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Retinal axon growth cones respond to EphB extracellular domains as inhibitory axon guidance cues

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SUMMARY

Axon pathfinding relies on cellular signaling mediated by growth cone receptor proteins responding to ligands, or guidance cues, in the environment. Eph proteins are a family of receptor tyrosine kinases that govern axon pathway development, including retinal axon projections to CNS targets. Recent examination of EphB mutant mice, however, has shown that axon pathfinding within the retina to the optic disc is dependent on EphB receptors, but independent of their kinase activity. Here we show a function for EphB1, B2 and B3 receptor extracellular domains (ECDs) in inhibiting mouse retinal axons when presented either as substratum-bound proteins or as soluble proteins directly applied to growth cones via micropipettes. In substratum choice assays, retinal axons tended to avoid EphB-ECDs, while time-lapse microscopy showed that exposure to soluble EphB-ECD led to growth cone collapse or other inhibitory responses. These results demonstrate that, in addition to the conventional role of Eph proteins signaling as receptors, EphB receptor ECDs can also function in the opposite role as guidance cues to alter axon behavior. Furthermore, the data support a model in which dorsal retinal ganglion cell axons heading to the optic disc encounter a gradient of inhibitory EphB proteins which helps maintain tight axon fasciculation and prevents aberrant axon growth into ventral retina. In conclusion, development of neuronal connectivity may involve the combined activity of Eph proteins serving as guidance receptors and as axon guidance cues.

Key words: Axon pathfinding, Ephrins, Extracellular domains, Retinal axons, Reverse signaling, Mouse

INTRODUCTION

Essential in the development of the nervous system is proper neuronal pathfinding of axons to their correct targets. This pathfinding is accomplished at the motile tip of the axon, the growth cone, as receptors respond to various cues, or ligands, in their environment. A variety of receptors and ligands have been implicated in controlling axon pathfinding, including members of the Eph and ephrin families. Eph proteins are a family of receptor tyrosine kinases that govern axon pathway development, including retinal axon projections to CNS targets. Eph receptors have been shown to bind to cell membrane-attached ligands called ephrins. Ephrins have been subdivided into two subclasses, the ephrin-As which are GPI-linked to the cell membrane, and the ephrin-Bs which are transmembrane proteins; likewise, the Eph receptors are also divided into two subclasses based on binding affinities to the ephrins (Eph Nomenclature Committee, 1997). Studies have shown that EphA receptor interactions with ephrin-A ligands underlie retinotectal topographic mapping (Feldheim et al., 2000; Brown et al., 2000; Wilkinson, 2000), and EphB2 and EphB3 receptors are involved in formation of brain commissures as well as retinal axon pathfinding to the optic disc (Henkemeyer et al., 1996; Orioli et al., 1996; Birgbauer et al., 2000).

During development, retinal ganglion cell (RGC) axons from all regions of the retina grow to the center of the retina where they exit through the optic disc to form the optic nerve. This precise axon pathfinding to the optic disc has been shown to involve netrin 1 (Deiner et al., 1997), Ig family cell adhesion molecules (Brittis et al., 1995; Ott et al., 1998), and more recently, EphB receptor tyrosine kinases (Birgbauer et al., 2000). In double mutant mice lacking both EphB2 and EphB3 receptors, RGC axons show pathfinding errors characterized by failure to exit at the optic disc and by abnormal axon growth into the opposite half of the retina (Birgbauer et al., 2000). Several aspects of this phenotype, however, are not easily explained by the traditional model of EphB proteins acting as guidance receptors. For example, pathfinding errors were found from dorsal but not ventral retinal axons, even though EphB3 expression appears uniform along the dorsoventral axis (Birgbauer et al., 2000) and ventral retina expresses higher levels of the EphB2 receptor (Henkemeyer et al., 1994; Holash and Pasquale, 1995; Henkemeyer et al., 1996; Birgbauer et al., 2000). In addition, in *EphB3* mutant animals, the presence of a truncated EphB2 receptor lacking the cytoplasmic kinase domain but containing an intact extracellular domain eliminated the pathfinding errors seen in *EphB2 EphB3* double null mutant animals (Birgbauer et al., 2000), indicating an

unexpected kinase-independent role for EphB ECDs in retinal axon guidance.

Previous biochemical experiments have shown that EphB receptor extracellular domains (ECDs) can trigger ephrin phosphorylation in ephrin-expressing cells, suggesting the occurrence of 'reverse signaling' during Eph/ephrin interactions (Holland et al., 1996; Brückner et al., 1997). Cell mixing experiments have implicated both 'reverse' as well as 'forward' signaling for efficient sorting of hindbrain cells (Mellitzer et al., 1999; Xu et al., 1999). Given the ability of EphB ECDs to promote reverse signaling, and the observation that in EphB mutant mice the affected dorsal retinal axons normally express ephrins and grow through an EphB environment on their way to the optic disc, we wished to investigate whether retinal growth cones navigate by responding to EphB ECDs as guidance cues. This model was tested directly by using EphB1, B2 and B3 ECDs in both substratum choice assays and by local delivery of protein to growth cones via a micropipette.

