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Ephrin-B Regulates the Ipsilateral Routing of Retinal Axons at the Optic Chiasm

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Summary

In *Xenopus* tadpoles, all retinal ganglion cells (RGCs) send axons contralaterally across the optic chiasm. At metamorphosis, a subpopulation of EphB-expressing RGCs in the ventrotemporal retina begin to project ipsilaterally. However, when these metamorphic RGCs are grafted into embryos, they project contralaterally, suggesting that the embryonic chiasm lacks signals that guide axons ipsilaterally. Ephrin-B is expressed discretely at the chiasm of metamorphic but not premetamorphic *Xenopus*. When expressed prematurely in the embryonic chiasm, ephrin-B causes precocious ipsilateral projections from the EphB-expressing RGCs. Ephrin-B is also found in the chiasm of mammals, which have ipsilateral projections, but not in the chiasm of fish and birds, which do not. These results suggest that ephrin-B/EphB interactions play a key role in the sorting of axons at the vertebrate chiasm.

Introduction

To establish binocular vision, information from both eyes must converge on the same area of the brain. The optic chiasm is the major midline choice point where the axons from specific regions of the retina are guided either contralaterally or ipsilaterally, enabling particular retinal ganglion cells (RGCs) that share visual space to project to the same side of the brain (Guillery et al., 1995; Mason and Sretavan, 1997). In the rodent chiasm, for example, retinal axons from the temporoventral crescent are directed to the ipsilateral optic tract, whereas axons from the rest of the retina project contralaterally (Guillery et al., 1995; Mason and Sretavan, 1997). The region of binocular overlap depends on the position of the eyes relative to the body, which leads to a distinct distribution of the ipsilaterally projecting RGCs in the retina of different animals: temporal half in cats (Stone, 1966), temporoventral crescent in rodents (Colello and Guillery, 1990; Sretavan, 1990), and ventral half extending temporodorsally in the frog *Xenopus* (Hoskins and Grobstein, 1985a). In the mouse, it is well established that the retinal axons encounter a specific cellular environment and show differential behaviors at the chiasm depending on whether they cross or not (Godement et al., 1990; Sretavan, 1990; Marcus and Mason, 1995; Marcus et al., 1995, 1999). However, the molecular mechanisms that differentially guide the crossed or uncrossed axons at the chiasm are not known.

The retinofugal projection in *Xenopus* provides a unique system to approach this question. During metamorphosis, laterally positioned tadpole eyes move dorsofrontally, giving rise to binocular vision which suits the frog's predatory lifestyle (Grant and Keating, 1986). In addition to these external morphological changes, a modification is made in the projection pattern of retinal axons to the brain target (Hoskins, 1990). All the retinal axons in the early tadpole project to the contralateral brain; however, a subpopulation of newly generated RGCs in the ventral and temporal margin send axons ipsilaterally to the thalamus in the metamorphosing frog (Hoskins and Grobstein, 1985a, 1985b). It has been shown that thyroid hormone is necessary for the generation of the ipsilateral retinal projection as well as a variety of other events that occur during the amphibian metamorphosis (Hoskins, 1990). When tadpoles are reared in the presence of propylthiouracil (PTU), an inhibitor of thyroxine synthesis, development is stalled at the premetamorphic stage (stage 54), and no ipsilateral retinal projections arise (Hoskins and Grobstein, 1984, 1985c). Local application of thyroxine into the eye of PTU-treated tadpoles induces the asymmetric growth of dorsal and ventral marginal retina (Hoskins and Grobstein, 1984, 1985c) characteristic of the metamorphic period (Hollyfield, 1971; Beach and Jacobson, 1979a). Thyroxine also induces uncrossed projections from the hormone-injected eye but not from the other eye, suggesting that the thyroxine acts on the eye itself (Hoskins and Grobstein, 1984, 1985c). However, other studies have shown that tri-iodothyronine, an active form of thyroid hormone, cannot induce a premature ipsilateral retinal projection in *Xenopus* tadpoles (Kennard, 1981). Considering the delayed generation of uncrossed retinal axons in the neotenic urodele *Ambystoma mexicanum*, which does not undergo metamorphosis due to the lack of circulating thyroid hormone (Kennard, 1981), additional changes independent of thyroid hormone are likely necessary to make the ipsilateral projection.

Recently, a variety of axon guidance molecules have been identified and these have been categorized into four groups; chemoattractive, chemorepulsive, contact-mediated attractive, and contact-mediated repulsive molecules (Tessier-Lavigne and Goodman, 1996). A previous study using collagen gel culture showed that chiasm cells secrete diffusible axon growth–inhibiting factors (Wang et al., 1996), but this effect was not specific to ipsilaterally projecting axons. On the other hand, the

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in vitro stripe assays have shown that membranes prepared from chiasm cells inhibit axonal growth from ventrotemporal retina but not from ventronasal retina in the rat (Wizenmann et al., 1993). This differential repulsive effect is also observed on monolayer cultures of chiasm cells in the mouse (Wang et al., 1995). These studies suggest that the molecules that guide the retinal projection ipsilaterally are contact-dependent repulsive molecules. To date, three groups of molecules are known to mediate contact-dependent repulsive responses; transmembrane semaphorins, extracellular matrix molecules such as tenacin or proteoglycans, and ephrins (Tessier-Lavigne and Goodman, 1996). None of these molecules has been shown to selectively affect ipsilaterally projecting retinal axons at the chiasm.

To address whether the early tadpole brain has guidance cues necessary to make the ipsilateral projection, we transplanted the ventrotemporal retina from metamorphic tadpoles, which normally project ipsilaterally, into embryonic eyes at stage 33/34. Almost all transplanted RGCs send axons contralaterally, suggesting that the cues guiding axons ipsilaterally are absent in the early embryo. We also show that transmembrane B-type ephrin is expressed strongly in the chiasm of metamorphosing frogs but is not expressed in the chiasm of premetamorphic tadpoles. By transfecting ephrin-B into the embryonic chiasm, we demonstrate that expression of ephrin-B at the chiasm is sufficient to cause EphB-expressing ventral retinal cells to project ipsilaterally. The concordance between ephrin-B expression at the chiasm and the formation of an ipsilateral projection among different vertebrate species strongly suggests that this class of molecule has played a central role in the evolution of subcortical binocularity.

Results

Transplantation of Metamorphic Retina into Embryonic Eyes

To find out whether the cues that guide retinal axons ipsilaterally are already present in the chiasm of young tadpoles, we transplanted the marginal zone tissue from the ventrotemporal retinas of metamorphosing frogs (stage 58–64; Nieuwkoop and Faber, 1994), which normally project ipsilaterally, into the retinas of stage 33/34 embryos. The transplants were labeled with the fluorescent dye PKH26. Isochronic control transplants were made by grafting ventrotemporal retinal tissue from GFP-expressing stage 33/34 donors to unlabeled stage 33/34 hosts. The operated tadpoles were reared with or without thyroid hormone for 2 days, and the retinal projection was observed in whole flat-mounted brains. Most of the axons from the transplanted tissues followed the normal contralateral retinal pathway and reached the optic tectum (Figure 1A). Although a few axons occasionally projected to the ipsilateral brain (Figure 1B), the number of such uncrossed axons was very small, and there was no significant difference between heterochronic and isochronic transplantation or in the presence or absence of thyroid hormone (Table 1). To confirm that the host environment had not changed the identity of the donor explants, we transplanted ventralmost retinas from stage 58 frogs into dorsal or ventral retinas of stage 33/34 tadpoles. In both types of transplant, the axons innervated the dorsalmost tectum (Figures 1C–1F), the normal target region of ventral RGCs (Holt and Harris, 1983), suggesting that the old retinal grafts kept their topographical information after the transplantation. Topography was similarly maintained when pieces of dorsal retina from stage 58 were transplanted into the dorsal or ventral retina of stage 33/34 tadpoles (data not shown). These results demonstrated that neuronal identity is conserved cell autonomously as previously described for the retina of early stage *Xenopus* (Willshaw et al., 1983), and suggested that the axons from the transplants crossed the chiasm in early tadpoles because of an absence of molecular information to guide them ipsilaterally.

