THE JOURNAL OF COMPARATIVE NEUROLOGY 452:51–64 (2002)

Expression of EphB Receptors and EphrinB Ligands in the Developing Chick Auditory Brainstem

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ABSTRACT

Nucleus magnocellularis (NM) in the avian auditory brainstem receives auditory input from nerve the VIIIth and projects bilaterally to nucleus laminaris (NL). This projection preserves binaural segregation in that ipsilateral NM projects to dorsal dendrites of NL and contralateral NM projects to ventral dendrites of NL. We have begun to examine the molecular signals that influence segregation of inputs onto discrete regions of NL cells. We previously showed that the Eph receptor, EphA4, is expressed selectively in the dorsal NL neuropil from embryonic day (E) 9 to E11, when NM axons grow into the NL neuropil. This asymmetric distribution suggests that EphA4 acts as a guidance molecule during binaural segregation. We report here on the developmental changes in the expression of two other Eph receptors, EphB2 and EphB5, and two ligands, ephrin-B1 and ephrin-B2, in the chick auditory brainstem. These proteins are expressed in the auditory nuclei during the maturation of the NM–NL projection. EphB2, EphB5, and ephrin-B1 are expressed in dorsal and ventral NL neuropil and at the midline of the brainstem at E10–E12. At this age, ephrin-B2, a ligand for EphB receptors and for EphA4, is expressed in NL cell bodies and NM–NL axons. The expression of these proteins diminishs in the posthatch ages examined. These results suggest that several members of the Eph family are involved in maturation of the nuclei and their projections. Moreover, ephrin-B2 in growing axons may interact with the asymmetrically expressed EphA4 during the establishment of binaural segregation. J. Comp. Neurol. 452:51–64, 2002.

Indexing terms: Eph receptor; ephrin; nucleus magnocellularis; nucleus laminaris; auditory brainstem

In the avian auditory brainstem, tonotopically organized auditory input from the cochlea is transmitted to n. magnocellularis (NM) and n. angularis (NA) via bifurcating axons from the VIIIth nerve (Boord, 1969; Rubel and Parks, 1975; Parks and Rubel, 1978; Jhaveri and Morest, 1982a,b). NM axons branch and project bilaterally and tonotopically to another auditory brainstem nucleus, n. laminaris (NL; Parks and Rubel, 1975; Young and Rubel, 1983). In chicks, NL is a monolayer of cells with symmetric bitufted dendrites oriented dorsally and ventrally (Smith and Rubel, 1979; Deitch and Rubel, 1984). The ipsilateral NM projection contacts only the dorsal part of NL, and the contralateral NM projection contacts only the ventral region. Each NL neuron thus receives segregated input from the two ears. The brainstem uses this organi-

Grant sponsor: NIDCD; Grant numbers: DC00395 and DC04661.

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Received 26 September 2001; Revised 26 March 2002; Accepted 9 July 2002

DOI 10.1002/cne.10399

Published online the week of August 19, 2002 in Wiley InterScience (www.interscience.wiley.com).

zation in the analysis of interaural time differences to compute the location of sounds in space (Parks and Rubel, 1975; Carr and Konishi, 1990; Overholt et al., 1992; Joseph and Hyson, 1993). The projection from NM to NL begins to grow at approximately embryonic day (E) 5 to E6 (Young and Rubel, 1986; Book and Morest, 1990), and synaptic connections form by E11 (Jackson et al., 1982). Binaural segregation is observed from the time of the earliest synaptic connections and is not the result of later developmental refinement (Young and Rubel, 1986). We are interested in identifying the mechanisms that direct the precise formation of synaptic connections at defined elements of single postsynaptic neurons during embryonic development.

Several families of molecules have been identified that influence axon outgrowth and guidance. One is the Eph family of receptor tyrosine kinases and their ligands, the ephrins. These proteins regulate cell migration (Xu et al., 1995; Krull et al., 1997; Wang and Anderson, 1997; Krull, 1998; Mellitzer et al., 1999) and axon outgrowth and guidance (Monschau et al., 1997; Feldheim et al., 1998; Frisen et al., 1998). Eph receptors and ephrins most often mediate chemorepulsive, or inhibitory, interactions (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999; O'Leary and Wilkinson, 1999; Klein, 2001). There are two classes of Eph receptors, the A class and the B class (Eph Nomenclature Committee, 1997). In addition, there are two classes of ligands. The ephrinA ligands bind to EphA receptors and have a glycosyl phosphatidylinositol linkage to the membrane. The ephrinB ligands bind to the EphB receptors and to EphA4 and have a transmembrane domain (Gale et al., 1996). Thus both types of ligands mediate cell–cell interactions with cells expressing appropriate Eph receptors. Interestingly, interactions between B ligands and their receptors display bidirectional signaling (Holland et al., 1996; Bruckner et al., 1997; Mellitzer et al., 1999, 2000; Lu et al., 2001), so that the ligands are phosphorylated on tyrosine residues in a manner dependent on binding with the corresponding receptor.

We previously reported that one member of the family, EphA4, is selectively expressed in the dorsal NL neuropil at E10–E11 (Cramer et al., 2000), when the NM axons make their initial connections with NL cells (Jackson et al., 1982; Young and Rubel, 1986). The labeling, which is most likely within dendrites of NL cells, becomes symmetric at E12 and then disappears by E18 (Cramer et al., 2000). The selective expression of EphA4 in dendrites that receive only ipsilateral NM inputs suggests that EphA4 may play an essential role in establishing the spatial segregation of the binaural connections to NL neurons. To evaluate this possibility, it is necessary to determine which ligands for EphA4 are expressed within the developing brainstem and to have a detailed understanding of their spatial organization. An interesting possibility is that growing axons from NM cells express ephrin ligands and respond to the asymmetry of EphA4 expression in the NL dendrites. Reverse signaling would then be required for Eph–ephrin interactions to direct axonal targeting. This mechanism would predict that NM axons express a B ephrin such as ephrin-B2, which has a strong affinity for EphA4 (Gale et al., 1996) and which undergoes bidirectional signaling (Holland et al., 1996; Bruckner et al., 1997; Mellitzer et al., 1999).

We have begun to examine the validity of these ideas by describing the expression of ephrins and other Eph receptors as a function of development in this system. We found that ephrin-B2 is indeed expressed on NM axons, consistent with the possibility that this ligand interacts with the asymmetrically distributed EphA4 to mediate binaural segregation. In addition, we found that ephrin-B1, EphB2, and EphB5 have dynamic expression patterns within the auditory brainstem nuclei over the early period of functional development. Because Eph–ephrin interactions play an important role in axon guidance at the midline (Bergemann et al., 1998; Cook et al., 1998; Cowan et al., 2000; Imondi et al., 2000; Kullander et al., 2001a; Yokoyama et al., 2001), we examined the expression of Eph family members in the midline of the brainstem during the time that these axons are growing. Ephrin-B1, EphB2, and EphB5 are expressed in developmental patterns consistent with a role in axonal guidance.

MATERIALS AND METHODS

Animals and tissue preparation

Chicken eggs were maintained in a humid, forced-air incubator at 37°C. Embryos were removed and staged; the ages reported correspond with the developmental age according to the stages of Hamburger and Hamilton (1951). We used tissue from E2, E7–E8, E10, E12, E14, and E18 and from posthatch day (P) 3 and P4. For all antibodies examined in this study, the results are representative of two to five animals per age group. For E2 tissue, whole embryos were processed. At older embryonic ages, brainstems were dissected from embryos before fixation. At posthatch ages, chicks were anesthetized with pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with 4% paraformaldehyde. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee (protocol 2048-02).

Antibodies

Ephrin-B1 polyclonal antibodies (Kalo et al., 2001) were generated in rabbit against the entire extracellular domain of the chick protein. The antigen was an Fe fusion protein fusion protein, and antibodies were affinity purified. These antibodies specifically bind ephrin-B1 and do not cross react with other B ephrins (Kalo et al., 2001). The preparation of the EphB2 antibody was described previously (Pasquale, 1991). Briefly, a β-galactosidase fusion protein containing amino acids 167–995 of EphB2 was used as the antigen. This region includes most of the extracellular domain and the entire transmembrane and catalytic domains. The rabbit polyclonal antisera were affinity purified and absorbed on a β-galactosidase column and did not cross react with other Eph receptors. We used affinity-purified rabbit polyclonal EphB5 antibodies directed against the 101 C-terminal amino acids of chick EphB5; these antibodies were specific for EphB5 (Soans et al., 1996). The EphA4 antibody was an affinity-purified rabbit polyclonal anti-peptide antibody directed against the carboxy terminus of the EphA4 receptor with demonstrated specificity for EphA4 (Soans et al., 1994).

