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EphB2 regulates axonal growth at the midline in the developing auditory brainstem

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Abstract

Eph receptors play important roles in axon guidance at the midline. In the auditory system, growth of axons across the midline is an important determinant of auditory function. The avian cochlear nucleus, n. magnocellularis (NM), makes bilateral projections to its target, n. laminaris (NL). We examined the time course of NM axon growth toward the midline, the expression of Eph proteins at the midline during this growth, and the effects of Eph receptor misexpression on axonal growth across the midline. We found that NM axons reach the midline at E4. At this age, EphB receptors are expressed at the ventral floor plate. Expression extends dorsally to the ventricular zone beginning at E5. NM axons thus grow across the midline at a time when EphB receptor expression levels are low. Overexpression of EphB2 at E2 resulted in misrouted axons that deflected away from transfected midline cells. This effect was observed when midline cells were transfected but not when NM cells alone were transfected, suggesting that EphB2 acts non-cell autonomously and through reverse signaling. These data suggest an inhibitory role for midline Eph receptors, in which low levels permit axon growth and subsequently high levels prohibit growth after axons have crossed the midline.

Keywords: Midline; Axon guidance; Auditory pathways; Auditory nerve; Nucleus laminaris; Nucleus magnocellularis; Eph receptor; Ephrin; Chick; Hindbrain; Brainstem

Introduction

Axon guidance at the midline of the nervous system is essential for the integration of information from both sides of the brain and thus has important consequences for perception and behavior. The relationship between bilateral neural architecture and sensory processing is exemplified in the auditory system. In the avian auditory brainstem, the first central nuclei that receive auditory information are the cochlear nuclei, nucleus magnocellularis (NM), and nucleus angularis (Jhaveri and Morest, 1982b; Parks and Rubel, 1978; Rubel and Fritzsch, 2002). NM axons bifurcate and project to nucleus laminaris (NL). The ipsilateral branch of NM axons contacts the dorsal dendrites of NL neurons, while the contralateral branch crosses the midline and contacts ventral dendrites of NL neurons (Jhaveri and Morest, 1982a; Smith and Rubel, 1979; Young and Rubel, 1983). Contralateral axons have orderly branches so that conduction delays are longer for more laterally projecting axon branches. These delay lines, together with coincidence detection in NL and the segregation of ipsilateral and contralateral NM axons to distinct sets of dendrites, form the neural basis for sound localization in the chick (Agmon-Snir et al., 1998; Carr and Konishi, 1990; Overholt et al., 1992; Young and Rubel, 1983). The length and branching of contralaterally projecting NM axons and the guidance of these axons to correct targets are thus essential for auditory function. However, very little is known about the mechanisms that control growth of axons in the auditory system or in the midline at the level of the hindbrain. In this study, we have examined the mechanisms that control initial growth of the contralateral branch of NM axons toward the midline.

Several families of axon guidance molecules have a demonstrated role in controlling decussation of axons and in preventing recrossing of axons after they reach their destination. The goal of the present study is to evaluate the role of Eph

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family proteins in midline guidance in the auditory pathways of the brainstem. These proteins are promising candidates in light of their demonstrated role in midline guidance in other regions of the nervous system and their strong expression in the auditory brainstem nuclei. Eph receptors constitute the largest known class of receptor tyrosine kinases, with ten receptors in the EphA class and six in the EphB class in vertebrates (Pasquale, 2005). Ephrin-A ligands generally bind EphA receptors, and ephrin-B ligands bind EphB receptors, but ephrin-B ligands can also bind EphA4 (Gale et al., 1996), and ephrin-A5 can also bind EphB2 (Himanen et al., 2004). An important aspect of Eph signaling is that ephrins are attached to membranes through a GPI linkage (ephrin-A) or a transmembrane domain (ephrin-B). Ephrins, particularly ephrin-B ligands can mediate bidirectional signaling (Bruckner et al., 1997; Henkemeyer et al., 1996; Holland et al., 1996; Knoll and Drescher, 2002; Kullander and Klein, 2002). In forward signaling, ephrin binding to Eph receptors induces signal transduction events in the cell expressing the receptor. In reverse signaling, the Eph receptor signals through the ligand, inducing changes in the cell expressing the ligand.

Both forward and reverse Eph protein signaling have been implicated in midline axon guidance. Eph proteins play a key role in the control of axon growth at the midline in corticospinal axons (Coonan et al., 2001; Dottori et al., 1998; Kullander et al., 2001a; Leighton et al., 2001; Yokoyama et al., 2001), in the anterior commissure (Henkemeyer et al., 1996), in commissural axons in the spinal cord (Imondi and Kaprielian, 2001), in decussating vestibular efferents (Cowan et al., 2000), and in the optic chiasm (Mann et al., 2004; Williams et al., 2003). An additional level of complexity arises from the fact that Eph/ephrin interactions can be repulsive or attractive (Davy and Soriano, 2005; Pasquale, 2005). Both types of interactions can operate within the same pathways (Eberhart et al., 2004; Hindges et al., 2002; McLaughlin et al., 2003). In order to characterize the mechanisms of Eph proteins in midline guidance, it is necessary to consider these multiple signaling modes and how they interact to form appropriate neural pathways.

We previously showed that Eph receptors and their ligands, ephrins, play an important role in the formation of auditory brainstem connections (Cramer et al., 2004), and that EphB2, EphB5, and ephrin-B1 are expressed in the midline at embryonic day 8 (E8) and later (Cramer et al., 2002). NM axons undergo their final mitotic division at about E2.5 (Rubel et al., 1976). While NM axons have been identified in the region of their contralateral NL target as early as E6, it is not clear when these axons approach the midline. Here, we have used two labeling methods to ascertain when NM axons first encounter the midline, and we have extended our immunohistochemical studies to determine EphB receptor and ephrin-B expression at this time. We found that EphB2 and EphB5 are absent from the midline until after the first NM axons cross on or after E4, and that these axons express ephrin-B2. Growth cones are thus attracted to the midline in the absence of these receptors. Upregulation of Eph receptors after the growth of axons is consistent with an inhibitory role for these proteins in midline guidance. We tested this role using spatially and temporally restricted misexpression of EphB2 constructs in NM cells or in the midline. The results suggest that a temporal window for axon growth is present in the early embryonic hindbrain, after which EphB2 stimulates reverse signaling through ephrin-B ligands to inhibit NM axon growth.

Materials and methods

Electroporation of E2 hindbrain

Eggs were windowed at E2 (48 h of incubation), staged according to Hamburger and Hamilton (1951), and injected with a small amount of India ink underneath the embryo to provide contrast. A small hole was made in the roof plate at the level of rhombomere 5 (r5), which contains precursors for the auditory brainstem nuclei (Cramer et al., 2000a; Marin and Puelles, 1995). A small drop of sterile PBS (5 to 10 μl) was placed over this opening and plasmid DNA (2 to 4 μg/μl in Tris/EDTA) was injected into the neural tube. A tungsten electrode was placed near the midline at the level of r5–6, and another electrode was placed to the left of the embryo. Current was delivered using a BTX electroporator, using a voltage of 25–50 V and 50-ms duration, in trains of 6 pulses with an interval of 100 ms. Five pulse trains were delivered, polarity was switched, and 5 more pulses were delivered. This sequence was repeated 3–5 times. The eggshell was taped closed, and the eggs were placed in a humid 37°C incubator for a survival period of 1 to 8 days.

Plasmids

Full-length EphB2 and kinase inactive EphB2 (kiEphB2; provided by E. Pasquale, Burnham Institute) were cut from pcDNA3 and cloned into the pMES vector (Cramer et al., 2004; Eberhart et al., 2004; Swartz et al., 2001) at the EcoRI site. EphB2 was also cloned into pCAX (provided by G. Mastick, University of Nevada) and co-transfected with pCAX-EGFP. Control transfected embryos were electroporated with pCAX-EGFP alone or with V5-EphA3 (provided by S. Pfaff, Salk Institute).