MATERIALS AND METHODS

Recombinant proteins

To produce EphB-Fc fusion proteins, DNA encoding the extracellular domains of EphB1 (aa 19-539), EphB2 (aa 27-548), and EphB3 (aa 30-552) (Ciossek et al., 1995) was cloned into the mammalian expression vector pEX.Fc (Exelixis) in frame with a signal sequence and the human IgG-Fc$_\gamma$ domain. The Fc domain served as a protein tag and also allowed dimerization of EphB•ECD-Fc molecules. Fc control protein was produced by expressing the IgG-Fc domain alone using the same vector. Stably transfected HEK 293 cell lines were transferred into serum-free medium 3 days before harvesting of supernatant, and Fc domain-containing proteins were isolated by protein A column chromatography (Amersham Pharmacia). Fractions were examined by PAGE and Coomassie Blue or silver staining as well as Western blotting with an anti-IgG (Fc$_\gamma$-specific) antibody (Jackson ImmunoResearch Laboratories, catalog #109-055-098). For heat-inactivated controls, purified EphB2•ECD-Fc was heated to 74°C for 20 minutes, similar to treatments devised for other collapse factors (Raper and Kapfhammer, 1990).

Retinal explants

Explants were obtained from dorsal or ventral retinas of E14 mouse embryos harvested from anesthetized C57/Bl6 timed pregnant mice (day of appearance of the vaginal plug is E0). Explants were grown overnight (16-24 hours) on polylysine- (1 mg/ml) and laminin-coated coverglass dishes at 37°C in 5% CO_2 with F12 medium containing N2 supplement (Gibco). Laminin (Gibco or EHS cell purified, gift from L. Reichardt) was used at the minimum concentration needed to support good outgrowth (2-10 μg/ml, depending on the lot).

Binding of EphB•ECD-Fc proteins to retinal axons

E14 retinal explants were cultured overnight to allow neurite outgrowth. Non-specific binding sites were blocked by adding BSA (1 mg/ml in F12 medium) to explant cultures and incubating for 30 minutes at 37°C. Following washes with Ringer's solution, cultures were incubated with 10 μg/ml EphB-ECDs or Fc control protein in Ringer's solution containing BSA (1 mg/ml) for 60 minutes at 4°C. Cultures were washed and then fixed in 2% paraformaldehyde and binding was detected using Cy3-conjugated anti-human IgG (Fc$_\gamma$-specific) antibody (Jackson ImmunoResearch, catalog #109-165-098).

Substratum choice assay

A mixture of EphB•ECD-Fc or Fc control protein (5-7 μg/ml), laminin (5-10 μg/ml), and Cy3-conjugated antibody as a fluorescent marker (1:500, Jackson ImmunoResearch) was prepared in PBS, and 1 μl drops were placed on polylysine-coated, glass coverslips dishes (Lab-Tek) for 2 hours at 37°C (humidified). After washing with PBS, laminin (same concentration as above) was applied uniformly over the entire surface for 2 hours at 37°C. Retinal explants from E14 dorsal or ventral retina were seeded onto prepared dishes and cultured overnight at 37°C and then fixed with paraformaldehyde. For quantitation, neurites were stained with Texas-Red Phalloidin (Molecular Probes), and only explants with neurites intersecting a border of laminin and EphB-ECD or Fc protein (with laminin) were selected for analysis. Neurites that reached the border were scored as either crossing over and growing onto the ECD or Fc substratum (non-responding), or stopping at the border or turning away from the border (responding). (Neurites that grew substantially over the border but then stopped or turned on the substratum were scored as 'non-responding' based on their behavior at the border.) A baseline 'response rate' without any border present was established by marking virtual spots on the uniform laminin only substratum in a similar size and position as EphB-ECD or Fc containing spots. A second observer then quantified the apparent response rate of axons turning or stopping at the virtual border (labeled 'none' in Fig. 3). The total response rate (Fig. 3A) was calculated by summing up all the axons 'responding' to a given border condition, while the mean response rate per explant (Fig. 3B) was obtained by calculating the percentage of 'responding' axons for each explant and then calculating the mean for all explants encountering a given border condition. There were 267-630 axons (19-39 explants) quantified per substratum border for dorsal retina, and 111-265 axons (9-17 explants) for ventral retina. Statistical tests were performed separately for dorsal and ventral retinal explant neurites using χ^2 analysis for total response rate and two-sample T-test for mean explant response rate.