The ephrin-B2 rabbit polyclonal antibody used in this study was made against amino acids GSSTDGNSAGHSGNNI of the spacer peptide region of the protein, which is unique to ephrin-B2. Before injection of rabbit for immunization, the antibody was conjugated to ovalbumin. Because the antibody was being used on chick tissue sec-

tions, ovalbumin was coupled to a cyanogen bromide Sepharose column (Sigma, St. Louis, MO) to absorb out anti-ovalbumin antibodies.

Western blot

Western blot analysis was performed to evaluate the specificity of the ephrin-B2 antibody and to verify that the ovalbumin immunoreactivity was removed. The cerebellum was isolated from E17 chick embryos. Tissue was immediately placed on ice into homogenization buffer (250 mM sucrose, 100 mM NaPO4, pH 7.0, 150 mM NaCl, 1 mM ethylene-diamine-tetraacetic acid, 4 mM ethylene glycol-bis(2 aminoethylether)-N,N,N′,N′-tetraacetic acid, 4 mM dithiothreitol, 0.5% Triton X-100, 2 μg/ml leupeptin, 3 μg/ml aprotinin, 0.2 mg/ml soybean trypsin inhibitor, and 1 mM 4-(2 aminoethyl)benzenesulfonyl fluoride hydrochloride), sonicated, and centrifuged at 16,000*g*, and the supernatant was collected and assayed for protein concentration with a Bradford assay (Bio-Rad, Richmond, CA). Protein was run on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Blots were then blocked overnight and probed with the antibody for 2 hours at room temperature. Blots were then washed and incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies and visualized using the Amersham ECLTM system (Arlington Heights, IL).

Immunohistochemistry on cryostat sections

Tissue was fixed by immersion or perfusion in 4% paraformaldehyde for 2–4 hours, rinsed in phosphate buffered saline (PBS; pH 7.4), and then placed in 30% sucrose in PBS overnight at 4°C. Brainstems were immersed in OCT and oriented within plastic molds before cutting on a cryostat at 12 μm. Sections were collected onto a series of eight slides, so that eight sets of serial sections could be used for immunohistochemistry. Each set contained a 1-in-8 series of sections.

Slides were stored at −20°C. For labeling, slides were allowed to equilibrate at room temperature. A PAP pen (The Binding Site Inc., San Diego, CA) was used to make small wells around sections. Slides were then rinsed in PBS to remove OCT, immersed in 100% methanol containing 0.3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity, rinsed, and incubated in blocking solution (5% nonfat dry milk, 0.1% Triton X-100 in PBS) for 1 hour. Primary antibodies were used at 1–5 μg/ml in blocking solution within PAP pen wells, and slides were stored in a sealed, humid container at room temperature overnight. Control sections were incubated overnight in blocking solution without primary antibody. Slides were rinsed, incubated in Vector biotinylated goat anti-rabbit secondary antibody diluted to 1:250 in blocking solution, rinsed, and incubated in Vector ABC solution (Vector Laboratories, Burlingame, CA). HRP was visualized with 3,3′ diaminobenzidine tetrahydrochloride. One series of sections from each brainstem was stained for Nissl to aid in the identification of brain nuclei.

Immunohistochemistry on paraffin sections

The methods used were described previously (Cramer et al., 2000). Briefly, embryonic brainstems from chicks were immersion fixed for 5–20 minutes in a solution containing 49% ethanol, 20% formalin, and 10% glacial acetic acid in dH_2O. Tissue was then dehydrated through increasing concentrations of ethanol followed by two incubations in Hemo-De clearing agent (Fisher Scientific, Pittsburgh, PA). Tissue was then immersed in three changes of paraffin and embedded in paraffin molds. Most brainstems were sectioned in the coronal plane at 12 μm. E2 embryos were sectioned at 12 μm in the horizontal plane. Sections were mounted and dried onto HCl/ethanol chrome alum–coated slides in five series of alternate sections so that adjacent sections could be stained with a variety of antibodies. Paraffin was removed with xylene, and sections were hydrated by immersion in decreasing concentrations of ethanol and then rinsed in PBS (pH 7.4). We used a PAP pen to make small wells around sections on slides for immunohistochemistry. For antigen retrieval, we used 1% sodium dodecyl sulfate in PBS for 5 minutes, and then we rinsed slides thoroughly in PBS. To quench endogenous peroxidase activity, the slides were placed in 0.3% H_2O_2 in 100% methanol for 10 minutes. Tissue was rinsed in PBS and incubated in a blocking solution containing 5% nonfat dry milk and 0.1% Triton X-100 in PBS for at least 1 hour. Tissue was then incubated overnight at room temperature in a humid chamber with antibody diluted in blocking solution at a concentration of 1 μg/ml. Control sections were incubated in blocking solution without the primary antibody. Sections were rinsed, incubated in a biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories) diluted to 1:200 in the blocking solution in a humid chamber at room temperature for 1–2 hours and then rinsed. Tissue was then incubated in ABC solution (Vector Laboratories) containing an avidin–HRP complex for 1 hour. HRP was visualized with a VIP substrate kit (Vector Laboratories). Slides were rinsed and immersed in increasing concentrations of ethanol and then xylene; slides were coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, UK).

Production of photomicrographs

Labeled sections were photographed on a Nikon Optiphot-2 microscope or on a Leitz Aristoplan microscope using a Spot IIe digital camera and acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI). Flat-field correction was used to compensate for any unevenness in the illumination of the field. Images were imported into Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA). The images were cropped and adjusted for brightness and contrast, and labels and scale bars were added.

RESULTS

Expression of ephrins and Eph receptors was examined at E7–E8, E10, E11, E12, E14, E18, and P3–P4. Immunolabeling was observed in auditory brainstem regions with ephrin-B1, ephrin-B2, EphB2, and EphB5 antibodies. Control sections in which primary antibody was omitted were unlabeled.

Expression of ephrin-B1

Photomicrographs of ephrin-B1 labeling in the auditory nuclei are shown in Figure 1. At E7–E8, the cells that make up the auditory nuclei were present within the auditory anlage, toward the dorsolateral region of the brainstem (Fig. 1A). Immunolabeling was evident in axons around the periphery of the anlage and in neuropil within the anlage (Fig. 1B). Cell bodies were not labeled. At E10–E14, ephrin-B1 immunolabeling was observed in dorsal and ventral NL neuropil (Fig. 1C,D). This labeling