Thyroid Hormone Is Not Sufficient to Generate the Ipsilateral Projection in Early Stage Tadpoles

Previous studies (Hoskins and Grobstein, 1984, 1985c) showed that retinal application of thyroxine is sufficient to generate an ipsilateral retinal projection in tadpoles developmentally arrested with PTU just prior to metamorphosis (stage 54). However, our experiments showed that even in thyroxine-reared embryos, the vast majority of postmetamorphic RGCs transplanted into stage 33/34 embryos projected to the contralateral tectum (Table 1). One explanation for this discrepancy may be that thyroid hormone cannot induce an ipsilateral projection until tadpoles near metamorphosis, possibly because the young optic pathway is not yet competent to receive the thyroxine signal. To test this idea, we reared stage 52 tadpoles for 4 days in the presence of thyroxine and analyzed the retinal projection pattern by placing a DiI crystal on a cut stump of the optic nerve. After hormone treatment, the tadpoles had reached stage 53, as judged by limb morphology (data not shown), and exhibited characteristic changes in brain morphology (Figures 2A and 2B) and asymmetric promotion of cell proliferation in the dorsal and ventral ciliary marginal zones of the retina (Figures 2C–2F), as reported previously (Beach and Jacobson, 1979b). However, no obvious change occurred in the projection pattern of the retinal axons, which mostly crossed the midline and projected to the contralateral brain in the hormone-treated tadpoles (Figure 2G). Occasionally, a few axons were found in the ipsilateral brain, but they entered the deep region of the optic tract (Figure 2G, arrowheads). Considering that old retinal axons are displaced into the deep optic tract by the newly added axons (Fawcett et al., 1984), the ipsilateral projection found in these tadpoles was most likely generated before the thyroid hormone treatment and was therefore nonspecific. To test whether hormone treatment can induce the ipsilateral projection in later stage tadpoles, we treated PTU-reared stage 54 tadpoles with thyroxine. The hormone treatment accelerated development, causing tadpoles to reach stage 57, as judged by limb morphology (data not shown), in 4 days though it normally takes 2 weeks (Nieuwkoop and Faber, 1994). The ipsilateral retinal projection was clearly increased in the hormone-treated tadpoles, and the ipsilateral axons entered the optic pathway just beneath the pial surface (Figure 2H), where

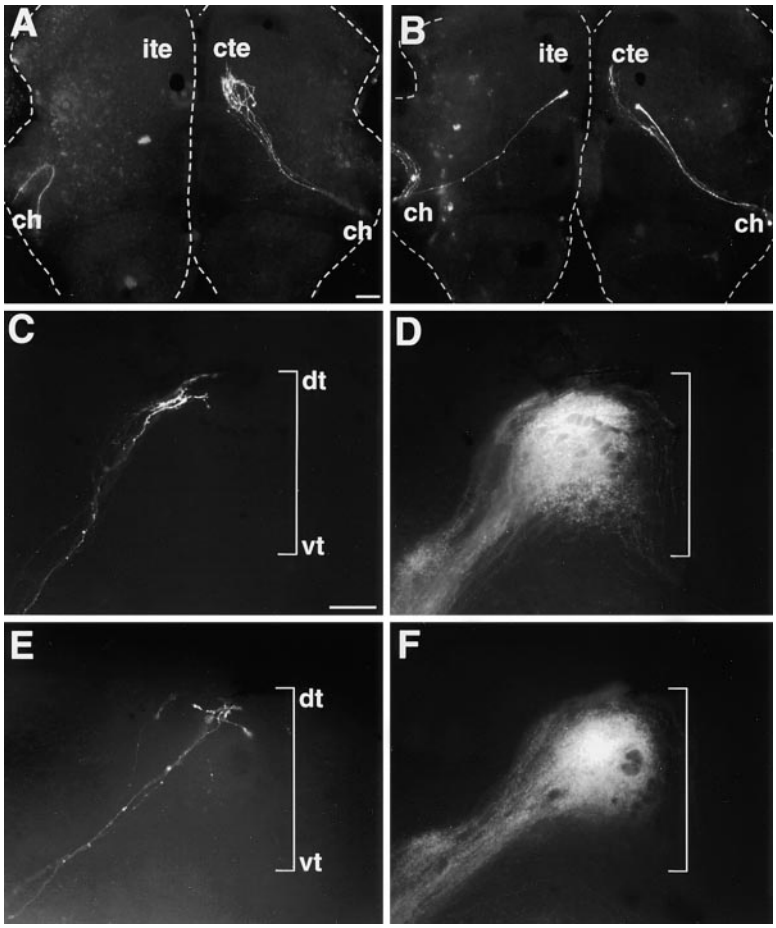

Figure 1. "Old" Retinal Neurons from Ipsilaterally Projecting Region of Frog Retina Send Axons Contralaterally in Young Tadpoles

(A and B) "Fillet" whole-mounted brains 2 days after the transplantation. Temporoventral retina of stage 64 frog was labeled with PKH26 and transplanted into temporoventral retina of stage 33/34 tadpole. Anterior is at the bottom; lateral is to the sides. More than 99% of axons navigate contralaterally as shown in (A), while less than 1% of axons go ipsilaterally as in (B). The dashed lines indicate the outline of the brains.

(C–F) Conservation of the topographical mapping after the transplantation. Ventral retina of stage 58 frog was labeled and transplanted into dorsal (C and D) or ventral (E and F) retina of stage 33/34 tadpoles. Axons from the transplants (C and E) projected to the dorsal part of the tectum in both cases at stage 43. Whole retinal projections at stage 43 are labeled with GFP (D and F) following grafts of eye primordia from GFP-RNA-injected embryos into unlabeled hosts.

Abbreviations: ite, ipsilateral tectum; cte, contralateral tectum; ch, chiasm; dt, dorsal tectum; vt, ventral tectum. Scale bars, 40 μm.

the newly generated axons are normally found (Fawcett et al., 1984). These results suggest that thyroxine alone is not sufficient to generate the ipsilateral projection prior to stage 52, and that the onset of responsiveness of the retinal projection to thyroid hormone occurs later in development, after stage 54.

Stage-Specific Expression of Ephrin-B at the Chiasm

We next tried to identify candidate axon guidance molecules that are not expressed in the chiasm of early tadpoles but are upregulated during metamorphosis. Ephrins and Ephs are ligand and receptor molecules involved in a variety of developmental processes (Holder and Klein, 1999; O'Leary and Wilkinson, 1999) including topographic ordering of the retinofugal projection (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Frisén et al., 1998; Feldheim et al., 1998). We noticed that the ventrotemporal RGCs from which the ipsilateral projection arises in *Xenopus* express high levels of Eph receptors (Cheng et al., 1995; Drescher et al., 1995; Holash and Pasquale, 1995; Kenny et al., 1995; Marcus et al., 1996; Braisted et al., 1997). This raised the possibility that ipsilaterally projecting axons might be routed to the same side of the brain through an interaction between Eph in the retina and ephrin in the chiasm. We therefore studied the expression pattern of several ephrin ligands using affinity probes with the extracellular domain of Eph receptors fused to alkaline phosphatase (AP tag staining) (Cheng and Flanagan, 1994). The ephrins and the Ephs fall largely into two binding specificity classes, A type and B type (Gale et al., 1996). We used EphA2-AP (Brennan et al., 1997) and EphB4-AP (Durbin et al., 1998) to detect ephrin-A and

Table 1. The Number of Axons Observed in Ipsi- or Contralateral Brain in the Presence or Absence of Thyroid Hormone

Donor Retina	n	Hormone	Ipsilateral	Contralateral	Ratio
stage 33/34	16	−	3	180	1.6%
stage 33/34	36	+	5	420	1.2%
stage 58–64	72	−	3	353	0.8%
stage 58–64	170	+	6	750	0.8%

n, the number of operated tadpoles; ipsilateral, the number of axons in ipsilateral brain; contralateral, the number of axons in contralateral brain; ratio, the number of ipsilateral axons divided by the total number of axons (the sum of ipsi- and contralateral).

