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Differential Expression of Eph Receptors and Ephrins in the Cochlear Ganglion and Eighth Cranial Nerve of the Chick Embryo

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ABSTRACT

The cochleovestibular ganglion of the chick differentiates at early embryonic stages as VIIIth nerve axons enter the brainstem. The tonotopic organization of the auditory portion of the VIIIth nerve can be discerned at the time axons initially reach their brainstem targets. The mechanisms underlying this early organization are not known. Eph receptor tyrosine kinases and their ligands, the ephrins, have a demonstrated role in guiding axons to topographically appropriate locations in other areas of the nervous system. In order to begin to test whether Eph proteins have a similar role in the auditory system, we investigated the tonotopic expression of several Eph receptors and ephrins in the VIIIth nerve during embryonic ages corresponding to the initial innervation of the auditory brainstem. Expression patterns of EphA4, EphB2, EphB5, ephrin-A2, and ephrin-B1 were evaluated immunohistochemically at embryonic days 4 through 10. Protein expression was observed in the cochlear ganglion and VIIIth nerve axons at these ages. EphB5, ephrin-A2, and ephrin-B1 were expressed throughout the nerve. EphA4 and EphB2 had complementary expression patterns within the nerve, with EphA4 expression higher in the dorsolateral part of the nerve and EphB2 expression higher in the ventromedial part of the nerve. These regions may correspond to auditory and vestibular components, respectively. Moreover, EphA4 expression was higher toward the low-frequency region of both the centrally and peripherally projecting branches of cochlear ganglion cells. Regional variation of Eph protein expression may influence the target selection and topography of developing VIIIth nerve projections. J. Comp. Neurol. 482:309–319, 2005.

Indexing terms: auditory pathways; auditory nerve; vestibular nerve; statoacoustic; brainstem

Auditory processing in the central nervous system (CNS) relies on highly ordered inputs from the periphery. During development, the cochlear ganglia and vestibular ganglia form distinct cell groups. Centrally projecting axons from both auditory and vestibular ganglion cells enter the brainstem through the VIIIth cranial nerve. From embryonic day 5.5 (E5.5), the rostral branch of the VIIIth nerve root contains vestibular components and the caudal branch contains both vestibular and auditory axons (Kubke et al., 1998; Kubke and Carr, 2000). Auditory nerve axons in the chick embryo enter the brainstem by E4.5, but do not invade the regions containing precursors for the auditory brainstem nuclei until E6 (Kubke and Carr, 2000; Molea and Rubel, 2003). Axons within the auditory nerve are arranged tonotopically, such that low-frequency selective axons enter the brainstem at the dorsolateral margin of the nerve with progressively higher frequencies represented more ventromedially. This projection forms a tonotopically organized map within the cochlear nucleus, or nucleus magnocellularis (NM) in the chick (Rubel and Parks, 1975). The tonotopic ordering is

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present from early embryonic ages, before VIIIth nerve axons form synaptic connections (Molea and Rubel, 2003).

A number of proteins have documented roles in the differentiation of cochlear ganglion neurons and guidance of centrally and peripherally directed axons. These include, but are not limited to, cell adhesion molecules (Kelley, 2003), transcription factors (Huang et al., 2001), neurotrophins (for review, see Fritzsch et al., 1998; Rubel and Fritzsch, 2002), and the Eph proteins, including the Eph receptor tyrosine kinases and their ligands, the ephrins, which have a role in guidance of cochlear ganglion cell axons in mammals (Bianchi and Gray, 2002; Brors et al., 2003).

Eph proteins are axon guidance molecules (Henkemeyer et al., 1994, 1996; Winslow et al., 1995; Ciossek et al., 1998; Imondi et al., 2000). Eph receptors include the EphA and EphB classes, and ligands are also classified into A and B groups (Eph Nomenclature Committee, 1997). Ephrin-A ligands have a glycosylphosphatidylinositol linkage and preferentially bind EphA receptors, while ephrin-B ligands have a transmembrane domain and preferentially bind EphB receptors. Exceptions to this specificity include EphA4, which also binds ephrin-B ligands (Gale et al., 1996) and EphB2, which also binds ephrin-A5 (Himanen et al., 2004). In addition to forward signaling from ligand to receptor, Eph signaling can also operate in the reverse direction, so that both proteins undergo phosphorylation at tyrosine residues and both cells respond with activation of signal transduction pathways (Holland et al., 1996; Bruckner et al., 1997). Interactions between ephrins and Eph receptors can be attractive or repulsive (Holmberg and Frisen, 2002; Murai and Pasquale, 2003).

Gradients of Eph receptor and ephrin expression have been shown to be essential for the formation of topographic maps in the visual system (Cheng et al., 1995; Drescher et al., 1995; Feldheim et al., 1998, 2000; Flanagan and Vanderhaeghen, 1998; Yates et al., 2001; Hindges et al., 2002; Mann et al., 2002b), the hippocamposeptal pathway (Gao et al., 1996; Yue et al., 2002), thalamic projections to sensory cortical areas (Dufour et al., 2003), and spinal cord projections to precise targets within muscles (Feng et al., 2000; Wang et al., 2001) or limbs (Helmbacher et al., 2000; Eberhart et al., 2002). These studies demonstrate the role of Eph proteins in the formation of topographic maps in a variety of neural pathways. Whether these proteins have a similar role within auditory projection has yet to be determined.

The molecules guiding early VIIIth nerve projections to the CNS are unknown. Two recent reports suggest a role for Eph proteins in establishing central auditory circuitry. First, we have recently shown that the Eph receptor EphA4 has a significant role in the formation of segregated ipsilateral and contralateral projections from NM to its target, nucleus laminaris (NL; Cramer et al., 2004). Second, while the role of EphA4 in the formation of the tonotopy in this projection is not known, the expression of EphA4 varies along the tonotopic axis of NL (Person et al., 2004). In the present study, we have begun to examine the role of Eph proteins in the formation of the tonotopic projections from the chick cochlear ganglion. We examined the expression of Eph proteins in the developing VIIIth nerve projection into the brainstem. We used a panel of specific antibodies during the time that VIIIth nerve axons grow into the brainstem and form topographic projections with their targets. The results suggest potential roles for individual Eph proteins in identifying appropriate target nuclei and in forming tonotopic projections within cochlear nuclei.

MATERIALS AND METHODS

Antibodies

We used a mouse monoclonal antibody for EphA4 that was generated using an Fc fusion protein of the extracellular domain of chick EphA4 (Ohta et al., 1996) and mouse monoclonal antibodies prepared using a similar approach that recognize the extracellular domains of EphA3, ephrin-A2, ephrin-A5 (Iwamasa et al., 1999). Rabbit polyclonal antibodies were affinity-purified and specific for EphB2 (Pasquale, 1991), EphB5 (Soans et al., 1996), and ephrin-B1 (Kalo et al., 2001). We confirmed the specificity of all antibodies used in this study with immunoprecipitation and Western blots.

