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# Developmental Regulation of EphA4 Expression in the Chick Auditory Brainstem

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ABSTRACT

The avian auditory brainstem nuclei nucleus magnocellularis (NM) and nucleus laminaris (NL) display highly precise patterns of neuronal connectivity. NM projects tonotopically to the dorsal dendrites of ipsilateral NL neurons and to the ventral dendrites of contralateral NL neurons. The precision of this binaural segregation is evident at the earliest developmental stage at which connections can be observed. We have begun to examine the possibility that Eph receptor tyrosine kinase signaling is involved in establishing these spatially segregated connections. The expression of the EphA4 tyrosine kinase was examined at several developmental stages. EphA4 is expressed in rhombomere 5, which contains progenitors for both NM and NL. In this rhombomere, the labeling becomes striped during the time that precursor cells migrate to the auditory anlage. At the precise time when NM-NL projections are forming, EphA4 expression in NL is asymmetric, with markedly higher expression in the dorsal NL neuropil than in the ventral neuropil, suggesting a possible role in guiding growing axons to the appropriate region. At later embryonic ages EphA4 expression is symmetric around NL, and is absent in NM. As auditory function matures, EphA4 expression decreases so that by 4 days after hatch no EphA4 antibody labeling is evident in the auditory brainstem nuclei. J. Comp. Neurol. 426:270–278, 2000. 

**Indexing terms: nucleus laminaris; nucleus magnocellularis; receptor tyrosine kinase; hindbrain; binaural segregation**

Precise connections in the auditory brainstem nuclei underlie accurate processing of auditory stimuli. In the chick, the nature of these highly ordered connections has been described in detail (Rubel and Parks, 1988). Auditory inputs from VIIIth nerve axons branch and project tonotopically to ipsilateral nucleus (n.) magnocellularis (NM) and n. angularis (NA). Each NM neuron projects tonotopically to n. laminaris (NL) on both sides of the brainstem (Young and Rubel, 1983). In the mature chick brainstem, NL is a laminar nucleus that is largely one cell thick, and each NL cell has symmetrical dorsal and ventral dendritic arbors (Smith and Rubel, 1979; Deitch and Rubel, 1984). The NM-NL projection exhibits extremely precise spatial segregation. Each NM neuron projects bilaterally. The ipsilateral NM axons project to dorsal dendrites and cell bodies of NL neurons and the contralateral NM axons project to the ventral dendrites and cell bodies of NL neurons. Thus, each NL cell receives segregated binaural input. Because the time of arrival of sound to each ear varies with the spatial location of the sound source, NL cells can use interaural time differences (ITDs) by comparing the timing of inputs from the two ears to compute sound location (Carr and Konishi, 1990; Overholt et al., 1992).

Given the importance of the specificity of these connections for auditory processing, we have begun to address how developmental events in the early embryonic chick lead to the formation of these precise connections. The auditory brainstem nuclei arise from a common auditory anlage that is evident at approximately embryonic day 5 (E5). Cells in the anlage migrate to their final positions in

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the individual nuclei, which are readily identifiable by approximately E9–10. Axonal connections from NM to NL begin forming at E9–10, and are subsequently refined so that the mature projection emerges by E14–15. Single cell reconstructions show that NM axons grow to NL and make initial projections to the appropriate region of the neuropil with very few errors from the outset (Young and Rubel, 1986). What are the molecular mechanisms that lead to this precision? Recently, it was discovered that the neurotrophin receptors TrkB and TrkC are present in and around NL during the formation of these connections (Cochran et al., 1999). Interestingly, TrkB expression is limited to the ventral dendrites of NL after E10. This selective expression pattern suggests a role for TrkB in establishing specificity in this projection.

In the present report, we have expanded our investigation of candidate molecules to include another receptor tyrosine kinase family, the Eph receptors. These receptors have been implicated in a broad range of developmental processes, including cell migration (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997), axon guidance (Henkemeyer et al., 1994, 1996; Winslow et al., 1995; Imondi et al., 2000), and the establishment of topographic maps (Cheng et al., 1995; Drescher et al., 1995; Gao et al., 1996; Zhang et al., 1996; Feldheim et al., 1998). Eph receptors are membrane-bound and contain a receptor tyrosine kinase domain. They are divided into A and B classes based on sequence similarity, and the numbers indicate the order in which they were discovered (Eph Nomenclature Committee, 1997). The ligands for Eph receptors, the ephrins, are also membrane-bound, either by a GPI linkage (the A class), or by a transmembrane domain (the B class). Ephrin ligands can initiate signal transduction events when bound to Eph receptors (Holland et al., 1996; Brückner et al., 1997; Mellitzer et al., 1999). Eph receptors are named according to the ephrin for which they show the highest binding affinity; however, there is a considerable amount of binding promiscuity between receptors and ligands (Friedman and O'Leary, 1996; Gale and Yancopoulos, 1997; Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999).