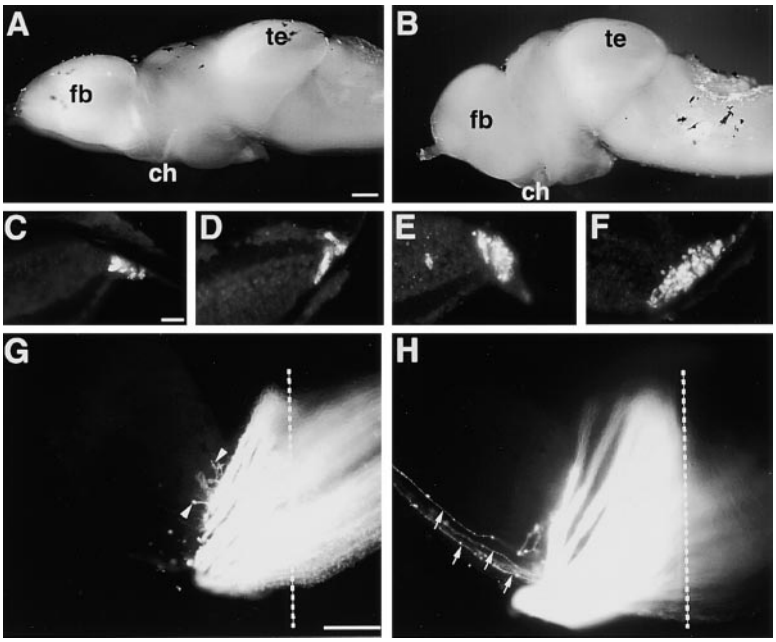

Figure 2. Thyroid Hormone Is Not Sufficient to Generate Ipsilateral Projection in the Early Tadpole

Stage 52 (A–G) or stage 54 (H) tadpoles were reared in the absence (A, C, and D) or presence (B and E–H) of thyroxine (10^{-9} M) for 4 days.

(A and B) Lateral view of whole-mount dissected brains.

(C–F) Cryo-section of the marginal region of retina showing BrdU incorporation in the dorsal (C and E) or ventral (D and F) marginal retinas.

(G and H) Transverse vibratome section at chiasm level. DiI was placed on a cut stump of optic nerve to label retinal projection. Arrowheads in (G) indicate the nonspecific ipsilaterally projecting axons in the deep optic pathway, which project to optic tectum on adjacent sections. Arrows in (H) indicate hormone-induced ipsilateral projection. Note that they are found close to the pial surface. Dashed lines in (G) and (H) indicate the midline.

Abbreviaitons: fb, forebrain; ch, chiasm; te, tectum. Scale bars: 200 μm (A and B), 20 μm (C–F), 100 μm (G and H).

ephrin-B, respectively. These probes are thought to detect all known ephrins (Gale et al., 1996). The conserved interaction between ephrin and Eph across species enabled us to detect the *Xenopus* ephrin/Eph proteins successfully using the AP tag from zebrafish genes (Figure 3). These initial experiments revealed that ephrin-B, but not ephrin-A, was expressed at the chiasm of stage 60 tadpoles when ipsilateral projections are being actively generated (Hoskins and Grobstein, 1985b). We therefore studied the expression pattern of ephrin-B and EphB during development.

The expression of ephrin-B at the chiasm was stage specific and could not be detected in embryos or premetamorphosing tadpoles earlier than stage 54 (Figure 3A), just before thyroid hormone–dependent metamorphosis begins (Hoskins and Grobstein, 1984, 1985c), while the expression was observed in other regions of the brain such as the olfactory bulb at this stage (Figure 3A, inset). Weak expression, however, was observed in the chiasm at stage 57 (data not shown), and a distinct AP tag signal was detected by stage 60 (Figures 3B and 3C). RNA in situ hybridization signals obtained with *Xenopus* ephrin-B1 and B2 probes at stage 60 mostly overlapped with the AP tag staining; however, the chiasm signal was located more dorsally in the ventricular zone (S. N. and C. E. H., unpublished data). This might be due to a difference in the distribution of ephrin-B mRNA and protein at the chiasm. As is evident in Figure 6D, dorsally located chiasm cell bodies send long processes ventrally that interdigitate with the optic axons. Alternatively, another type of ephrin-B, such as ephrin-B3, could be expressed.

The ephrin-B expression continued throughout and after metamorphosis and was also detected in the chiasm of frogs 2 months post metamorphosis (data not shown). Postmetamorphic brains tended to show low levels of uniform staining throughout the brain (compare Figures 3A and 3B), but it could not be determined whether this represented a real signal or background staining. To study the effect of thyroid hormone on the expression of ephrin-B in the chiasm, we reared stage 52 tadpoles or stage 54 tadpoles in the presence of thyroid hormone for 4 days and subsequently carried out AP tag staining. As described above, this hormone treatment caused rapid morphological changes, and the tadpoles reached stage 53 or 57, respectively. The ephrin-B protein was not detected in the stage 52 (53) tadpoles (Figure 3D), but weak expression was observed in the chiasm of stage 54 (57) tadpoles (Figure 3E) after the hormone treatment, which was consistent with the generation of ipsilateral projection by thyroxine described above (Figures 2G and 2H).

EphB Expression in the RGCs in the Ventrotemporal Retina

We then studied the expression pattern of the receptors for the ephrin-B ligands in the retina using ephrin-B2-AP probe. EphBs have been reported to be expressed in the ventral part of the retina in other species (Holash and Pasquale, 1995; Kenny et al., 1995; Marcus et al., 1996; Braisted et al., 1997), where most of the ipsilaterally projecting axons arise from in *Xenopus* (Hoskins and Grobstein, 1985a). At stage 35/36, well before the thyroid-dependent metamorphosis begins, a signal was observed in a small region of the ventralmost retina flanking the ventral choroidal fissure (Figure 4A). The expression was gradually upregulated and expanded to a wider ventral region, which resulted in the strong expression in the ventral third of the marginal region of the retina during metamorphosis (Figure 4B). We also studied the expression pattern of EphAs using ephrin-A5-AP probe, since a certain subtype of EphA (EphA4) binds to ephrin-B1/2 (Gale et al., 1996) and could thus be a possible receptor for the ephrin-Bs in the chiasm. Unlike in mouse or chicken (Cheng et al., 1995; Marcus et al., 1996), EphAs were most highly expressed in the