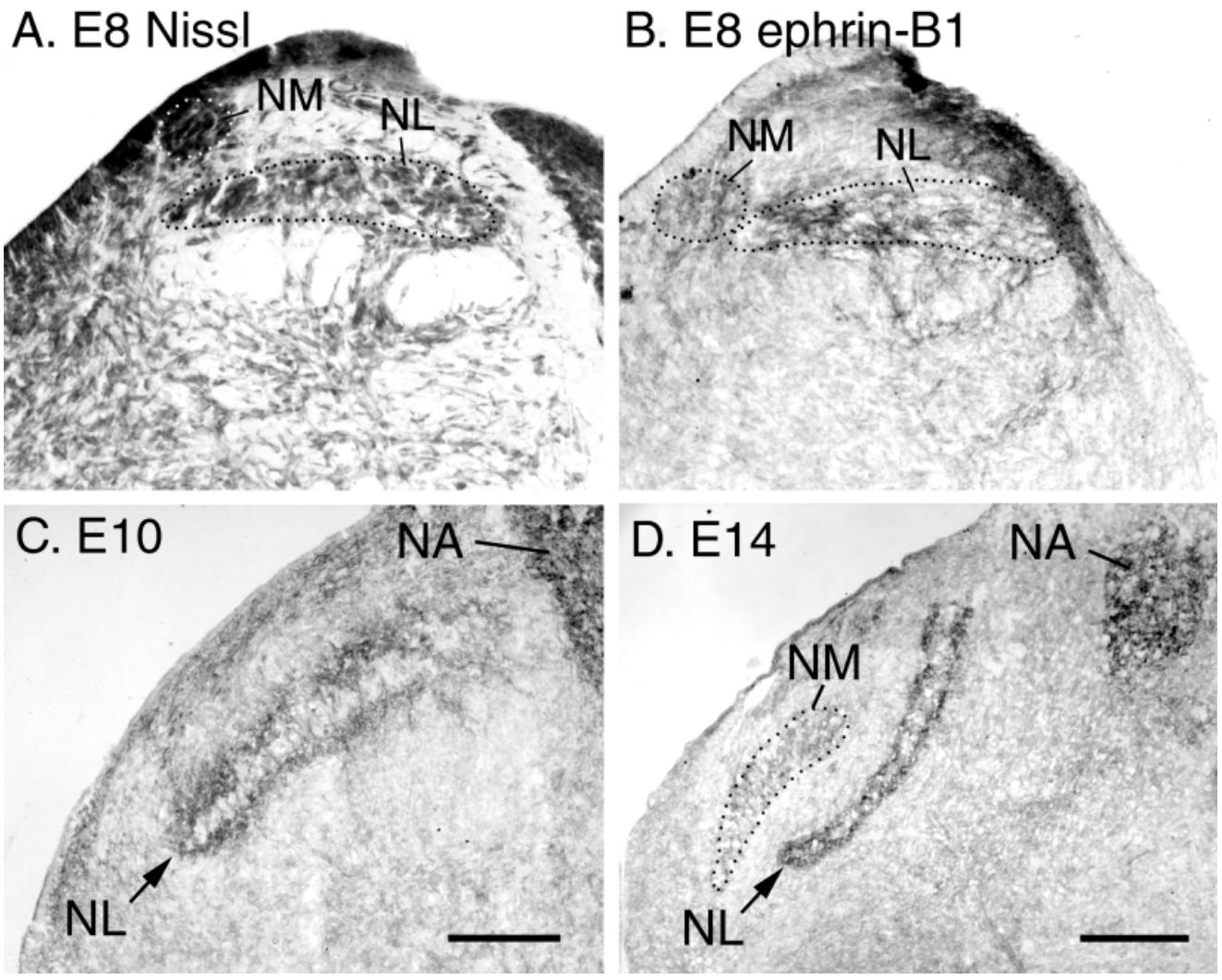

Fig. 1. Ephrin-B1 immunolabeling in the auditory brainstem. **A:** Coronal cryostat section from an embryonic day (E) 8 chick embryo with Nissl staining shows the location of the auditory anlage, containing the early nucleus magnocellularis (NM) and nucleus laminaris (NL; dotted lines). In these and all coronal sections showing one side of the brainstem, dorsal is up and medial is to the left. **B:** Section adjacent to that shown in A was labeled with an antibody to ephrin-B1. Dotted lines surround labeled axons in the region that will become NM and NL. **C:** Ephrin-B1 immunolabeling at E10 is evident in this section within the NL neuropil. The region within the NL, indicated by the arrow, is the line of cell bodies, which are unlabeled. Nucleus angularis (NA) is also labeled. NM is not present in this section. **D:** At E14, ephrin-B1 immunolabeling remains in the NL neuropil and is also seen in the NM (dotted lines) and NA. Scale bars = 100 μm for A–C, 200 μm in D.

was symmetric, with both regions labeled robustly. Cell bodies in NL were not labeled (arrow in Fig. 1C). Labeling in NM and NA was also evident (Fig. 1C,D); this label corresponded with auditory axons and neuronal cell bodies. In addition, VIIIth nerve axons entering the brainstem were labeled in sections that included this tissue (data not shown). By E18, ephrin-B1 labeling had greatly diminished. At this age, brainstem auditory nuclei were not labeled (data not shown). A small region of the VIIIth nerve, however, continued to show labeling at this age. At P3 and P4 (not shown), no labeling was observed in the auditory nuclei or in VIIIth nerve.

In addition to labeling within auditory brainstem nuclei, ephrin-B1 immunolabeling was observed within the midline of the brainstem through the region of the auditory nuclei (Fig. 2). At E8 a thin band of label was observed at the midline, extending across the dorsoventral axis (Fig. 2A). At E14 this band remained but had grown narrower (Fig. 2B); by E18 the midline labeling was restricted to a small region near the ventricular zone (Fig. 2C).

Characterization of ephrin-B2 antibody

The specificity of the ephrin-B2 antibody was examined using western blot analysis, summarized in Figure 3. The initial western blot before purification contained only two bands (Fig. 3A). One band (50 kDa) corresponded with ovalbumin immunoreactivity and the other with ephrin-B2 (32 kDa). Although ephrin-B2 is about the same size as ephrin-B3, it is unlikely that the band represents

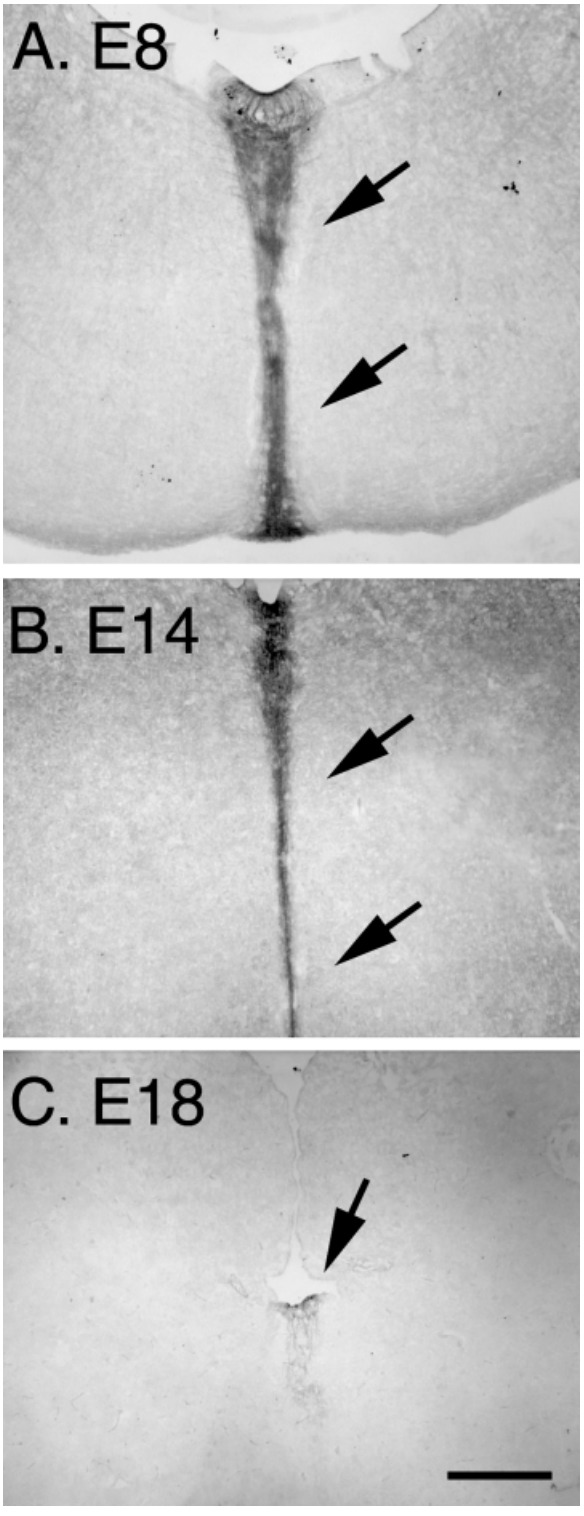

Fig. 2. Ephrin-B1 immunolabeling is seen in the midline of the developing brainstem in the region containing the auditory nuclei. **A:** Embryonic day (E) 8 brainstem section shows ephrin-B1 immunolabeling throughout the dorsoventral extent of the midline (arrows), including the ventricular zone. **B:** Ephrin-B1 immunolabeling remains within the midline at E14 (arrows), although the labeled region is narrower than at E8. **C:** At E18, the midline label is restricted to a very small region within the ventricular zone (arrow). Scale bar = 200 μm for A–C.

cross reactivity because the antibody was generated against a region of the ephrin-B2 protein that has no homology with ephrin-B3. After the antisera were run over an ovalbumin column to absorb out the anti-ovalbumin antibodies, the purified eluate reacted with only one band on a western blot (Fig. 3B), which corresponded with ephrin-B2, indicating that the ovalbumin immunoreactivity had been removed. Sections labeled with the antibody before purification had a greatly increased level of background staining compared with those labeled with the purified antibody (data not shown).

Another experiment to demonstrate the specificity of the ephrin-B2 antibody consisted of labeling hindbrain tissue at E2, when the pattern of labeling of ephrin-B2 is known to be within even rhombomeres, as shown by in situ hybridization (Bergemann et al., 1995; Kury et al., 2000). Paraffin sections in a plane horizontal to the hindbrain region were cut, and alternate sections were labeled with the EphA4 antibody, which labels rhombomeres 3 and 5 robustly (Becker et al., 1994; Irving et al., 1996; Cramer et al., 2000; Kury et al., 2000). EphA4 labeling allowed us to identify the rhombomeres unambiguously. In alternate sections immunolabeled for ephrin-B2, patches of label were evident in rhombomeres 4 and 6, but not in rhombomeres 3 and 5 (data not shown), consistent with what is expected based on previous studies of ephrin-B2 using in situ hybridization. Together, these results suggest that the ephrin-B2 antibody specifically recognizes ephrin-B2.

