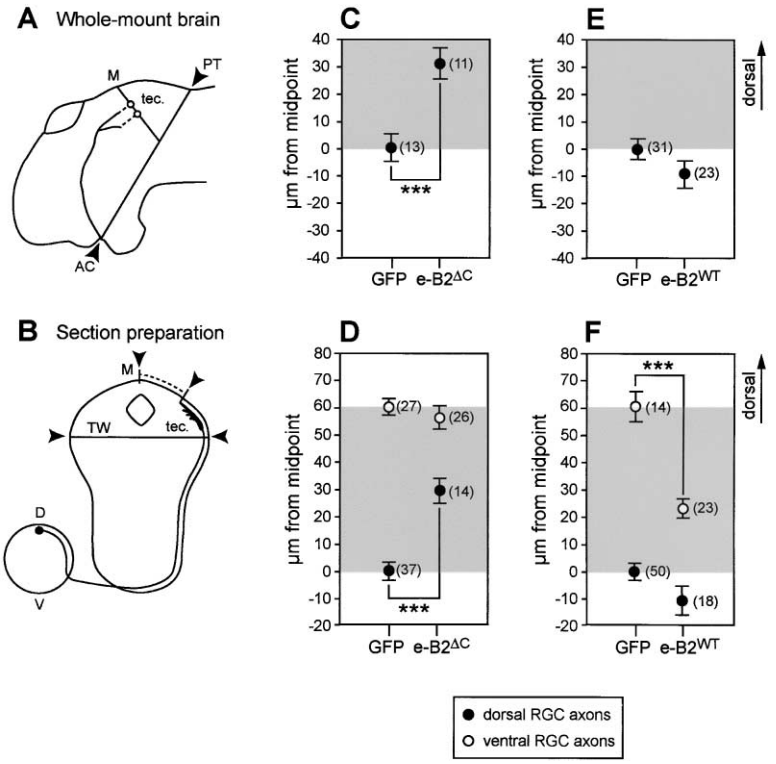

Figure 7. Dorso-Ventral Topography of Dorsal and Ventral Retinal Axons Expressing a Dominant-Negative and Full-Length Ephrin-B2 Ligand

(A and B) Systems for quantification of the D-V position of retinotectal terminals in whole-mount brain (A) and on cross-section through the optic tectum (B). (A) A reference line was drawn from the anterior edge of the chiasm (AC) to the posterior boundary of the tectum (PT). D-V axis is placed at $+70°$ to this line (Chien et al., 1995). Terminations of retinal axons were projected onto this D-V line and the dorso-ventral positions were measured from the dorsal midline of the tectum (M). To standardize measurements, distances were expressed as a fraction of the AC-PT distance. (B) For the analysis of dorso-ventral topography on cross-sections, axon terminations from RGCs located in either dorsal or ventral retina were localized, and the distances from the dorsal midline (M) to the branch tips were measured. To standardize measurements between samples, distances were expressed as a fraction of the width of the tectum (TW).

(C–F) Histograms showing the D-V position of dorsal and ventral RGC axons transfected with ephrin-B2$^{\Delta C}$ (C and D) and ephrin-B2^{WT} (E and F) compared to the average position of control dorsal RGC axons (midpoint). Positive values indicate more dorsal positions. The shaded area labels the region between the average position of ventral and dorsal RGC axons in control embryos. Data indicate mean ± SEM. Asterisks show significant differences from control projection by a two-tailed t test: ***$p < 0.001$; n = total number of embryos examined.

ephrin-B2$^{\Delta C}$ cDNA at four-cell stage. At stage 32, the transcription factors Vax 2 and ET are normally restricted to the ventral and dorsal sides of the retina, respectively (Li et al., 1997; Barbieri et al., 1999; Liu et al., 2001). Overexpression of ephrin-B2$^{\Delta C}$ throughout the eye from early stages did not induce the expression of the ventral marker Vax2 in dorsal retina nor did it repress the dorsal marker ET (Figures 6J and 6K).