Immunoprecipitation and Western blots

Brainstem tissue from E10 chick was homogenized on ice in Sten buffer (300 mM NaCl, 100 mM Tris, 4 mM EDTA, 0.4% NP-40, pH 7.6), then incubated with protein A beads (Roche Pharmaceuticals, Nutley, NJ) for 1 hour at 4°C, briefly centrifuged, and the supernatant was incubated with protein A beads coated with antibody at 4°C overnight. After centrifuging, pellets were washed and protein samples were separated by SDS polyacrylamide gel electrophoresis. Proteins were 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, incubated with secondary antibody, then enhancing solution (Bio-Rad).

Immunohistochemistry

Chicken eggs were incubated in a humid 37°C incubator (Lyon Electric, Chula Vista, CA). Three to five embryos at each embryonic age from E4 through E10 were included in the study. Embryos were removed from the egg and a segment of the embryo that included the brainstem, otocyst, and all overlying tissue was placed in 4% paraformaldehyde for 2 hours. Tissue was then rinsed in phosphate-buffered saline (PBS; pH 7.4) several times, then incubated in 30% sucrose in PBS overnight at 4°C. Specimens were blocked in molds and embedded in OCT mounting medium. Coronal sections were cut on a cryostat and thawed onto coated slides. Section thickness ranged from 12–20 μm. Slides were warmed on a slide warmer, then rinsed in PBS. Slides were placed in a solution of 0.3% H_2O_2 in methanol for 10 minutes to reduce labeling from endogenous peroxidase. After rinsing, a well was made around sections using a PAP pen (Binding Site, San Diego, CA). Sections were rinsed in PBS and blocking solution (5% nonfat dry milk and 0.1% Triton X-100 in PBS) was placed in each well for 1 hour. This solution was then removed and sections were incubated overnight at room temperature in primary antibodies (1–5 μg/ml in blocking solution). Negative controls were included in which the primary antibody was omitted. Sections were rinsed in PBS, then incubated for 2 hours at room temperature in secondary antibody, a biotinylated goat anti-mouse or goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted to 1:250 in blocking solution.

Sections were rinsed in PBS and incubated in Vector ABC kit for 1 hour, then rinsed, and HRP was visualized using 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO). After dehydration in a graded series of alcohols followed by xylene, slides were coverslipped using DPX mounting medium (BDH Laboratory Supplies, Poole, UK). One slide from each brain was stained with thionin to aid in identifying embryonic structures. In some cases, a second slide was immunolabeled for microtubule associated protein 2 (MAP2; Lab Vision, Fremont, CA) or glial fibrillary acidic protein (GFAP; Lab Vision) using this protocol in order to obtain additional information about these structures.

For double immunofluorescence, two primary antibodies generated in different species were used simultaneously. Secondary antibodies were goat anti-mouse conjugated to Alexa 594 and goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR). After rinsing secondary antibodies, slides were coverslipped with Glycergel (Dako, Carpinteria, CA). We viewed Alexa 594 with a Texas Red filter set and Alexa 488 with an FITC filter set (Chroma Technology, Rockingham, VT). Control experiments with single immunofluorescence verified that fluorescence did not bleed through to filters used for the second fluorophore.

Production of photomicrographs

Immunolabeled sections were viewed under a Zeiss Axioskop MOT 2 microscope with epifluorescence when applicable. Sections were photographed using a Zeiss Axiocam digital camera and Openlab software (Improvision, Lexington, MA). Images were imported into Adobe PhotoShop 7.0 (Adobe Systems, San Jose, CA) for contrast and brightness adjustment and labeling.

RESULTS

All antibodies were used in immunoprecipitation and Western blots on chick brainstem homogenates. A single band corresponding to the appropriate Eph family protein was seen in each case (data not shown). This specificity corresponded with previous reports (Pasquale, 1991; Ohta et al., 1996; Soans et al., 1996; Iwamasa et al., 1999; Kalo et al., 2001). In addition, the antibody used here for EphA4 had an identical labeling pattern in rhombomere 5 at E4–5 and in the brainstem auditory nuclei at E10 that we previously observed using a rabbit polyclonal antibody directed against the carboxy terminus of the protein (Soans et al., 1994; Cramer et al., 2000). Negative controls, in which the primary antibody was omitted, did not result in labeling.

Expression of EphA4

EphA4 was expressed in the VIIIth nerve at all of the ages examined in this study. At E4 expression was most intense within a narrow band at the dorsolateral margin of the nerve (Fig. 1A). At E5 expression was present in the dorsolateral portion of the VIIIth nerve, with a boundary near the middle of the nerve (Fig. 1B) that was more abrupt than that seen at E4. Immunolabeling was prominent near the entry point into the brainstem. Within the labeled region, expression was heaviest toward the dorsolateral edge. Immunolabeling was present in the nascent cochlear ganglion, a small group of cell bodies delaminating from the otocyst in the ventromedial region (Fig. 1C). The adjacent dorsal region of the otocyst was also labeled.

Expression of EphA4 was lighter in the cochlear ganglion than in the auditory fibers of the VIIIth nerve at all of the ages examined. Peripherally projecting fibers were immunopositive for EphA4 at all ages examined. An example from an E7 embryo is shown in Figure 1D. Fibers toward the ventromedial region, corresponding with lower frequencies, were more heavily labeled. A similar pattern was seen at E8; an example is shown with an adjacent Nissl-stained section in Figure 1E,F.

At E8 the gradient of expression within centrally projecting VIIIth nerve axons remained, although overall expression levels at later ages was less intense than at earlier ages. As with earlier ages, the dorsolateral region had the most intense labeling (Fig. 1G). The entry point of these axons to the brainstem was dorsolaterally positioned, consistent with the position of auditory axons at this stage. This region was immunolabeled for EphA4, and within this region the dorsolateral portion had the highest intensity (compare low-frequency (lf) region with high-frequency (hf) region in Fig. 1G). Labeled auditory nerve fibers curved around the region of the auditory anlage and did not extend beyond it. In some cases fibers were observed that were immunolabeled for EphA4 continuously from the VIIIth nerve entry to the brainstem to the region of the auditory anlage. This observation suggests that auditory axons within the VIIIth nerve express EphA4 from the initial entry into the synaptic target of these neurons, NM, while the latter is still undifferentiated within the anlage.

