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Bifunctional action of ephrin-B1 as a repellent and attractant to control bidirectional branch extension in dorsal-ventral retinotopic mapping

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SUMMARY

We report that the EphB receptor ligand, ephrin-B1, may act bifunctionally as both a branch repellent and attractant to control the unique mechanisms in mapping the dorsal-ventral (DV) retinal axis along the lateral-medial (LM) axis of the optic tectum. EphB receptors are expressed in a low to high DV gradient by retinal ganglion cells (RGCs), and ephrin-B1 is expressed in a low to high LM gradient in the tectum. RGC axons lack DV ordering along the LM tectal axis, but directionally extend interstitial branches that establish retinotopically ordered arbors. Recent studies show that ephrin-B1 acts as an attractant in DV mapping and in controlling directional branch extension. Modeling indicates that proper DV mapping requires that this attractant activity cooperates with a repellent activity in a gradient that mimics ephrin-B1. We show that ectopic domains of high, graded ephrin-B1 expression created by retroviral transfection repel interstitial branches of RGC axons and redirect their extension along the LM tectal axis, away from their proper termination zones (TZs). In contrast, the primary RGC axons are unaffected and extend through the ectopic domains of ephrin-B1 and arborize at the topographically correct site. However, when the location of a TZ is coincident with ectopic domains of ephrin-B1, the domains appear to inhibit arborization and shape the distribution of arbors. Our findings indicate that ephrin-B1 selectively controls, through either attraction or repulsion, the directional extension and arborization of interstitial branches extended by RGC axons arising from the same DV position: branches that arise from axons positioned lateral to the correct TZ are attracted up the gradient of ephrin-B1 and branches that arise from axons positioned medial to the same TZ are repelled down the ephrin-B1 gradient. Alternatively, EphB receptor signaling may act as a 'ligand-density sensor' and titrate signaling pathways that promote branch extension toward an optimal ephrin-B1 concentration found at the TZ; branches located either medial or lateral to the TZ would encounter a gradient of increasingly favored attachment in the direction of the TZ.

Key words: Axon attraction, Axon branching, Axon guidance, Axon repellents, Chick visual system, Electroporation, Optic tectum, Eph receptors, Gradients, Recombinant retrovirus, Topographic maps

INTRODUCTION

Topographic maps are a fundamental organizational feature of most axonal connections in the brain. The dominant model system for studying map development is the projection from the retina to its major midbrain target, the superior colliculus of mammals, or its non-mammalian homolog, the optic tectum. The precise spatial ordering of axonal arborizations of retinal ganglion cells (RGCs) maps the visual world along two sets of orthogonally oriented axes: the temporal-nasal (TN) axis of the retina along the anterior-posterior (AP) axis of the tectum, and the dorsal-ventral (DV) retinal axis along the lateral-medial (LM) tectal axis. Models that account for map development are based on graded distributions of axon guidance molecules and receptors (Sperry, 1963; Gierer, 1987). The mechanisms responsible, in part, for topographic mapping of the TN retinal axis along the AP tectal axis have been identified. In particular, ephrin-A2 and ephrin-A5, and a subset of their EphA receptors, are expressed in complementary graded patterns in the tectum and retina, and mediate axon repulsion that differentially affects temporal and nasal RGC axon guidance and interstitial branching along the AP tectal axis (Nakamoto et al., 1996; Monschau et al., 1997; Brown et al., 2000; Feldheim et al., 2000; Yates et al., 2001; McLaughlin et al., 2002).

The developmental mechanism by which the DV retinal axis maps along the LM tectal axis imposes unique requirements on the molecular activities that control this process (Fig. 1A). Initially, RGC axons from the same location in the retina enter the tectum at its anterior border over a broad LM extent. In rats and mice, RGC axons arising from the same DV retinal location are distributed across 80% of the LM axis of the SC (Simon and O'Leary, 1992a; Simon and O'Leary, 1992b; Hindges et al., 2002). In chick, RGC axons arising from the same DV retinal location are found millimeters medial and lateral to the correct location of their future termination zone (TZ) along the LM axis (Nakamura and O'Leary, 1989). Even along the AP tectal axis, the guidance of the primary axons is topographically inaccurate since they substantially overshoot

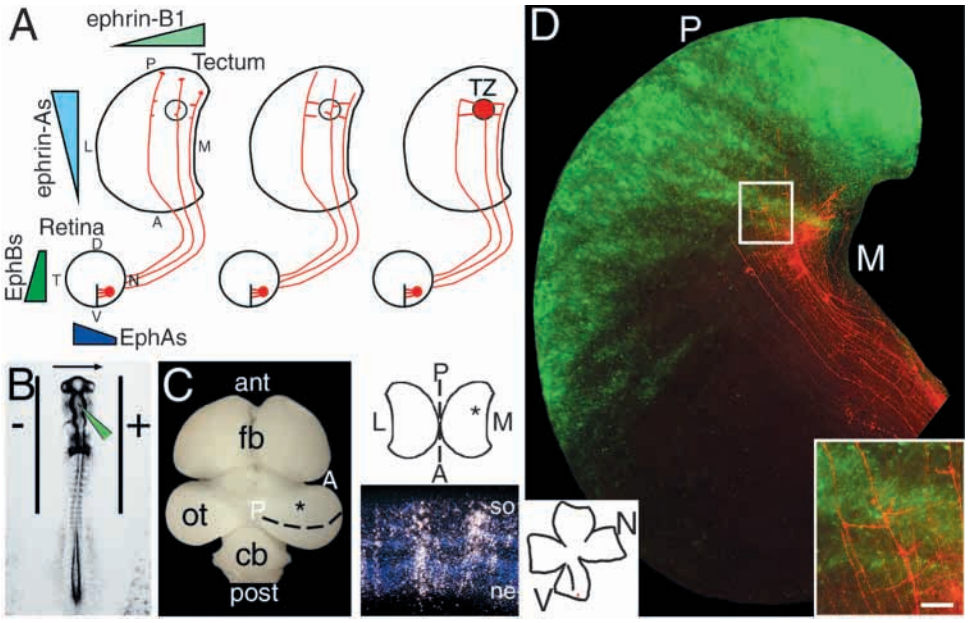

Fig. 1. Mechanisms that establish the retinotectal topographic map and lack of an effect of electroporation of a control RCAS vector on its development. (A) Normal development of the retinotopic map in the chick retinotectal projection. Initially, RGC axons extend posteriorly past the AP (anterior-posterior) location of their future TZ (circle). In addition, RGC axons originating from the same DV (dorsal-ventral) retinal location enter and extend across the tectum with a broad distribution along its LM (lateral-medial) axis. RGC axons form interstitial branches along their shafts at the AP level of their TZ; the branches are extended along the LM axis toward the TZ where they arborize. Later, overshooting segments of the primary axons are eliminated. Graded expression of Eph receptors in the retina and their ephrin ligands in the tectum are indicated. (B) Schematic of midbrain electroporation procedure on an E1.5 chick embryo. Cathode (+) and anode (–) electrodes were positioned on the opposite sides of the midbrain. (C) Dorsal view of an E12 chick brain. Between E6, when RGC axons first enter the tectum anteriorly, and E12, the tectal lobes rotate such that anterior tectum moves ventrally and away from the midline. This rotation results in the developmental AP (anterior-posterior) axis of the tectum (dashed line) being roughly perpendicular to the AP axis of the brain. For analysis, the optic tectum (ot) was removed, cut along the AP tectal axis, and the medial and lateral halves were mounted whole as shown in the drawing at right. The asterisk is in the same location in the photo and drawing. (C) In situ hybridization using an S^{35}-labeled ephrin-B1 probe on a coronal section through an E13 tectum transfected on E1.5 with an RCAS-ephrin-B1-IRES-eGFP. The transfection results in a columnar pattern of ectopic ephrin-B1 expression from the neuroepithelium (ne) to the stratum opticum (so). (D) Medial (M) half of an E13.5 tectum transfected with RCAS-eGFP at E1.5. Transfections domains express the green eGFP reporter. DiI was focally injected into NV (nasal-ventral) retina (red dot, left inset). DiI-labeled RGC axons (red) are visible in posterior tectum and arborize at the correct location for their TZ in mid-tectum. Branches are unaffected in areas of eGFP expression (right inset). RGC axons are also unaffected by the eGFP. cb, cerebellum; fb, forebrain. Scale bar in D: 300 μm and 100 μm in right inset.

the AP location of their TZ (Yates et al., 2001). RGC axons establish their appropriately ordered connections through interstitial branches that form perpendicular to the shaft of the primary RGC axon, with a topographic bias for the AP location of the TZ (Simon and O'Leary, 1992c; Yates et al., 2001). Subsequently, the interstitial branches extend medially or laterally across the tectum to reach and arborize at the topographically correct position along the LM axis (Nakamura and O'Leary, 1989; Simon and O'Leary, 1992b; Hindges et al., 2002) (present study). Thus, guidance of the primary RGC axon mainly serves to put the axon in the vicinity of the target, whereas the topographic formation and guidance of interstitial branches is the critical determinant in setting up ordered connections.