Misexpression of Ephrin-B Ligand in the Ventral Retina Causes In Vivo Mapping Errors of RGC Axons in the Optic Tectum

GFP-myc and ephrin-B2$^{\Delta C}$ cDNAs were lipofected at stage 15–16 near the midline of the separating single eye field which gives rise to the ventral retina (Eagleson et al., 1995), and the D-V position of transfected retinotectal axons was analyzed at stage 40 on section preparations. In control embryos lipofected with GFP-myc alone, axons from the ventral retina terminated in the dorsal part of the tectum, about 60 µm above the axons emanating from the dorsal retina ($+60.5 \pm 2.3$ µm, $p <$ 0.001; Figures 6D, 6G, 7D, and 7F). Ephrin-B2$^{\Delta C}$ expression appeared not to alter the topographic termination of ventral retinal cells (-4.8 ± 4.1 µm; Figures 6G, 6H, and 7D). In contrast, ectopic expression of the ephrin-B2^{WT} ligand into the ventral retina caused aberrant projections of retinotectal fibers that projected about 38 µm below their appropriate termination zone (-38.0 ± 3.5 µm; Figures 6G, 6I, and 7F). Thus misexpression of the full-length ephrin-B2 ^{WT} ligand was sufficient to cause D-V topographic errors.

Discussion

Our results show that EphB receptors and ephrin-B ligands play an important role in establishing dorsoventral topography. Previously it has been proposed that dorsoventral mapping is mediated via an attractive forward signaling mechanism through EphB/ephrin-B interactions based on the observations that EphB receptors in the retina are expressed in a decreasing ventral-to-dorsal gradient and ephrin-B ligand is expressed in an opposite dorsal-to-ventral gradient in the chick tectum (Braisted et al., 1997). However, a separate study failed to detect a gradient of ephrin-B ligand in the chick tectum (Holash et al., 1997), raising the question of whether forward signaling can mediate topography. Our findings support the idea of an attractive model via a mechanism in which EphB receptors in the tectum provide topographic guidance cues for ephrin-B-expressing RGC axons. Our results also demonstrate that the intracellular domain of ephrin-Bs in dorsal RGC axons is important for appropriate D-V mapping. Consistent with this interpretation, we found that misexpressing a full-length ephrin-B ligand in ventral RGCs causes them to terminate more ventrally in the tectum, whereas introducing a truncated ephrin-B ligand—that lacks a cytoplasmic domain—did not alter the appropriate D-V targeting of ventral RGC axons.

The expression of ephrin-B2$^{\Delta C}$ caused only a partial shift of dorsal RGC terminals, about 30% of the D-V extent of retinotectal termination zone. This suggests that the dominant-negative ephrin-B2 only partially

blocks the activity of the endogenous protein, or that blocking ephrin-B signaling is not sufficient to make dorsal retinal axons project to the extreme dorsal margin of the tectum. Disruption of D-V targeting in whole brains treated with soluble EphB2-Fc was more severe. This difference is consistent with a model in which the application of EphB2-Fc to the optic pathway, by affecting both forward and reverse signaling, causes ventral RGC axons to terminate further dorsally in the ephrin-B-expressing dorsal zone of the midbrain (see below). As a consequence, dorsal RGC axons can terminate further dorsally in the region of the tectum vacated by the ventral RGC axons. Single ephrin-B2$^{\Delta C}$-expressing dorsal axons, however, must compete for dorsal targets with the population of normal ventral retinal axons, which may have an advantage in terms of mass numbers and the expression of other ventral-specific factors involved in topographic guidance.

Birgbauer et al. (2001) have shown that the extracellular domains of EphB receptors induce growth cone collapse of RGC axons. In the present study, we found that dorsal retinal axons grow shorter on membrane particles expressing the EphB4 receptor than on control membranes. It remains unclear, however, if this effect is due to a repulsive/inhibitory activity of substrate-bound EphB receptor or if it is the consequence of an increased adhesion of growth cones to the substrate. There are some indications that ephrin-B/EphB interactions can mediate adhesive effects in vitro (Bohme et al., 1996; Holash et al., 1997; Stein et al., 1998). Dissociated cells from dorsal retina that express high levels of ephrin-B ligands adhere preferentially to a EphB2-Fc substrate (Holash et al., 1997). In the present study, the EphB1-Fc fusion protein elicited an attractive guidance activity on dorsal retinal neurites in the stripe assay. This effect could be due to adhesive interactions between ephrin-B-expressing axons and the substrate-bound EphB receptor. These distinct collapsing and adhesive/attractive responses to the EphB extracellular domain may reflect differences between transient versus persistent ephrin-B activation, differences in concentration, spatial distribution, or degree of clustering of EphB receptors.

We also show that expression of truncated ephrin-B2$^{\Delta C}$ in retinal cells completely abolished the EphB4-induced reduction in overall outgrowth of dorsal retinal explants, whereas overexpression of wild-type ephrin-B2 ligand rendered axons more sensitive to EphB4 activity. These results suggest that the effect of EphB receptors is mediated by the cytoplasmic domain of ephrin-Bs that actively signal in growing RGC axons, as the model of "reverse" signaling would predict. However, we can not rule out the possibility that the effects seen in this study are independent of ephrin-B reverse signaling, as the cytoplasmic tail of ephrin-B ligands could perhaps be needed for cell-cell adhesion mediated directly by Eph/ephrin binding.