Time-lapse microscopy and micropipette ECD application

E14 dorsal retinal explants were grown overnight in 35 mm coverslip culture dishes (MatTek Corporation). Cultures were overlaid with prewarmed mineral oil (Sigma or Fisher) and equilibrated on a 37°C microscope stage incubator with CO_2 influx. Glass micropipettes of 0.5-1 μm tip diameter were loaded with either 50 μg/ml EphB-ECD or Fc control along with fluorescein (Akron) and a large excess of BSA (1 mg/ml) in F12/N2 medium and then positioned 50-300 μm (mean=135 μm) away from individual growth cones. Time-lapse images were captured at one minute intervals with a CCD camera (PXL2, Photometrics) using Hoffman optics, and Deltavision image acquisition software (API). Baseline axon growth was first examined for 30-45 minutes without reagent release. (The occasional growth cone that showed a spontaneous response during baseline observation was not studied further.) EphB-ECD or Fc protein was then delivered by repeated pressure pulses (3-12 msecond duration) at 2 Hz intervals via a Picospritzer (General Valve) set at 9 psi, and establishment of a protein gradient verified by visualization of the fluorescent indicator. (Immunofluorescence detection revealed no EphB•ECD-Fc binding to the substratum after pipette delivery.) Time-lapse images were collected for another 45-60 minutes and the behavior of growth cones was classified as either 'not responding' (i.e., kept on growing) or as 'responding' and exhibiting one of the following. (1) Growth cone collapse and/or neurite retraction, (2) growth cone 'freezing' with cessation of motile activity, (3) cessation of axon elongation or reduction in elongation rate to <60% of baseline and by more than 25 μm/hour, (4) turning away from the pipette. Fisher's exact *t*-test was used for statistical analysis. Images were processed using Adobe Photoshop, and QuickTime movies were produced using Macromedia Director. QuickTime moves can be accessed at our web site at http://www.ucsf.edu/neurosc/faculty/Sretavan/Eph-timelapse.html.

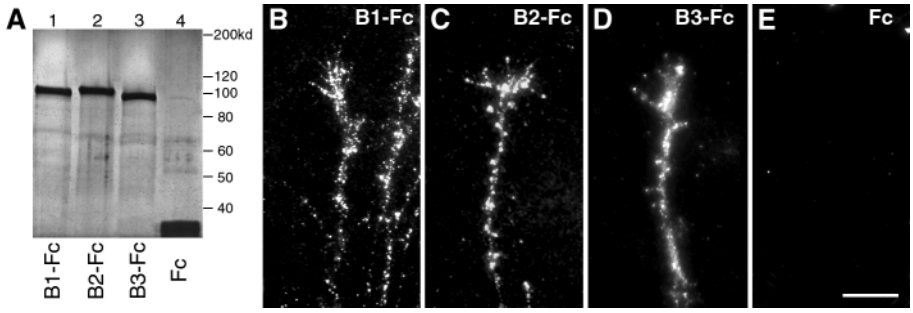

Fig. 1. Characterization of recombinant proteins consisting of EphB receptor extracellular domains fused to the immunoglobulin Fc$_\gamma$ domain. (A) Polyacrylamide gel electrophoresis and silver staining of protein A column-purified EphB1•ECD-Fc (lane 1), EphB2•ECD-Fc (lane 2), EphB3•ECD-Fc (lane 3), and Fc control protein (lane 4). (B-E) Binding of EphB1•ECD-Fc (B), EphB2•ECD-Fc (C), EphB3•ECD-Fc (D) and Fc control (E) onto E14 embryonic retinal axons and growth cones in culture, visualized with Cy3-conjugated anti-Fc antibody. No binding of the Fc control protein (E) was detected. Scale bar, 10 μm.

RESULTS

Generation of recombinant EphB extracellular domain proteins

Recombinant proteins consisting of the extracellular domains of EphB1, EphB2, or EphB3 fused to the human IgG Fc domain (abbreviated as EphB•ECD-Fc or EphB-ECD) were constructed and purified (Fig. 1A). In addition, a control protein consisting of just the Fc domain was produced and purified in the same manner (Fig. 1A, lane 4). All three recombinant EphB•ECD-Fc proteins were tested for binding activity to embryonic mouse retinal neurites and growth cones in vitro (Fig. 1B-D). No binding was detected using control Fc protein (Fig. 1E).

Retinal neurites avoid EphB ECDs

To determine whether EphB-ECDs can directly influence axon navigation, we examined how retinal axons growing on laminin behaved upon encountering substratum-bound EphB-ECD. Patterned substrata were prepared by spotting small quantities of laminin plus either EphB1-ECD, EphB2-ECD, EphB3-ECD, or Fc protein (and a fluorescent marker for visualization) onto glass-coverslip dishes followed by application of laminin to the entire dish. The behavior of axons from E14 mouse retinal explants was analyzed at the border region where axons were confronted with a choice between growth on laminin alone or a substratum containing both laminin and EphB-ECD, or laminin and Fc protein in control experiments.