RGC axons preferentially extend interstitial branches towards the appropriate location of their future TZ, regardless of whether they are located lateral or medial to it (Nakamura and O'Leary, 1989), indicating that the branches respond to graded molecular information that defines position along the LM axis. Therefore, the requirements for DV mapping along the LM tectal axis are distinct from those of TN mapping along the AP tectal axis, where all RGCs are repelled, albeit to different degrees, by ephrin-As (Monschau et al., 1997; Brown et al., 2000; Feldheim et al., 2000; Yates et al., 2001). The fundamental event leading to topography along the LM axis is the appropriate guidance of interstitial branches. Because RGC axons from the same DV retinal location are required to connect to the TZ from positions either lateral or medial to it, they must be able to preferentially extend branches medially or laterally.

Recent studies have shown that the EphB subfamily of receptor tyrosine kinases and their ephrin-B ligands act as attractants to control, in part, mapping of the DV retinal axis along the LM tectal axis (Hindges et al., 2002; Mann et al., 2002; McLaughlin et al., 2003). During map development in chicks and mice, EphB2, EphB3 and EphB4 are expressed in a low to high DV gradient by RGCs, EphB1 is expressed uniformly (Holash and Pasquale, 1995; Henkemeyer et al., 1996; Connor et al., 1998; Birgbauer et al., 2000; Hindges et al., 2002) and one of their ligands, ephrin-B1, is expressed in a low to high LM gradient across the tectum (Braisted et al., 1997; Hindges et al., 2002). RGC axons arising from the same DV retinal location, and therefore having similar levels of EphB receptors, extend interstitial branches either medially up the ephrin-B1 gradient or laterally down it to reach the LM location of their TZ (Fig. 1A). At least two distinct molecular activities are required to control this mapping behavior: one activity, accounted for at least in part by ephrin-B1-mediated attraction of branches medially up the gradient, shown by analyses of EphB2; EphB3 mutant mice (Hindges et al., 2002), and a second activity directs branches laterally. Modeling indicates that the additional activity is a branch repellent

expressed in a gradient similar to ephrin-B1 (Hindges et al., 2002).

We suggest that ephrin-B1 is not only an attractant for interstitial branches, but is also a repellent for them, and that a branch's response is context-dependent and depends upon the DV origin of its primary RGC axon, which determines its level of EphB receptors, and the origin of the branch relative to the LM location of its future TZ, and therefore its position along the ephrin-B1 gradient. Together, these parameters determine the level of EphB signaling experienced by a given branch, and whether it is less or more than that at the appropriate location of its TZ. The plausibility of a bifunctional action of ephrin-B1 is suggested by evidence that ephrin-Bs can have either a repellent or attractant affect on distinct populations of early and late migrating neural crest cells (Santiago and Erickson, 2002), and also findings that other guidance molecules, such as netrin 1, act as a repellent or an attractant for different types of axons (Colamarino et al., 1995) or for the same spinal axons depending upon the intracellular level of cyclic nucleotides (Ming et al., 1997; Song et al., 1997). To test this hypothesis, we have used RCAS vectors to ectopically express ephrin-B1 in the developing chick tectum and analyzed the effect of ectopic domains of ephrin-B1 on the trajectories and mapping of RGC axons, and the directional extension and arborization of their interstitial branches.

MATERIALS AND METHODS

In situ hybridization

^{35}S-labeled antisense riboprobes were synthesized from the full-length coding region of chick ephrin-B1 cDNA and a 1100 bp fragment of chick EphB2 cDNA (Wang and Anderson, 1997). In situ hybridizations were performed on 20 μm cryosections as described previously (Zhadanov et al., 1995).

Retroviral construction

We made three retroviral constructs based on the avian retroviral RCAS vector (Fekete and Cepko, 1993). Total RNA was harvested from E8 chick tectum using a QIAquick kit (Qiagen) and then cDNA was prepared by reverse transcription with random primers. PCR amplification of full-length ephrin-B1 was performed with nested primers specific for ephrin-B1. Four clones were sequenced; one of the four clones with the correct predicted protein sequence was used. For the RCAS-ephrin-B1-eGFP fusion construct, the stop codon of ephrin-B1 was replaced with the start codon of eGFP and inserted into the *Cla*I site of the RCAS vector. For the RCAS-ephrin-B1-IRES-eGFP construct, eGFP (Clontech) was inserted into the SLIRES11 shuttle vector (a gift from C. Cepko). Then the IRES-eGFP was amplified by PCR with primers containing a 5′ *Cla*I site and a 3′ *Cla*I site protected by a 5′ guanine, thus preventing *Cla*I cleavage when methylated. The PCR product was directly cloned into the *Cla*I site of RCAS, making RCAS-IRES-eGFP. Full-length ephrin-B1 was inserted into the RCAS-IRES-eGFP vector upstream of the IRES at the functional *Cla*I site. The third clone, RCAS-eGFP was made by cloning eGFP into the SLAX shuttle vector, then into the *Cla*I site of RCAS. All constructs were sequenced for orientation and fidelity.

Retroviral vector electroporation

Eggs of the white Leghorn strain of chicken were obtained from a local supplier (McIntyre Farms, Lakeside, CA) and incubated at 38°C for 40 hours prior to electroporation. At stages 10-12 (Hamburger and Hamilton, 1951), eggs were windowed, 2.5 ml of albumin removed, and they were injected in the mesencephalic ventricular space with a small amount of RCAS-eGFP, RCAS-ephrin-B1-eGFP, or RCAS-ephrin-B1-IRES-eGFP plasmid DNA at 1-4 μg/μl mixed with Fast Green (to visualize the solution) as described by Erkman et al. (Erkman et al., 2000). Parallel platinum-coated electrodes spaced at 4 mm were placed along the embryo such that the embryo was centered and its anterior – posterior axis was parallel to the electrodes. A small drop of L15 (Gibco) was placed on the embryo and five square pulses of 50 milliseconds at 25 volts were applied by a T820 Electrosquare porator (BTX). Nine to 16 days later embryos were perfused, dissected and examined with a confocal microscope (Zeiss). Typically, only one tectal lobe was transfected and only animals in which the entire infection was limited to one tectal lobe were analyzed further.

Anterograde axon labeling

Anterograde labeling of RGC axons was done as described by Yates et al. (Yates et al., 2001). Chicks were injected with a small amount of a 10% solution of DiI (Molecular Probes) in DMF into the retina and perfused 1 day later. Whole mounts of the optic tectum contralateral to the injected eye were examined on a Zeiss confocal microscope. Two-channel confocal microscopy was performed to record all DiI and eGFP present in the optic tectum.