Whole-mount preparation

Embryos were removed at E3 following electroporation at E2. The location of the transfection was verified under a fluorescence stereomicroscope (Leica). Embryos with transfection in r5 were dissected to include the hindbrain, otocysts, and surrounding tissue, and the roof plate was removed to permit flattening of the hindbrain. Tissue was fixed in 4% paraformaldehyde for 20 min, rinsed, and mounted in glycerol with DABCO to prevent bleaching of fluorescence.

In vitro axon labeling

Dextran dyes were used to label NM axons in embryos ranging from E3 to E10 using methods adapted from previous studies (Burger et al., 2005; Cramer et al., 2004; Young and Rubel, 1983; Young and Rubel, 1986). Briefly, chick embryos were removed from the egg, and the brainstem together with surrounding tissue was dissected in Tyrode's solution (8.12 g/l NaCl, 0.22 g/l KCl, 1.43 g/l $NaHCO_3$, 0.2 g/l $MgCl_2$, 0.333 g/l $CaCl_2$, and 22 g/l dextrose) infused with 95% O_2/5% CO_2. Rhodamine dextran amine (RDA), MW = 3000 (Molecular Probes) in a 6.25% solution containing 0.4% Triton-X100 in phosphate-buffered saline (PBS) was pressure injected using several 10-ms pulses at 10 psi into NM on only one side of the brain. At the younger ages, injections were made in the region containing precursors for NM, in the rhombic lip close to the otocyst. In some cases, current was passed through the tissue to promote dye transfer (Burger et al., 2005). The tissue was then immersed in Tyrode's solution continuously perfused with 95% O_2/5% CO_2 for 2–4 h, then fixed for 1–3 h in 4% paraformaldehyde at 4°C. Tissue was cryoprotected in 30% sucrose then embedded in OCT medium. Cryostat sections were cut in the coronal plane at 12–14 μm, and sections were coverslipped using Glycergel mounting medium (Dako). Alternate sections were counterstained using bisbenzimide.

Immunoprecipitation and immunoblotting

The specificity of all the antibodies used in this study was confirmed using immunoprecipitation and immunoblotting. Chick brainstem tissue was homogenized on ice in Sten buffer (300 mM NaCl, 100 mM Tris, 4 mM EDTA, 0.4% NP-40, pH 7.6), incubated with protein A beads (Roche Pharmaceuticals) for 1 h at 4°C then briefly centrifuged, and the supernatant was incubated with antibody-coated protein A beads overnight at 4°C. Proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), blocked with 5% nonfat dry milk, then incubated in primary antibody overnight at 4°C. Membranes were washed then incubated with an appropriate secondary antibody followed by enhancing solution (Bio-Rad).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Siddiqui and Cramer, 2005). Briefly, tissue including the brainstem, otocyst, and all overlying structures were dissected from embryos aged E3 to E8, fixed in 4% paraformaldehyde for 2 h, rinsed, then incubated in 30% sucrose in PBS overnight at 4°C. Tissue was embedded in OCT mounting medium, and 14-μm sections were cut on a cryostat and thawed onto coated slides. Slides were rinsed in PBS and treated with 0.3% H_2O_2 in methanol for 10 min to reduce background peroxidase labeling. A PAP pen (The Binding Site Inc., San Diego, CA) was used to make small wells around mounted tissue sections. Sections were treated with blocking solution (5% nonfat dry milk and 0.1% Triton X-100 in PBS) for 1 h and were then incubated overnight at room temperature in primary antibodies (1–5 μg/ml in blocking solution). We used polyclonal antibodies that recognize EphB2 (Pasquale, 1991), EphB5 (Soans et al., 1996), ephrin-B1 (Kalo et al., 2001), and ephrin-B2 (Cramer et al., 2002; Person et al., 2004). We used commercially available antibodies that recognize ephrin-B3 and a pan-ephrin-B antibody that recognizes ephrin-B1, ephrin-B2, and ephrin-B3 (both from Zymed Laboratories, Invitrogen Corporation). Negative controls in which the primary antibody was omitted were included, and Western blots here and in our previous studies (Cramer et al., 2002; Siddiqui and Cramer, 2005) verified that the antibodies specifically recognized these proteins. Sections were incubated in a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted to 6 μg/ml in blocking solution, rinsed, and incubated in Vector ABC kit for 1 h. HRP was visualized using 3,3′diaminobenzidine tetrahydrochloride (DAB; Sigma).

Cryosections with labeled axons were used for immunofluorescence to reveal EphB2 and EphB5. The fluorescent secondary antibodies, goat anti-rabbit conjugated to Alex 594 or Alexa 488 (Molecular Probes, Eugene, OR), were used at 2 μg/ml. Alexa 594 was used in tissue with axons labeled using EGFP electroporation, while Alexa 488 was used in tissue with axons labeled using in vitro RDA injections. Alexa 594 was visualized with a Texas Red filter set and Alexa 488 with a FITC filter set (Chroma Technology Corp.). Some sections were counterstained with bisbenzimide to facilitate identification of auditory nuclei. Photomicrographs were produced using a Zeiss Axiocam digital camera and Openlab software (Improvision), and figures were prepared using Photoshop (Adobe Systems).

Analysis of misexpression studies

Untransfected control embryos, EGFP and EphA3 transfected control embryos, and EphB2 and kiEphB2 transfected experimental embryos were analyzed by a single investigator who was blind to the treatment group. Sections with labeled NM axons were included in the analysis. The location of transfected cells was classified as being in the midline or in NM axons without transfection in the midline. Within EGFP-positive regions of the brainstem, the percent of sections with abnormal axonal trajectories was recorded in each case. Abnormal trajectories included additional turns, poor fasciculation, and diversion from the ventricular zone. Embryos in different treatment groups were compared using a Wilcoxon/Kruskal-Wallis test. Significant differences were those with P values less than 0.05.

Results

Timing of axon growth at the midline of the auditory brainstem

A difficulty in identifying auditory axons at the time of their initial projection is that auditory structures have not yet formed. We thus used two methods to ascertain the time course over which NM axons cross the midline. EGFP transfection of precursors has been used to identify projections of motor neurons from identified rhombomeres (Prin et al., 2005). Here, we used a similar method in which the regions of the hindbrain containing precursors for NM were transfected with EGFP at E2, and tissue survived to varying ages. We took advantage of the known locations of precursors for NM in medial r5 and r6 (Cramer et al., 2000a) to selectively transfect these regions. Transfected embryos reliably showed EGFP expression in NM when they survived to E10 or later, ages at which NM is clearly visible and distinct from NL. This method thus results in labeling of NM axons. The second method was in vitro labeling of tissue taken at different ages, with RDA dye placement in the region of NM progenitors in the auditory anlage. At the ages when the anlage is present, from about E5 to E7 (Harkmark, 1954), it is a small region of cells in the dorsolateral hindbrain. The accuracy of the RDA placement is supported by labeling of axons in the dorsal midline and also by the presence of retrograde labeling in the VIIIth nerve and cochlear ganglion, which provide synaptic input to NM, in tissue E6 and older (data not shown). We found very similar results using these two labeling methods.

E3 embryos were examined in whole-mounted embryos following EGFP transfection at E2. Axonal trajectories were evaluated near the midline only at the level of r5–6, where NM progenitors are found. Many growth cones were observed at the ends of axons near the midline but putative auditory axons did not traverse the midline at this level. (Fig. 1A). At E4, some prospective NM axons had already reached the midline region. Figs. 1B and C show examples of coronal sections through E4 embryos transfected with EGFP in r5. Axons in Fig. 1B are seen at the midline (arrow) but have not traversed this region (arrowheads). Some embryos at this age had axons that crossed the midline and reached the contralateral side of the brainstem (Fig. 1C). By E5, all of the embryos examined had axons traversing the midline (not shown). By this time, the auditory anlage is formed, and we could verify that labeled axons arose from this region.