Retinal neurites, when confronted with substratum containing Fc protein, appeared unaffected and grew onto the Fc control region in a pattern resembling that of growth on laminin alone (Fig. 2A,C). EphB-ECD containing substratum regions elicited a different pattern of neurite outgrowth characterized by the turning away of axons from the border region and reduced axon growth in the substratum region containing EphB-ECDs (Fig. 2B,D). This difference in response to EphB-ECDs versus Fc control was robust, and individual explants of a given condition all showed similar behavior. We quantified the results for each substratum type by examining all axon encounters with substratum borders and counting the number of axons that turned or stopped (responding axons). The number of responding axons was then expressed as a percentage of all axon encounters for that substratum type to derive an axon response rate (Fig. 3A). In 10 separate experiments involving all substrata, 2,148 axons

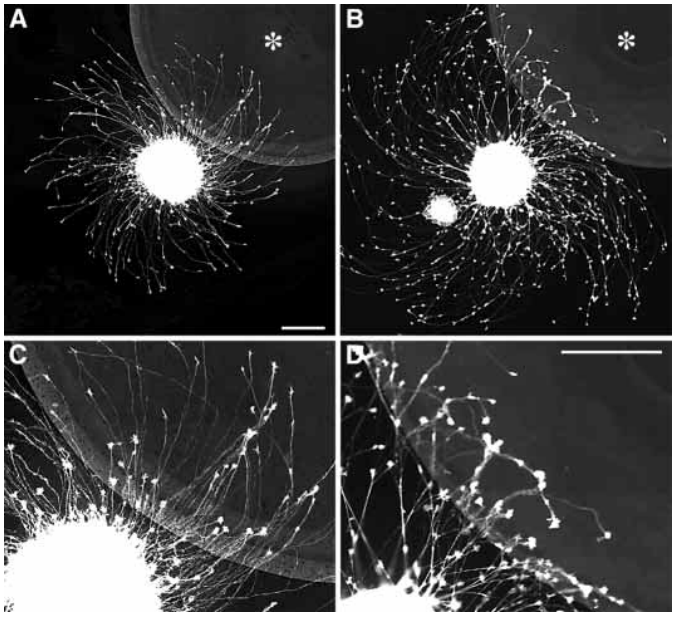

Fig. 2. Retinal axon responses to EphB•ECD-Fc proteins in substratum choice assays. (A,C) Retinal axons, visualized with TR-phalloidin, grow freely into regions containing Fc control protein. (B,D) Reduced retinal axon growth on EphB2-ECD substratum region. Note tendency of individual axons to stop or turn at the border. C,D are higher magnification views of A,B at substratum border. Asterisks in A,B indicate test substratum region. Scale bars, 200 μm (A and B are same magnification, as are C and D).

encountering a border were analyzed from 151 explants (see Materials and Methods). In control conditions with Fc protein alone, most axons freely crossed the border (Fig. 3A), but a few appeared to turn or stop, most likely reflecting the natural curved growth of retinal axons on laminin (see Fig. 2A-D) or simply the recent arrival of particular growth cones at the border. This apparent 'Fc response rate' was comparable to a 'response rate' of axons simply growing on the laminin substratum in which a virtual border was drawn (Fig. 3A, 'none'), indicating that Fc protein had little or no influence on retinal axon behavior in this assay.

EphB1-ECD, EphB2-ECD, and EphB3-ECD all elicited stop or turn response rates much greater than Fc control (P<0.01; Fig. 3A). From dorsal retinal axons, EphB1-ECD and EphB2-ECD elicited somewhat greater responses compared to