Quantification of branch orientation

Confocal images were taken with a 10× objective from which montages were made and aligned with Adobe Photoshop software to create a full view of each tectum. The medial and lateral borders of the emerging termination zone (TZ) were marked using Adobe Photoshop software. The image was expanded so individual DiI-labeled axons and their branches could be easily visualized and unambiguously identified. All branches and their orientation were marked on the image prior to knowledge of infection domain and blind to their precise location within the tectum. All domains of eGFP in chicks electroporated with RCAS-eGFP or RCAS-ephrin-B1-IRES-eGFP were marked, blind to location in tectum, on each whole-mount montage. All eGFP marks were made blind to all DiI labeling. The interstitial branch and eGFP tracings were overlayed and branches in contact with eGFP markings were considered to be in a domain of infection. The tectum was separated into three sections based on the exact location of the nascent TZ. The orientation, length, relation to eGFP expression (whether eGFP alone or eGFP coexpressed with ephrin-B1), and position of the primary axon for all branches was recorded. RGC axons were labeled with DiI and analyzed between E11 and E14 for the branch directionality data presented in Fig. 3. E10-E13 chicks were used to collect data that relates branch directionality to branch length.

Receptor affinity probe staining

Recombinant mouse EphB2 fused to the Fc portion of human IgG (R&D Systems) was used to stain E7-E15 chick tecta as previously described (Braisted et al., 1997). A cy3-conjugated goat-anti-human secondary antibody (Jackson) was used to reveal EphB2-Fc binding sites.

RESULTS

Effects of transfection domains of control and ephrin-B1 RCAS vectors on retinotectal topography

To test the proposed bifunctional role for ephrin-B1 in retinotectal mapping, we made several RCAS-based, replication-competent retroviral expression vectors to electroporate in ovo into the developing chick midbrain. As a control, we used an RCAS-eGFP vector, which in addition to viral proteins, expresses the green fluorescent marker protein,

eGFP. We used two distinct RCAS vectors to express ephrin-B1. One is an RCAS-ephrin-B1-eGFP vector that expresses a full-length ephrin-B1 fused to eGFP at its C terminus. Therefore, the detection of eGFP reveals the precise location of the virally expressed ephrin-B1. In addition, we used an RCAS-ephrin-B1-IRES-eGFP that produces a single ephrin-B1-IRES-eGFP transcript that is translated into two distinct proteins: the native version of ephrin-B1 and the reporter eGFP. Thus, each cell expressing eGFP also expresses the virally introduced native ephrin-B1. Our findings are similar using either ephrin-B1 vector, indicating that fusing eGFP to the C terminus of ephrin-B1 does not significantly alter the functions of ephrin-B1 analyzed here. Therefore, we consider the data obtained with the two vectors together.

Each vector was transfected into the mesencephalic vesicle at E1.5 using in ovo electroporation (Fig. 1B). Transfection domains were limited to a single tectal lobe (not shown). We were able to obtain persistent expression of eGFP alone, or eGFP and ephrin-B1, to at least E17 with no detectable reduction in expression. Because the RCAS vector encodes a replication competent avian retrovirus, in addition to the viral vector being integrated into the genome of the host progenitor cells and being passed on to its progeny, the transfected/infected cells produce viable viral particles, which likely infect nearby progenitors, leading to larger domains of vector-expressing cells. Electroporated tecta were characterized by transfection/infection domains in columnar patterns extending from the ventricular zone to the pial surface (Fig. 1C), ensuring the presence of exogenous ephrin-B1 in the stratum opticum, the intratectal path of RGC axons and their branches, and the superficial layers of the tectum where the interstitial branches arborize, throughout the development of retinotectal topography. For simplicity, we will refer to the ectopic expression domains as transfections. To analyze the effects of the expression vectors on retinotectal mapping, a small number of RGC axons were anterogradely labeled by the fluorescent axon tracer DiI, injected into the eye contralateral to the transfected tectum, 16 hours before fixation.

Transfection domains of control RCAS vectors have no effect on retinotectal development

As expected, the RCAS-eGFP vector had no effect on RGC axon trajectory, the directional extension of interstitial branches, or location of the TZ (n=3 tecta) (Fig. 1D). Even within domains of eGFP, interstitial branches were evident and were preferentially directed along the LM axis towards their TZ (Fig. 1D inset). In contrast, as described below, ectopic domains of ephrin-B1 created by transfection with either the RCAS-ephrin-B1-eGFP vector or the RCAS-ephrin-B1-IRES-eGFP vector had substantial effects on the arborization of RGC axons and the directional extension of interstitial branches.

Ectopic domains of ephrin-B1 have no influence on the trajectories of primary RGC axons

Surprisingly, the ectopic domains of ephrin-B1 in the tectum had no influence on the growth or trajectories of primary RGC axons regardless of whether they arose from ventral retina with high levels of EphB expression, or from more dorsal retina with lower levels of EphB expression (Fig. 2A-A″; n=11 tecta). The topographic positioning of the TZ formed by the DiI-labeled RGC axons in the ephrin-B1 transfected cases appears to be at the topographically correct site, regardless of whether the TZ formed in a location within the tectum heavily transfected with ectopic domains of ephrin-B1, or lacking them entirely. This lack of effect differs from the strong repellent effect that ectopic domains of ephrin-A2 have on temporal RGC axons and on the topographic positioning of their TZs (Nakamoto et al., 1996).

Ectopic domains of ephrin-B1 restrict or inhibit the arborization of RGC axons

In contrast, we found that the ectopic domains of ephrin-B1 restrict or inhibit the arborization of RGC axons in each of the 10 cases in which the domains co-localize with the DiI-labeled TZs. In six of the 10 cases, the ectopic domains of ephrin-B1 circumscribed a well-defined, densely labeled TZ, with little or no overlap between areas of dense arborization and the ectopic domains of ephrin-B1 (Fig. 2B-B″,C-C″). These complementary distributions of arbors and ectopic domains of ephrin-B1 suggest that high levels of ephrin-B1 act as a repellent for branches that form the dense arborizations of RGC axons, and thereby hem in the arbors and restrict their extent. This effect of ephrin-B1 closely resembles the repellent effect of ectopic domains of ephrin-A2 on the arborizations of temporal RGC axons [see figure 4 in Nakamoto et al. (Nakamoto et al., 1996)].

Perhaps the most dramatic examples of the repellent action of the ectopic domains of ephrin-B1 are their apparent ability to shape and distribute an arbor. Two such examples are illustrated in Fig. 2. In one example, a comet-like TZ had a bulbous head abutting an ectopic domain of ephrin-B1, and a narrow tail confined to a space hemmed by ectopic domains of ephrin-B1 (Fig. 2C-C″). In another example, an ectopic domain of ephrin-B1 splits what would likely have been a single high density arbor into two distinct high density components with a domain of substantially reduced density between them that is coincident with an elongated ectopic domain of ephrin-B1 (Fig. 2D-D″).

In the remaining four of the 10 ‘co-localized’ cases, the DiI-labeled TZ was found at a tectal location coincident with small ectopic domains of ephrin-B1. These TZs were not consistently dense as in controls, but contained areas devoid of significant branching or arborization. In most instances, these areas of diminished branching and arborization corresponded precisely to small ectopic domains of ephrin-B1 (Fig. 2E-E″). Taken together, these findings indicate that high levels of ephrin-B1 act as a repellent or inhibitor of the branching process that leads to the formation of dense arborizations of RGC axons within the tectum.