With in vitro labeling using RDA, the dorsolateral region of the brainstem containing the auditory progenitors (prior to E5) or the auditory anlage (E5 or later) were targeted for injection. Axons were not observed in the midline region at E3, consistent with our observations on EGFP transfected hindbrains. Fig. 1D shows the absence of axons at the midline in the auditory region of the brainstem, counterstained with bisbenzimide (Fig. 1E) After E4, axons reached or exceeded the midline (Figs. 1F and G). These results are very similar to those obtained using EGFP transfection. Overall, no E3 embryos had significant axonal growth to the midline (n = 3), and 5 out of 9 E4 embryos had axons at or past the midline. All embryos at E5 (n = 4), E6

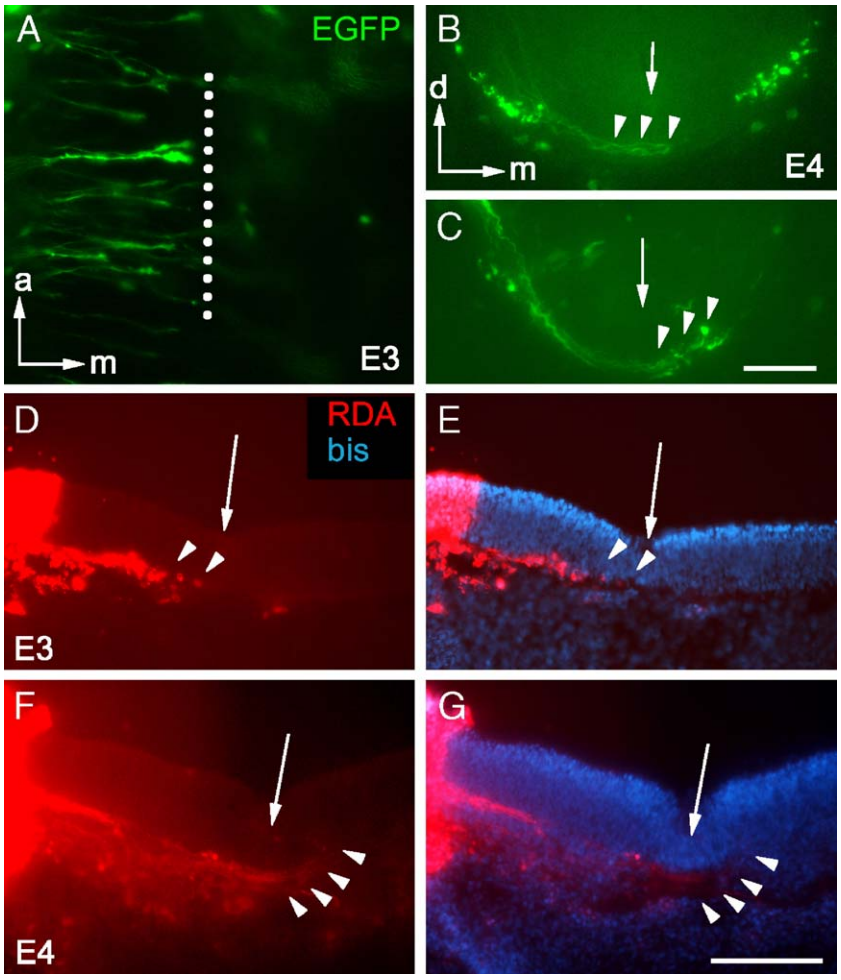

Fig. 1. Axons emanating from NM precursors reach the midline of the floorplate by E4. (A–C) Axons labeled by electroporating EGFP into regions containing NM precursors in E2 embryos. (A) Whole-mounted E3 embryo hindbrain viewed from the dorsal surface at the level of rhombomeres 5–6. Axons approach but do not cross the midline at this age. Dotted line indicates the location of the midline. Axes: a = anterior, m = medial. (B and C) Two examples of hindbrains at E4 in coronal sections. Axes: d = dorsal, m = medial. Arrows indicate position of the midline, and arrowheads indicate the position of axons emanating from NM. Axons have just reached the midline in panel B and have extended beyond the midline in panel C. (D–G) Axonal growth at the midline demonstrated with RDA labeling. (D) At E3, NM axons (arrowheads) have not yet reached the midline (arrow). (E) Bisbenzimide counterstain reveals the distribution of cell nuclei. Axons grow at the base of the ventricular zone. (F and G) Axons have just crossed the midline in this E4 embryo. At this age, the mantle has begun to form and axons remain at the base of the ventricular zone. Scale bar in panel C, 100 μm, applies to panels A through C. Scale bar in panel G, 100 μm, applies to panels D–G.

(n = 4), and E7 (n = 2) showed axons growing past the midline to the contralateral side.

Developmental expression of EphB receptors at the midline

We previously reported that the EphB proteins EphB2, EphB5, and ephrin-B1 are expressed at the midline of the brainstem at E8 and later, during the formation of connections between the auditory brainstem nuclei. These proteins are absent from decussating axons, whereas ephrin-B2 is present in axons but not in the midline (Cramer et al., 2002). To begin to evaluate the role of these proteins in early axon guidance at the midline, we investigated the expression of these proteins at the time when NM axons first encounter the midline. We found that neither EphB2 nor EphB5 is expressed in the midline or in NM axons at E3. At E4, midline expression of EphB2 is limited to a small patch at the floor plate (Fig. 2A). This patch excludes the ventricular zone and is flanked by regions lacking expression, beyond which lateral regions of the brainstem express EphB2. At E5, E6, and E7 (Figs. 2B–D), the midline patch of labeling elongates and reaches the ventricular zone, forming flanks around the central region (arrowheads in Fig. 2B). During these ages, expression includes the entire dorsoventral extent of the midline within the mantle zone. At all the ages examined, the ventral portion of the immunolabeled region shows slightly higher expression levels than the dorsal portion adjacent to the ventricular zone. Ventral expression was also more pronounced than dorsal expression at later embryonic ages (Cramer et al., 2002).

EphB5 expression was examined at ages E3–E7. A small ventral region (indicated by arrowhead) is immunolabeled at E4 (Fig. 2E), and this region expands to fill the midline, excluding the ventricular zone, at later ages (Figs. 2F–H). Expression at these ages is much weaker than those seen for EphB2. In addition, expression in the region outside the midline is uniformly low. Like EphB2, the labeled region becomes