In this paper, we do not directly address the role of forward signaling. Our expression results indicate that at least one EphB receptor, EphB2, is expressed in RGC axons from the ventral retina, and ephrin-B1 and ephrin-B2 are found in the dorsal tectum, suggesting that forward signaling could play an important role in retinotectal targeting in vivo. Although ephrin-B1 shows a high-dorsal to low-ventral gradient distribution in the midbrain, the expression is largely restricted to the region dorsal and medial to that normally innervated by retinal axons. It is unlikely, therefore, to act as a topographic cue within the tectum. An alternative function of ephrin-Bs in the dorsal midbrain is that of a boundary "stop signal." Such a role would parallel that of ephrin-B in the chiasm, where it prevents ipsilateral-projecting axons from entering the contralateral optic tract (Nakagawa et al., 2000). In the present study we found that applying EphB2-Fc to the optic pathway resulted in mistargeting of the retinotectal projection, causing retinal axons to extend dorsally toward the dorsal midline. This result supports a possible role of ephrin-Bs in restricting the dorsal expansion of retinotectal axons (see Figure 8). The fact that these axons do not cross the dorsal midline might indicate the presence of additional stop cues (Irie et al., 2001).

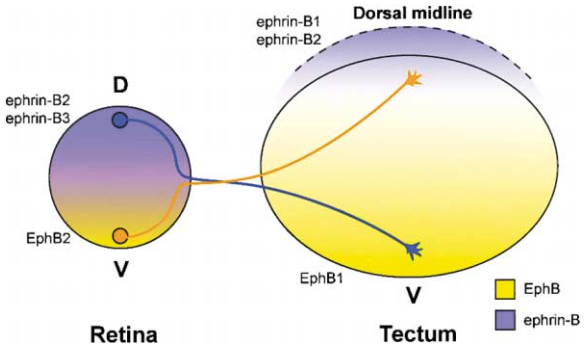

Figure 8. Proposed Model for Topographic Retinotectal Mapping along the D-V Tectal Axis

EphB receptors and ephrin-B ligands are expressed in opposite D-V gradients in the retina, with EphB2 high in ventral retina and ephrin-B2 and ephrin-B3 abundant in dorsal retina. EphB1 is in a matching gradient in the tectum, and ephrin-B1 and ephrin-B2 are expressed most heavily in the region dorsal to that normally innervated by retinal axons. EphB1 expressed in the ventral tectum, by interacting with ephrin-B ligands expressed on dorsal RGCs, attracts dorsal RGC axons to their appropriate target region possibly through a reverse signaling mechanism. Retinotectal fibers from the ventral retina are restricted from growing too far dorsally by the expression of ephrin-B1 and ephrin-B2 that act as "stop" signals through repulsive forward signaling.

In the *Xenopus* visual system, retinal axons expressing high amounts of ephrin-Bs project to regions of the tectum expressing high amounts of EphB receptors. This, together with the observation that dorsal retinal fibers grow preferentially on EphB1-Fc stripes, suggests that RGC axons from the dorsal retina are guided to the ventral tectum by an attractive mechanism (Figure 8). This situation is similar to the mouse olfactory system where vomeronasal axons that express high levels of ephrin-A5 ligand project to regions of the accessory olfactory bulb that express high levels of EphA6 receptor (Knoll et al., 2001). Another recent study of *EphA4*$^{-/-}$ mice suggests that EphA4 is an attractive/adhesive signal for ephrin-expressing cortical axons that form the anterior commissure (Kullander et al., 2001b).

In the mouse retina, removal of EphB2 and EphB3 causes pathfinding errors of some dorsal RGC axons that fail to exit the eye and instead extend aberrantly into the ventral retina (Birgbauer et al., 2000). The model