Bi-functional effects of ephrin-B1 on directional extension of interstitial branches

In chicks, as in rodents, arbors are formed by interstitial branches that extend directionally from the shafts of primary RGC axons either medially up the ephrin-B1 gradient, or laterally down it, to reach the LM location of their TZ (Fig. 1A). Recent work has shown that EphB forward signaling acts as an attractant to guide interstitial branches medially up the gradient of ephrin-B1, which is consistent with ventral RGCs, expressing high levels of EphBs, mapping to medial tectum with high levels of ephrin-B1 (Hindges et al., 2002). Although our findings above show that ectopic domains of ephrin-B1 do

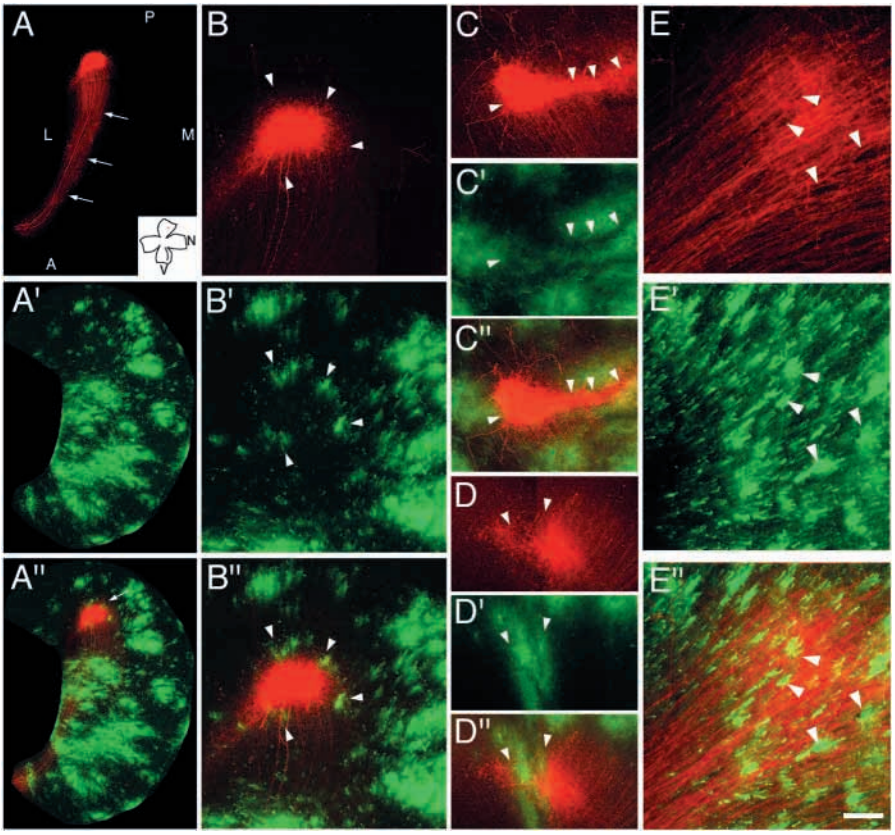

Fig. 2. Ectopic domains of ephrin-B1 inhibit/repel the arborization of RGC axons, but do not affect their trajectories. Ectopic domains of ephrin-B1 are marked by the green eGFP reporter and RGC axons and arborizations are labeled red by anterogradely transported DiI. (A″,B″,C″,D″ and E″ are merged images of A and A′ etc.) (A-B″) Lateral (L) half of an E14 tectum transfected with RCAS-ephrin-B1-IRES-eGFP. DiI was focally injected into nasal (N) dorsal retina. (A-A″) RGC axons extend without deviation through ectopic domains of ephrin-B1. (B-B″) Close up views reveal that ectopic domains of ephrin-B1 ring the TZ (arrowheads). (C-D″) Ventral RGC axons in tecta transfected with RCAS-ephrin-B1-eGFP. In both cases, a dense TZ is present in the appropriate location. However, the TZs appear to be shaped by the domains of ectopic ephrin-B1-eGFP. (C-C″) Ectopic domains of ephrin-B1 hem in the TZ; dense arborizations of the TZ fill areas with little or no ectopic expression of ephrin-B1 (arrowheads). (D-D″) The TZ is split into a larger and smaller component of dense arborization by an area of sparse arborization coincident with an ectopic domain of ephrin-B1 (arrowheads). (E-E″) A TZ in medial tectum at E13 interspersed with small patches of ectopic domains of ephrin-B1. The tectum was transfected with RCAS-ephrin-B1-IRES-eGFP. The usually uniformly dense TZ is perforated with areas of sparse arborization (arrowheads in E) that in most instances are coincident with ectopic domains of ephrin-B1 (arrowheads in E′). V, ventral. Scale bar in E″: 900 μm (A), 300 μm (B,C,D) and 200 μm (E).

not affect the trajectories of primary RGC axons, they do have a repellent or inhibitory effect on their arborizations. Thus, in addition to ephrin-B1 being an attractant for interstitial branches, it may also be a repellent for them, and therefore have a bifunctional role in guiding interstitial branches along the LM axis.

To address this issue, we analyzed the directional extension of all visible interstitial branches in control and ephrin-B1-transfected tecta of E11-E14 chicks 1 day after focal DiI injections were made into the retina. High-resolution confocal images were analyzed, often in three-dimensional projections to confirm the points of origin of branches and their directionality. All branches were digitally traced, blind to infection domains, from confocal images and their length, location, and orientation recorded. We also digitally marked all areas of eGFP, blind to RGC axon labeling, in all RCAS-ephrin-B1-IRES-eGFP animals. The quantification scheme is outlined in Fig. 3A.

Directional extension of branches in control tecta

In control tecta, temporal axons (n=14 tecta; Fig. 3B) and nasal-ventral axons (n=11 tecta; Fig. 3C) exhibited similar preferences in the directional extension of interstitial branches towards the LM location of their future TZ. For both populations, RGC axons located lateral to their future TZ preferentially extended interstitial branches medially toward their future TZ, whereas axons located medial to the TZ preferentially extended branches laterally (Fig. 3C). At the LM location of the TZ, we observed no bias in branch orientation. As expected, outside of the TZ, directional branch extension is related to branch length. Branches shorter than 25 μm in length are randomly oriented (53% extend towards their future TZ; n=21 cases, 146 branches; χ^2 test P=0.51). Branches greater than 100 μm in length are preferentially directed towards their future TZ (69% extend towards the TZ; n=200 branches; P<<0.001). These data indicate that initial branch formation along the primary axon occurs without a directional response to molecular information that encodes position along the LM axis, but that branches initially directed toward their TZ preferentially elongate toward it.

Essentially no difference was observed in the directional extension of branches toward the TZ from axons displaced lateral or medial to their TZ (Fig. 3B,C), indicating that equally effective mechanisms account for their guidance. The similarities in these data from different retinal sites, and to published data from peripheral temporal retina (Nakamura and O'Leary, 1989) suggest that RGC axons throughout the retina use the same mechanism. Therefore, for the analysis of the effects of ephrin-B1, we focused on ventral RGCs, which express high levels of EphBs and normally arborize in medial tectum, which expresses high levels of endogenous ephrin-B1.

Directional branch extension is biased laterally within ectopic domains of ephrin-B1

Within ectopic domains of ephrin-B1, regardless of the location within the tectum and their relationship to the TZ,