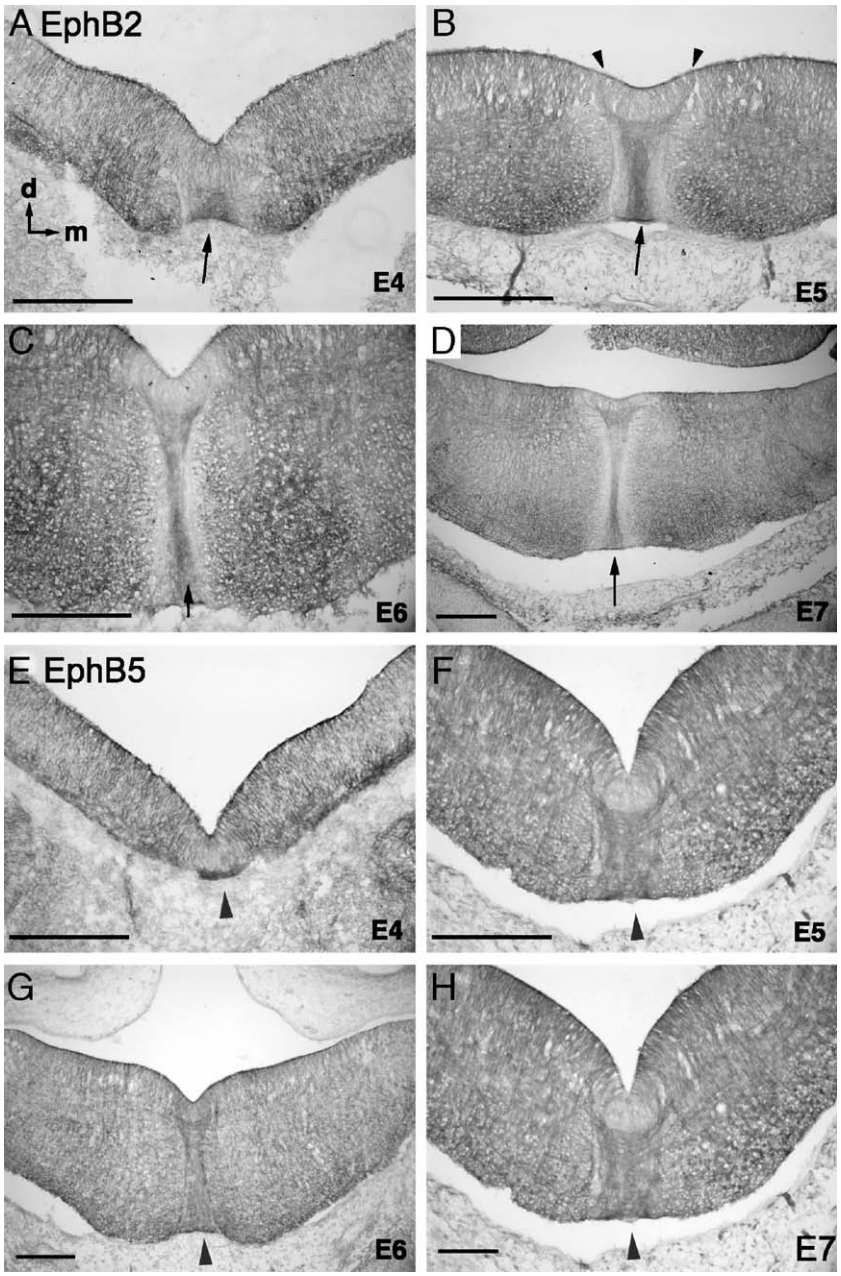

Fig. 2. EphB proteins are expressed at the midline of the early auditory brainstem. (A) Expression of EphB2 is limited to a narrow region at the ventral midline at E4 (arrow). For this and all panels, sections are coronal through the auditory region of the hindbrain, with dorsal (d) up, and medial (m) toward the center. (B) At E5, expression extends through the midline of the floorplate and into the ventricular zone around the central region (arrowheads). (B–D) Expression becomes pronounced in more dorsal regions at E5 (B), E6 (C), and E7 (D). (E) EphB5 is expressed in a narrow region at the ventral margin of the midline at E4 (arrowhead). (F and G) A band of low level expression is seen at E5 and E6. (H) A band of EphB5 expression spanning the midline is present at E7. The intensity of the label is uniform along the dorsoventral axis. Scale bars, 200 μm.

narrower but includes a wider extent of the dorsal midline. Neither EphB2 nor EphB5 is expressed in NM axons at these ages.

Ephrin-B proteins are expressed in the midline and in NM axons

To complement studies of EphB protein expression, we examined the expression of ephrin-B proteins in the midline and in decussating branches of NM axons at E4–5. We used a pan-ephrin-B antibody and found extensive labeling of a wide region through the midline as well as strong labeling in axons decussating at the dorsal region of the midline below the ventricular zone (Figs. 3A and B, arrowheads).

We then used antibodies specific for each ephrin-B to determine the distribution of individual family members. Ephrin-B1 is strongly expressed in the midline of the brainstem. At E4, a wide region is labeled throughout the dorsoventral extent of the floor plate, including the ventricular zone (Fig. 3C). At E5, ephrin-B1 expression includes a slightly less

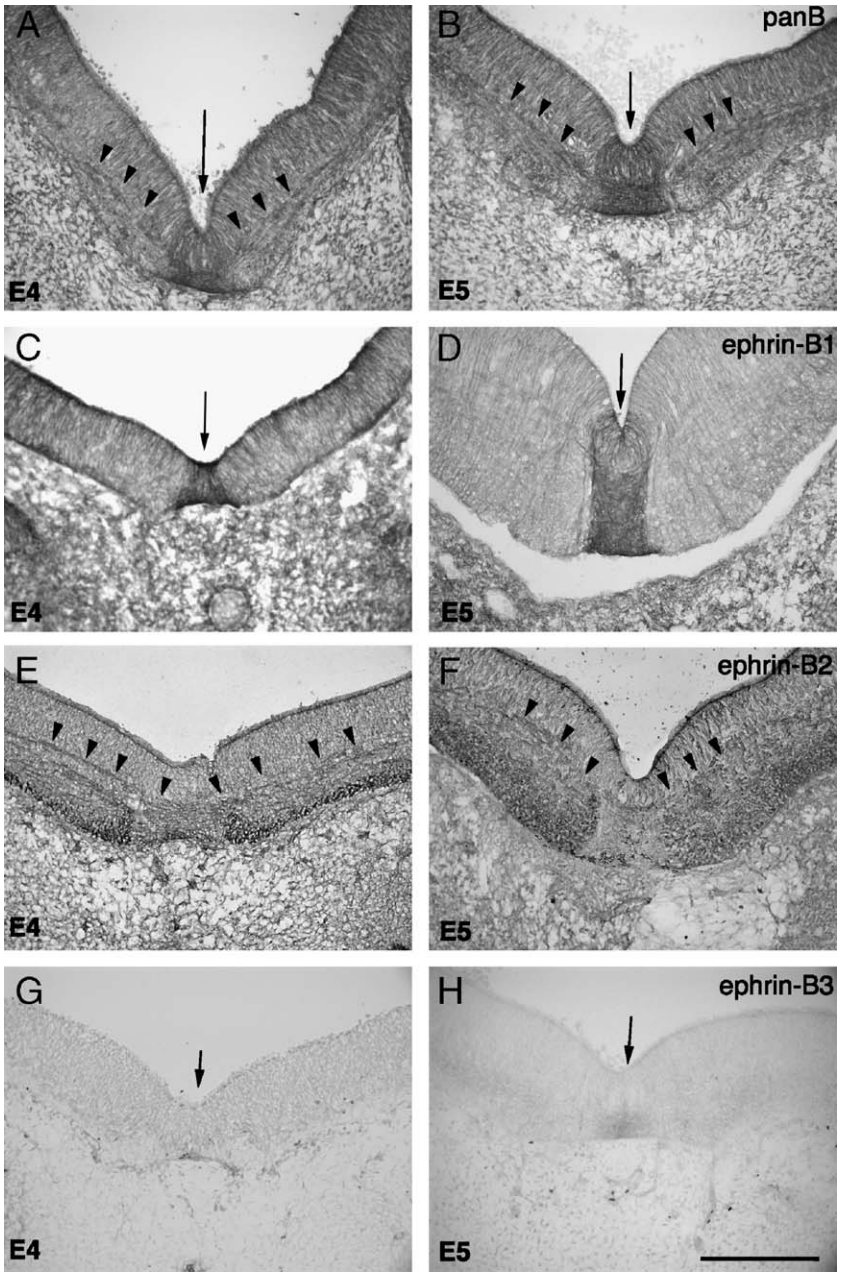

Fig. 3. Ephrin-B proteins are expressed in the midline and in decussating axons in the hindbrain, shown in coronal sections as in Fig. 2. (A) A pan-ephrin-B antibody shows a broad band of expression at the midline (arrow) and labeled axons traversing the midline (arrowheads). (B) A similar pattern of expression is seen at E5. (C) An antibody that recognized only ephrin-B1 was used to label E4 tissue. A dense band at the midline shows that ephrin-B1 is expressed in the floorplate and includes the ventricular zone (arrow). (D) At E5, ephrin-B1 remains strongly expressed at the midline (arrow). (E) Ephrin-B2 immunohistochemistry at E4. Axons are labeled (arrowheads), but the midline has low expression levels. (F) E5 embryos show a similar pattern of labeling. (G) Ephrin-B3 immunohistochemistry does not show significant expression at E4. (H) At E5, ephrin-B3 is expressed at low levels in a small region of the midline but not in decussating axons. Scale bar, 200 μm, applies to all panels.

extensive region of the ventricular midline (Fig. 3D). Expression levels at E6 and E7 (not shown) remain high throughout the midline but narrow with age. The narrowing of this expression region continues through late embryonic development (Cramer et al., 2002).