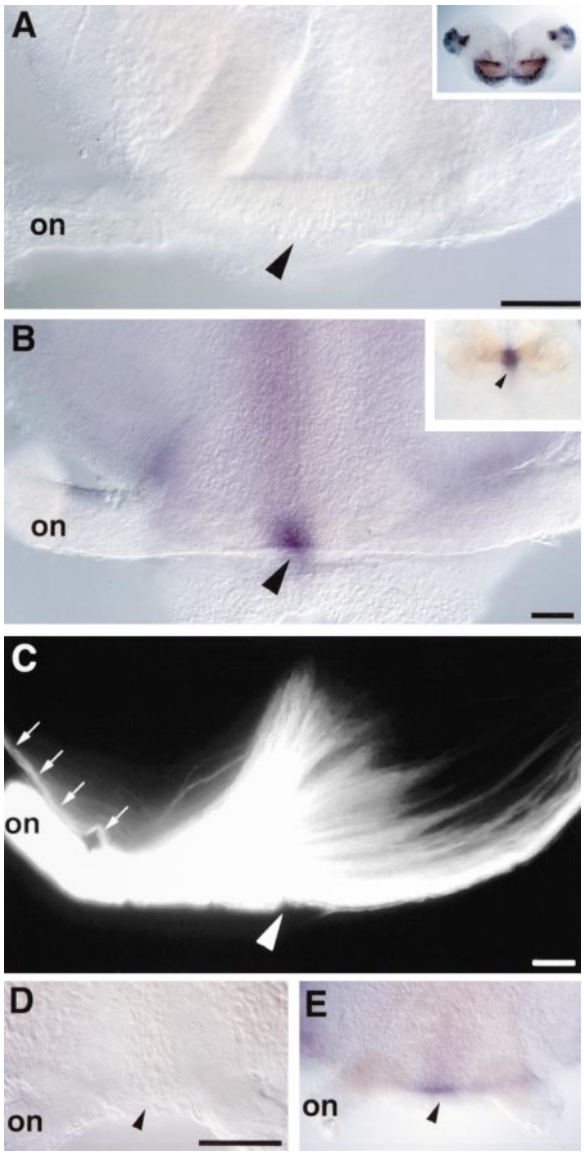

Figure 3. Ephrin-B Expression at the Chiasm Begins at Metamorphosis

Expression pattern of ephrin-B was revealed by AP tag staining on transverse vibratome sections except for the inset in (B), which is ventral view. Arrowheads indicate the position of the chiasm midline.

(A and B) Ephrin-B in stage 54 (A) and stage 60 (B) tadpoles. Inset in (A) shows the ephrin-B staining in the olfactory bulb of the same tadpole. These samples were processed under identical conditions at the same time.

(C) Distribution of retinal axons at the chiasm. Sections were taken from the same stage and the same axial level as (B). The DiI-labeled retinal axons cross the ephrin-B-expressing region. Arrows indicate the ipsilaterally projecting axons.

(D and E) Expression of ephrin-B in hormone-treated tadpoles. Stage 52 (D) or stage 54 (E) tadpoles were reared in the presence of thyroxine for 4 days.

Abbreviation: on, optic nerve. Scale bars, 100 μm.

ventrotemporal region, gradually decreasing in a "clockwise" manner toward the ventronasal region (Figure 4C), making a sharp boundary at the optic fissure (Figure 4C, arrow).

To compare the EphB-expressing region and the distribution of the ipsilaterally projecting RGCs, we injected DAPI into the dorsal diencephalon as a retrograde labeling tracer (Marsh-Armstrong et al., 1999). Since this labeling method was not compatible with AP tag staining, we detected EphB proteins using a specific antibody to EphB2 (Holash and Pasquale, 1995). Successful retrograde labeling was confirmed by the presence of labeled cells in the contralateral eye where they were distributed throughout the retina (Figures 4J and 4K). In the ipsilateral retina, DAPI-labeled RGCs were first observed in the peripheral margin of the temporal retina at stage 56 (Figure 4D). At this stage, EphB2 was expressed in the ventral third of the marginal retina (Figure 4E), higher in the temporal side than the nasal side, resulting in the asymmetrical left–right distribution of EphB2-positive retinal axons at the optic nerve head (Figure 4E, inset). At stage 66, the DAPI-labeled RGCs were found in a wider ventral region extending ventronasally (Figure 4F), which largely overlapped with EphB2-expressing region (Figure 4G). Sections of the ipsilateral retina revealed that EphB2 protein was localized mainly in the inner plexiform layer and RGC axons in the ventral retina (Figure 4H) but not in the dorsal retina (Figure 4I). Similarly, the retrogradely transported DAPI signal was found in the RGC layer of ventral retina (Figure 4H) but not in the dorsal retina (Figure 4I). The EphB2 signal in the ganglion cell bodies was very weak, and we could not detect obvious differences in the expression level between DAPI-positive and -negative cells (Figures 4H and 4J).

Ectopic Gene Expression at the Chiasm Using In Vivo Lipofection

To investigate the function of the late expression of ephrin-B at the chiasm, we designed an experiment to induce precocious expression of ephrin-B at the chiasm of early tadpoles. Using the in vivo lipofection technique for targeted overexpression (Holt et al., 1990), we microinjected DNA–lipid complex into the presumptive chiasm, the most anterior region of the neural tube at stage 19 (Figure 5A). At stage 40, the expression was mostly localized to the chiasm cells, although a few cells in the neural tube and head mesenchyme also expressed the lipofected GFP (Figure 5C). The expression was sometimes observed in the ventral retina at the level of the optic nerve head (Figure 5C, inset). On the other hand, we could not detect any expression at the chiasm when we microinjected DNA–lipid complex into the eye field (Figure 5B) except for the axonal signal coming from the lipofected eye (Figure 5D) that contained a large number of lipofected cells (Figure 5D, inset). Successful overexpression was obtained only when early embryos (stage 18–20) were used; injection at later stages yielded very poor expression (data not shown). We then studied the time course of the ectopic gene expression in relation to the growing retinal axons by injecting HRP into the retina and double staining for GFP and HRP. At stage 33/34, when the first retinal axons reach the chiasm (Holt, 1984), overexpressed proteins were detected in the chiasm region (Figures 5E and 5G). This expression continued for at least 48 hr after the lipofection (Figures 5F and 5H), when the initial retinotectal projections are first established (Holt, 1984).