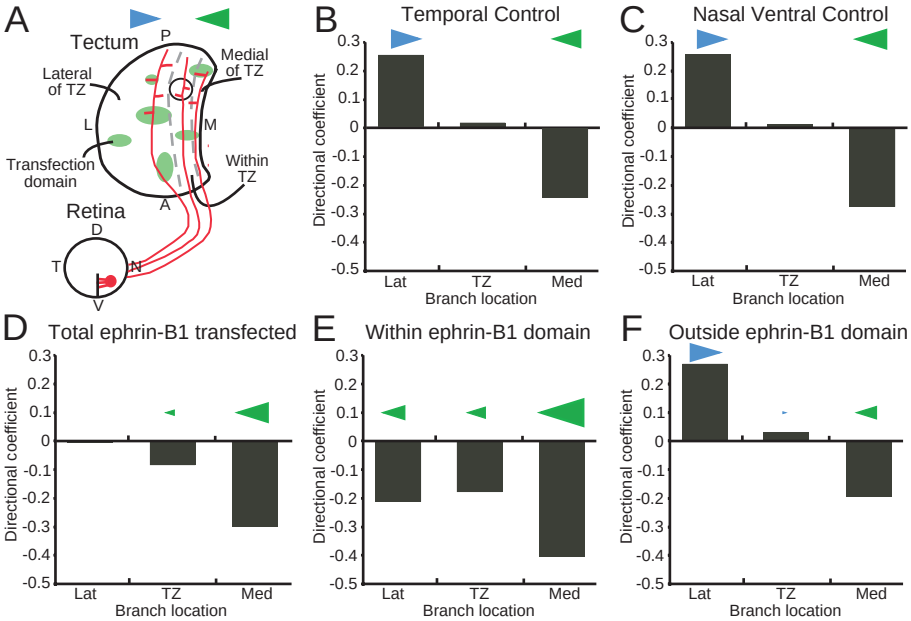

Fig. 3. The normal preferential extension of interstitial branches toward their TZ is altered by ectopic domains of ephrin-B1 consistent with a repellent action. (A) Quantification scheme. The tectum was divided into three domains: medial of the TZ, within the LM extent of the TZ, and lateral of the TZ. Branches in each of these bins were scored as either directed laterally or medially, and as within or outside a transfection domain. The blue and green arrowheads represent the relative strength and direction of the branching preference. A Directional Coefficient (DC) was calculated by subtracting the percentage of branches directed laterally from the percentage of branches directed medially. A positive DC indicates a preference to branch medially, whereas a negative DC indicates a preference to branch laterally. (B,C) In control E11-E14 chicks most branches formed along temporal RGC axons, outside the LM extent of the TZ, extend towards the TZ. At the LM position of the TZ branches show no preference in orientation. The temporal control cases (B) included 14 normal, non-transfected tecta (n=499 branches). The nasal-ventral control cases (C) included 3 RCAS-eGFP-transfected tecta, 1 tectum electroporated with RCAS-ephrin-B1-IRES-eGFP, but in which no eGFP reporter labeling was detected, and 7 normal non-transfected tecta (n=399 branches). (D) In chicks transfected with RCAS-ephrin-B1-IRES-eGFP (n=11 tecta, 700 branches), quantitation of branch directionality, irrespective of their relationship to ectopic domains of ephrin-B1, shows a disruption in normal directionality and a bias to extend laterally. (E) Branches from RCAS-ephrin-B1-IRES-eGFP-transfected cases that were located within an ectopic domain of ephrin-B1 expression were directed laterally, regardless of position (n=11 tecta, 386 branches). (F) Branches in RCAS-ephrin-B1-IRES-eGFP transfected cases that were located outside an ectopic domain of ephrin-B1 exhibited normal branching preferences toward their TZ (n=11 tecta, 314 branches). Statistical tests of significance of quantitation of directional extension of interstitial branches: Nasal-ventral controls (C): in the lateral bin, more branches were directed medially than laterally, Student's paired t-test, $P<0.01$; at the LM location of the nascent TZ, there is no difference in branch directionality, $P=0.87$; in the medial bin, more branches are directed laterally than medially, $P<0.04$. Temporal controls (B) show the same branch directionality as the nasal-ventral controls (C): lateral bins, $P=0.92$; TZ bins, $P=0.80$; medial bins, $P=0.84$ (χ^2 test). Test of significance for all interstitial branches in ephrin-B1 transfected tecta (D) compared to nasal-ventral controls (C): lateral bins, $P<<0.001$ (χ^2 test); TZ and medial bins are not significantly different. Test of significance in ephrin-B1-transfected tecta for branch directionality within ectopic domains of ephrin-B1 (E) compared to nasal-ventral controls (C): lateral bins, $P<<0.001$; TZ bins, $P<0.04$; medial bins, $P=0.367$ (χ^2 test). Test of significance in ephrin-B1-transfected tecta for branch directionality within ectopic domains of ephrin-B1 (E) versus outside of the domains (F): lateral bins, $P<<0.001$; TZ bins, $P<0.04$; medial bins, $P<0.1$ (χ^2 test). Test of significance in ephrin-B1 transfected tecta for branch directionality outside of ectopic domains of ephrin-B1 (F) compared to nasal-ventral controls (C): lateral bins, $P=0.863$; TZ bins, $P=0.85$; medial bins, $P=0.52$ (χ^2 test).

branches tended to extend laterally, down the gradient of endogenous ephrin-B1. Even in cases where the primary axon is adjacent to the forming TZ, but within an ectopic domain of ephrin-B1, branches were directed away from the TZ (Fig. 4). In addition, branches extended from the same primary axon located lateral to the future TZ can exhibit different directionalities that relate to whether they extend within or outside of an ectopic domain of ephrin-B1: branches outside an ectopic domain of ephrin-B1 preferentially extended medially towards the TZ up the gradient of endogenous ephrin-B1, whereas branches within an ectopic domain of ephrin-B1 preferentially extended laterally away from the TZ down the gradient of endogenous ephrin-B1 (Fig. 4B-D, arrowheads). These findings suggest that high levels of ephrin-B1 act as a repellent for interstitial branches.

As an initial quantitative analysis of this phenomenon, in tecta transfected with RCAS-ephrin-B1-IRES-eGFP (n=11) we scored the directional extension of all branches, irrespective of their relationship to ectopic domains of ephrin-B1. We found that a higher percentage of branches extended laterally than in controls (compare Fig. 3D with 3B,C). Thus, in spite of this indiscriminating analysis, the overall directional extension of interstitial branches is altered in a manner consistent with high levels of ephrin-B1 acting as a repellent for interstitial branches, even though the number and extent of the ectopic domains of ephrin-B1 differ substantially from case to case, and cover only a portion of the tectum.

To examine directly the effect of high levels of ephrin-B1 on directional branch extension, we divided the pool of branches into those that were in ectopic domains of ephrin-B1 and those that were not. Within ectopic domains of ephrin-B1, branches exhibited a significant bias to be directed laterally, down the endogenous ephrin-B1 gradient, regardless of their location along the LM axis (Fig. 3E). In contrast, in the same tecta, branches found outside the ectopic domains of ephrin-B1 exhibited the same directionality as in control tecta: those lateral to the TZ preferentially extended medially towards it and up the ephrin-B1 gradient, whereas those medial to the TZ preferentially extended laterally towards it and down the ephrin-B1 gradient (Fig. 3F, compare with 3B,C). Thus, the effect of the ectopic domains of ephrin-B1 on branch directionality is limited to the transfection domains.

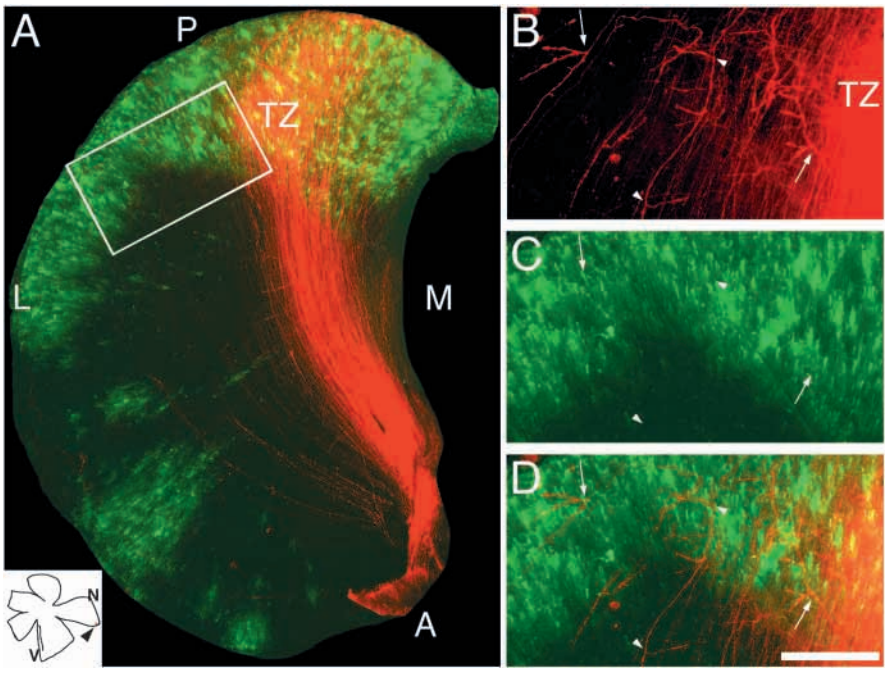

Fig. 4. Interstitial branches extended within ectopic domains of ephrin-B1 are directed away from their correct TZ. (A) Medial half of an E13 tectum transfected with RCAS-ephrin-B1-IRES-eGFP. A focal injection of DiI was made in NV (nasal-ventral) retina (arrowhead, inset). RGC axons form a TZ in MP (medial-posterior) tectum. Ectopic domains of ephrin-B1 are marked by the green eGFP reporter and RGC axons, interstitial branches and arborizations are labeled red by anterogradely transported DiI. The boxed area lateral to the TZ is enlarged in B-D. (B-D) Most branches that extend within an ectopic domain of ephrin-B1 are directed laterally, away from their TZ (arrows). Branches that extend outside ectopic domains of ephrin-B1 are appropriately directed toward their TZ, as most branches are in control cases. The disruption in the guidance of interstitial branches is confined to ectopic domains of ephrin-B1. Along a single axon, a branch outside an ectopic domain of ephrin-B1 extends toward the TZ (lower arrowhead) whereas a branch inside a domain of ectopic ephrin-B1 is directed aberrantly away from the TZ (upper arrowhead). Scale bar in D: 800 μm (A) and 250 μm (B-D).