Ephrin-B2 immunohistochemistry on E4 (Fig. 3E) and E5 (Fig. 3F) brainstem sections showed that decussating axons express ephrin-B2 (arrowheads). These axons traverse the midline dorsally and extend to dorsolateral region of the brainstem. Based on their anatomical organization, these axons are likely crossing branches of NM axons. This interpretation is strengthened by our previous demonstration of ephrin-B2 expression in brainstems E8 and older, in which NM axons are more clearly identifiable (Cramer et al., 2002). Ephrin-B2 is not expressed in the midline region.

Ephrin-B3 immunolabeling was absent from the midline at E4 (Fig. 3G) and was observed in a limited region of the midline at E5 (Fig. 3H). Ephrin-B3 did not appear to be expressed in

decussating axons in this region of the brainstem. The results of expression studies for individual ephrin-B proteins are consistent with the pattern seen using a pan-ephrin-B antibody.

Relative timing of axon growth and Eph receptor expression

Expression of EphB2 and EphB5 is limited when auditory axons first enter the brainstem but expands along the dorsoventral axis after axons have crossed. These results suggest that the timing of Eph receptor expression at the midline may be an important factor regulating the growth of crossing axons. To examine the relative timing more accurately, we determined the relative states of axon growth and Eph receptor expression simultaneously using double labeling within individual embryos. We used immunofluorescence on sections in which axon labeling had been performed using either EGFP transfection or in vitro axon tracing; examples using EGFP are shown in Fig. 4. At E4, when axons are just reaching the midline (Fig. 4A, arrow), EphB2 is expressed only at the ventral margin of the midline (Figs. 4B and C). At this age,

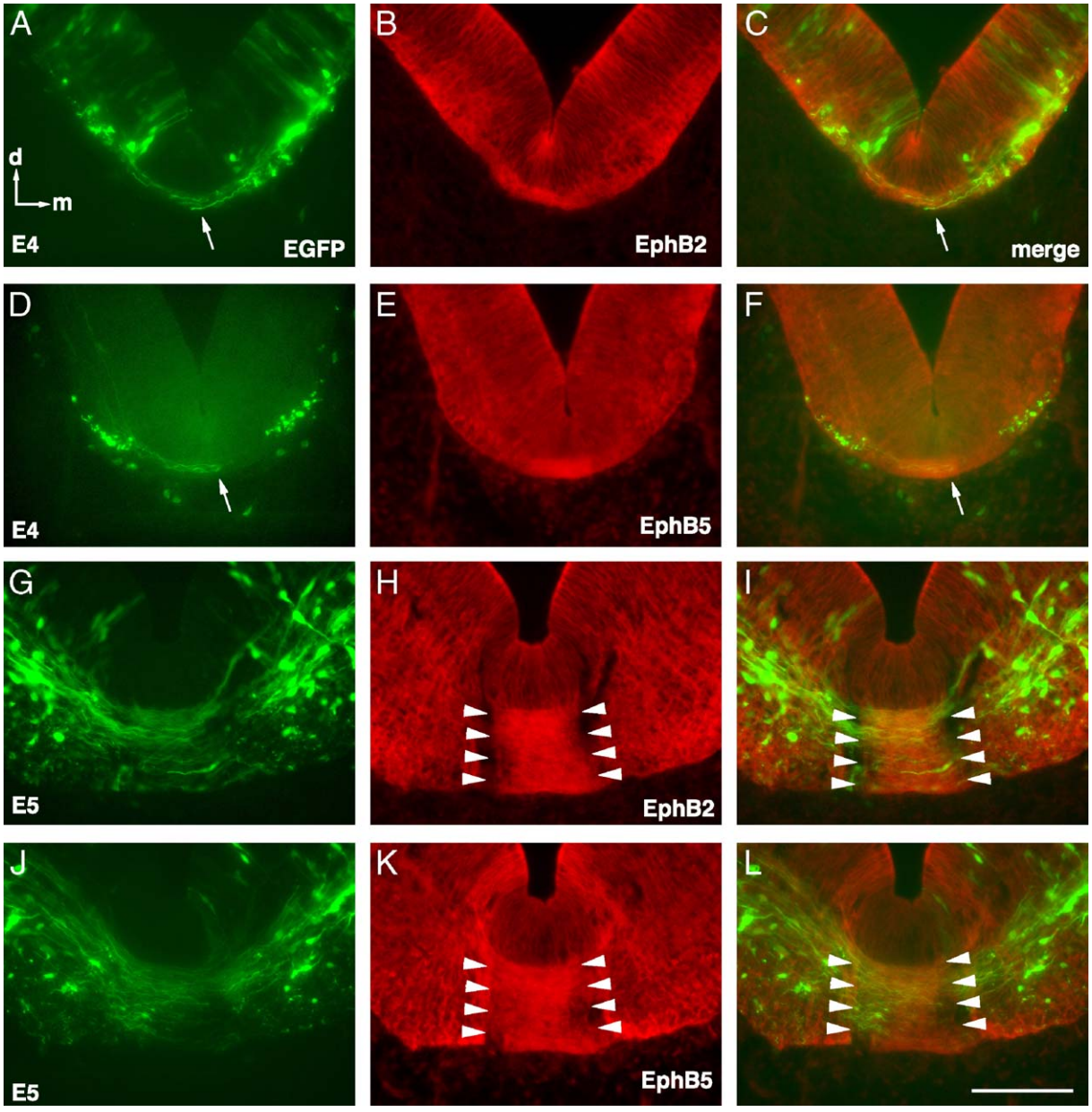

Fig. 4. EphB receptor expression increases at the midline after axons grow across the midline. (A) Axons labeled by EGFP transfection at E2 can be seen within the midline at E4 (arrowhead). Coronal sections through the hindbrain at the level of the future auditory nuclei. Axes indicate that dorsal is up, and medial is toward the center in all panels. (B) EphB2 immunofluorescence shows a small patch of label at the dorsal margin of the tissue. (C) Merged image from panels A and C. (D) Axons within the midline at E4 shown with EGFP fluorescence. This embryo had fewer transfected neurons than that shown in panels A–C. (E) EphB5 immunofluorescence in the same section as that shown in panel D. A small region is labeled in the ventral region. (F) Merged image showing axons at the ventral margin of the ventricular zone. (G) EGFP labeled axons in an E5 brainstem section. (H) EphB2 immunofluorescence shows labeling in the midline. (I) Merged image shows axons at the dorsal part of the EphB2 labeled region, ventral to the ventricular zone. (I) EGFP labeled axons in an E5 brainstem section. (K) EphB5 immunofluorescence at E5 in the section shown in panel I. (L) Merged image showing axons within the midline after EphB5 expression begins in the midline. Scale bar, 200 μm, applies to all panels.

EphB5 expression is similarly limited (Figs. 4D–F). At E5, when axons have crossed the midline in all labeled embryos, EphB2 and EphB5 expression extend dorsally through the midline (Figs. 4G–L, arrowheads). Thus, at the ages when axons traverse the midline, EphB expression is very limited, while after axons cross, the expression extends and remains in the midline. These results suggest that the absence of EphB receptors is permissive for axon growth, and that EphB receptors may prevent recrossing of axons at the midline.