In this study, we have examined the expression of one Eph receptor, EphA4, in the chick hindbrain from E2 to posthatch day (P) 4. This member of the Eph receptor class was especially promising because it is expressed early in the hindbrain segments, or rhombomeres, r3 and r5 (Nieto et al., 1992; Theil et al., 1998), and r5 contains precursors for both NM and NL (Marín and Puelles, 1995; Cramer et al., 2000). We report here that EphA4 expression is restricted to longitudinal bands in r3 and r5 during early development. Once NM and NL have formed and axonal projections are developing, EphA4 is restricted to the dorsal neuropil, and for a few days in development has a staining pattern that is complementary to that of TrkB. Subsequently, EphA4 is expressed symmetrically around NL, until it disappears after E18. Our results suggest a role for EphA4 in regulating the spatial segregation of connections between NM and NL.

## MATERIALS AND METHODS

### Immunohistochemistry on paraffin sections

Chick embryos were staged according to Hamburger-Hamilton (HH) staging criteria to minimize variations in developmental age. Brainstem tissue from chicks (number of animals in parentheses) at E5 (3), E6 (2), E8 (2), E9 (2), E10 (5), E11 (3), E12 (3), E14 (1), E15 (3), E18 (1), and posthatch day (P) 4 (1) were dissected and immersion fixed in a solution of 49% ethanol, 20% formalin, and 10% glacial acetic acid in $dH_20$. Tissue was then dehydrated by immersion in increasing concentrations of ethanol for 1 hour in each change, followed by 2 changes of 1 hour each in Hemo-De clearing agent (Fisher Scientific, Pittsburgh, PA). Tissue was then embedded in paraffin. Most tissue was serially sectioned in the coronal plane at 12 μm. Two E5 embryos were sectioned at 12 μm in the horizontal plane, and two E10 brainstems and one E14 brainstem were sectioned at 12 μm in the sagittal plane. Sections were mounted and dried onto HCL/ethanol chrome alum subbed slides in four or five series of alternate sections so that adjacent sections could be stained with various methods. Slides were deparaffinized with xylene, hydrated in a graded series of ethanol, then rinsed in phosphate buffered saline (PBS), pH 7.4. For immunohistochemistry, a PAP Pen (The Binding Site Inc., San Diego, CA) was used to make small wells around sections on slides. Tissue was treated for 5 minutes with 1% sodium dodecyl sulfate (SDS) in PBS to expose antigenic epitopes. Sections were then rinsed in PBS, and endogenous peroxidase activity was reduced with a 10-minute incubation in 0.3% $H_2O_2$ in 100% methanol. 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 an affinity-purified rabbit polyclonal antipeptide antibody directed against the carboxy terminus of the EphA4 receptor (Soans et al., 1994) diluted 1:100 in the blocking solution, or an antibody to the TrkB receptor (Lefcort et al., 1996; Cochran et al., 1999). After several rinses with PBS, sections were incubated in a biotinylated goat anti rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in the blocking solution for 1–2 hours at room temperature then rinsed extensively with PBS. Tissue was then incubated in an avidin-horseradish peroxidase (HRP) complex made from an ABC kit (Vector Laboratories) for 1 hour and rinsed with PBS. We used a VIP substrate kit (Vector Laboratories) to visualize the HRP then rinsed sections in PBS. Sections were dehydrated in alcohol and xylene, and then slides were cover-slipped with DPX mountant (BDH Laboratory Supplies, Poole, England). One series of sections from each brainstem was Nissl stained using thionin so that alternate sections could be used to help identify brainstem nuclei.

On each slide one section was used as a negative control with primary antibody omitted. The cerebellum, which is known to stain heavily for EphA4, was used as a positive control. In addition, in E5 tissue, the presence of rhombomeres allowed us to use r3 and r5 as a positive control.