At all ages examined an intensely immunolabeled region of the VIIIth nerve was seen at the entry point of the nerve into the brainstem; EphA4 was expressed in a gradient within this band, with high expression dorsolaterally (Fig. 1A,B,G). This region may represent a transition from peripheral to CNS components, which are myelinated by distinct types of glial cells. Figure 1H shows that GFAP, expressed in oligodendrocytes, is seen in this brainstem region at E10, and expression is uniform within the band. EphA4 expression is likely axonal, because labeling is seen at early ages when axons first grow toward the brainstem; however, this protein may be expressed on glia as well.

Expression of EphB2

EphB2 was expressed in the VIIIth nerve at all the ages examined. At E4–5 immunolabeling in VIIIth nerve axons was more intense toward the ventromedial portion of the nerve, corresponding with vestibular regions and higher-frequency regions of the auditory portion of the nerve (Fig. 2A). Many labeled axons entered the brainstem and turned toward the midline. This pattern remained at E6 (Fig. 2B), where in addition a band of labeling at the nerve entry point was visible, and EphB2 labeling was also observed in the dorsolateral edge of this band and of the VIIIth nerve. While the ventromedial region was also intensely labeled in this region, the dorsolateral region contained a labeled patch that was more limited in extent than that seen with EphA4 immunohistochemistry. While EphA4 and EphB2 were largely complementary at this age, the region of EphA4 labeling varied in intensity along the nerve, while the region of EphB2 labeling appeared more uniform, with slightly less labeling in dorsolateral regions.

EphB2 immunolabeling was observed in the cochlear ganglion in the perimeter of cell bodies; this labeling could

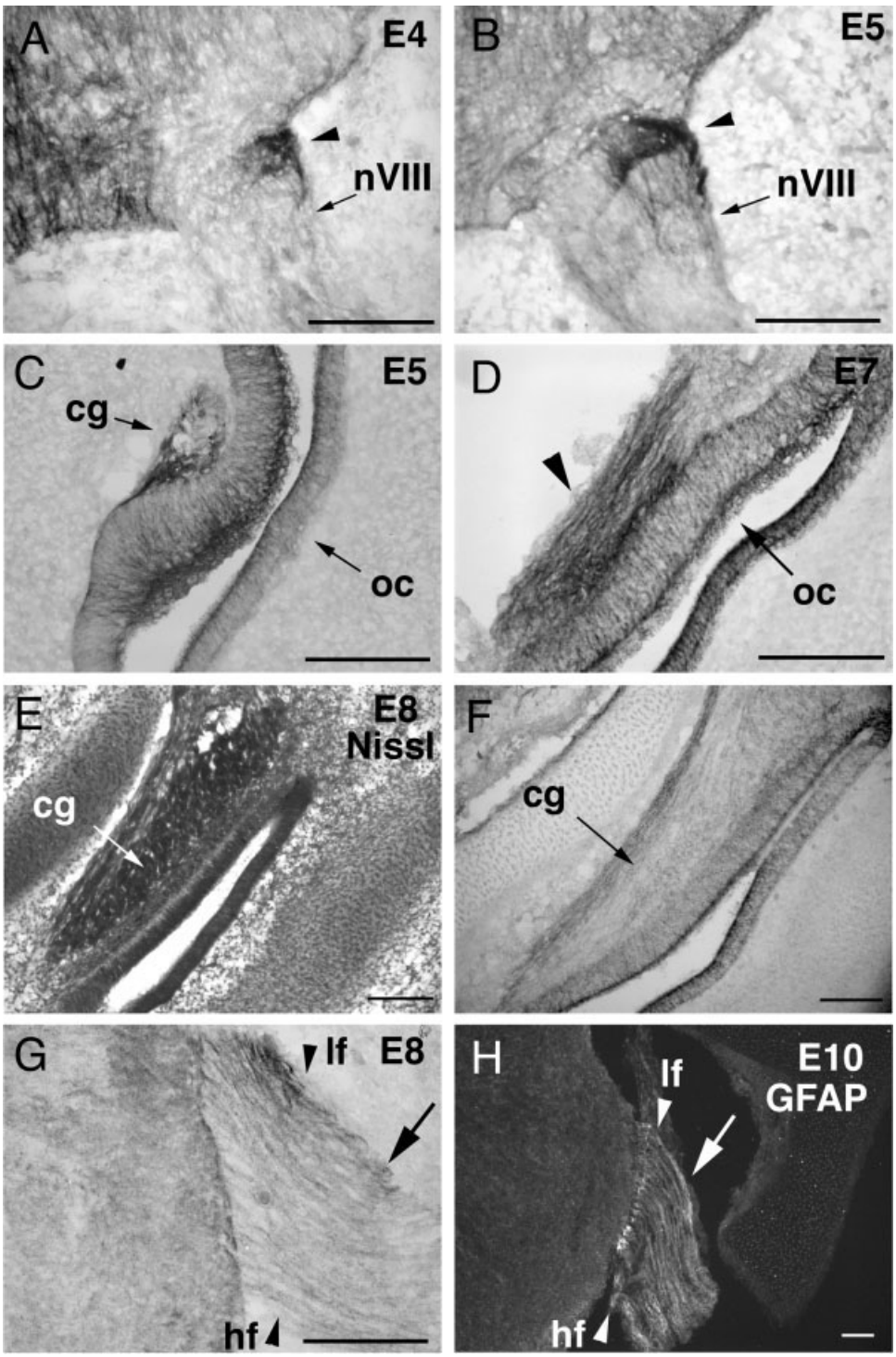

Fig. 1. EphA4 immunolabeling in the cochlear ganglion and VIIIth nerve. All sections are coronal with the dorsal region toward the top. **A:** EphA4 immunolabeling is present at E4 in the lateral portion of the VIIIth nerve (arrow) and also in a band at the entry point of the nerve into the brainstem (arrowhead). **B:** EphA4 immunolabeling at E5. The VIIIth nerve is labeled, along with a band of labeling showing a gradient of expression. Labeling in the nerve is limited to the dorsolateral-most part of the nerve, and ends abruptly at the center. **C:** EphA4 immunolabeling in the cochlear ganglion at E5. Fibers are labeled but cell bodies show limited levels of expression. **D:** At E7 peripherally projecting fibers are labeled (arrowhead). **E:** Nissl-stained section showing the cell bodies of the cochlear ganglion at E8. **F:** Section adjacent to E showing that expression of EphA4 is low in the cell bodies of the cochlear ganglion but fibers are labeled at E8. **G:** Cochlear nerve fibers are labeled in E8 tissue. Large arrow indicates VIIIth nerve. Labeling is more intense in the low-frequency region than in the high-frequency region. **H:** Glial fibrillary acidic protein is expressed in a band where the nerve enters the brainstem, with some expression in portions of the VIIIth nerve (large arrow). nVIII, VIIIth nerve; cg, cochlear ganglion; oc, otocyst; lf, low frequency; hf, high frequency; GFAP, glial fibrillary acidic protein. Scale bars = 100 μm.

represent receptor expression within the cell membranes. Labeling was prominent in peripherally directed branches of cochlear ganglion cells at E8 (Fig. 2C,D, arrowheads). At this age centrally directed axons were also labeled (Fig. 2E,F), with the highest intensity labeling in the dorsolateral (lf) region and a narrow zone in the ventromedial region. Auditory nerve fibers (anf) are also seen near the auditory anlage (aa) at this age.