Expression of ephrin-B2

Representative sections labeled with ephrin-B2 immunohistochemistry are shown in Figure 4. At E7–E8, immature NM and NL were identified in sections stained for Nissl (Fig. 4A). Ephrin-B2 immunohistochemistry showed labeled cells within this region (Fig. 4B). Although this labeling was modestly above background, it suggests that ephrin-B2 is expressed in cell bodies of the auditory nuclei and is distinct from the fiber labeling observed with ephrin-B1 immunohistochemistry (cf. Fig. 1B). At E10, ephrin-B2 expression was observed within cell bodies of NM (Fig. 4C,D) and NL (Fig. 4C,E) neurons. There was also label in the axons that arise in NM and project to NL (Fig. 4C, arrowheads). Label was observed in the axons that innervate NM and in NM neuropil (Fig. 4D). In addition, labeling was seen in small axons dorsal and ventral to the cell body line in NL (Fig. 4E). NA neuronal cell bodies also were labeled (not shown). At E12 and E14, this pattern of labeling remained similar, although less intense, with visible axon labeling in the regions dorsal and ventral to NL containing the NM axonal projections (Fig. 4F, arrowheads). NM and NL cell bodies were labeled (Fig. 4F), but the neuropil region around the row of NL cell bodies was not heavily labeled.

At E18 (not shown), the pattern of ephrin-B2 labeling had changed so that axon labeling was very pale. NM, NL, and NA cell bodies remained labeled. By P3, the NM–NL fiber tract was not labeled with ephrin-B2 immunohistochemistry, whereas cell bodies remained robustly labeled within NM, NL, and NA (Fig. 4G). Immunolabeling in the neuropil around NL was pale.

The midline of the brainstem was not labeled with ephrin-B2 immunohistochemistry, but the auditory axons that traverse the dorsal midline were labeled at embryonic ages. This labeling is shown in Figure 5. At E8 (Fig.

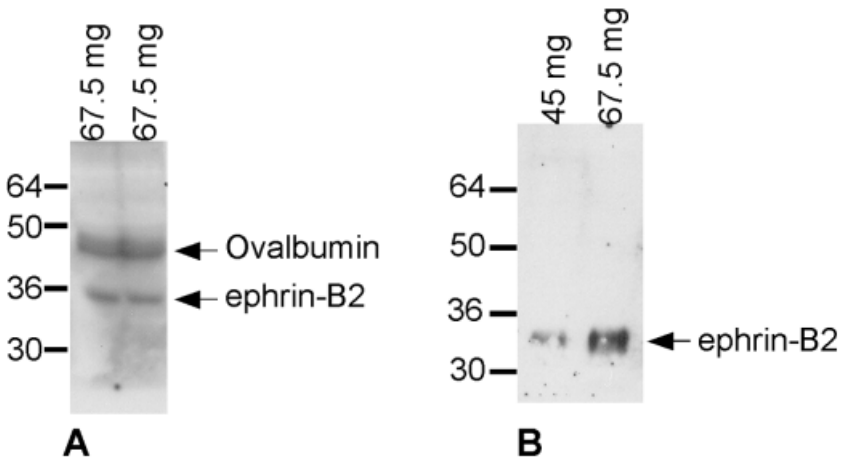

Fig. 3. Western blot analysis of the ephrin-B2 antibody. **A:** The antibody was tested in a western blot and it labeled two bands in chick cerebellar homogenates. The 50-kDa band corresponds with ovalbumin, and the 32-kDa band corresponds with ephrin-B2. Left and right lanes were each loaded with 67.5 mg of protein. **B:** After the antisera were absorbed on an ovalbumin column, the western blot revealed only one band, the 32-kDa band corresponding to ephrin-B2. The left and right lanes were loaded with 45 mg and 67.5 mg of protein, respectively. This purification thus removed the immunoreactivity for ovalbumin.

5A), faintly labeled axons were observed at the dorsal midline (arrowheads). These axons originate in NM cells of the auditory anlage on one side and project contralaterally. At E10 (Fig. 5B), labeled axons were observed at the dorsal midline (arrowheads); these axons arise from NM and project to contralateral NL. Labeling in these axons was more intense than at E8. The midline structures themselves were not labeled. A similar pattern of labeling was seen in E14 tissue (Fig. 5C). At E18 and P3, axon labeling was no longer observed at the dorsal midline (data not shown).

Expression of EphB2

At some of the ages examined, the pattern of EphB2 labeling was very similar to that of ephrin-B1 labeling. Figure 6 illustrates patterns of EphB2 labeling during development. At E7–E8, labeling was robust in the auditory anlage and appeared to be concentrated within processes (Fig. 6A,B). At E11, EphB2 was expressed robustly and symmetrically in the neuropil surrounding NL, but not in NL cell bodies (Fig. 6C). Labeling was observed in NA (not shown) and NM (Fig. 6C), where labeling included NM processes and axons but excluded cell bodies (Fig. 6D). In addition, immunolabeling was present in axons originating in NM and projecting to NL (Fig. 6C, arrowheads) and in axons dorsolateral to NM that appeared to be auditory nerve axons (Fig. 6C). This pattern of labeling remained similar but somewhat less intense at E12 and E14. Figure 6E shows labeling in the neuropil around NL at E14. At E18, the pattern of neuropil labeling was greatly diminished. No NM, NL, or NA cell bodies were labeled at this age, but a very faint pattern could be discerned in the neuropil around NL (Fig. 6F). By P3, no labeling was observed in the auditory nuclei or in the region of axonal projection between them (not shown).

Midline labeling was observed with EphB2 immunohistochemistry (Fig. 7). At E8–E10, the midline was labeled throughout the dorsoventral extent of the floor plate, with somewhat weaker labeling at the ventricular zone (Fig. 7A). Axons at the dorsal midline were lightly labeled. Labeling in the midline was less intense at E14 (Fig. 7B). At E18, the midline labeling was restricted to approximately the ventral half of the brainstem (Fig. 7C).

Expression of EphB5

EphB5 immunolabeling in the auditory brainstem nuclei was consistently less pronounced with respect to background than the labeling seen with the other antibodies used in this study (Fig. 8). At E8, lightly labeled regions were observed in and around the auditory anlage (data not shown). Some regions were labeled in the portion of the auditory nerve close to the brainstem. At E10, there was very pale labeling around the NL neuropil (Fig. 8A). This minimal labeling was present in dorsal and ventral regions of the neuropil, with no appreciable difference in the intensity of label on the two sides. Labeling was pale within NL cell bodies, and NM and NA were not labeled (Fig. 8A). Labeling within VIIIth nerve axons was still apparent. At E12, the neuropil labeling was absent (Fig. 8B). At E18, NL and NA were unlabeled, and NM cells had very pale labeling. At P3, cell bodies were labeled in NM, NL, and NA (Fig. 8C), with immunoreactivity higher in the cell nuclei (Fig. 8C, inset).

At the midline, a stripe of distinct label was visible throughout the dorsoventral extent at E8. The labeling in the midline at E10 (Fig. 9A) suggests that the pale labeling in the auditory nuclei reflects low expression levels rather than artifactually low labeling due to histologic methods. Labeling throughout the entire dorsoventral midline persisted at E14 (Fig. 9B), although it was narrower than in younger embryos. At E18, there was a small amount of labeled tissue in the most dorsal region of the midline at the ventricular zone. This label persisted in sections from P3 tissue (Fig. 9C).