proposed to explain this phenotype is that in wild-type retina, dorsal RGC axons navigating to the optic disc encounter an increasing gradient of EphB receptors that serve as repulsive guidance cues to prevent axons from extending into the opposite ventral half of the retina (Birgbauer et al., 2001). Such repulsive interactions between ephrin-B- and EphB-expressing axons may also play an important role in sorting dorsal from ventral RGC axons in the optic tract. It is possible that EphB receptors induce opposite responses in different parts of the optic pathway, where different molecular contexts might regulate ephrin-B function. Indeed, growth cone responses to EphB1-Fc in vitro were found to critically depend on the nature of the growth substratum: dorsal retinal axons showed an attractive response to EphB1-Fc in the presence of a low concentration of laminin (1 μg/ml), whereas no guidance effect was observed on fibronectin or on high laminin concentration (10 μg/ml) (F.M. and C.E.H., unpublished data). Alternatively, it is possible that ephrin-B does not directly control growth cone guidance, but rather, acts to regulate other guidance cues. Recently, ephrin-B was found to bind constitutively to a PDZ binding protein, PDZ-RGS3, which regulates heteromeric G protein receptors through its RGS domain (Lu et al., 2001). In cultures of cerebellar granule cells, ephrin-B activation by soluble EphB2-Fc was found to activate PDZ-RGC3 and to silence the cytokine SDF-1 chemotropic attraction (Lu et al., 2001). A similar mechanism could act in dorsal RGC axons to silence a chemotropic signal that attracts visual axons toward the dorsal tectum. Finally, activation of ephrin-A ligands by EphAs has been shown to result in a β1-integrin-dependant increase in adhesion of ephrin-A-expressing cells (Davy and Robbins, 2000; Huai and Drescher, 2001), whereas activation of both EphA and EphB receptors leads to inhibition of integrin function and reduced cell substrate adhesion (Huynh-Do et al., 1999; Zou et al., 1999; Becker et al., 2000; Miao et al., 2000). It will be interesting to investigate whether activation of ephrin-B ligands up-regulates the function of cell adhesion molecules that may account for the "attractive" effect of EphB receptors in retinotectal mapping.

Experimental Procedures

Animals

Embryos came from in vitro fertilization of eggs obtained from adult female *Xenopus laevis* injected with human chorionic gonadotropin hormone (Sigma, UK). Embryos were dejellied in 2% cysteine (pH 8.0) and reared in 0.1× Modified Barth's Saline (MBS). Stages were determined according to Nieuwkoop and Faber (1967).

Whole-Mount and Cell Culture Staining with Receptor/Ligand Affinity Probes

In situ staining using the zebrafish ephrin-B2a and EphB4b ectodomains fused to alkaline phosphatase (ephrin-B2-AP and EphB4-AP respectively [Durbin et al., 1998]) was performed as previously described (Nakagawa et al., 2000). In zebrafish, some genes have been duplicated: *ephrin-B2a* and *ephrin-B2b*, or *EphB4a* and *EphB4b* are paralogs of mammalian *ephrin-B2* and *EphB4,* respectively. For staining retina, eyes were dissected out and the pigmented epithelium was removed before processing.

In Situ Hybridization

Plasmids used for probe synthesis have been described previously: ephrin-B1 (Xlerk) (Jones et al., 1997), ephrin-B2 (Smith et al., 1997), ephrin-B3 (Helbling et al., 1999), EphB1 (Smith et al., 1997), EphB2 (Tanaka et al., 1998), EphB3 (TCK) (Scales et al., 1995), EphB4 (Helbling et al., 1999), Vax2 (Barbieri et al., 1999; Liu et al., 2001), and ET (Li et al., 1997). *Xenopus* embryos were fixed in 4% PFA and processed for cryostat section as previously described (Riehl et al., 1996). In situ hybridization was made according to Shimamura et al. (1994). Sections were viewed using an Axioplan compound microscope (Zeiss) and photographed using a Coolpix 990 digital camera (Nikon). No staining was obtained when the sense probes were used.

Expression of Recombinant Receptor and Ligand in L Cells and Membrane Preparation

The full-length zebrafish *EphB4b* gene (formerly *EphB-rtk8* [Durbin et al., 1998]), cloned into the pCS2+ plasmid, was cotransfected with a pGK-Neomycin plasmid into L cells with Lipofectamine (GIBCO-BRL, UK) according to the manufacturer's instructions. Cells were grown in DMEM/F12 (1:1), 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 0.2 mg/ml G418 (GIBCO), and G418-resistant colonies were then screened for EphB4b expression using AP-tag ligand probe as described above. Cell membranes were purified as previously described (Mann et al., 1998) using transfected cell lines that exhibited high levels of AP-tag fusion protein binding.

In Vivo cDNA Injection and Lipofection

cDNA constructs encoding one of the wild-type zebrafish ephrin-B2a (ephrinB2^{WT}) and a truncated ephrin-B2a ligand which lacks the cytoplasmic domain (ephrinB2$^{\Delta C}$) were cloned into pCS2+ and used for in vivo expression studies (Durbin et al., 1998). Ephrin-B2^{WT} and ephrinB2$^{\Delta C}$ cDNAs were mixed with GFP-myc cDNA at a ratio of 1:1 for visualization of expressing cells. For culture experiments, a borosilicate glass needle was used to inject 250 pg of cDNA into the two antero-dorsal blastomeres at the four-cell stage using a Picospritzer (General Valve Corporation, Fairfield, NJ). For analysis of dorso-ventral topography in vivo, cDNA were introduced in retinal precursor cells by lipofection using previously published methods (Holt et al., 1990).