Double immunofluorescence for EphA4 and EphB2

The pattern of EphB2 expression was distinct from that seen with EphA4. In adjacent sections, VIIIth nerve axons had a dorsolateral region immunopositive for EphA4 and a ventromedial region immunopositive for EphB2, with little or no overlap. While expression patterns were con-

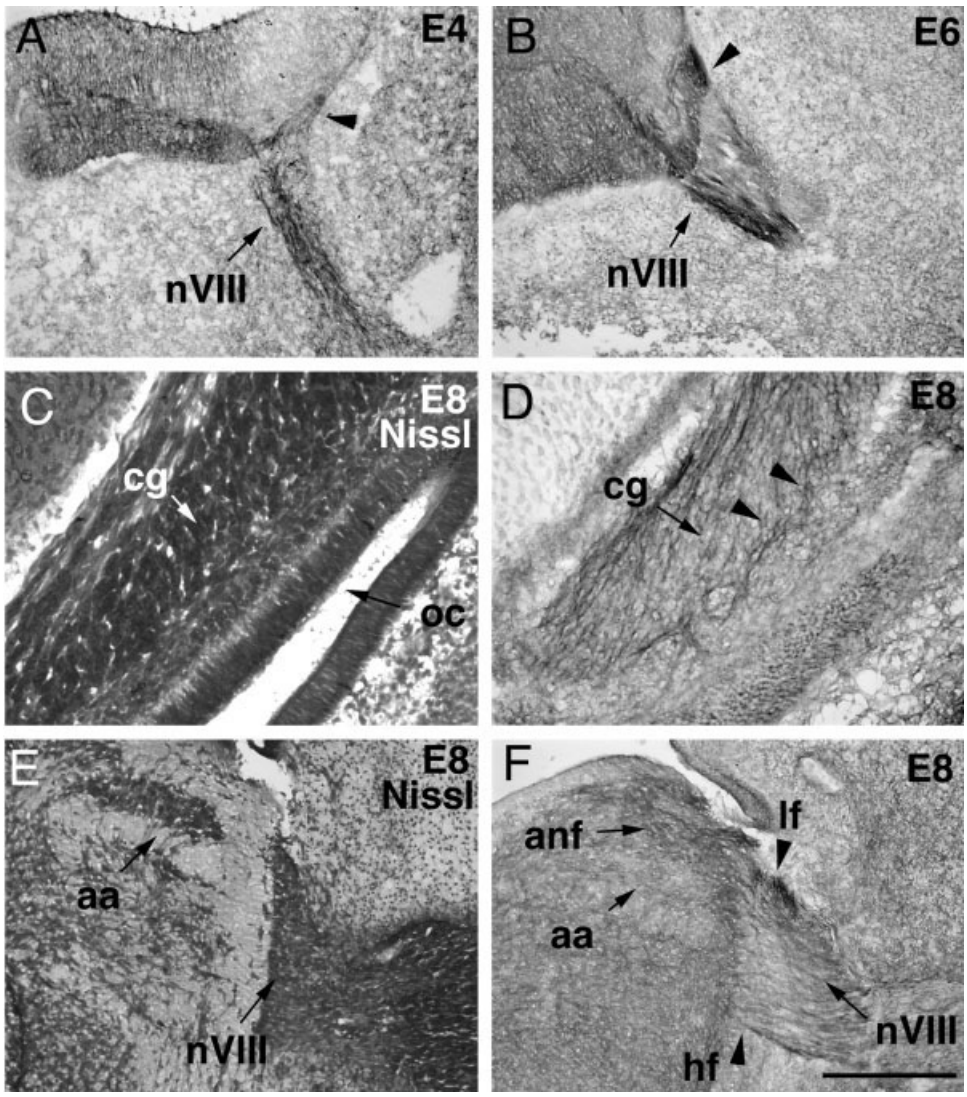

Fig. 2. Expression of EphB2 in the cochlear ganglion and VIIIth nerve. **A:** Expression of EphB2 in the VIIIth nerve is confined to the ventromedial fibers at E4. The dorsolateral region of the nerve lacks expression. A small region at the entry to the brainstem is also labeled (arrowhead). **B:** At E6 the ventromedial region is heavily immunolabeled. A wide band at the entry to the brainstem is also labeled, with heavier expression in the dorsolateral part of this band (arrowhead). **C:** Nissl-stained section throughout the cochlear ganglion of an E8 embryo. **D:** Adjacent section immunolabeled for EphB2. Expression is present in fibers but not in cell bodies (arrowheads). **E:** Nissl-stained section showing the brainstem and centrally projecting regions of the cochlear ganglion at E8. **F:** Adjacent section showing EphB2 immunolabeling. Expression is uniform in the auditory nerve except for a high-intensity region at the dorsolateral edge near the nerve entry point, corresponding with low-frequency fibers. In addition, fibers around the auditory nuclei are also immunopositive. aa, auditory anlage; anf, auditory nerve fibers; others as in Figure 1. Scale bars = 200 μm in F (applies to A,B,E,F); 100 μm for C,D.

sistent across animals for EphA4 and EphB2 single immunohistochemistry, the use of adjacent sections was limited due to variations in the coursing of VIIIth nerve axons, as well as potential variations in the plane of section. To unambiguously determine the relative expression patterns of these proteins within individual sections, we performed double immunofluorescence.

As previously reported (Cramer et al., 2000; Kury et al., 2000), EphA4 was expressed at E5 in longitudinal bands within rhombomeres 3 and 5. At E6 the bands were relatively more laterally positioned (Fig. 3A) and were observed in the region of the auditory anlage. At this age the complementary expression patterns of EphA4 and EphB2 within the VIIIth nerve could be discerned. EphB2 was more heavily expressed in the ventromedial region, while EphA4 was more heavily expressed in the dorsolateral region (corresponding to VIIIth nerve regions that transmit preferentially low frequencies). A very narrow band of EphB2 immunopositive fibers was also seen in the dorsolateral margin. The complementarity extended to the band of heavily labeling at the entry point to the brainstem. A similar pattern was seen in the E6 cochlear ganglion (Fig. 3B). Here, EphA4 was more heavily expressed ventromedially, in projections to low-frequency-selective cells in the basilar papilla, while EphB2 was more heavily expressed in the dorsolateral region. A narrow ventromedial band was double-labeled. At E7 the pattern persisted (Fig. 3C), and the band of label at the nerve entry point was very intensely labeled. An example of labeling at E8 is shown in Figure 3D. The relative patterns of labeling were seen in portions of the nerve emanating from the cochlear ganglion. The differences in intensity in fluorescent images were similar to those seen with immunohistochemistry, and were observed in the same patterns in multiple sections and in several brains.