DISCUSSION

The results of this study demonstrate that several members of the Eph family are expressed in the developing auditory nuclei within the chick brainstem. Ephrin-B1,

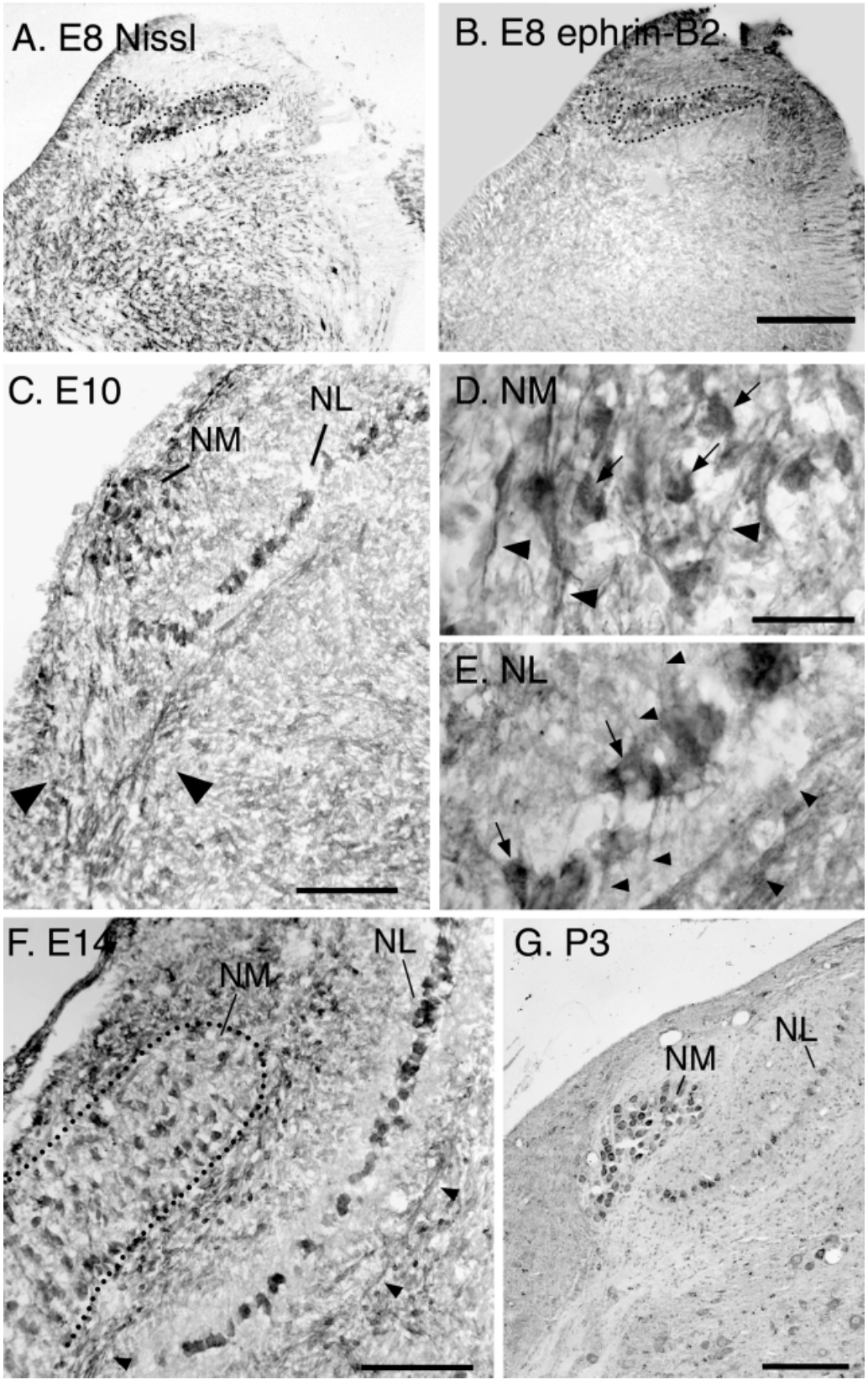

Fig. 4. Immunolabeling with ephrin-B2 antibodies in the developing auditory brainstem nuclei in coronal sections. **A:** Nissl-stained embryonic day (E) 8 cryostat section shows prospective nucleus magnocellularis (NM) and nucleus laminaris (NL; dotted lines). **B:** Adjacent sectioned was labeled immunohistochemically for ephrin-B2 expression. Diffuse label appears within the NM and NL anlage and is evident within the neuropil and cell bodies. **C:** Ephrin-B2 expression at E10. Label is seen in NM and within the cell bodies in NL. In addition, label is seen within the axons projecting from NM to NL (arrowheads). **D:** Higher power photograph of NM from the section shown in C. NM cell bodies are labeled (arrows), and the neuropil within NM is also labeled (arrowheads). **E:** Higher power photograph of NL from the section shown in C. NL cell bodies are labeled (arrows), as are axons projecting to NL (arrowheads). **F:** Ephrin-B2 expression in the E14 auditory brainstem. NM (dotted lines) cell bodies and NL cells bodies are labeled. Some label is evident in the axons projecting from NM to NL (arrowheads). **G:** Ephrin-B2 labeling in a posthatch day 3 (P3) paraffin section. NM and NL cell bodies are labeled, but labeling within axons has diminished. Scale bars = 200 μm in B (applies to A), and G, 100 μm in C and F, 25 μm in D and E.

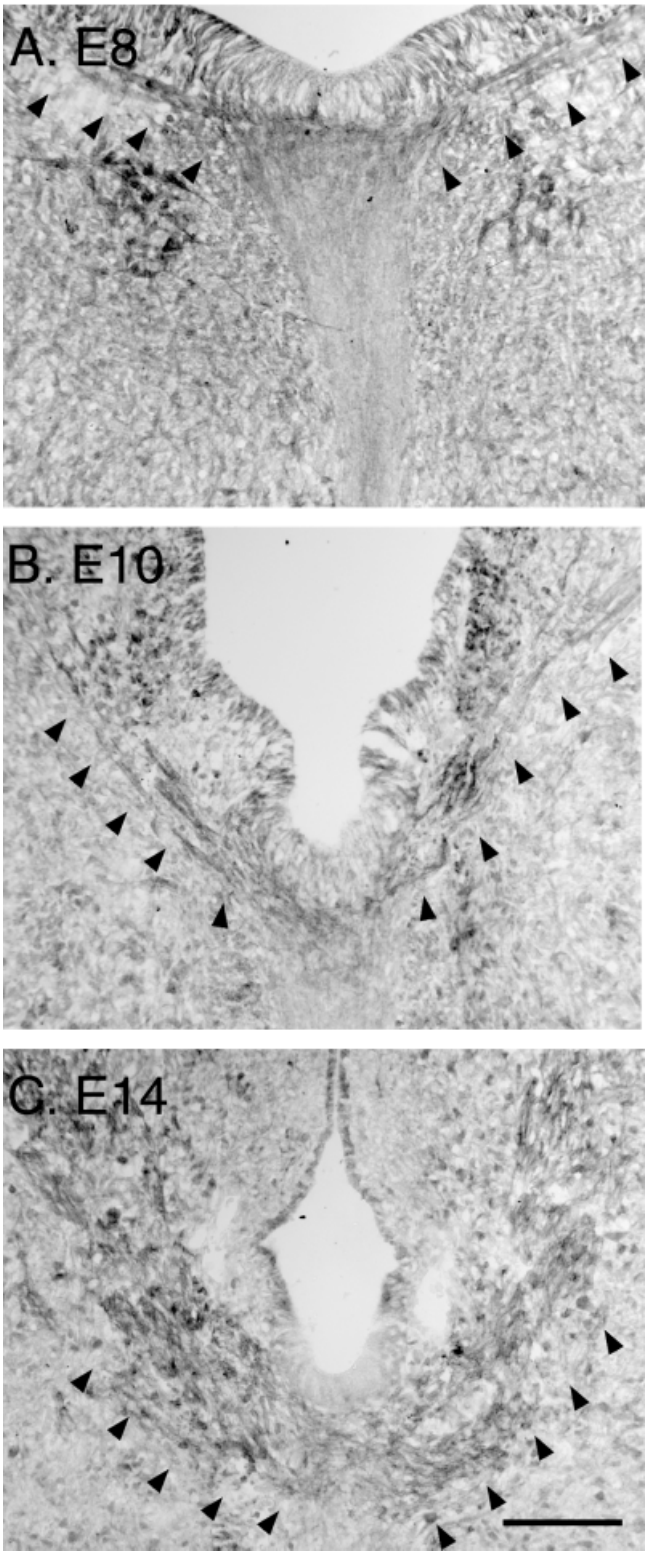

Fig. 5. Ephrin-B2 expression in the crossing axons of the dorsal midline. **A:** At embryonic day (E) 8, axons traversing the dorsal part of the midline in the auditory brainstem are visible (arrowheads). The midline region per se is not labeled. **B:** A similar pattern of labeling is seen at E10. Axons projecting from the nucleus magnocellularis traverse the dorsal midline (arrowheads). **C:** At E14, axon labeling is still present (arrowheads) and slightly less intense than at earlier ages. Scale bar = 100 μm for A–C.

EphB2, EphB5, and EphA4 are expressed in NL neuropil during embryonic development, whereas ephrin-B2 is expressed in all the auditory nuclei and in the axon tracts that innervate them. These proteins are expressed during the period of axon growth, synapse formation, and synaptic maturation. They then appear to be rapidly downregulated at approximately E18 and early posthatch ages, when the connections in the auditory brainstem are mature. Although the present study addresses only the expression of these proteins, the observed patterns will be instructive in designing experiments to test the function of these proteins in the development of the auditory brainstem.