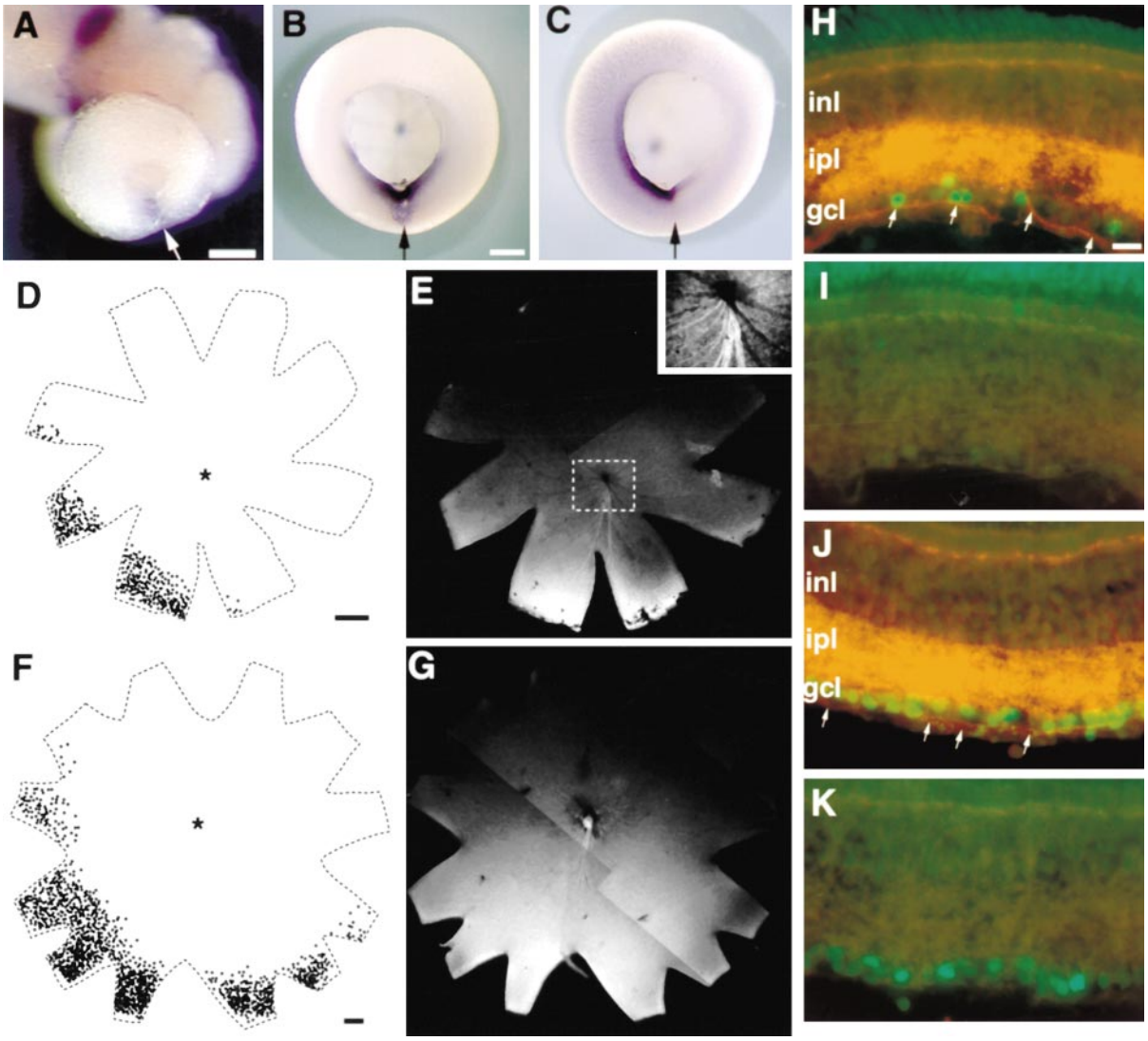

Figure 4. Receptors for Ephrin-B Are Expressed in the Ipsilaterally Projecting Region of the Retina

Expression patterns of EphBs (A and B) or EphAs (C) in intact whole-mount retinas of stage 35/36 (A) and stage 62 (B and C) tadpoles revealed by AP tag staining. Arrows indicate the position of the ventral fissure.

(D–G) Distribution of DAPI-positive ipsilaterally projecting cells (D and F) and immunolabeled EphB2-expressing cells (E and G) of stage 57 (D and E) or stage 66 (F and G) flat-mounted retinas. Asterisks in (D) and (F) indicate the position of the optic nerve head. The inset in (E) shows a higher magnification view of the optic nerve head (dashed box in [E]).

(H–K) Double staining of EphB2 (red) and retrogradely transported DAPI (green).

(H and I) Ipsilateral eyes.

(J and K) Contralateral eyes.

(H and J) Transverse cryo-sections of ventral retina.

(I, K) Transverse cryo-sections of dorsal retina.

Small arrows indicate EphB2 expression on RGC axons. Note that ipsilaterally projecting RGCs are located only in ventral retina (H) but not in dorsal retina (I), while contralaterally projecting cells are found both in ventral (J) and dorsal (K) retina. Dorsal is up in (A) through (G).

Abbreviations: inl, inner nuclear layer; ipl, inner plexiform layer; gcl, RGC layer. Scale bars: 100 μm (A), 200 μm (B–G), 20 μm (H–K).

Precocious Expression of Ephrin-B at the Chiasm Induces Ipsilateral Retinal Projection in the Embryo

To test if ephrin-B expression at the chiasm is sufficient to drive axons differentially to the ipsilateral side of the brain, we precociously expressed zebrafish ephrin-B2, which we used for detecting receptor expression in the tadpole retina by AP tag staining, or GFP, in the chiasm of early tadpoles (Figures 5A and 5C). Both of the proteins were myc-tagged at the C terminus to identify the lipofected cells. HRP was subsequently placed into one of the eyes to label the whole retinal pathway. In control stage 41 tadpoles, most axons crossed the midline and projected to the contralateral brain (Figures 6A and 6C). In ephrin-B2-lipofected tadpoles, however, a substantial number of axons failed to cross the ephrin-B-expressing midline cells and projected into the ipsilateral side of the brain (Figures 6B and 6D; Table 2). To analyze the results more quantitatively, we counted the number of ipsilaterally projecting axons either in the ventral, mid-

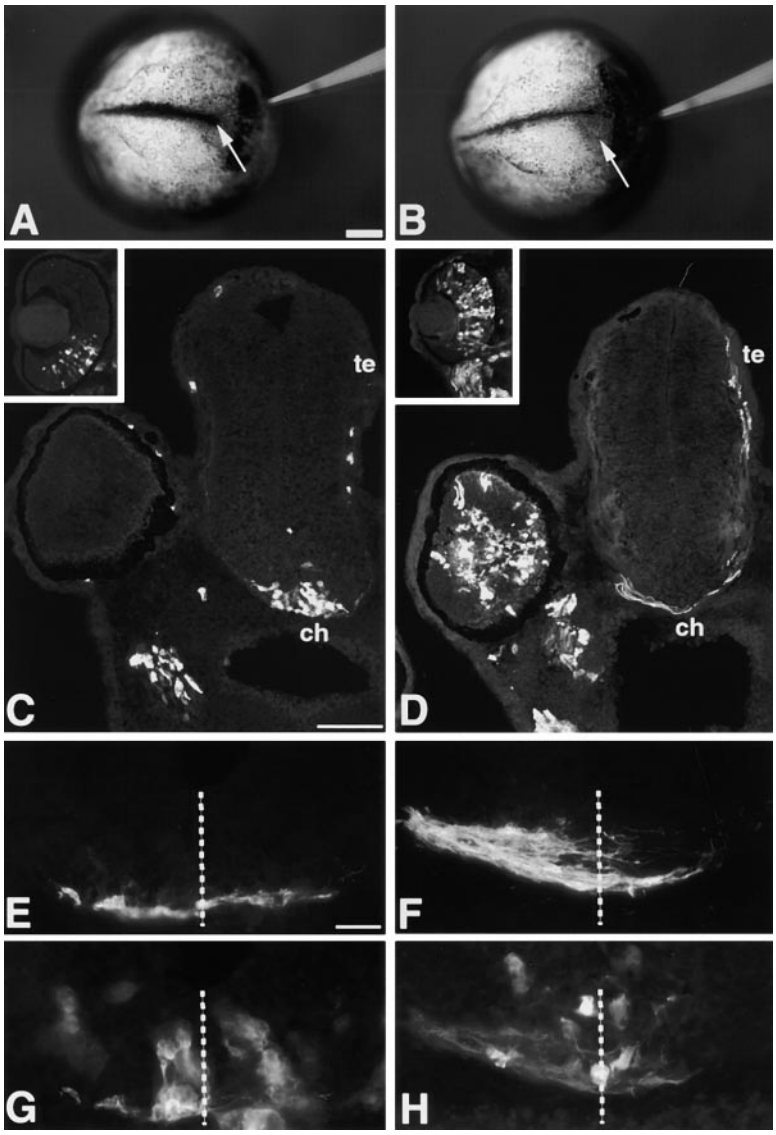

Figure 5. Ectopic Gene Expression in the Chiasm Region by Targeted Lipofection

(A and B) DNA–lipid complex was injected into either the future chiasm region (A) or the retinal primordium (B). Arrows indicate the position of the tip of the injection needle.