Expression of EphB5

Immunolabeling for EphB5 was observed in the VIIIth nerve at all ages examined. There was no systematic variation in labeling intensity within the nerve. The labeled region was wider than that seen in adjacent sections labeled with EphA4 antibodies, and most likely contained

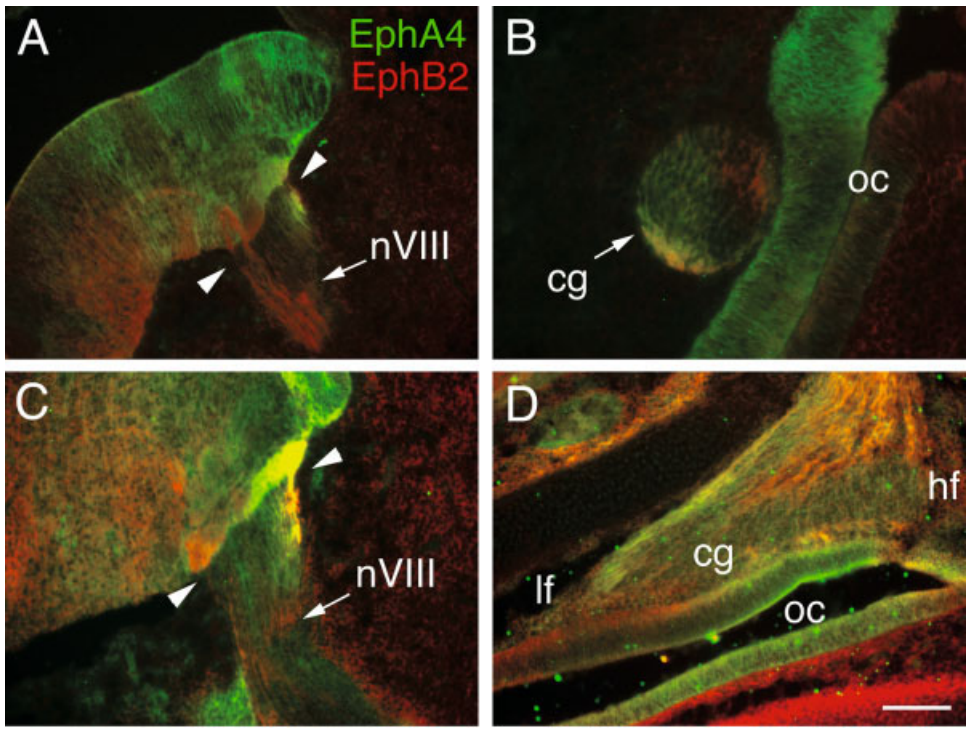

Fig. 3. Double immunofluorescence using EphA4 and EphB2 antibodies. **A:** E6 brainstem and VIIIth nerve immunolabeled for EphA4 (green) and EphB2 (red). EphA4-immunopositive fibers are found in the dorsolateral part of the nerve, corresponding with the auditory regions. Within this region, the intensity declines toward the middle of the nerve. EphB2-immunopositive fibers are mostly localized to the ventromedial part of the VIIIth nerve, corresponding to the vestibular region; an additional narrow EphB2-immunopositive region is seen in the lateral margin of the nerve. Arrowheads indicate the margins of the nerve with the highest intensity of immunolabel for both proteins. **B:** Cochlear ganglion labeling at E6. The dorsolateral portion is more intensely labeled for EphB2, while the ventromedial region is has more EphA4 expression. The ventromedial edge of the ganglion, which corresponds to low frequencies, is double-labeled. **C:** E7 brainstem and nerve. The pattern of labeling in the nerve is similar to that seen at E6, but the band of label at the nerve entry point is more intensely labeled. Here, the ventromedial region expresses EphB2 but not EphA4, while the dorsolateral region expresses both proteins, with the EphA4 positive region more extensive. **D:** E8 tissue labeled with double immunofluorescence. The centrally projecting fibers from the cochlear ganglion express EphB2 in the dorsolateral, high-frequency region, and EphA4 in the ventromedial, lower-frequency region. Scale bars = 100 μm.

vestibular and auditory axons. A representative example of an E7 brainstem shown together with an adjacent Nissl stained section is shown in Figure 4A,B. VIIIth nerve fibers are labeled in the region entering the brainstem. At E10 the labeling is sparse (Fig. 4C), and pale labeling is observed in the neuropil around NL, consistent with our previous report (Cramer et al., 2002).

At E4, EphB5 was expressed in a small cluster of cells dorsomedial to the otocyst; these cells are likely delaminating neurons that will contribute to the cochlear ganglion; similar patterns were seen at later ages. Figure 4D shows labeling within cell bodies of the cochlear ganglion at E6. Here, the expression seems to reside within cell bodies rather than fibers.

Expression of ephrin-A2

Ephrin-A2 was observed in the early VIIIth nerve projection. Figure 5A shows labeling throughout the VIIIth nerve projection. Labeling remains uniform at E6 (Fig. 5B), with no consistent variation in the expression levels in relation to tonotopic position. A slightly more intense pattern is seen at the nerve entry point. At E7, ephrin-A2 is expressed in auditory nerve fibers (Fig. 5C) around the region of the auditory anlage, as well as uniformly through the VIIIth nerve. At E10 (Fig. 5D), these fibers remain labeled and, in addition, immunolabel is seen in the neuropil around NL.

Expression of ephrin-B1

Ephrin-B1 was expressed in the cochlear ganglion at E5 (Fig. 6A). In addition, VIIIth nerve fibers were immunopositive throughout the nerve at this age (Fig. 6B), where the most intense labeling was seen outside the brainstem and terminating at the nerve entry point. At this age no consistent difference in staining intensity was noted according to tonotopic position.