A summary of Eph family staining patterns in the auditory nuclei during the formation of synaptic connections (E10–E12) is shown in Figure 10. In this figure, indicated compartments within the brainstem include NM cell bodies and surrounding neuropil, NL cell bodies and surrounding neuropil, the midline, and axons projecting from NM to NL. Darker areas represent areas with more intense immunolabeling. From this summary, it is evident that, although several family members have similar patterns of label, the distribution of receptor tyrosine kinases is different for each family member during development. Thus far, only EphA4 and TrkB have been shown to be expressed asymmetrically in NL. TrkB and TrkC are expressed in the neuropil around NL at early embryonic ages. At about E10–E11, TrkB expression becomes restricted to the ventral NL dendrites, which receive contralateral NM input. Thus, for Eph receptors and Trk receptors, one family member of those examined thus far is restricted in its expression to a zone in NL that receives input from only one side of the brain, whereas other family members are expressed symmetrically within dorsal and ventral dendrites of NL.

Coexpression of Eph receptors and ephrins

Coexpression of several Eph receptors and ligands (Hornberger et al., 1999) is observed in many developing brain areas. During the formation of the retinotectal projection in the chick, gradients of ephrin-A2 and ephrin-A5 are present in the tectum, and a gradient of EphA3 in retinal axons (Cheng et al., 1995; Monschau et al., 1997) is thought to instruct the topographically organized innervation of the tectum by growing retinal ganglion cell axons. EphrinA ligands also are expressed on these axons, and their presence may modulate the function of EphA3 (Hornberger et al., 1999; Menzel et al., 2001). The patterns of expression in the cerebellum are similar to those we observed in the embryonic hindbrain; ephrin-B1 and EphB2 are coexpressed on granule cells in a manner that could interact with regions that express EphA4 (Karam et al., 2000). However, EphB2 and ephrin-B1 are not always coexpressed. For example, EphB2 and ephrin-B1 are coexpressed in cell bodies of rat primary olfactory neurons at E18.5 but not at earlier ages. (St. John and Key, 2001). In addition, in the developing retina, EphB2 and ephrin-B1 are expressed in opposing gradients (Braisted et al., 1997; Holash et al., 1997).

The coexpression of several Eph family members in the developing auditory brainstem also might reflect a chemoadhesive interaction (reviewed in Klein, 2001). In the developing vascular system, ephrins and Eph receptors have adhesive interactions (Stein et al., 1998; Huynh-Do et al., 1999). In the nervous system, there is evidence for adhesive interactions between ephrinA ligands and receptors.

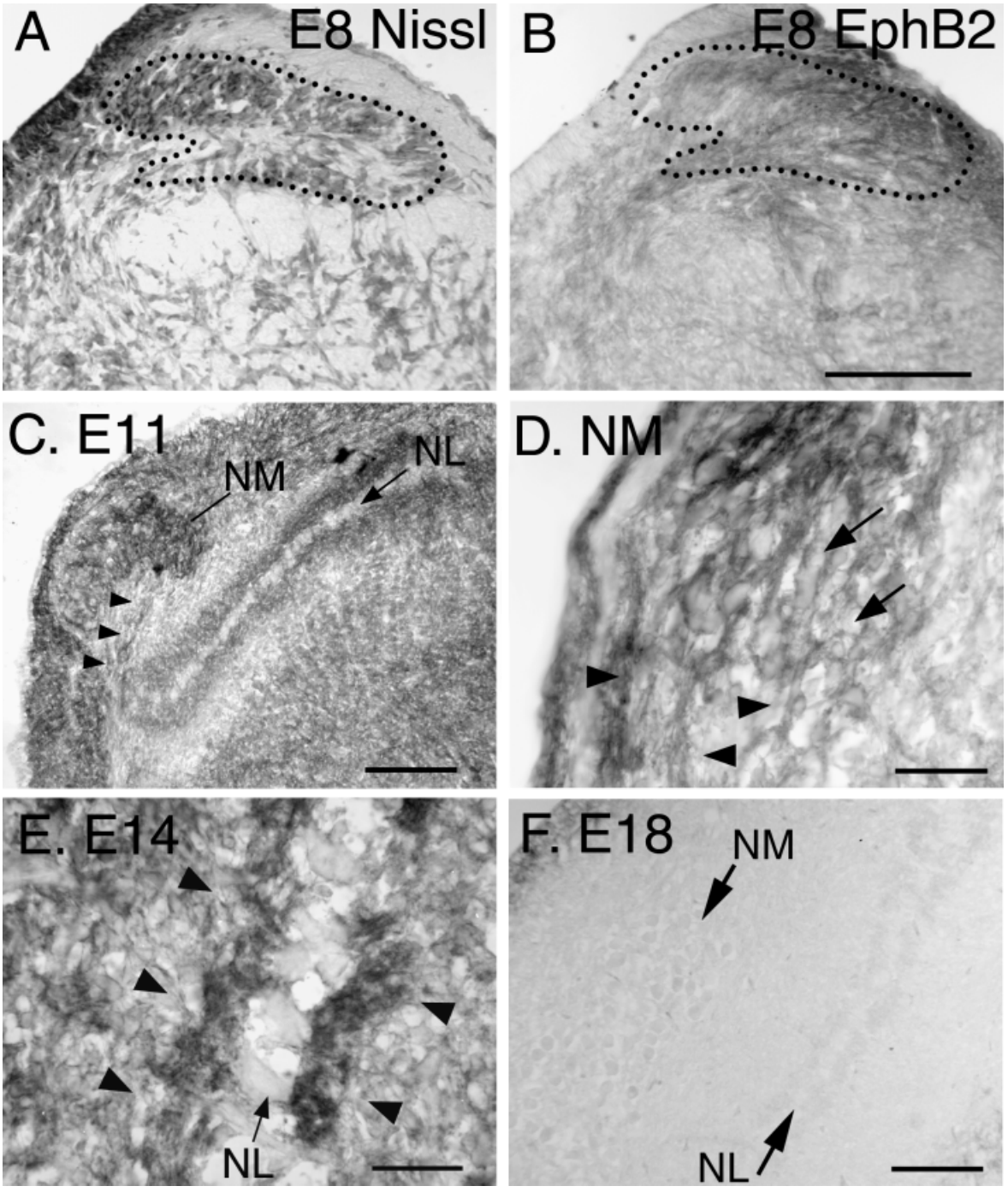

Fig. 6. EphB2 expression in the auditory brainstem nuclei. **A:** Nissl-stained section through an embryonic day (E) 8 brainstem with the auditory anlage highlighted in dotted lines. **B:** Adjacent section labeled with EphB2 immunohistochemistry shows labeled fibers within the auditory anlage, whose borders are shown in dotted lines. **C:** EphB2 immunolabeling at E10. Expression is seen in the nucleus magnocellularis (NM) and nucleus laminaris (NL) neuropil and in axons that emerge from the NM (arrowheads) and axons that appear to be auditory nerve axons. Label is not evident in NL cell bodies (arrow). **D:** Higher power photograph of labeled areas in NM. NM neuronal somata (arrows) are pale. Neuropil within NM and axons that course toward the midline are labeled (arrowheads). **E:** EphB2 expression in NL at E14. The arrow indicates the unlabeled line of NL cell bodies. The neuropil on both sides of the NL cell bodies is labeled (arrowheads). **F:** At E18, EphB2 immunolabeling has greatly diminished in NM and NL. In the neuropil around NL, very faint labeling is observed on both sides. The cell bodies of NL neurons are not labeled. Scale bars = 100 μm for B (applies to A, C, F), 25 μm for D and E.