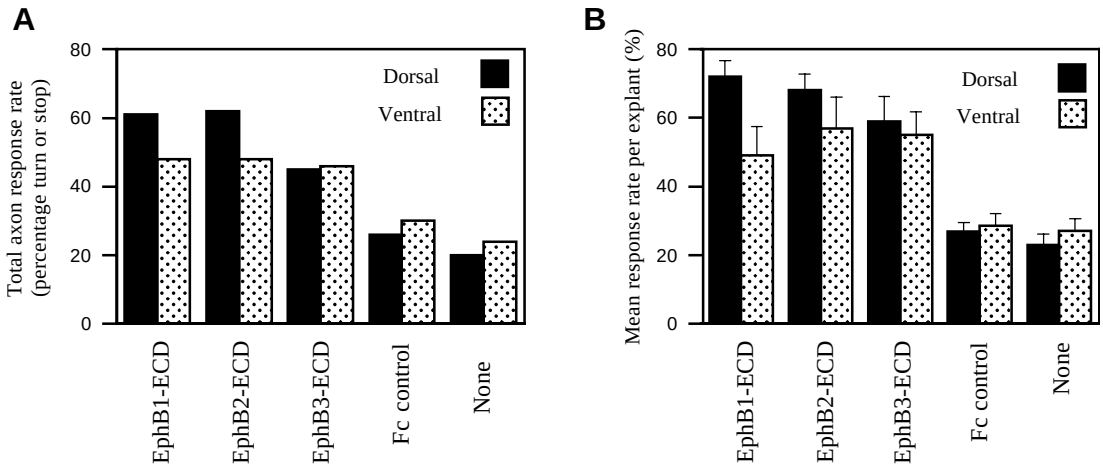

Fig. 3. Quantitation of substratum choice assay. The response rate expressed as the percentage of axons turning or stopping at the border of EphB-ECD or Fc control containing substratum. 'None' shows the results of quantitation of a virtual border drawn on a uniform laminin substratum. Results from dorsal and ventral retinal explants were quantified separately. (A) Response rate (percentage either stopping or turning at border) from pooling all individual axon encounters of a given condition. (B) Mean percentage of axons stopping or turning at the border for each explant. Error bars show s.e.m.

EphB3-ECD (P<0.01). Since in vivo, in *EphB2 EphB3* mutant animals, dorsal retinal axons have pathfinding errors but ventral axons do not (Birgbauer et al., 2000), we also compared the behavior of axons from explants of dorsal and ventral retina (Fig. 3A). The responses of ventral retinal axons were similar to dorsal axons, although dorsal retinal axons appeared somewhat more sensitive than ventral axons to EphB1-ECD (P=0.02) and EphB2-ECD (P=0.01).

Since there is some variability between individual explants in the amount of axon outgrowth and the number of axons encountering a border, we also quantified the results based on response rate per explant. The mean response rate per explant (Fig. 3B) for all three EphB ECDs was significantly higher than for Fc control (P≤0.05). Analysis by mean explant response rate showed dorsal explants to be slightly more responsive than ventral explants to EphB1-ECD (P=0.03) but, by this analysis, not statistically more sensitive to EphB2-ECD (P=0.3). Thus, the substratum choice assays, analyzed both by pooling all axon encounters for a given substratum type or by averaging the response rate of individual explants, showed that retinal axons avoided substrata containing the extracellular domains of any of the three EphB proteins tested.

Retinal growth cones are directly inhibited by EphB ECDs

To investigate whether this avoidance behavior reflected growth cone responses to an inhibitory activity, we used time-lapse video microscopy to examine the effects of soluble EphB-ECDs applied locally by micropipettes onto individual growth cones. The application method was similar to that used by others to examine growth cone signaling responses (Zheng et al., 1994; Ming et al., 1997; de la Torre et al., 1997). Fc control protein application typically did not affect retinal axon extension or growth cone motility (Fig. 4A; see also video in supplementary materials on the web at http://www.ucsf.edu/neurosc/faculty/Sretavan/Eph-timelapse.html). In contrast, EphB-ECD delivery to growth cones resulted in significant changes in growth cone behavior. The most common response was a loss of lamellipodia and filopodia accompanied by retraction of the growth cone, characteristic of growth cone collapse (Fig. 4B; see video on web site). A second but less common behavior was a cessation of growth cone motility with maintenance of lamellipodia and filopodia (Fig. 4C; see video on web site). This 'freeze' response was accompanied by continued movement of cytoplasmic material from the axon into the base of the growth cone (Fig. 4C, white arrowheads). Less frequently axon elongation stopped, or slowed down, but the growth cone remained motile, or the growth cone turned away from the pipette (Table 1). Compared to the major response of growth cone collapse, which was seen at a much higher frequency following EphB-ECD treatment, the other types of growth cone behaviors were not observed at high enough frequency to be certain of their specificity as true responses to EphB-ECD treatment.

The percentage of all inhibited growth cone responses after EphB1-ECD (79%) or EphB2-ECD (72%) treatment was

Table 1. Growth cone responses to soluble EphB•ECD-Fc protein

Protein in pipette	*n* total	Percent (*n*) response	Response type			
			Collapse	Freeze	Stop/slow	Turn
Fc control	28	25% (7)	3	2	2	0
EphB1•ECD-Fc	28	79% (22)	14	4	3	1
EphB2•ECD-Fc	36	72% (26)	19	3	4	0
EphB3•ECD-Fc	24	42% (10)	7	2	0	1
Heat-inactivated (EphB2•ECD-Fc)	25	20% (5)	5	0	0	0

Collapse: growth cone collapse and/or neurite retraction; freeze: cessation of growth cone motile activity; stop/slow: cessation of axon elongation or slowing of growth rate, but with continued growth cone motility; turn: turning away from the pipette.