The uniform pattern of labeling for ephrin-B1 was maintained throughout the ages examined. Figure 6C,D shows immunolabeled sections from an E10 embryo together with an adjacent Nissl-stained section. Immunolabeling is intense in the VIIIth nerve, and is also seen in auditory nerve fibers and neuropil around NM and NL. At this age immunolabeling is also present in the cochlear ganglion, with uniform expression throughout the tonotopic axis. Figure 6E,F shows a section through the ganglion together with an adjacent Nissl-stained section showing the position of the ganglion cell bodies and otocyst. Ephrin-B1 is expressed

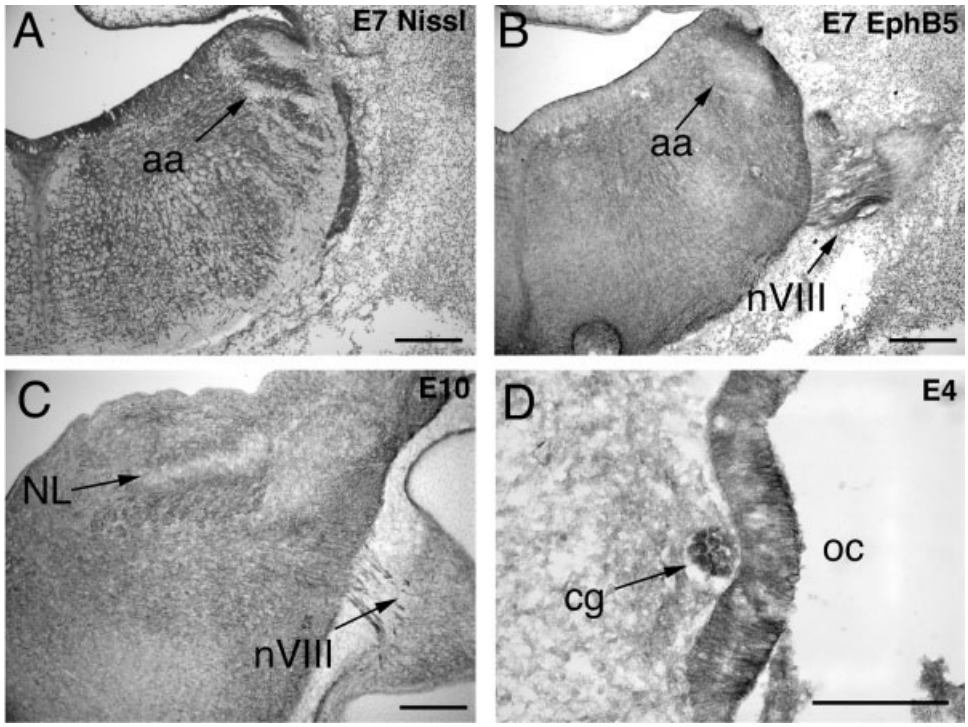

Fig. 4. Immunolabeling for EphB5. **A:** E7 Nissl-stained section showing the location of the auditory anlage. **B:** Adjacent section to that shown in A, showing expression pattern of EphB5. Immunolabeling is present in the VIIIth nerve and is absent from the auditory anlage. **C:** At E10 EphB5 is expressed sparsely in the VIIIth nerve and, as previously shown, has low-expression levels around nucleus laminaris (NL). **D:** In the E6 embryo, EphB5 is expressed in the cell bodies of the cochlear ganglion. Labeling is slightly higher in intensity than in the immediately surrounding mesenchyme. Scale bar = 200 μm in A–C; 100 μm in D.

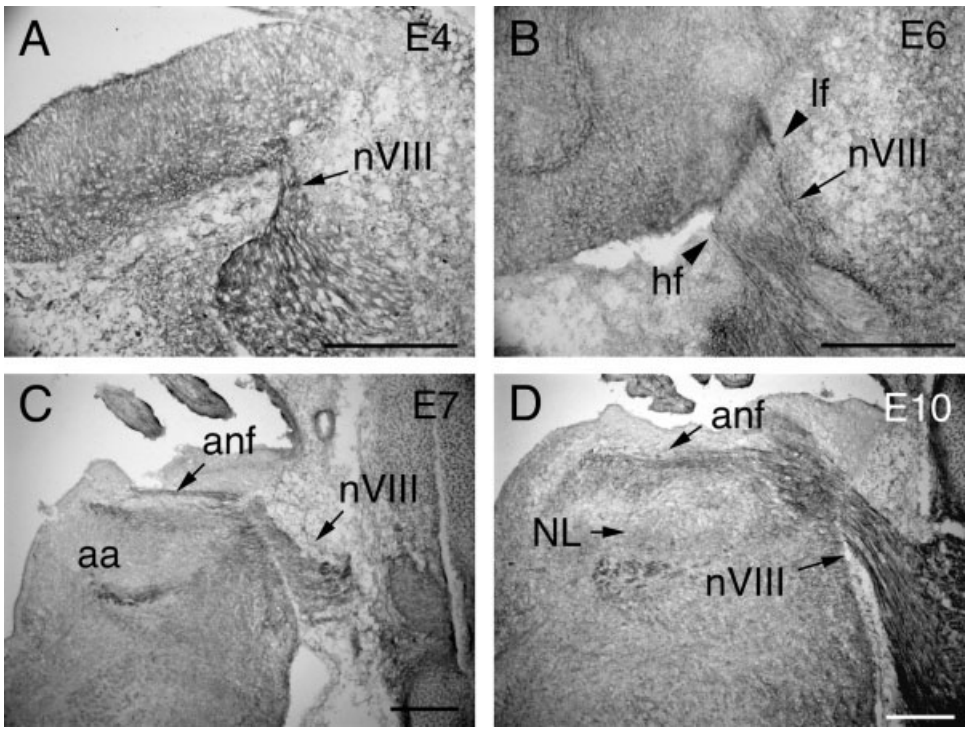

Fig. 5. Immunolabeling for Ephrin-A2. **A:** At E4 ephrin-A2 is expressed in fibers projecting to the brainstem through the VIIIth nerve. **B:** At E6 ephrin-A2 is expressed uniformly through the dorsolateral–ventromedial extent VIIIth nerve fibers. At the entry site to the brainstem, the dorsolateral edge is somewhat more intensely labeled than other regions. **C:** At E7 ephrin-A2 is expressed in VIIIth nerve and in fibers that approach the auditory anlage region. **D:** At E10 ephrin-A2 remains expressed uniformly through the VIIIth nerve and is expressed in auditory nerve fibers that invade NM, and in the neuropil region around NL. Scale bars = 200 μm.

throughout the ganglion and in both centrally and peripherally projecting fibers. These results agree with those reported by Bianchi and Gray (2002). While the labeling is of moderate intensity, it is greater than that seen in the ventral portion of the otocyst, which is unlabeled.