Exposed Brain Preparation

The exposed brain preparation was performed as described (Chien et al., 1993), with slight modification. Briefly, stage 35/36 embryos were anaesthetized with 0.4 g/l MS222 in 1× MBS, and the skin and eye over the left side of the brain were removed. Embryos were transferred in experimental or control solutions and allowed to develop for 24 hr until stage 40. Control solution consists of 1× MBS supplemented with 0.1 g/l MS222, 100 U/ml penicillin, and 100 μg/ml streptomycin. For experimental solutions, purified EphB2-Fc fusion protein (R&D Systems, UK) or purified Fc fragment alone (R&D Systems) were added to the control solution. EphB2-Fc protein was used at concentrations of 4, 8, and 16 μg/ml without clustering by antibodies. In some experiments, EphB2-Fc was heat inactivated at 74°C for 20 min (Birgbauer et al., 2001). To visualize retinal projections, RGC axons from the remaining eye were labeled with horseradish peroxidase (HRP; Sigma) using previously published methods (Cornel and Holt, 1992). After fixation in 1% PFA and 0.5% glutaraldehyde (1 hr at RT), brains were dissected out, reacted with diaminobenzidine (DAB, Sigma), and mounted in PBS.

Retinal Explant Cultures

Glass coverslips were coated with 10 μg/ml of laminin and 20 μg/ml of poly-L-lysine in PBS (1 hr at 37°C), washed with PBS, and then incubated with 50 μg/ml of membrane suspension purified from L cells (2 hr at 37°C). Coated coverslips were placed in a Nunc 4-well plate in 500 μl of culture medium (60% L15 media, 40% H_2O, supplemented with 0.1% BSA, 0.4%–0.2% methylcellulose, 100 U/ml penicillin, and 100 μg/ml streptomycin). Explants from either dorsal or ventral retina were dissected from stage 28–32 embryos and pipetted onto the membrane substrate. Cultures were fixed after 40 hr in vitro in 4% paraformaldehyde with 15% sucrose. Quantitation of neurite outgrowth was done by measuring the length of retinal axons from the external border of the explant to the tip of their growth cone using IPLab Spectrum P software. Statistical compari-

sons between average axonal lengths under different culture conditions were determined using a two-tailed Student's t test.

Stripe assay was performed using a special silicon matrix obtained from Juergen Jung (Max Planck Institut, Tuebingen, Germany) as previously described (Vielmetter et al., 1990). Briefly, a poly-L-lysine- (20 μg/ml) coated glass coverslip was placed onto the silicone matrix. Purified EphB1-Fc (5–15 μg/ml; R&D Systems) was preclustered with an anti-Fc antibody (50–150 μg/ml; Jackson ImmunoResearch, London, UK) for 1 hr at room temperature. To visualize the stripes, an Alexa488-conjugated antibody (Molecular Probe, The Netherlands) was added to the mixture (1:500) as a fluorescent marker. About 50 μl of the solution were injected into the silicone matrix channels and incubated 2 hr at 37°C. After three washes with PBS, the glass coverslip was removed from the matrix, placed in a Nunc 4-well plate and coated with 1 μg/ml laminin for 2 hr at 37°C. After washing out the laminin solution, the stripe substrate was covered with culture medium and stage 28–30 dorsal retinal explants were placed onto it. After 24 hr in vitro, cultures were fixed, and axons were labeled with Alexa594-conjugated phalloidin (Molecular Probes). For quantitative analysis, cultures were examined under an inverted microscope with 20× phase-contrast objective. Growth preference for each explant was scored as either "attraction" on fluorescent labeled stripes, "repulsion," or "no response." Analysis was performed blind. Data are presented as the percent of explants ± SEM from four independent experiments. Statistical differences were determined with a Mann-Whitney U test.