The directional extension of branches is controlled, at least in part, by the graded expression of ephrin-B1 (Hindges et al., 2002) (present study). Because branch directionality is not random within the ectopic domains of ephrin-B1, but is shifted toward a lateral bias, we suspected that within the ectopic domains of ephrin-B1 protein, the overall level of endogenous and transgene ephrin-B1 parallels the normal ephrin-B1 gradient, albeit at higher levels than the graded distribution in positions adjacent to the transfection domains. To address this issue, we stained chick tecta with an EphB2-Fc receptor affinity probe to reveal the distribution of ephrin-B1 (Fig. 5). We found that ephrin-B1 protein is concentrated in the stratum opticum, as previously reported (Braisted et al., 1997), and exhibited a low to high LM gradient similar to ephrin-B1 transcripts (Braisted et al., 1997). In addition, EphB2-Fc staining in tecta transfected with RCAS-ephrin-B1-IRES-eGFP revealed the combined distribution of endogenous and transgene ephrin-B1 protein. Qualitatively, the level of EphB2-Fc labeling within the ectopic domains of ephrin-B1 appeared to equal or exceed the level of staining in medial tectum, which has the highest level of endogenous ephrin-B1. When an ectopic domain of ephrin-B1 is sufficiently large to detect a gradient, a low to high LM gradient is evident, but at a higher overall level of ephrin-B1 (Fig. 5D-F). Taken together these findings suggest that at high levels, ephrin-B1 still acts as a directional cue for interstitial branches of RGC axons, but rather than being an attractant, it acts as a repellent.

DISCUSSION

Bifunctional action of ephrin-B1 in DV retinotopic mapping

RGC axons arising from a given retinal location are broadly distributed across the LM tectal axis relative to their appropriate TZ, and overshoot their TZ along the AP axis. Two critical events in the development of the retinotopic map in rodents and chicks are topographic interstitial axon branching along the AP axis, and the directed extension of these branches along the LM axis to the appropriate site of their future TZ, where they subsequently arborize. A prominent role for EphAs and ephrin-As in mapping appears to be to inhibit interstitial branching of RGC axons posterior to their correct TZ (Roskies and O'Leary, 1994; Yates et al., 2001). EphBs and ephrin-B1 subsequently act to direct interstitial branches along the LM axis to the correct position of their TZ (Hindges et al., 2002) (present study). We have recently shown, using EphB2/EphB3 loss-of-function analyses in mice, that ephrin-B1 acts through EphB forward signaling as an attractant in DV retinal mapping to direct RGC axon branches that are positioned lateral to their correct TZ, medially up the increasing gradient of ephrin-B1 (Hindges et al., 2002). Furthermore, modeling presented in that study showed that proper mapping requires a repellent activity in a gradient that mimics that of ephrin-B1 attractant activity. In principle, ephrin-B1 itself may be this required repellent activity and acts bifunctionally as both a repellent and an attractant in a context-dependent manner to direct branch extension along the LM axis. In this model, RGC axons positioned medial to their correct TZ would be in a higher concentration of ephrin-B1 than that at their correct TZ, and their branches would be repelled by this high level of ephrin-B1 and grow down its gradient laterally towards their TZ.

Our gain-of-function studies reported here were designed to test this hypothesis. If correct, branches that form in ectopic domains of ephrin-B1, created by transfection, would experience higher levels of ephrin-B1 than those found at their appropriate TZ, and therefore would be repelled laterally whether or not this would direct them towards their appropriate TZ. We find that within ectopic domains of ephrin-B1, interstitial branches of RGC axons show an aberrant bias to extend laterally down the gradient of ephrin-B1 irrespective of the DV origin of the primary RGC axon or the LM origin of the branch; even branches extended by RGC axons positioned

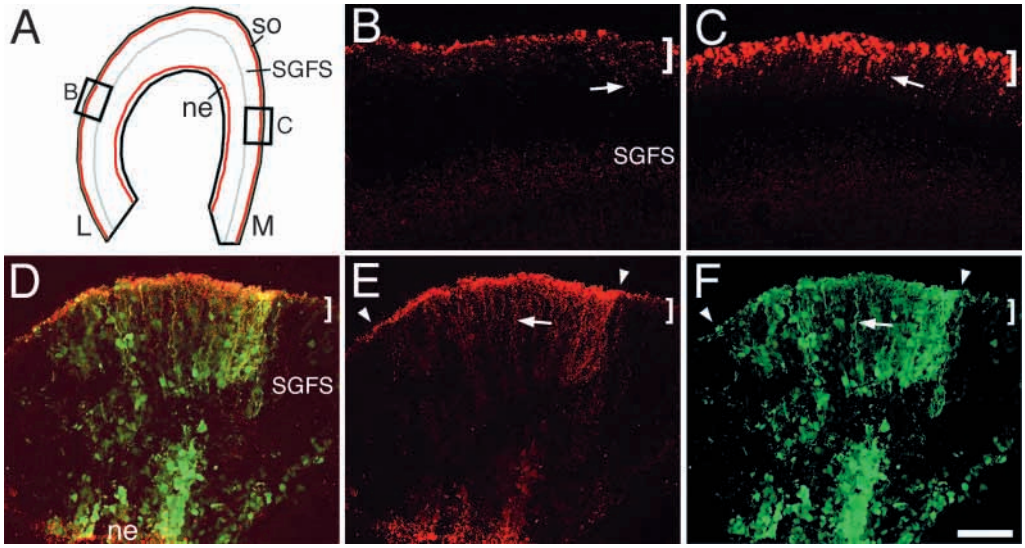

Fig. 5. Overall ephrin-B1 protein in transfection domains exhibits a graded distribution that parallels the endogenous ephrin-B1 gradient. (A) Schematic of a coronal section through an E10 tectum. Dividing cells are present in the neuroepithelium (ne) and stratum griseum et fibrosum superficiale (SGFS). RGC axons extend through the stratum opticum (SO) at the pial surface of the tectum. Boxes indicate areas shown in B and C. (B,C) E10 tectum incubated with EphB2-Fc reveals the distribution of ephrin-B1 along radially aligned processes (arrows) and in the SO (brackets). (B) Lateral tectum has low levels of ephrin-B in the SO. (C) Medial tectum has high levels of ephrin-B in the SO. The images in B and C are of the same section, taken sequentially using the same confocal settings and processed identically. (D-F) Coronal section through E7 lateral tectum after electroporation at E1.5 with RCAS-ephrin-B1-IRES-eGFP. Tectum was stained with EphB2-Fc to reveal the distribution of ephrin-B1. Infected cells and processes are in green and EphB2-Fc staining is red. Many infected cells are present in the ne as well as the SGFS. Lateral is to the left and medial is to the right of each panel. (E) EphB2-Fc reveals the presence of ephrin-B1 in the SO (bracket) and along radially aligned processes (arrow). Within the transfection domain (between arrowheads) a gradient of ectopic ephrin-B1 that parallels the endogenous ephrin-B1 gradient is apparent. (F) The eGFP reveals the extent of the transfection (between arrowheads). The level of eGFP is relatively consistent across most of the transfection domain. At these ages (E7-E10), only ephrin-B1 is expressed within the tectum; therefore the EphB2-Fc staining reveals the distribution of ephrin-B1 protein selectively (Braisted et al., 1997). Scale bar: 40 μm (B,C) and 50 μm (D-F).

lateral to their appropriate TZ are preferentially directed laterally away from it (Fig. 6A). Consistent with this aberrant lateral bias in directional branch extension, we show that within the ectopic domains the overall ephrin-B1 protein (endogenous and transgene) is distributed in a gradient that parallels the low to high LM gradient of endogenous ephrin-B1, but at a higher level of protein. Branches outside the ectopic domains of ephrin-B1 exhibit their normal directional extension such that they are preferentially directed medially or laterally toward the TZ depending on whether they originate lateral or medial to it.