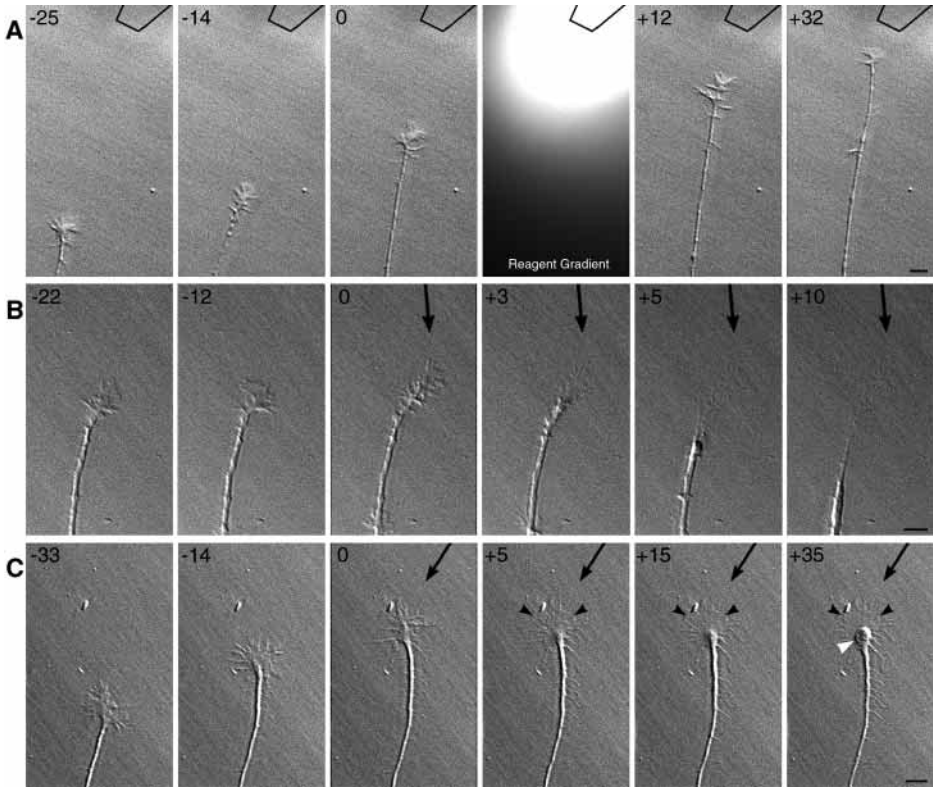

Fig. 4. Time-lapse microscopy of retinal growth cone responses to soluble EphB-ECDs or Fc protein applied by micropipette. Time in minutes relative to beginning of reagent application (0) is indicated in top left of panels. (A) Retinal axon elongation and growth cone behavior before and after application of soluble Fc protein. The growth cone was unaffected and continued towards the micropipette tip (outline in upper right). The 4th panel shows the fluorescent marker gradient after expulsion of reagent from the pipette. (B) Growth cone collapse triggered by EphB1-ECD. The micropipette tip was located at the top approx. 180 μm away from the growth cone and the direction of EphB1-ECD dispersal is indicated by the arrow. (C) Example of cessation of growth cone motile activity after application of EphB2-ECD. Black arrow indicates direction of protein dispersal from the micropipette tip located ~70 μm away. Within 5 minutes of application, the growth cone ceased filopodial and lamellipodial movements (black arrowheads) and remained in this 'frozen' state without retraction. Cellular material (white arrowhead) accumulated within the growth cone body, indicating continuing axonal transport. Scale bars, 10 μm.

significantly higher than in Fc control (25%; $P<0.01$; Fig. 5, Table 1). Application of EphB3-ECD, however, elicited an intermediate response (42%; Fig. 5). Heat inactivation of EphB2-ECD resulted in a significantly reduced growth cone response (20%), which was similar to the level seen in Fc control conditions (Table 1). Thus, we conclude that the majority of the growth cone responses were likely due to specific functional activity of EphB-ECDs. A portion of the responses observed, including under control conditions, was likely due to the approx. 10% spontaneous growth cone collapse rate seen in time-lapse studies of retinal axon growth cones without protein expulsion (E. B. and D. W. S., unpublished). In sum, the extracellular domains of EphB receptor proteins can trigger inhibitory responses in retinal axon growth cones; EphB1 and EphB2 share this property, while EphB3 may be less efficacious in this role.

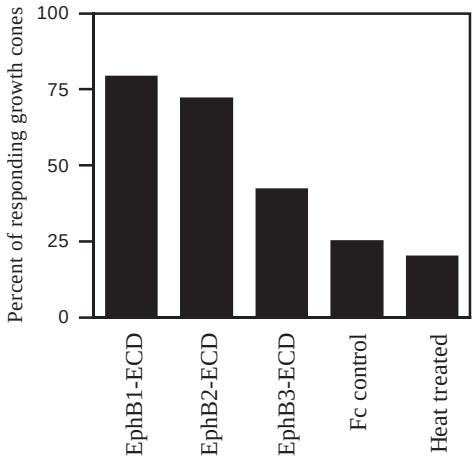

Fig. 5. Graph quantifying the percentage of retinal growth cones inhibited by micropipette application of specific EphB-ECD or Fc control reagents. 'Heat treated' shows the responses to heat-inactivated EphB2-ECD. Responses include growth cone collapse, cessation of growth cone motility, cessation or significant reduction in axon elongation rate, or growth cone turning (see Table 1).