Misexpression of EphB2

Our expression studies showed that EphB receptors, particularly EphB2, show marked increase in expression at the floorplate after NM axons reach the midline, whereas ephrin-B1 expression was high throughout the midline during the growth of axons. Our previous study showed that EphB2 is present to some extent in NM axons at later ages and could potentially respond to ephrin-B1 both at the midline and within the target. However, we did not detect significant levels of EphB2 in NM axon branches at the midline at early ages. High levels of ephrin-B1 expression persist during the period of midline axon growth, suggesting that ephrin-B1 does not inhibit NM axons. In this study, we therefore focused on the role of EphB2 as a midline guidance molecule. This protein is also a likely candidate based on its role in other areas of the nervous system. We postulated that low expression of EphB2 at early ages is permissive for axon growth across the midline. Moreover, higher expression levels in the ventral region of the midline could guide auditory axons toward the dorsal midline, where they form their normal trajectory towards the contralateral NL. To determine the role of Eph receptors in midline guidance in the brainstem, we used in ovo electroporation to misexpress EphB2. This method has the advantage that transfection can be selectively directed to focal regions within the embryo at a time preceding the normal expression of EphB2. In order to distinguish between cell autonomous and non-cell autonomous effects, we expressed EphB2 in the midline and/or in NM axons at E2, 2 days before endogenous expression occurs at the midline. We then compared axonal trajectories at several ages following transfection to those seen in control embryos.

NM axons send contralateral projections through the dorsal midline adjacent to the ventricular zone. Fig. 5A shows an example of axons labeled with an in vitro RDA injection into NM in untransfected control embryos (n = 9) at E10. Axons make a smooth trajectory in a narrow region, indicated by arrows. Tissue sections through the region of the brainstem containing labeled axons were evaluated. Errors were observed in 6.1% ± 2.5% of these sections from these cases. In transfected embryos, sections through the auditory brainstem containing EGFP labeling in the midline and/or in NM axons and RDA labeling in axons were included in our analysis. In control transfected embryos (n = 6; Fig. 5B), plasmids containing EGFP expressed in the midline and/or brainstem did not have significantly abnormal trajectories of dorsal midline axons; disorganized trajectories were observed in 6.5% ± 4.9% of sections. When EphB2 was misexpressed in NM axons (n = 3), trajectories appeared normal at E5 (Fig. 5C) and at E10 (Fig. 5D). The mean percentage of sections with errors was 10% ± 10%. However, when EphB2 was misexpressed in the midline (n = 11), axonal trajectories were aberrant, with 78.5% ± 5.9% of sections showing disorganized axons at the midline. An example from an E7 embryo is shown in Fig. 5E, and examples from E10 embryos are shown in Figs. 5F–G, with aberrant axonal trajectories indicated by arrows. Axons were more sparsely organized (Figs. 5E and F; compare with Figs. 5A and C), had additional turns, deflected away from transfected midline cells (e.g., asterisk in Fig. 5E), and did not course smoothly along the ventricular zone (e.g., Figs. 5F–G). Because axon trajectories were altered when EphB2 was transfected into the midline and not into axons, the effects of the EphB2 plasmid are non-cell autonomous. This observation suggests that EphB2 acts as an axon guidance molecule for NM axons, on which ephrin-B ligands may mediate reverse signaling. As an additional control for the specificity of these effects, we transfected embryos with EphA3, which does not bind to any ephrin-B ligands (Fig. 5H). We found that after transfection of EphA3 in the midline (n = 3), these embryos had normal NM axonal trajectories, with abnormal trajectories in 6.7% ± 6.7% of sections.

Expression of kiEphB2

We used a form of EphB2 lacking a functional kinase domain, kiEphB2 (Ethell et al., 2001), to further test the role of EphB2 in midline guidance. This plasmid has a normal extracellular domain but a mutation in the cytoplasmic domain that inhibits kinase function. This protein encoded by this construct acts as a dominant negative, which produces a loss of function for forward signaling (Ethell et al., 2001), but may produce a gain of function for reverse signaling. The expression patterns of EphB2 and ephrin-B2 suggest that EphB2 signals to axons via reverse signaling through ephrin-B2. If forward signaling mediates NM axon guidance at the midline, then we would expect opposing effects of EphB2 and kiEphB2 expression. Conversely, if EphB2 at the midline instructs axons via reverse signaling, then we would expect that misexpression of EphB2 and kiEphB2 would have similar effects on axonal trajectories.

Transfection into NM only (n = 4) did not appear to change axon trajectories (arrows in Figs. 6A and B), with disorganized projections in 18.8% ± 12% of sections. However, transfection in the midline (n = 6) was associated with aberrant turns in axonal growth patterns in 68.3% ± 11% of sections (Figs. 6C–F), and turns were often seen near transfected midline cells, indicated by asterisks in Figs. 6D and F. These alterations in trajectory likely resulted from disruptions in axon guidance, as neither EphB2 nor kiEphB2 resulted in changes in the gross morphology of the brainstem. Because kiEphB2 and EphB2, which have identical extracellular domains, both increased the errors in axonal trajectories when expressed at the midline, our results suggest that forward signaling through EphB2 receptors in NM axons is not used in NM axon guidance at the midline.