Antibodies and Immunostaining

The following antibodies were used: mouse anti-myc monoclonal antibody 9E10 (Sigma) at 1:2000 (in sections) and 1:1000 (in whole mount), Cy3-conjugated donkey anti-mouse IgG (Chemicon, UK) at 1:500 (in sections) and 1:1000 (in whole mount), or Alexa-488-conjugated goat anti-mouse IgG (Molecular Probes) at 1:1000 (in whole mount). Standard immunochemical procedures were used for cultures, cryostat, and whole-mount preparations. Briefly, samples were washed in PBS, permeabilized in methanol (10 min, −20°C), rinsed in PBS, and incubated with the primary antibodies (1 hr at room temperature [RT] or overnight at 4°C) in blocking solution (4% skim milk in PBS). After rinses in PBS, tissues were incubated with the secondary antibodies (45 min at RT or overnight at 4°C), washed and mounted in FluorSave reagent (Calbiochem, CN Biosciences, UK).

Analysis of Dorso-Ventral Topography

Samples were visualized and photographed with an Orca Snapper DIG 24 CCD camera mounted on a Nikon epifluorescence microscope, and analyses were performed using OpenLab software (Improvision system). D-V position of retinal axon terminals was measured from lateral views of whole-mount brains. Quantification was carried out using a polar coordinate system as previously described (Chien et al., 1995). A reference line was drawn through two easily and reliably identified landmarks: the anterior edge of the chiasm (AC) and the dorsal midline of the tectum (PT). In the tectum, the D-V axis was placed at +70°. Terminations of labeled axons were projected onto this axis and the D-V positions were measured from the dorsal midline of the tectum. Distances were measured as a fraction of the AC-PT distance. Means and SEs were converted to approximate real distance by multiplying by 620 μm (the average AC-PT distance) (Chien et al., 1995). For the analysis of D-V topography in section preparation, GFP-myc-immunopositive RGCs were visualized on 20 μm thick cross-sections through the eye. In sections through the tectum, identified by the widening of the third ventricle, axon terminations were localized, and the distances from the dorsal midline to the branch tips were measured. To standardize measurements, distances were expressed as a fraction of the width of the tectum (TW) at the widest point. Average distances and SEs were converted to approximate real distances by multiplying by 198 μm (the average TW). Statistical comparisons between experimental conditions were determined using a two-tailed Student's t test.

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References

Adams, R.H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U., and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. Cell *104*, 57–69.

Baier, H., Klostermann, S., Trowe, T., Karlstrom, R.O., Nusslein-Volhard, C., and Bonhoeffer, F. (1996). Genetic dissection of the retinotectal projection. Development *123*, 415–425.

Barbieri, A.M., Lupo, G., Bulfone, A., Andreazzoli, M., Mariani, M., Fougerousse, F., Consalez, G.G., Borsani, G., Beckmann, J.S., Barsacchi, G., et al. (1999). A homeobox gene, vax2, controls the patterning of the eye dorsoventral axis. Proc. Natl. Acad. Sci. USA *96*, 10729–10734.

Becker, E., Huynh-Do, U., Holland, S., Pawson, T., Daniel, T.O., and Skolnik, E.Y. (2000). Nck-interacting Ste20 kinase couples Eph receptors to c-Jun N-terminal kinase and integrin activation. Mol. Cell. Biol. *20*, 1537–1545.

Birgbauer, E., Cowan, C.A., Sretavan, D.W., and Henkemeyer, M. (2000). Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. Development *127*, 1231–1241.

Birgbauer, E., Oster, S.F., Severin, C.G., and Sretavan, D.W. (2001). Retinal axon growth cones respond to EphB extracellular domains as inhibitory axon guidance cues. Development *128*, 3041–3048.

Bohme, B., VandenBos, T., Cerretti, D.P., Park, L.S., Holtrich, U., Rubsamen-Waigmann, H., and Strebhardt, K. (1996). Cell-cell adhesion mediated by binding of membrane-anchored ligand LERK-2 to the EPH-related receptor human embryonal kinase 2 promotes tyrosine kinase activity. J. Biol. Chem. *271*, 24747–24752.

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

Brandli, A.W., and Kirschner, M.W. (1995). Molecular cloning of tyrosine kinases in the early Xenopus embryo: identification of Eck-related genes expressed in cranial neural crest cells of the second (hyoid) arch. Dev. Dyn. *203*, 119–140.

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

Chien, C.B., Rosenthal, D.E., Harris, W.A., and Holt, C.E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. Neuron *11*, 237–251.

Chien, C.B., Cornel, E.M., and Holt, C.E. (1995). Absence of topography in precociously innervated tecta. Development *121*, 2621–2631.

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.

Cornel, E., and Holt, C. (1992). Precocious pathfinding: retinal axons can navigate in an axonless brain. Neuron *9*, 1001–1011.

Davy, A., Gale, N.W., Murray, E.W., Klinghoffer, R.A., Soriano, P., Feuerstein, C., and Robbins, S.M. (1999). Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes Dev. *13*, 3125–3135.