DISCUSSION

Retinal axon pathfinding within the retina to the optic disc has been previously shown to involve a kinase-independent function of EphB proteins. In the present study, through the use of both substratum choice assays and time-lapse analysis of retinal axon responses to EphB ECD proteins, we have found that the extracellular domain of EphB receptors can act as an inhibitory guidance cue for retinal axons. In the substratum choice assay, retinal neurites growing on laminin tended to avoid regions containing EphB-ECDs. In the time-lapse analysis, retinal growth cones were inhibited by direct presentation of soluble EphB-ECDs causing growth cone collapse and neurite retraction. Results from this present study demonstrate that in addition to their role as guidance receptors, EphB proteins have an unusual ability to act as inhibitory axon guidance cues via an activity intrinsic to their extracellular domains.

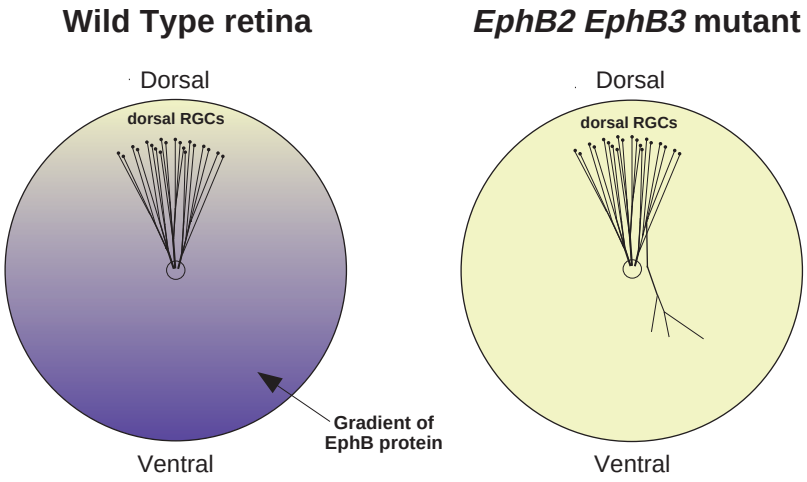

Fig. 6. Model of EphB proteins acting as inhibitory axon guidance cues governing dorsal retinal axon pathfinding to the optic disc. In wild-type retina (left), the high-ventral to low-dorsal gradient of EphB protein expression (based primarily on EphB2 protein expression) is depicted in purple. Dorsal RGC axons growing ventrally towards the optic disc encounter an increasing gradient of EphB protein extracellular domains serving as inhibitory axon guidance cues. It is proposed that this inhibition leads to tight axon fasciculation as RGC axons approach the optic disc and that the high EphB protein expression in the ventral retina prevents inappropriate dorsal axon growth into the opposite half of the retina. Both these constraints are removed in the *EphB2 EphB3* null mutant mice (right) resulting in the observed dorsal RGC axon defasciculation defects around the optic disc and the aberrant axon growth into the ventral half of the retina.

Effects on laminin substratum

The inhibitory effects of EphB-ECDs were observed with retinal neurites growing on a laminin substratum. Recent studies have suggested that growth cone responses to cues could be dependent on the specific growth substratum (Hopker et al., 1999), and therefore the responses to EphB-ECDs in the context of other substrata will be important to examine. However, we do consider that laminin is a relevant substratum for examining retinal growth cone responses to EphB-ECDs. First, laminin is a potent neurite growth promoting factor for retinal axons in vitro. Second, RGC axons in vivo normally grow through a region rich in laminin to reach the optic disc (Cohen et al., 1987; Liesi and Silver, 1988; Hopker et al., 1999), suggesting that laminin is a natural substratum for these axons. Lastly, as retinal axons traverse this laminin-rich region, their growth cones are also in a position to come into contact with EphB ECDs, leading us to infer that the inhibitory effect of EphB ECDs on retinal neurites growing on laminin likely reflects an in vivo situation.