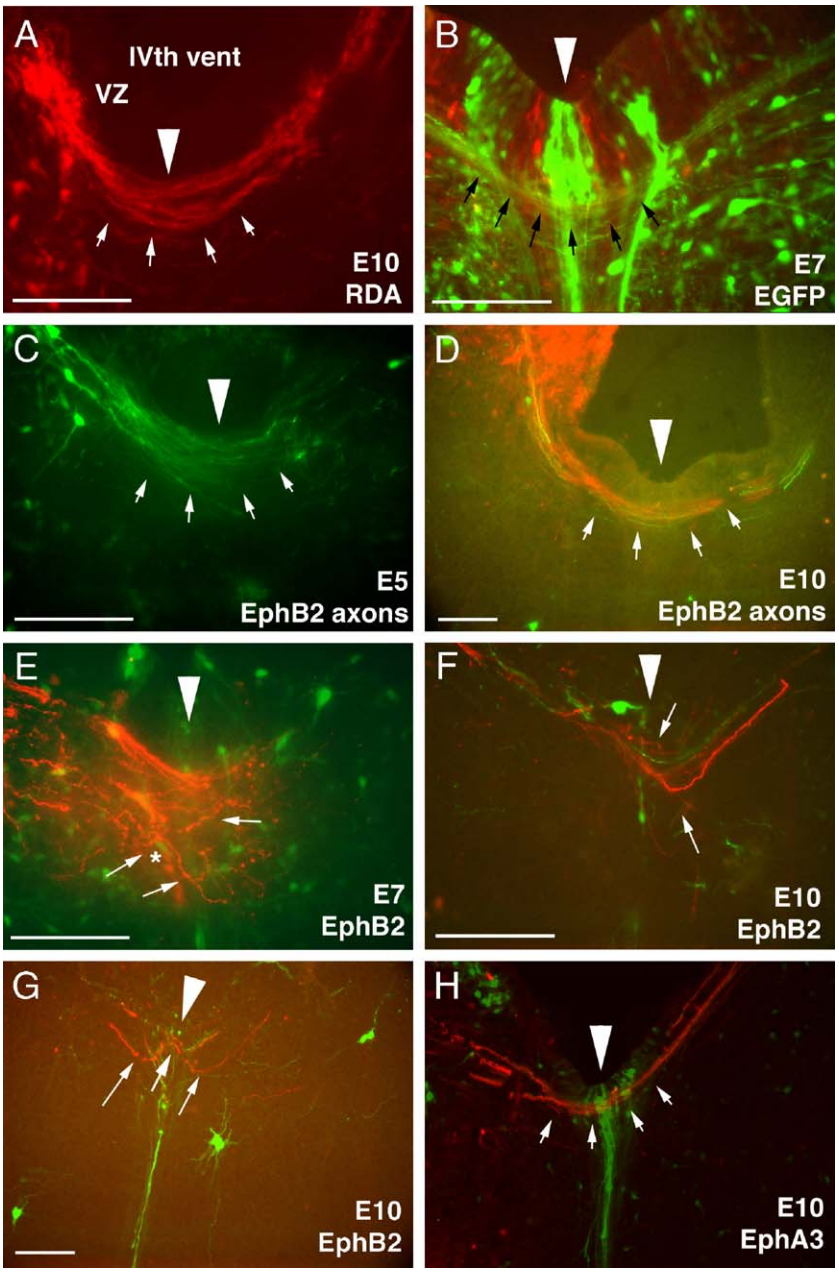

Fig. 5. Misexpression of EphB2 alters axonal trajectories at the midline. (A) Untransfected control axons labeled with RDA at E10. Axons, shown in red, form a smooth bundle (arrows) just ventral to the ventricular zone (VZ) ventral to the fourth ventricle (IVth vent). Large white arrowheads denote position of the midline in all panels. (B) EGFP transfected embryo at E7, with a normal trajectory of axons at the midline. EGFP labeled axons (arrows) are seen coursing just ventral to the ventricular zone, and transfected regions in the midline also express EGFP. (C) At E5, EphB2 transfected axons (green) have a smooth trajectory (arrows) at the midline when the midline is not transfected. (D) A second example of EphB2 misexpressed in NM axons but not the midline, shown at E10. Axons are labeled with RDA (red) and EGFP (green) indicates transfected axons. These axons have a normal trajectory. (E) EphB2 misexpressed at the midline results in disorganized NM axon growth (arrows). Example shown is at E7. Deflections occur near transfected cells at the midline (asterisk), and the tract is poorly fasciculated. (F–G) EphB2 midline transfection and abnormal axon trajectories at E10. Axons have additional turns (arrows) and do not course smoothly near the ventricular zone. (H) Midline transfection with EphA3 (arrowhead) does not disrupt the normal trajectory of crossing NM axons. Axons course smoothly (arrows) through the transfected midline region. Scale bars, 100 μm.

The similarity in the effects of EphB2 and kiEphB2 together with the expression patterns of these proteins provide additional evidence that the extracellular domain of the protein mediates reverse signaling through ephrin-B2 during axon guidance at the midline.

Results from all groups in the misexpression studies are summarized in Fig. 7. We used Wilcoxon/Kruskall-Wallis tests to show that groups differed significantly in the percent of sections with abnormal trajectories. Pairwise comparisons showed that EphB2 in the midline and kiEphB2 in the midline were both significantly different from each of the other groups ($P < 0.05$) but not significantly different from each other ($P > 0.35$). Electroporation in the midline with EphB2 and kiEphB2 thus resulted in significantly greater disorganization than that seen in

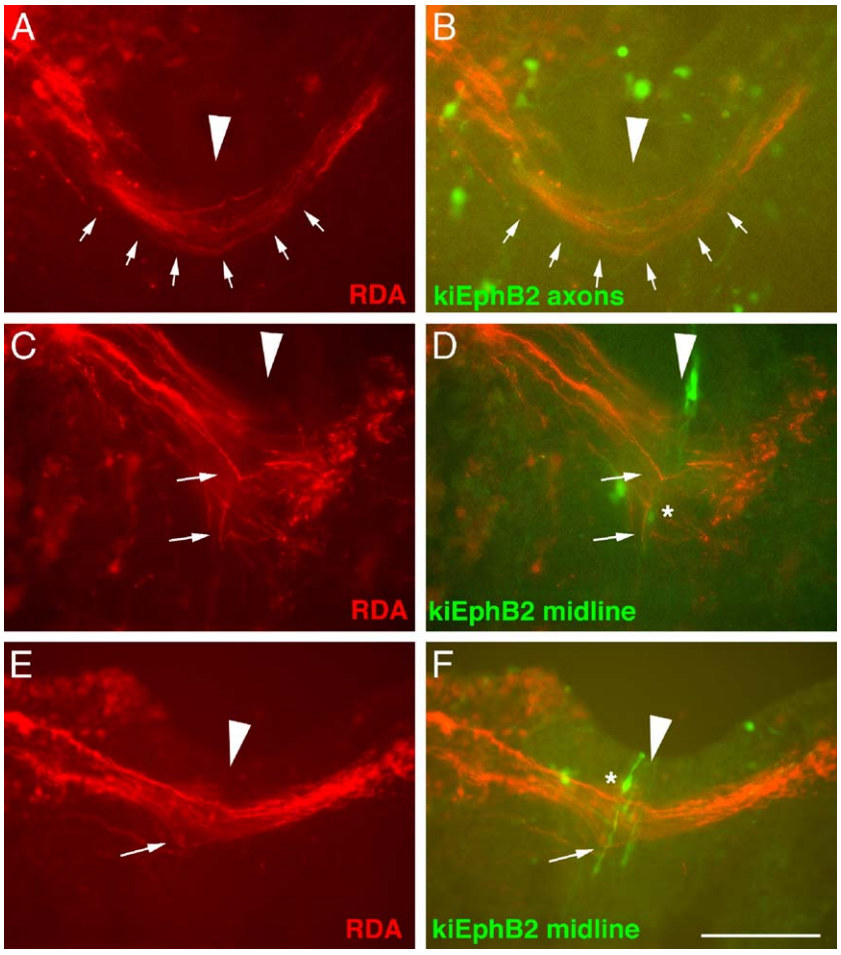

Fig. 6. Transfection with kiEphB2 alters axonal trajectories at the midline. Coronal sections through auditory region of the brainstem. (A) Axonal trajectories in E10 embryo with kiEphB2 expressed in NM axons. Arrows indicate smooth trajectories taken by crossing NM axons. White arrowheads denote position of the midline in all panels. (B) Same section as panel A showing location of transfection (green) and axonal trajectories (red) together. (C and D) E10 embryo with transfection in the midline. Axons have additional turns (arrows) and do not form a smooth bundle. Turns are often seen adjacent to transfected cells (asterisk). (E and F) Additional example of kiEphB2 transfection in the midline. Turns occur near transfected midline cells (asterisk). Scale bar, 100 μm, applies to all panels.

untransfected controls, EGFP controls, EphA3 controls, or embryos transfected with either plasmid in NM but excluding the midline.

Discussion

In this study, we identified the time course of axonal growth in the developing auditory system and provided the first evidence that Eph signaling is essential for axon guidance at the midline in the chick hindbrain. We found that NM axons first reach the midline at E4, after which EphB2 and EphB5 expression at the midline increases. Ephrin-B2 is expressed in NM axons as their branches approach the midline. This expression relative to NM axon growth suggested that EphB receptors in the midline are inhibitory for growing NM axons. Misexpression of EphB2 and kiEphB2 beginning at E2 resulted in abnormal axonal trajectories when exogenous protein was expressed in the midline. These trajectories included additional turns and poor fasciculation in the crossed dorsal cochlear tract. These findings support the hypothesis that EphB2 reverse signaling influences midline axonal growth. Moreover, EphB2 has a developmentally regulated inhibitory influence on NM axon growth.

Growth of auditory axons at the midline

Our finding that axons reach the midline at E4 is consistent with data showing that the earliest NM axons reach their contralateral target in the auditory anlage at E6 (Book and Morest, 1990; Young and Rubel, 1986). The present data suggest that these axons reach the midline before NM cell bodies have migrated into the mantle zone and shortly after NL neurons undergo their final mitotic division. NM axon midline crossing occurs before NM cells are contacted by VIIIth nerve axons (Kubke and Carr, 2000; Molea and Rubel, 2003). The initial midline pathway is seen at the boundary between the ventricular zone and the mantle zone, where it remains in the mature auditory system. This crossed dorsal tract thus develops early and in its appropriate position during its initial formation.