Davy, A., and Robbins, S.M. (2000). Ephrin-A5 modulates cell adhe-

sion and morphology in an integrin-dependent manner. EMBO J. *19*, 5396–5405.

Drescher, U., Bonhoeffer, F., and Muller, B.K. (1997). The Eph family in retinal axon guidance. Curr. Opin. Neurobiol. *7*, 75–80.

Durbin, L., Brennan, C., Shiomi, K., Cooke, J., Barrios, A., Shanmugalingam, S., Guthrie, B., Lindberg, R., and Holder, N. (1998). Eph signaling is required for segmentation and differentiation of the somites. Genes Dev. *12*, 3096–3109.

Eagleson, G., Ferreiro, B., and Harris, W.A. (1995). Fate of the anterior neural ridge and the morphogenesis of the Xenopus forebrain. J. Neurobiol. *28*, 146–158.

Feldheim, D.A., Kim, Y.I., Bergemann, A.D., Frisen, J., Barbacid, M., and Flanagan, J.G. (2000). Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron *25*, 563–574.

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

Helbling, P.M., Saulnier, D.M., Robinson, V., Christiansen, J.H., Wilkinson, D.G., and Brandli, A.W. (1999). Comparative analysis of embryonic gene expression defines potential interaction sites for Xenopus EphB4 receptors with ephrin-B ligands. Dev. Dyn. *216*, 361–373.

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.

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 signaling through the EPH-family receptor Nuk and its transmembrane ligands. Nature *383*, 722–725.

Holt, C.E., and Harris, W.A. (1983). Order in the initial retinotectal map in Xenopus: a new technique for labelling growing nerve fibres. Nature *301*, 150–152.

Holt, C.E., Garlick, N., and Cornel, E. (1990). Lipofection of cDNAs in the embryonic vertebrate central nervous system. Neuron *4*, 203–214.

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.

Huai, J., and Drescher, U. (2001). An ephrin-A-dependent signaling pathway controls integrin function and is linked to the tyrosine phosphorylation of a 120-kDa protein. J. Biol. Chem. *276*, 6689–6694.

Huynh-Do, U., Stein, E., Lane, A.A., Liu, H., Cerretti, D.P., and Daniel, T.O. (1999). Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through alphavbeta3 and alpha5beta1 integrins. EMBO J. *18*, 2165–2173.

Irie, A., Yates, E.A., Turnbull, J.E., and Holt, C.E. (2001). Specific heparan sulfate structures involved in retinal axon targeting. Development *129*, 61–70.

Jones, T.L., Karavanova, I., Maeno, M., Ong, R.C., Kung, H.F., and Daar, I.O. (1995). Expression of an amphibian homolog of the Eph family of receptor tyrosine kinases is developmentally regulated. Oncogene *10*, 1111–1117.

Jones, T.L., Karavanova, I., Chong, L., Zhou, R.P., and Daar, I.O. (1997). Identification of XLerk, an Eph family ligand regulated during mesoderm induction and neurogenesis in *Xenopus laevis*. Oncogene *14*, 2159–2166.

Jones, T.L., Chong, L.D., Kim, J., Xu, R.H., Kung, H.F., and Daar, I.O. (1998). Loss of cell adhesion in *Xenopus laevis* embryos mediated by the cytoplasmic domain of XLerk, an erythropoietin-producing hepatocellular ligand. Proc. Natl. Acad. Sci. USA *95*, 576–581.

Klein, R. (1999). Bidirectional signals establish boundaries. Curr. Biol. *9*, R691–R694.

Knoll, B., Zarbalis, K., Wurst, W., and Drescher, U. (2001). A role for the EphA family in the topographic targeting of vomeronasal axons. Development *128*, 895–906.

Koshiba-Takeuchi, K., Takeuchi, J.K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K., et al. (2000). Tbx5 and the retinotectum projection. Science *287*, 134–137.

Kullander, K., Croll, S.D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S., DeChiara, T.M., Klein, R., Yancopoulos, G.D., et al. (2001a). Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. Genes Dev. *15*, 877–888.

Kullander, K., Mather, N.K., Diella, F., Dottori, M., Boyd, A.W., and Klein, R. (2001b). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron *29*, 73–84.

Li, H., Tierney, C., Wen, L., Wu, J.Y., and Rao, Y. (1997). A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. Development *124*, 603–615.

Liu, Y., Lupo, G., Marchitiello, A., Gestri, G., He, R.Q., Banfi, S., and Barsacchi, G. (2001). Expression of the Xvax2 gene demarcates presumptive ventral telencephalon and specific visual structures in Xenopus laevis. Mech. Dev. *100*, 115–118.