Lack of expression of EphA3 and ephrin-A5

EphA3 and ephrin-A5 expression were not observed in the brainstem or in the VIIIth nerve at any of the ages we examined (data not shown). As a positive control for our antibody and our immunohistochemical methods, we per-

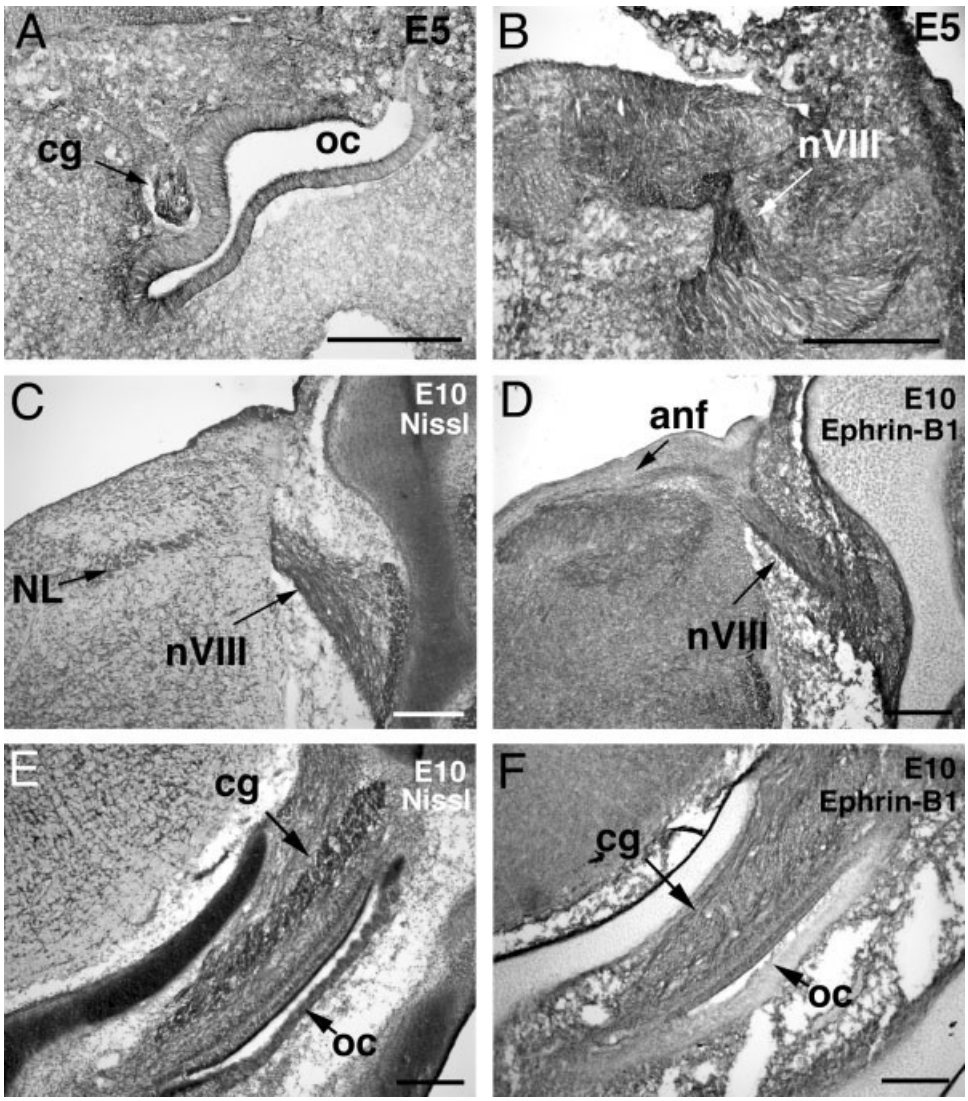

Fig. 6. Expression of Ephrin-B1. **A:** Ephrin-B1 is expressed in the cochlear ganglion at E5. Some regions of the surrounding mesenchyme are also labeled. **B:** At E5, labeling is evident uniformly through the VIIIth nerve toward the brainstem; labeling intensity diminishes as axons enter the brainstem. **C:** Nissl-stained section from an E10 embryo showing the brainstem and VIIIth nerve. **D:** Adjacent section to that shown in C. Immunolabeling is prominent within the VIIIth nerve approaching the brainstem. Immunopositive fibers are also seen approaching nucleus magnocellularis and in neuropil around NL. **E:** Nissl-stained section from an E10 embryo showing the cochlear ganglion. **F:** Adjacent section to that shown in E immunolabeled for ephrin-B1. Labeling is present in the cochlear ganglion and in centrally and peripherally projecting fibers. Labeling intensity is similar for cochlear ganglion cells bodies and fibers and is greater than that seen in the ventrolateral portion of the otocyst. Scale bars = 200 μm.

formed immunohistochemistry using identical methods on cryostat sections of E9 optic tectum. We found intense labeling consistent with published reports of expression in this region at this age (Cheng et al., 1995; Drescher et al., 1995; Marin et al., 2001). We thus conclude that our negative finding in the brainstem indicates that ephrin-A5 is not expressed in the auditory brainstem pathways from E4–10.

DISCUSSION

Auditory nerve fibers enter the region of the cochlear nucleus early in embryonic development, and this initial projection is tonotopically ordered in both birds and mammals (Leake and Snyder, 1989; Leake et al., 2002; Molea and Rubel, 2003). Tonotopy was demonstrated in the chick at E6 using lipophilic dyes to label apical and basal regions of the nerve (Molea and Rubel, 2003). This tonotopy precedes the formation of synaptic connections, which occurs at E11 (Saunders et al., 1973; Jackson and Parks, 1982). While activity-dependent mechanisms have important roles in cell survival in this pathway (Levi-Montalcini, 1949; Parks, 1979; Jackson and Parks, 1982; Trune, 1982; Hashisaki and Rubel, 1989), neuronal activity is unlikely to play a major role in the formation of the tonotopic arrangement of inputs (Friauf and Lohmann, 1999; Rubel and Cramer, 2002; Molea and Rubel, 2003). Instead, the principal mechanisms underlying the development of this tonotopic projection likely involve molecular cues. We have begun to evaluate the hypothesis that Eph proteins represent such cues.

We have demonstrated that several Eph proteins are expressed in the VIIIth cranial nerve during the growth of axons to the brainstem and into the region of the auditory nuclei. EphB5, ephrin-A2, and ephrin-B1 were expressed uniformly throughout the axons of the VIIIth nerve, while EphA3 and ephrin-A5 were not expressed in the VIIIth nerve or the brainstem. In contrast, EphA4 was heavily expressed in the dorsolateral region of the nerve, and progressively weaker expression toward the center of the nerve. EphB2 expression in this region was present but at lower levels than that seen with EphA4. EphB2 had

higher expression levels in the ventromedial portions of the nerve, with progressively weaker expression toward the center. The expression patterns seen for EphA4 and EphB2 along the dorsolateral region represent gradients that vary along the tonotopic axis of the nerve.