Reverse signaling and role of ephrins

Previous biochemical studies in cell lines have shown that EphB-ECD binding to transmembrane B-ephrins can trigger ephrin phosphorylation (Brückner et al., 1997; Holland et al., 1996), indicating a potential for 'reverse signaling' from receptor through ligand. Cell sorting experiments in zebrafish (Mellitzer et al., 1999) have shown that signaling through Ephs and ephrins can proceed bidirectionally to produce a biological response, suggesting that restrictions on cell mixing in the hindbrain (Fraser et al., 1990; Birgbauer and Fraser, 1994) are mediated by bidirectional Eph/ephrin signaling (Xu et al., 1999). In this study, we have demonstrated that EphB extracellular domains can act in axon guidance, providing experimental evidence for the previous suggestion, based on genetic studies, that important aspects of anterior commissure and retinal axon guidance operate by a reverse signaling mechanism (Henkemeyer et al., 1996; Birgbauer et al., 2000). Although we have not shown that this effect is specifically mediated by B-ephrins as in a true reverse signaling paradigm, B-ephrins are the only known partners of EphB proteins (Eph Nomenclature Committee, 1997; Gale et al., 1996) and have highly homologous cytoplasmic domains consistent with a conserved role in signaling (Holland et al., 1996; Torres et al., 1998).

Model for EphB ECD function in RGC axon guidance to the optic disc

This study, along with our previous analysis of retinal pathfinding errors in *EphB2 EphB3* double mutant animals (Birgbauer et al., 2000), leads to a model for Eph/ephrin reverse signaling during axon guidance in the retina (see Fig. 6). We previously found that retinal axons in *EphB2 EphB3* double mutant mice exhibited guidance defects as they approached the optic disc, characterized by abnormal defasciculation and aberrant growth into the opposite half of the retina (Birgbauer et al., 2000). Unexpectedly, these pathfinding errors were seen in axons from dorsal but not ventral retina despite the fact that dorsal retina normally expresses lower levels of these receptors than ventral retina (Henkemeyer et al., 1994; Holash and Pasquale, 1995; Henkemeyer et al., 1996; Birgbauer et al., 2000). This paradox can now be explained by proposing that as dorsal RGC axons grow in the ventral direction towards the optic disc, they normally encounter and respond to increasing levels of extracellular domains of EphB proteins acting as guidance cues rather than as receptors. The high degree of axon fasciculation as they approach the disc may reflect their behavior in this increasingly inhibitory environment. The high levels of EphB proteins that are normally found in the ventral half of the retina would serve as a corrective mechanism so that any dorsal RGC axons that may have grown aberrantly into the ventral retina would be inhibited and prevented from growing further. Loss of both EphB2 and EphB3 proteins in null mutants would remove these inhibitory constraints, resulting in dorsal axon defasciculation and bypass at the optic disc as well as aberrant dorsal retinal axon growth into the ventral half of the retina. In this model, the absence of the EphB2 cytoplasmic kinase domain would not affect pathfinding to the disc or lead to aberrant axon growth into the ventral retina since the inhibition intrinsic to the ECD remains intact (Birgbauer et al., 2000).

Although this model explains many of our observations, several aspects still require further analysis. In our in vitro assays, we found that both dorsal and ventral retinal neurites

responded to all three EphB-ECDs tested, although it appeared that dorsal retinal neurites were more sensitive than ventral ones to EphB1 and EphB2 ECDs. This finding is in contrast to the dorsal-ventral difference in retinal axon sensitivity to EphB ECDs in vivo and suggests that this in vivo difference in responsiveness is not simply due to an intrinsic difference in the ability of dorsal versus ventral retinal axons to respond to EphB ECDs. Rather, it raises the possibility of additional factors within the in vivo retina that account for the pathfinding differences of dorsal versus ventral RGC axons.

It is also worth noting that while EphB3 is important in retinal axon pathfinding in vivo, in our in vitro assays it produced a reduced effect compared to EphB1 and EphB2. It is possible that this is related to the method of production and purification of the EphB3•ECD-Fc protein. However, it is also possible that there are additional activities of EphB3 that are not revealed by our in vitro assays.

Dual function of Ephs and ephrins in axon pathfinding

Several lines of evidence now suggest that both Eph and ephrin molecules serve a dual function as receptors and ligands in a number of developmental events. Intriguingly, in the visual system, RGC axons appear to express both Ephs and ephrins (Marcus et al., 1996; Holash et al., 1997; Braisted et al., 1997; Connor et al., 1998; Hornberger et al., 1999; Birgbauer et al., 2000) leading to the possibility that Ephs and ephrins may serve as both receptors and ligands within the same cell. In the retina, RGC axons navigate to the optic disc in response to EphB proteins acting as guidance cues, while in the visual target, the superior colliculus, RGC axons have been shown to respond to ephrins, likely using EphA proteins as guidance receptors, during the formation of the retinotopic map (Wilkinson, 2000). Although EphA and EphB proteins may differ in their direction of signaling, recent studies have suggested that the EphA/ephrin-A system may also show reverse signaling (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001; Knoll et al., 2001), and therefore EphA as well as EphB proteins may act both as receptors and guidance cues. Thus, there arises the intriguing possibility that a given axon may regulate Eph/ephrin signaling such that either forward or reverse signaling is utilized for pathfinding at different points along the pathway.

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