(C and D) Spatial distribution of the ectopic expression. GFP expression vector/lipid complex was microinjected into the chiasm (C) or retinal (D) region of stage 19 embryos. Transverse cryostat sections were stained with anti-GFP antibody. Insets show the retina at the level of the optic nerve head.

(E–H) Time course of the ectopic expression in the chiasm region. Dashed lines indicate the midline of the ventral neural tube. HRP was injected into the retina to visualize the growing retinal axons (E and F). The tadpoles were fixed at stage 33/34 (E and G) or stage 41 (F and H) and double stained for HRP (E and F) and GFP (G and H).

Abbreviations: te, tectum; ch, chiasm. Scale bars: 200 μm (A–D), 20 μm (G–H).

dle, or dorsal optic tract (Figure 6G). The increase in the number of ipsilaterally projecting axons was statistically significant when ephrin-B2 was ectopically expressed in the chiasm (Figure 6G). To exclude the possibility that ectopic expression in the retina, but not in the chiasm, was responsible for the effect, we overexpressed ephrin-B2 in the retina (Figures 5B and 5D). In this case we could not see any increase in the number of ipsilaterally projecting retinal axons (Figure 6G). Overexpression of GFP in the retina also did not increase the ipsilateral projection (data not shown).

To confirm that the precocious ipsilaterally projecting axons originated from the ventral population of EphB-expressing RGCs, a small DiI crystal was placed either in the ventral- or dorsalmost region of retina, which does or does not express EphB at this stage (Figure 4A), respectively. A prominent ipsilateral projection was observed when DiI was placed in the ventral retina, but not the dorsal retina (Figures 6E and 6F; Table 2), of the ephrin-B2-lipofected tadpoles. This suggests that the uncrossed axons arise as a result of a specific interaction between EphB and ephrin-B, and not by nonspecific perturbation of the midline-crossing behavior of retinal axons.

Expression of Ephrin-B at the Chiasm in Other Vertebrates

Finally, we carried out a comparative expression study of ephrin-B at the chiasm in zebrafish, chicken, and mouse. In zebrafish and chicken, almost all RGCs send axons across the chiasm and project to the contralateral brain (Guillery et al., 1995; Mason and Sretavan, 1997). There are only a few ipsilateral projections in these species (O'Leary et al., 1983), which are temporary and are removed during development. Interestingly, these species without the ipsilateral retinal projection had no ephrin-B expression in the chiasm (Figure 7A) at the stage when the majority of retinal axons are crossing it, which was consistent with previous studies (Braisted et al., 1997). Because the ephrin-B protein was detected

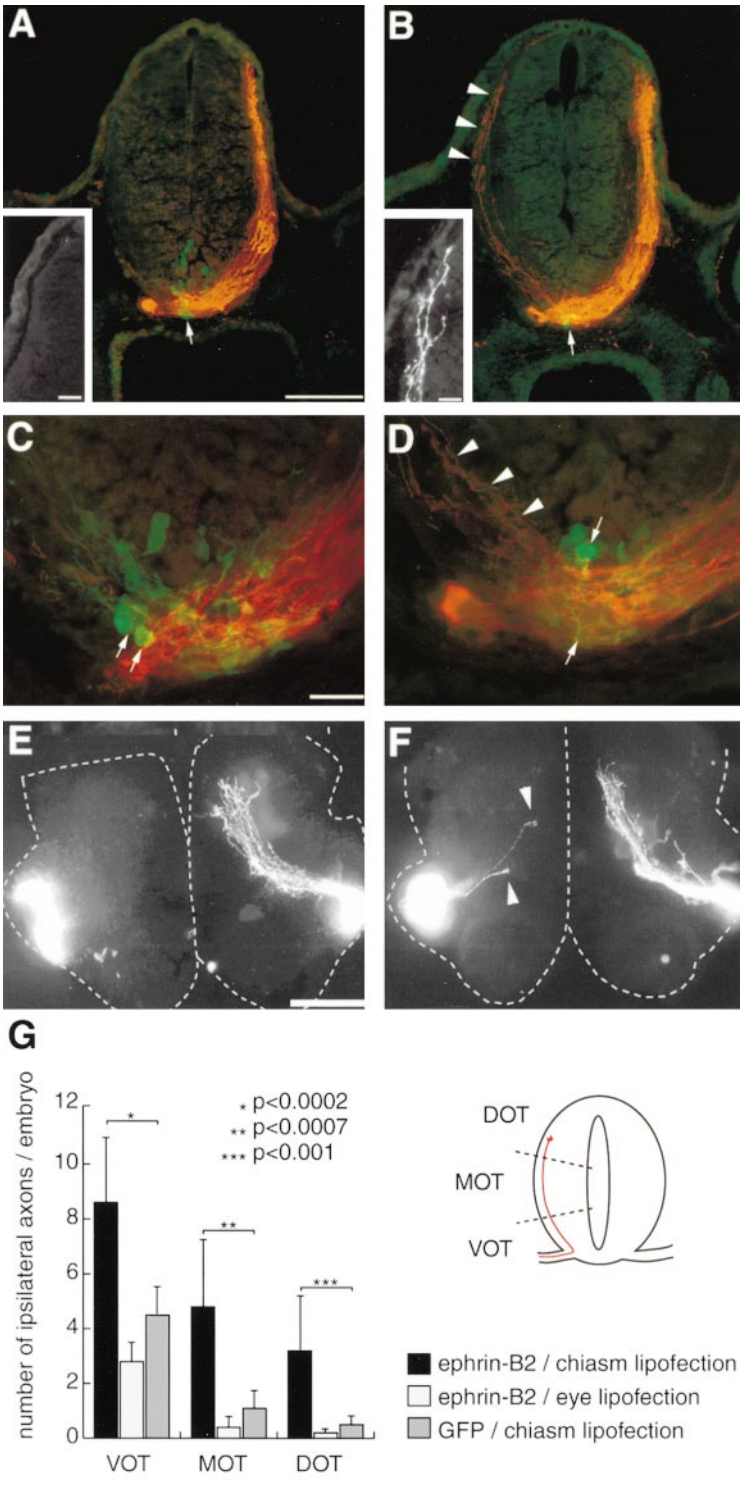

Figure 6. Ephrin-B Overexpression at the Chiasm Induces Precocious Ipsilateral Projection in the Early Tadpole

(A–D) Double staining for the HRP-labeled retinal axons (red) and lipofected cells (green). GFP-myc (A and C) or ephrin-B2-myc (B and D) was lipofected at stage 19, and HRP was injected in the retina at stage 41. Arrows indicate the lipofected chiasm cells and arrowheads indicate the ipsilaterally projecting axons. Since this region of the chiasm is largely composed of crossing axons, only a few lipofected cells are observed on these sections. Insets in (A) and (B) show the tectal region of the ipsilateral brain.

(E and F) DiI was placed on dorsal (E) or ventral (F) regions of the retina in the ephrin-B-lipofected tadpole. Arrowheads indicate ipsilaterally projecting axons to the tectum (top) and to the diencephalic nuclei (bottom), respectively. The dashed lines indicate the outlines of the brains. The orientation of the brains is the same as in Figures 1A and 1B.

(G) Statistical analysis (Student's t test) of the number of ipsilaterally projecting axons in the lipofected embryos. The ipsilateral optic tract was divided in three regions—dorsal (DOT), middle (MOT), and ventral (VOT)—and the number of axons was counted.