Our impression from EphB2-Fc staining is that the ectopic domains of ephrin-B1 have overall levels of ephrin-B1 that equal or exceed the highest level of endogenous ephrin-B1, which is found in the most medial part of the tectum. Nonetheless, we cannot exclude the possibility that in some instances an RGC axon in an ectopic ephrin-B1 domain may encounter an overall level of ephrin-B1 similar or lower than that found at the location of its TZ and thus either exhibit random extension (if the level is similar) or a medially-directed growth indicative of attraction (if the level was lower). If true, this scenario would likely only involve the branches of axons that arise from peripheral ventral retina (which normally map to the highest levels of ephrin-B1 in medial tectum) and are positioned within an ectopic domain of ephrin-B1 in the most lateral part of the tectum (with the lowest levels of endogenous ephrin-B1). Although RGC axons from a given DV location are broadly distributed across the tectum, with a peak centered on the LM location of the correct TZ, few if any axons originating from peripheral ventral retina are found in the far lateral tectum. Thus, even if this scenario is accurate, it would only involve a small percentage of branches, and would actually result in an underestimate of the lateral bias in directional branch extension within ectopic domains of ephrin-B1 lateral to the TZ.

In contrast to their interstitial branches, the primary RGC axons themselves do not respond to either the endogenous ephrin-B1 gradient or the ectopic domains of ephrin-B1 by altering their trajectories or stopping their growth. These findings suggest that during normal development, ephrin-B1 acts as a bifunctional guidance molecule to control selectively the directional extension of interstitial branches extended by RGC axons arising from the same DV position and presumably expressing the same subtypes and levels of EphB receptors (Fig. 6C). In addition, the ectopic domains of ephrin-B1 inhibit the arborization of RGC axons and shape the distribution of arbors (Fig. 6B). These findings are consistent with a mechanism in which a high level of ephrin-B1 signaling repels RGC axon branches. Therefore, ephrin-B1 may cooperate with ephrin-As to restrict the size and shape of arbors formed by interstitial branches (Fig. 6D). Our findings indicate that ephrin-B1 may help limit arbors along the LM axis, whereas the findings from other studies suggest that ephrin-As may serve to limit the posterior extent of an arbor (Nakamoto et al., 1996; Yates et al., 2001).

Although the bifunctional action of ephrin-B1 on the directional extension of interstitial branches can in principle explain LM mapping, our findings do not rule out that other, as yet unidentified, guidance molecules may contribute to topographic mapping along this axis (see Mui et al., 2002). Reverse signaling of ephrin-Bs, which mediates RGC axon attraction in the frog retinotectal system (Mann et al., 2002), may potentially contribute to this mapping; a caveat however

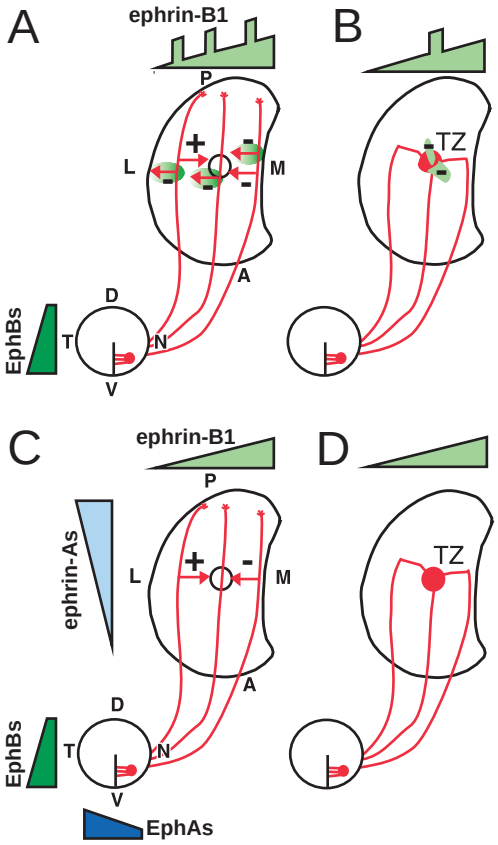

Fig. 6. Bifunctional action of ephrin-B1 as a repellent and attractant to direct interstitial axon branches and limit arborization to develop the DV retinotectal map. A and B summarize our findings and C and D summarize a model of the bifunctional action of ephrin-B1 during normal map development in chick and mouse, and the graded expression of EphB and EphA receptors by RGCs and ephrin-B1 and ephrin-As in the tectum (or SC). (A) Interstitial branches of primary RGC axons are normally directed towards their future termination zone (TZ; open circle) either medially or laterally, dependent upon primary axon location along the LM tectal axis. Within ectopic domains of ephrin-B1 (green ovals, and indicated as peaks on the endogenous LM gradient of ephrin-B1), normal bidirectional branch extension is disrupted and branches preferentially extend laterally, regardless of axon position. (B) Ectopic domains of ephrin-B1 shape and inhibit the arborization of interstitial branches at the TZ (red shape). These data suggest that a high level of ephrin-B1 is a repellent for interstitial branches and their arbors. (C) Proposed bifunctional action of ephrin-B1 during normal development of the DV retinotopic map. RGC axons initially extend posteriorly past their future TZ and preferentially form branches along their shafts at the level of the AP location of their TZ (Yates et al., 2001). Both the initial axon overshoot and the formation of interstitial branches are controlled, in part, by a repellent action of ephrin-As on EphA-expressing RGC axons (Yates et al., 2001). Branches extended by RGC axons positioned lateral to their TZ are attracted medially by ephrin-B1 up its gradient toward the TZ (Hindges et al., 2002). Branches extended by RGC axons positioned medial to their TZ are repelled laterally by ephrin-B1 down its gradient toward the TZ. Together these findings suggest that the response to ephrin-B1 of interstitial branches extended by the same DV population of RGC axons, and therefore expressing the same subtypes and levels of EphB receptors, is context-dependent. If an interstitial branch forms along an axon positioned lateral to its TZ, the branch initially extends in a domain of lower ephrin-B1 than found at its TZ. At this level of ephrin-B1, for a given axon, it acts as an attractant and guides branches medially up the gradient of ephrin-B1. Conversely, an interstitial branch that forms along an axon positioned medial to its TZ, encounters a level of ephrin-B1 higher than that at its TZ. At this level, ephrin-B1 acts as a repellent and directs branches laterally down the gradient of ephrin-B1. Therefore, ephrin-B1 may act as a bifunctional guidance molecule to control the position-dependent bidirectional extension of interstitial branches of RGC axons originating from the same DV retinal site. Alternatively, EphB receptor signaling may act as a 'ligand-density sensor' and titrate signaling pathways that promote branch extension toward the optimal ephrin-B1 concentration found at the TZ; branches located either medial or lateral to the TZ would encounter a gradient of increasingly favored attachment in the direction of the TZ. (D) Arbors are formed at the TZ exclusively by interstitial branches (Yates et al., 2001). Overshooting segments of the primary RGC axons are eliminated during this process. Based on our findings, ephrin-B1 may also function to help restrict the extent of an arbor along the LM tectal axis. Ephrin-As may help restrict the posterior extent of the arbor (Nakamoto et al., 1996; Yates et al., 2001). Retinal axes: D, dorsal; V, ventral; T, temporal; N, nasal. Tectal axes: L, lateral; M, medial; A, anterior; P, posterior.

is that although ephrin-Bs are expressed in a countergradient to EphBs in the RGC layer of chick retina, in contrast to EphBs, ephrin-Bs are not detected along the length of RGC axons in vivo (Braisted et al., 1997). In addition, other activities may be required to initiate branching along the RGC axon shaft (Yates et al., 2001), and promote the arborization and laminar patterning of interstitial branches (Cohen-Cory and Fraser, 1995; Inoue and Sanes, 1997).

ephrin-B1 selectively affects directional branch extension and arborization

During normal development, the primary RGC axons do not respond to ephrin-B1, or any LM guidance information, by changing their trajectories; nor are their trajectories affected by the ectopic domains of ephrin-B1. However, the extension of interstitial branches, which extend either up or down the ephrin-B1 gradient, are affected by ephrin-B1. Since short branches extend randomly, whereas the orientation of long branches is biased towards their TZ, we conclude that ephrin-B1 does not promote the formation of interstitial branches, but directs their extension along the LM axis.