Lu, Q., Sun, E.E., Klein, R.S., and Flanagan, J.G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. Cell *105*, 69–79.

Mann, F., Zhukareva, V., Pimenta, A., Levitt, P., and Bolz, J. (1998). Membrane-associated molecules guide limbic and nonlimbic thalamocortical projections. J. Neurosci. *18*, 9409–9419.

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.

Meima, L., Moran, P., Matthews, W., and Caras, I.W. (1997). Lerk2 (ephrin-B1) is a collapsing factor for a subset of cortical growth cones and acts by a mechanism different from AL-1 (ephrin-A5). Mol. Cell. Neurosci. *9*, 314–328.

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

Miao, H., Burnett, E., Kinch, M., Simon, E., and Wang, B. (2000). Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat. Cell Biol. *2*, 62–69.

Nakagawa, S., Brennan, C., Johnson, K.G., Shewan, D., Harris, W.A., and Holt, C.E. (2000). Ephrin-B regulates the ipsilateral routing of retinal axons at the optic chiasm. Neuron *25*, 599–610.

Nieuwkoop, P.D., and Faber, J. (1967). Normal Table of *Xenopus Laevis* (Daudin), Second Edition (Amsterdam: North Holland Publishing Company).

O'Leary, D.D., and Wilkinson, D.G. (1999). Eph receptors and ephrins in neural development. Curr. Opin. Neurobiol. *9*, 65–73.

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.

Riehl, R., Johnson, K., Bradley, R., Grunwald, G.B., Cornel, E., Lilienbaum, A., and Holt, C.E. (1996). Cadherin function is required for axon outgrowth in retinal ganglion cells in vivo. Neuron *17*, 837–848.

Scales, J.B., Winning, R.S., Renaud, C.S., Shea, L.J., and Sargent, T.D. (1995). Novel members of the eph receptor tyrosine kinase subfamily expressed during Xenopus development. Oncogene *11*, 1745–1752.

Schulte, D., Furukawa, T., Peters, M.A., Kozak, C.A., and Cepko, C.L. (1999). Misexpression of the Emx-related homeobox genes cVax and mVax2 ventralizes the retina and perturbs the retinotectal map. Neuron *24*, 541–553.

Shimamura, K., Hirano, S., McMahon, A.P., and Takeichi, M. (1994). Wnt-1-dependent regulation of local E-cadherin and alpha N-catenin expression in the embryonic mouse brain. Development *120*, 2225–2234.

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.

Sperry, R.W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA *50*, 703–710.

Stein, E., Lane, A.A., Cerretti, D.P., Schoecklmann, H.O., Schroff, A.D., Van Etten, R.L., and Daniel, T.O. (1998). Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. Genes Dev. *12*, 667–678.

Tanaka, M., Wang, D.Y., Kamo, T., Igarashi, H., Wang, Y., Xiang, Y.Y., Tanioka, F., Naito, Y., and Sugimura, H. (1998). Interaction of EphB2-tyrosine kinase receptor and its ligand conveys dorsalization signal in Xenopus laevis development. Oncogene *17*, 1509–1516.

Trowe, T., Klostermann, S., Baier, H., Granato, M., Crawford, A.D., Grunewald, B., Hoffmann, H., Karlstrom, R.O., Meyer, S.U., Muller, B., et al. (1996). Mutations disrupting the ordering and topographic mapping of axons in the retinotectal projection of the zebrafish, Danio rerio. Development *123*, 439–450.

Vielmetter, J., Stolze, B., Bonhoeffer, F., and Stuermer, C.A. (1990). In vitro assay to test differential substrate affinities of growing axons and migratory cells. Exp. Brain Res. *81*, 283–287.

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.

Wilkinson, D.G. (2000). Eph receptors and ephrins: regulators of guidance and assembly. Int. Rev. Cytol. *196*, 177–244.

Wilkinson, D.G. (2001). Multiple roles of EPH receptors and ephrins in neural development. Nat. Rev. Neurosci. *2*, 155–164.

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.

Yokoyama, N., Romero, M.I., Cowan, C.A., Galvan, P., Helmbacher, F., Charnay, P., Parada, L.F., and Henkemeyer, M. (2001). Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. Neuron *29*, 85–97.

Zou, J.X., Wang, B., Kalo, M.S., Zisch, A.H., Pasquale, E.B., and Ruoslahti, E. (1999). An Eph receptor regulates integrin activity through R-Ras. Proc. Natl. Acad. Sci. USA *96*, 13813–13818.