### Immunohistochemistry on whole-mounted embryos

Embryos (ages E3 and E5; HH stages 19 and 25) were dissected into chilled 4% paraformaldehyde in PBS. They were then transferred to a fresh solution of 4% paraformaldehyde and allowed to fix for 3 to 4 hours at 4°C. Embryos were washed in PBS, post-fixed in a series of increasing strengths of methanol, and in some cases frozen at −20°C. Before staining, endogenous peroxidase activity was quenched with a 6-hour incubation in a solu-

tion of 80% methanol and 6% $H_2O_2$. Embryos were rehydrated, rinsed in PBS, and incubated in blocking/extracting solution containing 1% saponin, 10% goat serum, and 2% bovine serum albumin (BSA) for 1–4 hours at 4°C with agitation. Tissue was rinsed in PBS and then incubated with anti-EphA4 antibody at 1 μg/ml in a wash solution containing 1% normal goat serum, 1% BSA, and 0.1% saponin in PBS for 48 hours. Tissue was rinsed in wash solution and incubated in presorbed secondary antibody (Vector biotinylated goat anti-rabbit, 1:400 in wash solution) for 24–48 hours at 4°C. Tissue was rinsed and incubated in a Vector ABC solution for 24 hours at 4°C, rinsed, and HRP reaction product was visualized using diaminobenzidine (DAB) as the chromagen.

### Production of photomicrographs

Color figures were produced from color slides that were scanned using a Nikon 35-mm Film Scanner LS-1000 and imported into Adobe PhotoShop (Adobe Systems, Inc., San Jose, CA). This program was used to make small adjustments in color, contrast, and brightness, and to add labels. Gray-scale images were acquired directly from the microscope by using a SPOT-2 digital camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI) and imported into Adobe PhotoShop with flatfield corrections to compensate for uneven illumination. Adobe PhotoShop was again used to add labels and to make small adjustments in contrast.

## RESULTS

The EphA4 polyclonal antibody used in this study was prepared using the 11 carboxy-terminal amino acids of EphA4 and was characterized for specific binding using immunoblotting (Soans et al., 1994). It was shown to be specific for EphA4 and did not bind to closely related proteins of the Eph family (Soans et al., 1994). In the present study, we have successfully used the antibody to label whole-mounted tissue and sections of paraffin embedded tissue. Immunohistochemical labeling for EphA4 was present in the brainstem at most of the ages examined. Negative controls in which the primary antibody was omitted never appeared labeled. In addition, the expression pattern we observed at E3 and E5 is similar to patterns of mRNA expression previously described (Küry et al., 2000). Its expression was dynamic and was specific to distinct regions. A description of the labeling at each age is given below.

### E3–E6

At these ages EphA4 is expressed in rhombomeres 3 and 5, similar to the pattern seen in several other species (Nieto et al., 1992; Theil et al., 1998) and to the pattern described in chick embryos (Küry et al., 2000). Figure 1 shows whole-mounted embryos that have been stained with EphA4. At E3 EphA4 is expressed in rhombomeres 3 and 5 (Fig. 1A). This pattern supports the specificity of the antibody in our tissue. At E5–6, staining in these rhombomeres is not uniform; alternating stripes of more and less intense EphA4 staining can be seen reliably (arrows, Fig. 1B). Figure 2A shows a horizontal section through rhombomere 5 at E5. EphA4 is also present in the otocyst. In a coronal section through r5 in an E5 embryo (Fig. 2B), striped regions of EphA4 expression are evident as well