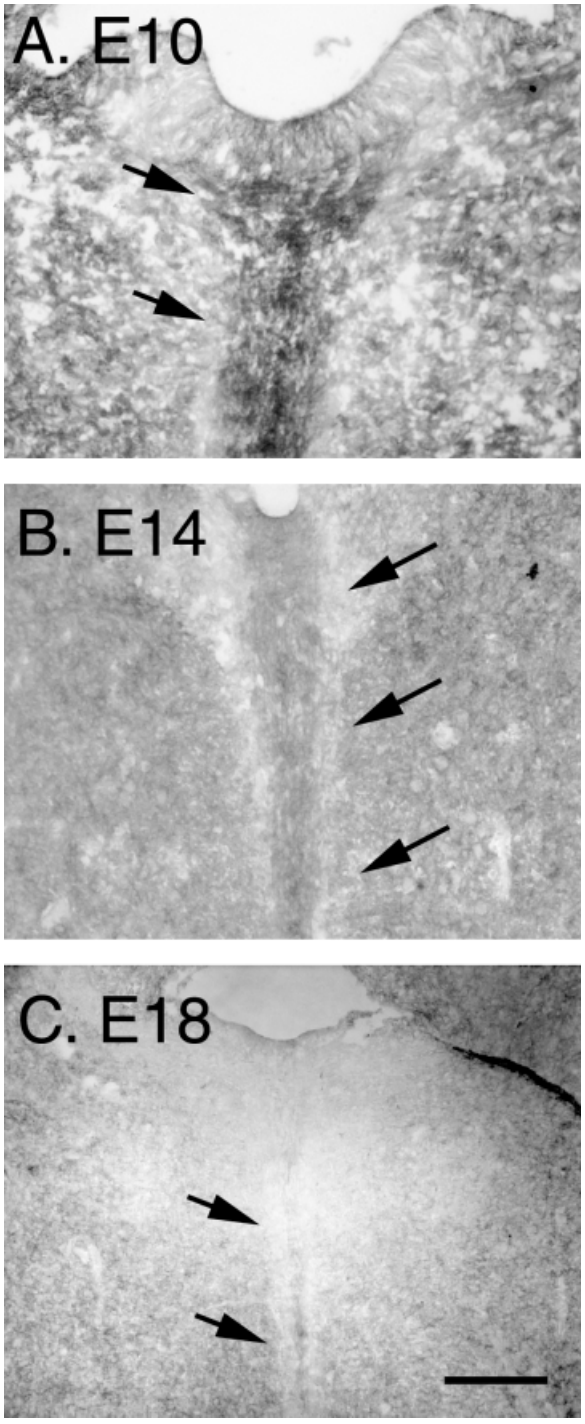

Fig. 7. EphB2 expression in the midline of the auditory brainstem. **A:** Embryonic day (E) 10 coronal section is stained with an antibody that recognizes EphB2. Labeling is evident along the dorsoventral extent of the midline (arrows), with lighter labeling in the ventricular zone. **B:** EphB2 expression in the midline within a section from an E14 brainstem. The labeled region (arrows) has narrowed and is less robust than at E10. **C:** Midline labeling in an E18 brainstem. The labeled region (arrows) has become a narrow strip in the ventral portion of the brainstem. Scale bar = 200 μm in C and 100 μm in A and B.

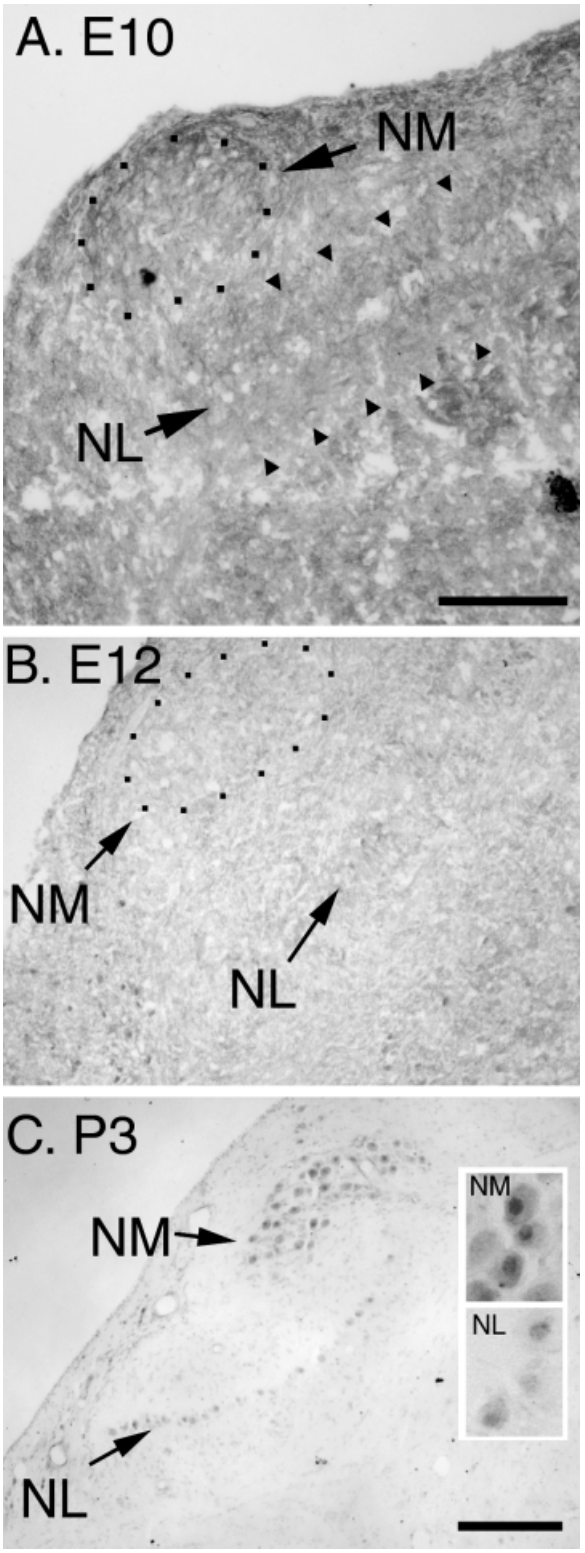

Fig. 8. EphB5 expression in the auditory brainstem nuclei. **A:** Light labeling with EphB5 immunohistochemistry is observed in the neuropil around the nucleus laminaris (NL) at embryonic day (E) 10 (arrowheads). Nucleus magnocellularis (NM) boundaries are indicated by the dotted line. The line of cell bodies in NL is indicated by the arrow. **B:** EphB5 immunolabeling is greatly diminished in the E12 brainstem. **C:** At posthatch day 3 (P3), pale labeling within NM and NL appears strongest in the cell nuclei, as shown in the inset. Scale bars = 100 μm in A, 200 μm in C (applies to B), 50 μm for inset.

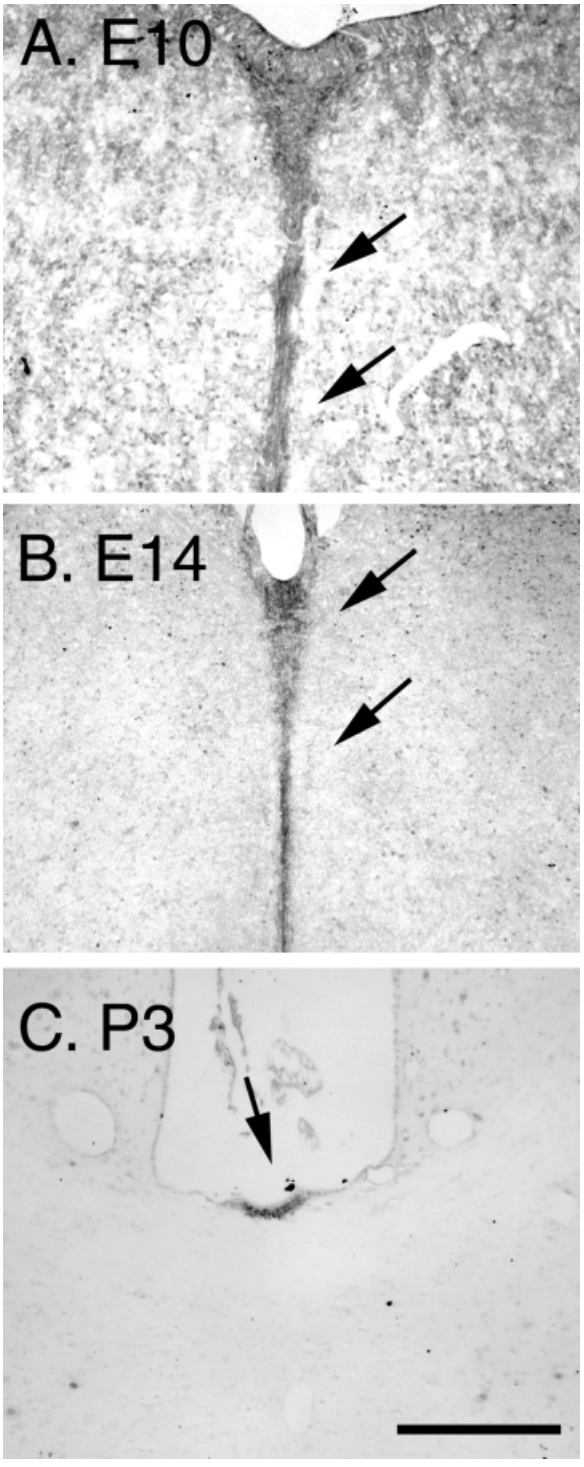

Fig. 9. EphB5 expression in the midline of the auditory brainstem. **A:** At embryonic day (E) 10, a band of label is seen throughout the dorsoventral extent of the auditory brainstem (arrows). **B:** At E14, the band of midline label has thinned but still extends throughout this region of the brainstem (arrows). **C:** At posthatch day 3 (P3), the midline label is confined to the ventricular zone (arrow). Scale bar = 100 μm in A and B, 200 μm in C.