Scale bars: 20 μm (C and D) (insets in [A] and [B]), 100 μm (A, B, E, and F).

in the hypothalamus of zebrafish and chicken embryo, our AP tag probe was thought to detect the ephrin-B protein in these species (Figures 7A and 7B). In the mouse, ephrin-B was not expressed in the chiasm at early stages up to E12.5 (data not shown). Weak expression, however, was detected in E13.5 embryo (Figure 7C) and gradually upregulated in the older embryo, resulting in strong expression in the chiasm midline cells at E16.5 embryo (Figure 7D).

Discussion

The results presented here suggest that the discrete expression of ephrin-B at the chiasm during and after metamorphosis may play a key role in directing the growth of EphB-expressing retinal axons into the ipsilateral brain. Ephrin-B may therefore be responsible for the acquisition of the uncrossed retinothalamic projection in postmetamorphic *Xenopus*. The following observations

Table 2. The Number of Tadpoles that Have Axons in the Ipsilateral Brain

Lipofected Gene/Labeling method	n	Number of Uncrossed Axons 0	1–5	6+
GFP-myc/whole eye (HRP)	102	38	47	17
ephrin-B2-myc/whole eye (HRP)	69	9	21	39
ephrin-B2/whole eye (HRP)	101	12	32	57
GFP-myc/dorsal eye (DiI)	17	15	2	0
GFP-myc/ventral eye (DiI)	17	15	2	0
ephrin-B2-dorsal eye (DiI)	28	24	4	0
ephrin-B2/ventral eye (DiI)	44	17	27	0

Chiasm cells were lipofected with ephrin-B2/ephrin-B2-myc or GFP-myc, and the retinal axons were labeled with either HRP (whole-eye labeling) or DiI (region-specific labeling). Only tadpoles that have expression in the chiasm cells were counted; these were identified by anti-myc antibody or colipofected GFP.

support this idea. First, ephrin-B is not expressed in the chiasm of premetamorphic tadpoles and is switched on during metamorphosis. Second, EphB is expressed in the ipsilaterally projecting region of the retina. Third, misexpression of ephrin-B in the chiasm of early tadpoles induces a precocious ipsilateral projection from the EphB-expressing region of the retina. We recognize that the function of ephrin-B remains to be confirmed in complementary loss-of-function experiments, although it could be argued that the premetamorphic chiasm that does not express ephrin-B is similar to a conditional knockout.

Eph and ephrin are expressed in the adjacent regions in mouse embryos (Flenniken et al., 1996; Gale et al., 1996), and they regulate the formation of rhombomere boundaries in zebrafish (Xu et al., 1999). Similarly, the principal role of ephrin-B at the chiasm could be to restrict the chiasm cells from mixing with the surrounding cells and thus to establish the special cellular organization found in that region (Marcus and Mason, 1995; Marcus et al., 1995, 1999). For these reasons, it would be intriguing to analyze the retinal projection pattern in ephrin-B/EphB knockout mice that have defects in the forebrain commissural axons (Henkemeyer et al., 1996; Orioli et al., 1996; Wang et al., 1998), although conditional knockout mice would be necessary for the analysis of ephrin-B2 knockout mice because they die before the generation of the retinal projection.

The production of ipsilaterally projecting retinal axons is dependent on thyroid hormone and previous evidence suggests that the action of thyroid hormone is on the eye itself rather than on the chiasm or other brain regions (Hoskins and Grobstein, 1984, 1985c). Our results, however, reveal the importance of changes in the chiasm for directing axons ipsilaterally. Since a growth burst of the ventral retina occurs during metamorphosis (Hollyfield, 1971; Beach and Jacobson, 1979a; Marsh-Armstrong et al., 1999), it is possible that the normal role of thyroid hormone in the eye is to increase the number of EphB-expressing RGCs by stimulating their proliferation. Ephrin-B expression at the chiasm is not under the sole control of thyroid hormone because stage 52 tadpoles reared in thyroxine fail to express ephrin-B, while treatment at later stages causes ephrin-B upregulation. Thus, the dependency of ephrin-B expression on thyroid hormone is stage specific.

The ipsilaterally projecting axons were found to originate from the EphB-expressing region of the peripheral retina in postmetamorphic frogs. The absence of ipsilaterally projecting cells in the nonmarginal retina that express EphB can be explained by the growth mode of the *Xenopus* retina in which newly born cells are continuously added at the periphery (Glücksmann, 1940). Since ephrin-B is first expressed at the chiasm during metamorphosis, only the more peripheral EphB-expressing axons that cross the midline after the upregulation of ephrin-B would be affected. However, there are two other discrepancies in the distribution of the ipsilaterally projecting cells and the expression pattern of EphB. First, at the beginning of metamorphosis (stage 57), ipsilaterally projecting cells were found exclusively in the temporal but not nasal half of the ventral retina, while EphB was also expressed in the ventronasal retina. Second, the ipsilaterally projecting cells extended further dorsally in the temporal half than EphB expression. Interestingly, EphA4, one of the EphA receptors expressed in the retina of other species (Cheng et al., 1995; Marcus

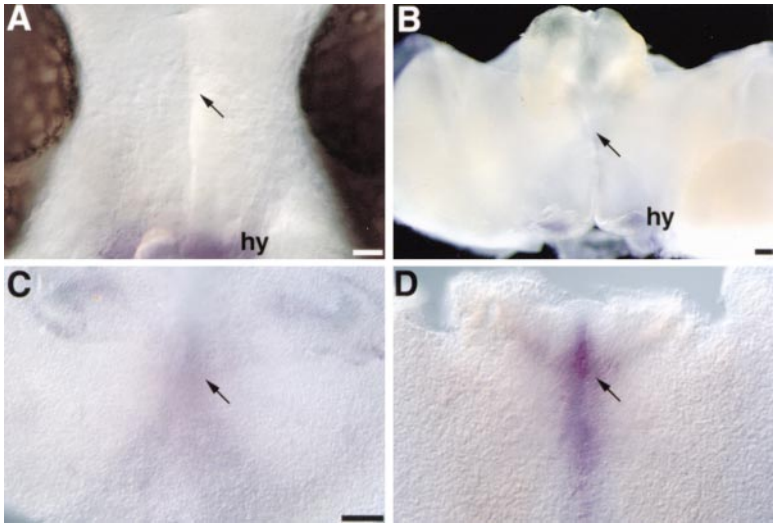

Figure 7. Expression of Ephrin-B at the Chiasm of Other Species from Ventral View

Expression pattern of ephrin-B was revealed by AP tag staining on horizontal vibratome sections (A, C, and D) or dissected whole-mount ventral diencephalon (B). In all cases, anterior is top and posterior is bottom.
(A) 48 hr zebrafish.
(B) E8 chicken.
(C) E13.5 mouse.
(D) E16.5 mouse.
The arrowheads indicate the position of the chiasm. Abbreviation: hy; hypothalamus. Scale bars: 20 μm (A), 200 μm (B–D).

et al., 1996; Brennan et al., 1997), has been reported to bind to ephrin-B1/B2 in addition to A-type ephrins (Gale et al., 1996). Our AP tag staining revealed that EphAs were expressed in the ventrotemporal region but not in the ventronasal region, raising the possibility that signals through both A-type and B-type receptors are involved in generating the ipsilateral projection from the correct region of the retina. This could also explain the dorsal extension of ipsilaterally projecting RGCs in the temporal half, where EphB receptor expression is fairly weak. It should also be noted that only a subpopulation (10%–15%) of the RGCs in the ventrotemporal retina send axons ipsilaterally in *Xenopus* (Hoskins and Grobstein, 1985a), while EphB expression in this region is rather uniform, implying the existence of additional mechanisms that cooperate with EphB.