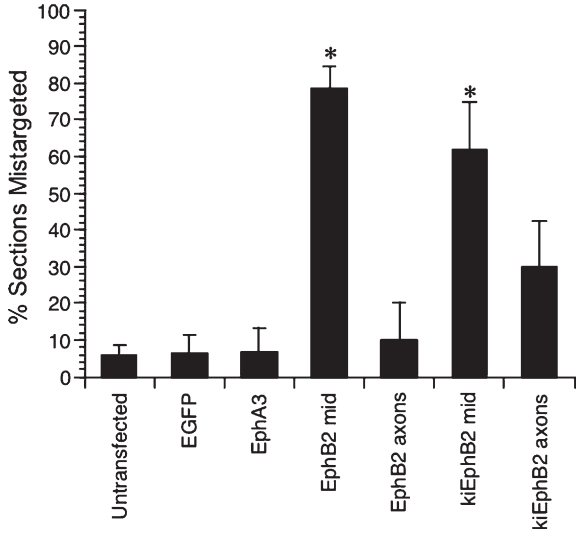

Fig. 7. Summary of data in misexpression experiments. Each bar shows the percent of sections containing turns or abnormal bundling of axons in the midline projection, ±SEM. Untransfected controls, EGFP control transfected, EphB2 transfection in NM axons, kiEphB2 transfection in axons, and transfection with EphA3 were not significantly different from each other and differed significantly from both EphB2 in midline and kiEphB2 in midline. Asterisk indicates significant difference from untransfected control or EGFP control, Wilcoxon/Kruskal-Wallis test.

Expression of Eph proteins in the midline of the hindbrain

Our expression studies demonstrate that individual EphB receptors and ephrin-B ligands are expressed in a complementary manner, such that none of these proteins is expressed in both the midline and the decussating axons. This complementarity is similar to that described in the chick spinal cord (Imondi et al., 2000); however, in the spinal cord, the axons express EphB receptors, and ephrin-B ligands are expressed in the midline. This difference suggests that while the organization of midline projections may use the same class of molecules, the nature of the interactions differs in different regions of the neuraxis.

Although expression patterns of individual Eph proteins are frequently compartmentalized and complementary, it is common for axons or target regions to express both ephrins and Eph receptors (Holash et al., 1997; Hornberger et al., 1999; Menzel et al., 2001). In our study, we found both EphB receptors and ephrin-B ligands expressed in the midline during development. A complex, extensive expression pattern of EphB receptors and ephrin-B ligands was also described in the mouse corpus callosum, in which development requires both forward and reverse signaling (Mendes et al., 2006). Expression of both ligands and receptors within a single cell may allow for interactions in *cis* for EphA proteins (Marquardt et al., 2005; Yin et al., 2004). These interactions may regulate the responsiveness of axons to different Eph family proteins.

EphB2 regulates axon guidance at the midline

In this study, we tested the role of EphB2 in axon guidance at the midline of the hindbrain. The expression of this protein is upregulated just after NM axons grow into the midline and is higher in the ventral midline. In the auditory brainstem, axons may encounter lower levels of Eph receptor en route to the midline; these levels do not appear to be inhibitory for growth. Greater expression levels or combinations of Eph proteins at the midline may provide instructive cues for growing axons. An interesting possibility is that the interactions at the midline may alter expression of Eph family proteins (Brittis et al., 2002). Misexpression in the midline disrupts axon growth using EphB2 and kiEphB2. Axons turn away from transfected midline, and the tract spreads more widely in transfected embryos than in control, consistent with a role for EphB2 in axon fasciculation and in inhibiting growth. While repulsion is supported by our findings, we did not observe axons that turned back toward their side of origin and recrossed the midline. However, it is likely that several Eph receptors work together to coordinate appropriate trajectories at the midline. Similar responses of NM axons to both EphB2 and kiEphB2 suggest that the extracellular domain mediates the response, and that signaling operates in the reverse direction. In support of this conclusion, no significant effect was seen when NM axons but not midline cells misexpressed the exogenous proteins.

An additional rationale for examining EphB2 is the demonstrated role for this protein in midline axon guidance in other systems. A non-cell autonomous inhibitory role for EphB2, signaling in the reverse direction through ephrin-B2, has been demonstrated in the posterior portion of the anterior commissure in mice (Cowan et al., 2004; Henkemeyer et al., 1996). EphB2 also regulates the formation of contralateral inner ear efferents in the vestibular system (Cowan et al., 2000). In this system, lack of EphB2 prevents contralateral projections, indicating that EphB2 may be a permissive signal during normal mouse development. Thus, EphB2 can have both attractive and inhibitory roles in midline guidance.

Ephrin signaling in midline pathways

Eph protein-mediated guidance cues for some commissural pathways may be conserved across species. In both the chick and the mouse spinal cord, ephrin-B ligands expressed at the midline are inhibitory and prevent recrossing of axons (Imondi and Kaprielian, 2001; Imondi et al., 2000; Yokoyama et al., 2001). In the visual pathway, ephrin-B ligands expressed at the optic chiasm inhibit growth of retinal ganglion cell axons expressing EphB receptors in both *Xenopus* and mice (Mann et al., 2004; Nakagawa et al., 2000; Williams et al., 2003, 2004).

Interestingly, EphB2 is expressed in the midline of the hindbrain during growth of vestibular efferents at embryonic ages (Cowan et al., 2000). It is not known whether EphB2 regulates growth of auditory midline axons in mammals or whether its expression is uniform along the dorsoventral aspect of the midline. While auditory brainstem circuitry in birds and mammals have overall functional similarities, there are important differences (McAlpine and Grothe, 2003), which may arise from differences in neural circuitry and development. One difference to consider in the context of our study is that

mammalian cochlear nucleus axons cross the midline at the ventral surface, while the avian tract crosses dorsally, suggesting that midline guidance may be controlled by distinct mechanisms in these classes.

Ephrin-B signaling through EphA4 appears to be an additional midline guidance mechanism. In the decussating corticospinal tract, EphA4 mutations lead to bilateral projections that result in mirror movements (Dottori et al., 1998; Kullander et al., 2001b; Leighton et al., 2001). Both forward and reverse signaling have been implicated in this pathway (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001). Axons express EphA4, and repulsion by ephrins requires kinase activity but may also require additional levels of ephrin-mediated regulation, such as higher order clustering (Egea et al., 2005). Interestingly, the corticospinal decussation occurs at the caudal medulla in a restricted region lacking ephrin-B3 (Yokoyama et al., 2001), suggesting a spatial window through which axons are permitted to cross the midline. This pattern is reminiscent of the temporal window described here, in which EphB2 and EphB5 are not expressed at the midline during a limited time period coinciding with initial axon growth through the midline. EphA4 is not expressed in NM axons or in the midline of the auditory hindbrain, so it is unlikely to have a role in midline guidance in this system. However, it is selectively expressed in the ipsilateral recipient zone of the target, NL (Cramer et al., 2000b), and appears to have a role in binaural segregation of NM inputs to NL (Cramer et al., 2004).

Other mechanisms controlling midline guidance

Studies of Eph proteins in axon guidance at the midline support a role for the B class of ephrins and their receptors, the EphB receptors as well as EphA4. Here, we have shown that the hindbrain regions that give rise to the auditory brainstem in the chick also use EphB signaling to regulate midline growth, and that EphB expression is closely linked to the timing of axon growth. Whether or not this type of signaling is a general feature of midline guidance remains to be seen. Several other families of proteins have important roles in controlling axonal midline crossing, including netrins, DCC, semaphorins, neuropilins, slits, and robos (Kaprielian et al., 2001; Tessier-Lavigne and Goodman, 1996). Differences in embryogenesis and protein expression between midline structures indicate that guidance cues are diverse and complex (Charron and Tessier-Lavigne, 2005; Williams et al., 2004). An important challenge will be to understand how Eph proteins cooperate with other cues to establish precise, functional circuitry in the nervous system.

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