These findings are in strong contrast to the demonstration that primary RGC axons, as well as their branches and arbors, are repelled or inhibited by ephrin-As that control, in part, TN retinal mapping along the AP tectal axis (Nakamoto et al., 1996; Yates et al., 2001). The selective influence of ephrin-B1 on branches rather than primary RGC axons, and the bifunctional action of ephrin-B1 on directional branch extension, may underlie the inability to show differential DV retinal responses to LM tectal cells or membranes using in vitro axon guidance assays and chick tissues (Bonhoeffer and Huf, 1982; Walter et al., 1987) or differential DV retinal responses of chick retina to membranes of heterologous cells transfected with ephrin-B1 or substrates of artificially clustered ephrin-B1-Fc (T.McL., J. E. Braisted, D.D.M.O'L. unpublished observation). The same types of in vitro assays effectively reveal the repellent or inhibitory effect of posterior tectal membranes and ephrin-As on chick or rodent temporal retinal axons (Walter et al., 1987; Simon and O'Leary, 1992a; Drescher et al., 1995; Nakamoto et al., 1996; Monschau et al., 1997; Feldheim et al., 1998) and their interstitial branches (Roskies and O'Leary, 1994; Yates et al., 2001).

Why primary RGC axons and their branches respond to ephrin-A2 whereas only the branches respond to ephrin-B1 is unclear. One possible explanation for this is that EphB receptors may be differentially distributed on axons and

branches, and preferentially found on branches. This possibility is suggested by the finding that EphA2 receptors are predominantly distributed to the distal part of spinal commissural axons by a mechanism of RNA translation within the axon and insertion of the locally synthesized EphA2 into the distal part of the growing axon (Brittis et al., 2002). This mechanism appears to account for the change in commissural axon responsiveness to guidance cues at different points in their pathway. By analogy, RNAs encoding EphBs may be preferentially translated at branch points and exported to the membrane of newly formed branches which could account for the selective effect of ephrin-B1 on directional branch extension and arborization. Interestingly, in several systems, interstitial branches have been found to extend from varicosities on the primary axon (Bastmeyer et al., 1998), and that these varicosities form de novo as a prelude to branch formation (Bastmeyer and O'Leary, 1996). The varicosities may act as pools enriched with RNAs and the machinery for RNA translation into protein. Alternatively, EphBs may be transported intra-axonally from the cell body, for example in association with vesicles, and preferentially exported to the membrane of developing branches.

Potential mechanisms for context-dependent ephrin-B1-mediated branch attraction and repulsion

The context in which a branch extends is a critical determinant in the choice between attraction and repulsion. Our findings indicate that this differential response is a locally controlled phenomenon since interstitial branches extending from the same primary axon exhibit different responses depending on whether they extend within or outside of ectopic domains of ephrin-B1. Local changes in the intracellular environment of the axon and its branches may be a critical parameter in determining these differential responses to ephrin-B1, since in vitro studies have shown that changes in cyclic nucleotides can change the response of axonal growth cones from attraction to repulsion, or vice versa (Song and Poo, 1999). Furthermore, at least in some instances, these responses require local protein synthesis (Campbell and Holt, 2001). The substrate upon which an axon grows can also be an important factor in determining its response to a guidance cue (Hopker et al., 1999), which in principle may be affected by changes in concentrations of ephrin-B1.

Our findings indicate that for RGC axons originating from the same DV position, and therefore expressing the same levels of EphB receptors, whether an interstitial branch is repelled or attracted by ephrin-B1 depends upon where along the LM tectal axis (and therefore the gradient of ephrin-B1) the branch originates from the primary axon. It is possible that this is controlled by a single guidance molecule with an attractant and repellent function, dependent on distinct receptors. Dual activities (attraction and repulsion) of one guidance molecule depending on the receptor complexes with which it interacts have been shown for netrin (Hong et al., 1999) and semaphorins (Liu and Strittmatter, 2001; Castellani and Rougon, 2002). The ephrin-B ligands present in the dorsolateral migratory path of melanoblasts to the skin act as an attractant for these EphB-expressing cells, but act as a repellent for an earlier migrating population of EphB-expressing neural crest cells that take a ventral path (Santiago and Erickson, 2002). In addition, the repellent activity of ephrin-A5 mediated by EphA7 can be suppressed and even changed to adhesion by co-expression of splice variants of EphA7 that lack the kinase domain (Holmberg et al., 2000). During the postnatal period of branch extension, RGCs express EphB2, EphB3 and EphB4 in a DV gradient, and EphB1 uniformly. EphB2 and EphB3 have been shown to transduce an attractant signal upon binding ephrin-B1 (Hindges et al., 2002), whereas EphB1 may transduce a repellent signal upon binding ephrin-B1. Consistent with this possibility is the finding that ephrin-B1 interaction with EphB1 or EphB2 results in different signaling complexes (Stein et al., 1998), suggesting different cellular responses.

Alternatively, activation of each of the EphB receptors may result in a similar response, but the response switches from attraction to repulsion at a threshold level of EphB/ephrin-B1 signaling. A single axon guidance molecule can act in vitro as an attractant or a repellent depending on the intracellular cyclic nucleotide levels in the axon (Ming et al., 1997; Song et al., 1997). Also the degree of response (e.g. repulsion/growth cone collapse) can be modulated by other signaling pathways such as neurotrophin/trk pathways (Tuttle and O'Leary, 1998). The threshold at which the switch from branch attraction to repulsion occurs could be determined by the proportion of EphB receptors occupied by ephrin-B1, by absolute levels of EphB signaling, or possibly by differences in ephrin-B1 concentration that may affect its oligomer state. Support for this latter suggestion comes from the work of Stein et al. (Stein et al., 1998) who show that the oligomer state of ephrin-B1 (dimers, tetramers, and higher order multimers) results in the formation of markedly different EphB1 and EphB2 signaling complexes, as well as differences in receptor phosphorylation and cell attachment. Consistent with this mechanism, the concentration of ephrin-B1 dimers or tetramers in a substrate of extracellular matrix molecules has been shown to be a critical factor in EphB1-induced, integrin-mediated attachment of various cell lines (Huynh-Do et al., 1999). Within a critical concentration range, cells attach to their substrate in an integrin dependent fashion at a much higher density; if the concentration of ephrin-B1 is either above or below this optimal level, cell attachment is decreased. The ephrin-B1 concentration at which maximal attachment is observed is oligomer dependent, with tetramers being most effective at a lower concentration than dimers (Huynh-Do et al., 1999).

It is intriguing to speculate that an analogous mechanism of EphB receptor signaling acts as a 'ligand-density sensor' to control DV retinotectal mapping. In such a model, the DV gradient of EphB receptors in the retina and the LM gradient of ephrin-B1 in the tectum would set the critical range of ephrin-B1 concentration at the appropriate LM position for DV retinotopic mapping. An interstitial branch would sense ephrin-B1 concentration through EphB receptors, which would titrate signaling pathways that promote branch extension toward the optimal ephrin-B1 concentration, for example by controlling the density of receptors (e.g. integrins) on its surface that mediate attachment to ECM components and cells in the tectum and cytoskeletal changes required for branch extension. The level of ephrin-B1 at the TZ would be the optimal concentration for maximal attachment; therefore a branch located either medial or lateral to the TZ would encounter a gradient that increasingly favored attachment in the direction of the TZ. If in principle this model is correct, it may warrant

a reconsideration of the mechanisms of axon guidance by graded molecules and the terminology used to describe axonal responses to them.

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