If some EphA-expressing RGCs are able to respond to ephrin-B at the chiasm, then it is possible that misexpression of ephrin-A at the chiasm would also cause an embryonic ipsilateral projection. Our preliminary results indicate that this is indeed the case (S. N. and C. E. H., unpublished data). Nevertheless, since ephrin-A is not normally expressed at the chiasm, although it is expressed in the hypothalamus posterior to the chiasm (Marcus et al., 2000), it is unlikely that ephrin-As normally reroute axons ipsilaterally. Recently, it has been reported that overexpression of ephrin-A in chick retina causes abnormal ipsilateral projections to form (Dütting et al., 1999). In the light of the present study, it is possible that the ipsilateral projections were induced by the ectopic ephrin-A at the chiasm, because the whole optic pathway from retina to the optic chiasm was infected by the ephrin-A-expressing virus in this experiment (Dütting et al., 1999).

All the ipsilateral retinal projections in the metamorphic *Xenopus* innervate diencephalic nuclei, but not other target regions such as the optic tectum (Hoskins and Grobstein, 1985b). Therefore, it might be possible that some cofactors exclusively expressed in the thalamic-projecting cells are required to control the decision at the chiasm. If this is the case, RGCs projecting to the tectum cross the chiasm independent of EphB expression, while cells projecting to the thalamus cross the chiasm only when they do not express EphB. In our lipofection experiments, the precociously induced ipsilateral axons projected to the tectum rather than the thalamus (Figures 6B and 6F). Because the retinothalamic projections develop slightly later than the retinotectal projection (Holt and Harris, 1983), it is possible that the target region in the thalamus was immature and lacked the signals to tell the axons to stop at the stage when the precociously induced ipsilateral axons were passing through.

Prominent ipsilateral retinal projections may have evolved more than once in vertebrate evolution, since such projections are present in postmetamorphic lampreys, frogs, and mammals but are absent in fish, reptiles, and birds (Sarnat and Netsky, 1981). It is noteworthy that both mice and frogs express ephrin-B at the chiasm, which raises the possibility that they acquired the system independently using the same molecules. Alternatively, the prototypical retinal projection may have been bilateral, and ephrin-B was subsequently lost in certain vertebrate species. The transient ipsilateral projection in chick embryos (O'Leary et al., 1983) might arise due to weak ephrin-B expression in the chiasm that is below the level of detection with our methods, or from temporary expression after E8. Interestingly, ephrin-B3 is expressed in the ventral midline of spinal cord (Bergemann et al., 1998) and Eph/ephrin signaling controls midline crossing in *C. elegans* (Zallen et al., 1999). Considering the fact that EphB receptor knockout mice have defects in the commissural axons in the forebrain (Henkemeyer et al., 1996; Orioli et al., 1996), it might be possible that ephrin-B/EphB signaling at the midline provides a general mechanism for regulating midline crossing both in vertebrates and invertebrates.

Experimental Procedures

Animals

Stages were determined according to normal tables (Nieuwkoop and Faber, 1994). For hormone treatment experiments, tadpoles were reared in 0.1× MMR containing 10^{-9} M thyroxine (Sigma) or 0.01% PTU (Sigma).

Labeling Techniques

Metamorphosing frogs (stage 58–64) were anesthetized with MS222 in MMR. After decapitation, eyes were dissected out and incubated with 0.05% trypsin (1:250, Difco) in MMR for 5 min. The marginal region of the retina was dissected free, cut into small pieces, and then incubated with 1% PKH26 in Diluent C for 5 min (Sigma). The labeling reaction was stopped by adding 1% BSA in MMR and labeled explants were washed several times with MMR. Since PKH-26 labeling was not compatible with the young retinal tissues containing a large amount of yolk, 1 ng of GFP-myc mRNA was injected at the 2-cell stage using standard procedures (Chien et al., 1995). The injected embryos were reared in 0.1× MMR to reach stage 33/34, and specific regions of the retina were dissected out. Horseradish peroxidase (HRP) was used as previously described (Chien et al., 1995) to label the entire retinal projection in the lipofected tadpoles. Transplantation-mediated whole pathway labeling was performed for the topographical mapping experiment as previously described (Chien et al., 1995). For DiI labeling, a small crystal of DiI was placed either in the dorsal or ventral retina of stage 41 tadpoles following lens removal. They were fixed in 4% paraformaldehyde for 1 hr at room temperature and kept in the dark for 2 days. To label the optic pathway of later stage tadpoles, DiI was placed at the cut stump of the optic nerve after fixation and the brain was kept in the dark for 2 weeks. Retrograde labeling of retinal axons was performed following the protocol recently developed by Dr. Marsh-Armstrong (Marsh-Armstrong et al., 1999). To label proliferating cells, 1 μl injections of BrdU (50 mM, Sigma) were made intraabdominally in anesthetized tadpoles. After 2 hr of survival, the retina was fixed and processed for immunohistochemistry.

Transplantation of the Retinal Pieces

Host embryos (stage 33/34) were anesthetized with MS222. A small slit was made between the lens primordium and the retinal margin, and the labeled explants were inserted between them using sharpened pins. The embryos were transferred into a clay-bottomed dish, and a small piece of glass was placed on top of the wound for 30 min to help healing. Then they were reared in 0.1× MMR for two days to reach stage 43. The operated tadpoles were then anesthetized and fixed, and the brains were dissected out. After cutting the ventral midline with fine scissors, the brains were flat mounted with Fluorsave (Calbiochem).

Immunostaining

Antibodies used were as follows: rabbit anti-Cek5 (a kind gift of E. B. Pasquale), mouse anti-myc monoclonal antibody 9E10 (Sigma), rabbit polyclonal antibody to HRP (Sigma), Cy3-conjugated goat anti-rabbit IgG (Chemicon), and Alexa488-conjugated goat anti-mouse and anti-rabbit IgG (Molecular Probes). Embryos were fixed in 4% paraformaldehyde in HEPES-buffered (pH 7.6) salt solution

(HBSS) at room temperature for 1 hr. Immunohistochemistry was carried out following the procedures described previously (Chien et al., 1995).

AP Tag Staining

The preparation of the EphA2-AP, EphB4-AP, and ephrin-B2-AP, has been described previously (Brennan et al., 1997; Durbin et al., 1998). Whole-mount alkaline phosphatase binding studies were performed as described (Cheng and Flanagan, 1994) with slight modification. Briefly, unfixed brains were sliced into thick sections (200–300 μm) using a vibratome (Technical Products International), incubated with probes for 2 hr, washed five times with MMR, fixed in 4% paraformaldehyde for 1 hr on ice, rinsed three times with HBSS, and heat-inactivated for 1 hr at 65°C. They were cooled and processed for NBT-BCIP color reaction. For staining retina, dissected retina freed from the pigment epithelium was used.

In Vivo Lipofection

The cDNA constructs used were pCS2-ephrin-B2 (Durbin et al., 1998), pCS2-GFP-myc, and pCS2-ephrin-B2-myc. pCS2-GFP-myc and pCS2 ephrin-B2-myc were made by subcloning whole coding sequence of GFP or ephrin-B2 into BamHI-ClaI site of pCS2-MT (Turner and Weintraub, 1994). In vivo lipofection was carried out as described previously (Holt et al., 1990). Two micrograms of plasmid DNA (1 μg/ml) were mixed with 6 μl of DOTAP (Roche) and injected into the future chiasm region in the anterior midline of the neural tube at stage 19. For colipofection, 1.8 μg of ephrin-B2 was mixed with 0.2 μg of GFP-myc. Ten nanoliters of DNA–lipid complex were injected using a picospritzer (General Valve), and embryos were reared in MMR for several hours and 0.1× MMR for 2 days to reach stage 41.

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