Truncated EphA7 splice variants favor adhesion with ephrin-A5 in cell cultures, and these adhesive interactions may have a significant role in neurulation (Holmberg et al., 2000). In addition, adhesion may prevail over repulsion when metalloproteases are not present or activated (Hattori et al., 2000).

Bidirectional signaling

One goal of this study was to test whether ephrin-B2, a ligand that binds EphA4 with strong affinity, is expressed in axons emerging from NM neurons. The finding that ephrin-B2 is in fact expressed in NM axons during the time that the terminal arbors form and synapses begin to form suggests that ephrin-B2 acts as a ligand for EphA4 within the neuropil of NL. Because Eph receptors appear to be expressed within the dendrites and ephrin-B2 is on the axons, it is attractive to speculate that, in the development of the NM–NL pathway, reverse signaling between EphA4 and ephrin-B2 is involved in regulating axonal arbor growth and specifying binaural segregation. Specifically, this model suggests that EphA4 in the dorsal neuropil would be inhibitory for growing axons from contralateral NM, which terminate on ventral NL dendrites. Ephrin-B2–expressing axons could grow and synapse in the narrow region ventral to NL cell bodies in which EphA4 expression is very low at E10. Because ipsilateral NM cells appear to be in contact with their NL targets during the migratory stage, the appearance of EphA4 may follow the arrival of appropriate axons in the dorsal NL neuropil. EphA4, in addition to preventing axon growth across the cell body lamina, would induce branching of ipsilateral axons via interactions with ephrin-B2.

Although this is an attractive model that is testable by using misexpression of EphA4 in NL, other pairs of ephrin ligands and Eph receptors might mediate forward signaling within the NM–NL pathway. For example, at E10, growing NM axons that express EphB2 might be inhibited by ephrin-B1 in the neuropil around NL and by ephrin-B2 in NL cell bodies. Although these ephrins are expressed with dorsoventral symmetry in NL, they may prevent NM axons from growing across the cell body line to reach the inappropriate side of NL neuropil.

Expression of ephrin-B1, EphB2, and EphB5 at the midline

We have also documented the developmental changes in expression of ephrin-B1, EphB2, and EphB5 precisely at the midline of the embryonic brainstem in the region of the auditory nuclei. Previous studies using in situ hybridization (Kury et al., 2000) demonstrated the presence of ephrin-B1 and EphB2 mRNA in the ventricular region of the midline through about E7 and expression of ephrin-B2 until about E4.5. We extend these periods by showing protein expression through most of the dorsoventral extent until about E15. Also, in agreement with Kury et al., we did not observe ephrin-B2 along the midline. The presence of these proteins at the midline is consistent with a role for Eph–ephrin signaling in the guidance of decussating axons. The earliest age at which NM axons have been observed crossing the midline is at E6 (Young and Rubel, 1986; Book and Morest, 1990), and most crossing NM axons have reached the region of contralateral NL by E9 (Rubel et al., 1976; Young and Rubel, 1986). Taken together, the data on B ephrins and EphB receptors over this period of development are consistent with a role for

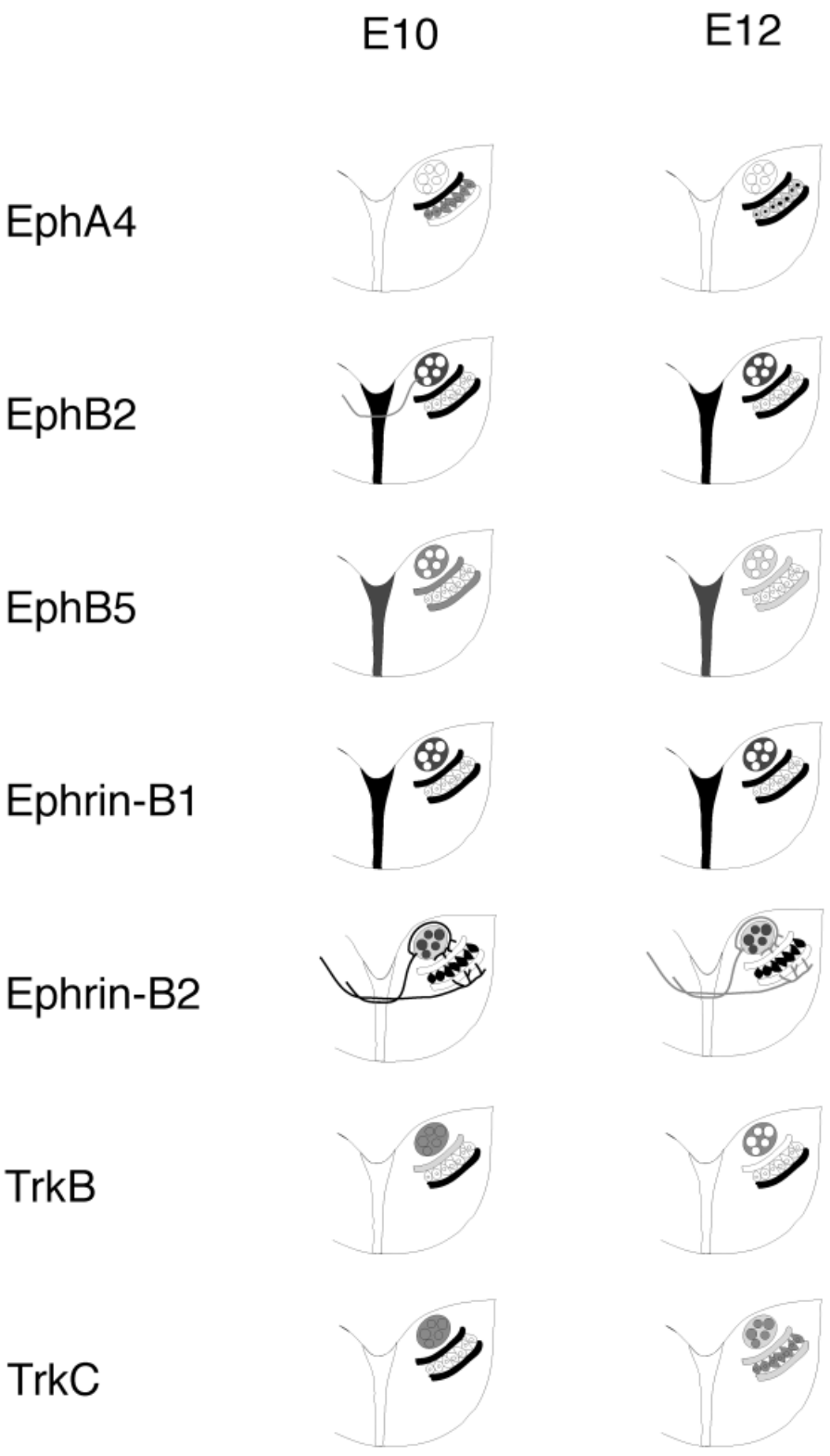

Fig. 10. Summary of immunolabeling patterns for receptor tyrosine kinases in the auditory region of the brainstem during the formation of synaptic connections. The regions displayed include nucleus magnocellularis (NM) cell bodies and the surrounding neuropil, nucleus laminaris (NL) cell bodies and nuclei and the surrounding neuropil, the midline, and the axons projecting from NM to NL. Darker regions denote areas of more intense immunolabeling. Several family members are expressed in each region, but a variety of patterns is observed. Only EphA4 and TrkB are expressed asymmetrically in the neuropil around NL. Data on TrkB are from Cochran et al. (1999) and Cramer et al. (2000). Data on TrkC are from Cochran et al. (1999).

Eph signaling in the guidance of NM axons that innervate contralateral NL, but this role has not been verified experimentally. A similar role has been demonstrated experimentally in the vestibular system (Bergemann et al., 1998; Cowan et al., 2000), the spinal cord (Coonan et al., 2001; Kullander et al., 2001a,b; Leighton et al., 2001; Yokoyama et al., 2001), and the optic chiasm of *Xenopus* tadpoles (Nakagawa et al., 2000).

ACKNOWLEDGMENTS

The authors thank Dale Cunningham for assistance with histochemical procedures, Glen MacDonald for help with production of photomicrographs, Laurie Johnson for assistance with article preparation, and Abigail Person for helpful comments on the article. Edwin W Rubel received grants DC00395 and DC04661 from NIDCD.

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