Tonotopic expression patterns

The spatial distribution of EphA4 immunolabeling corresponds to low-frequency regions of the nerve. Support for this assertion comes from the fact that labeling is highest in the region that will contain low-frequency fibers, and that labeled fibers are seen in this region as early as E4, when high-frequency axons have not yet grown into the brainstem (Molea and Rubel, 2003). Moreover, within the peripherally directed projection, EphA4 was more heavily expressed in apical regions, which correspond with the low-frequency area of the basilar papilla. Within the cochlear ganglion and peripheral projections, EphB2 immunolabeling was complementary to that of EphA4, suggesting that EphB2 is expressed in high-frequency areas. These gradients of expression suggest a possible role for EphA4 and EphB2 in the formation of tonotopic maps in this pathway. The pattern of EphA4 labeling contrasts with tonotopic gradients described in NL at E10 (Person et al., 2004), in which high-frequency areas were more intensely labeled than low-frequency areas. Moreover, in the present study, expression of these receptors is in axons, while in NL expression is likely in dendrites. This difference in expression suggests the possibility that the NM-NL projection relies on reverse Eph signaling, while the VIIIth nerve projection relies on forward signaling.

Nerve entry point

In addition to expression within the nerve, several Eph proteins were expressed heavily in a band within the brainstem near the nerve entry point. This region corresponds with the PNS–CNS junction. Peripheral myelination of the VIIIth nerve arises from Schwann cells, while central myelination arises from oligodendrocytes; markers specific for these types of myelinating cells have been identified within the VIIIth nerve (Knipper et al., 1998). Neuronal EphA receptors can interact with ephrins expressed in glia (Davenport et al., 1998; Murai et al., 2003). Here we report that GFAP is expressed within the region of the brainstem where the nerve enters. The EphA4 and EphB2 labeling we observed within VIIIth nerve fibers most likely represents expression within axons at early ages. Axons are unmyelinated prior to E13 (Fermin and Cohen, 1984; Sun et al., 1998); at later times, both axons and glia may express these proteins. An interesting possibility is that Eph proteins mark the location of nerve entry, and that EphB2 and EphA4 together guide VIIIth nerve axons to appropriate (i.e., auditory or vestibular) targets within the brainstem by selective routing at this nerve entry point. Eph proteins on VIIIth nerve axons may thus interact with glial cells as axons enter the central nervous system. Whether or not these interactions aid in axon guidance to appropriate targets remains to be determined.

Auditory versus vestibular pathways

The mechanisms that maintain the segregation of auditory and vestibular components have not been identified. In mice, the transcription factor GATA3 marks auditory components, while NeuroD marks vestibular neurons (Lawoko-Kerali et al., 2004). It is not clear whether these proteins have similar expression patterns in avian embryos. However, they could not be used in the present study to identify pathways because their expression is restricted to cell bodies, while differential labeling of Eph proteins was observed within the nerve. Because of the anatomical arrangement of expression patterns, both within the junction of axons from cochlear and vestibular nuclei and at the entry to the brainstem, we can conclude that EphA4 and EphB2 had differential distribution patterns that varied with auditory vs. vestibular targets. Moreover, EphB2 labeled axons entered the brainstem and turned medially, while EphA4 immunopositive axons coursed dorsolaterally, indicating that at early ages these axons took largely distinct trajectories. Functional studies will be required to evaluate the role of these proteins in establishing appropriate connections from the auditory and vestibular divisions of the VIIIth nerve.

Roles for Eph proteins

Interactions between ephrins and Eph receptors are known to be important in the formation of tonotopic projections in several areas of the nervous system. The best-studied example is the retinotectal projection, where gradients of EphA3 receptors are present in retinal axons and opposing gradients of ephrin-A2 and ephrin-A5 are present in the tectum (Cheng et al., 1995; Drescher et al., 1995). Because of axonal repulsion mediated by interactions between ephrins and Eph receptors, axons with high levels of EphA3 terminate selectively in regions with low levels of ephrin-A2 and ephrin-A5. Conversely, axons with low levels of EphA3 project to regions of high levels of ephrins. However, this mechanism does not entirely explain the interactions between Eph proteins during the formation of topography. Several Eph proteins are coexpressed in the retina (Holash et al., 1997; Hornberger et al., 1999; Menzel et al., 2001), some of which are not seen in a topographic gradient. Moreover, the role of EphB receptors in dorsoventral mapping in the retinotectal projection appears to rely on attractive signals between ephrin-B ligands and EphB receptors (Hindges et al., 2002; Mann et al., 2002a; Pittman and Chien, 2002).

In the auditory system, gradients of expression have been observed in NL (Person et al., 2004) and in the VIIIth nerve in the present study. In both structures, Eph receptors and ligands are coexpressed, most of which are not graded along the tonotopic axis. Interactions between coexpressed Eph receptors and ephrins may serve to change the responsiveness of cells to Eph proteins in other cells; these *cis* interactions have been shown to operate through functional binding domains (Yin et al., 2004).

The presence of tonotopic gradients of Eph receptors in VIIIth nerve axons may suggest a mechanism analogous to that seen with EphA signaling in the retinotectal projection. This analogy predicts the presence of opposing tonotopic gradients of ligands within NM. That no such gradient has been found to date may simply indicate that the appropriate protein has not yet been examined. Alternatively, it is possible that the gradients observed within the nerve are sufficient to establish a tonotopic map without a complementary gradient in NM. The opposing gradients of two different classes of Eph receptor may represent a variation of the mechanism proposed for retinotopic development.

Comparison with mammalian studies

The present study, together with several other expression and functional studies, support a role for Eph proteins in the early organization of auditory brainstem pathways. Several groups have examined the auditory periphery of mammals. Ephrin-A ligands and EphA receptors are expressed in the gerbil in both vestibular and auditory neurons in patterns that vary with developmental stage (Bianchi and Liu, 1999). Ephrin-B ligands are expressed in the statoacoustic ganglion of chicks and mice (Bianchi and Gray, 2002). Additionally, ephrin-A2 is expressed in the mouse spiral ganglion but EphA4 is not (Pickles et al., 2002). However, EphA4 is expressed in the spiral ganglion of guinea pigs (van Heumen et al., 2000). Ephrin-B ligands are expressed in these neurons as well (Pickles et al., 2002; Brors et al., 2003) and treatment in vitro with EphA4 induces turning of these axons, suggesting repulsive, reverse signaling mediated by ephrin-B2 and ephrin-B3 (Brors et al., 2003). Similarly, in vitro studies in chick suggest that outgrowth of ephrin-B-positive statoacoustic ganglion is inhibited by reverse signaling (Bianchi and Gray, 2002). In the mouse EphB2 is expressed in the early vestibo-acoustic ganglion, and mutations cause vestibular deficits (Cowan et al., 2000).

Thus, the distribution of individual Eph family members differs between chicks and mammals, and tonotopic gradients of expression have not been described in the projections from the periphery through the VIIIth nerve in mammals. Nonetheless, together these studies support a broad role for Eph proteins in peripheral auditory and vestibular development.

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