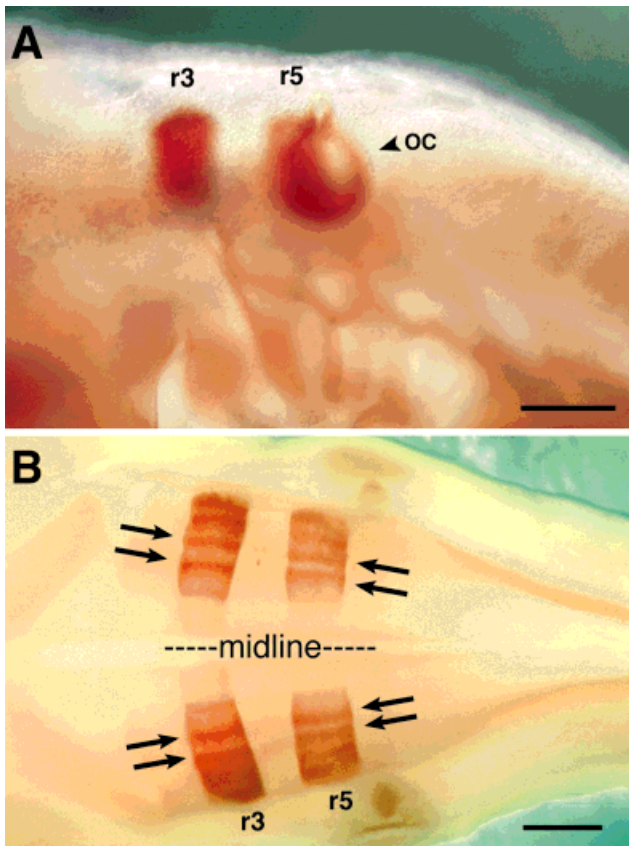


Fig. 1. EphA4 immunolabeling in whole-mounted tissue at early stages. **A:** Lateral view of an embryonic day (E) 3 embryo at Hamburger-Hamilton stage 19. The roof plate has been dissected away and the hindbrain has been exposed. Label is evident in r3 and r5. The otocyst (oc) is visible lateral to the hindbrain region containing r5 and r6. **B:** Dorsal view of the hindbrain of an E5 (stage 25) embryo. EphA4 labeling is seen in the alar plate region of r3 and r5. At this age, labeling in these rhombomeres has become restricted to longitudinal bands that are in register for both labeled regions. Arrows indicate areas of high intensity labeling. The basal region of the neural plate is not labeled. Anterior is to the left in both panels. Scale bars = 200 μm in A,B.

but are less dramatic. Arrows in Figure 2A,B indicate areas of more intense staining.

### E8–9

At E8 and E9, the auditory anlage is clearly identifiable at the dorsolateral edge of the brainstem (Fig. 3A), but the specific localization of NM and NL neurons within this area is still unclear. EphA4 is not expressed in the cell bodies of the anlage, but is present in the neuropil surrounding the cell bodies in the medial region of the anlage (Fig. 3B). In the region below the anlage, cerebellar fibers are darkly stained.

### E10–11

By E10, the cells from the auditory anlage have migrated to their mature positions within NM and NL. The Nissl-stained section in Figure 4A clearly shows the auditory nuclei in an E10 brainstem. At this age, EphA4 is not expressed in NM somata, although there is robust stain-

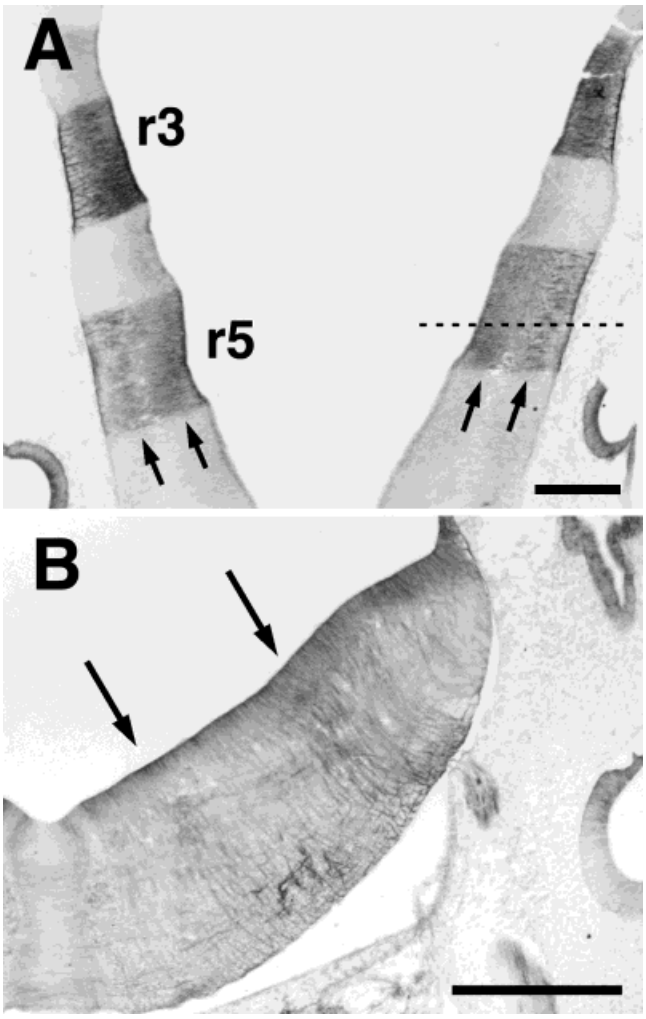


Fig. 2. EphA4 immunohistochemistry on paraffin sections of embryonic day (E) 5 brainstems. **A:** A horizontal section showing the labeling pattern along the rostrocaudal extent of the brainstem. Labeling is seen in r3 and r5. Longitudinal bands of more intense staining are observed. **B:** Right side of a coronal section through r5 of an E5 brainstem at approximately the level shown in dashed line in A. Longitudinal bands of labeling are indicated by arrows. Scale bars = 200 μm in A,B.

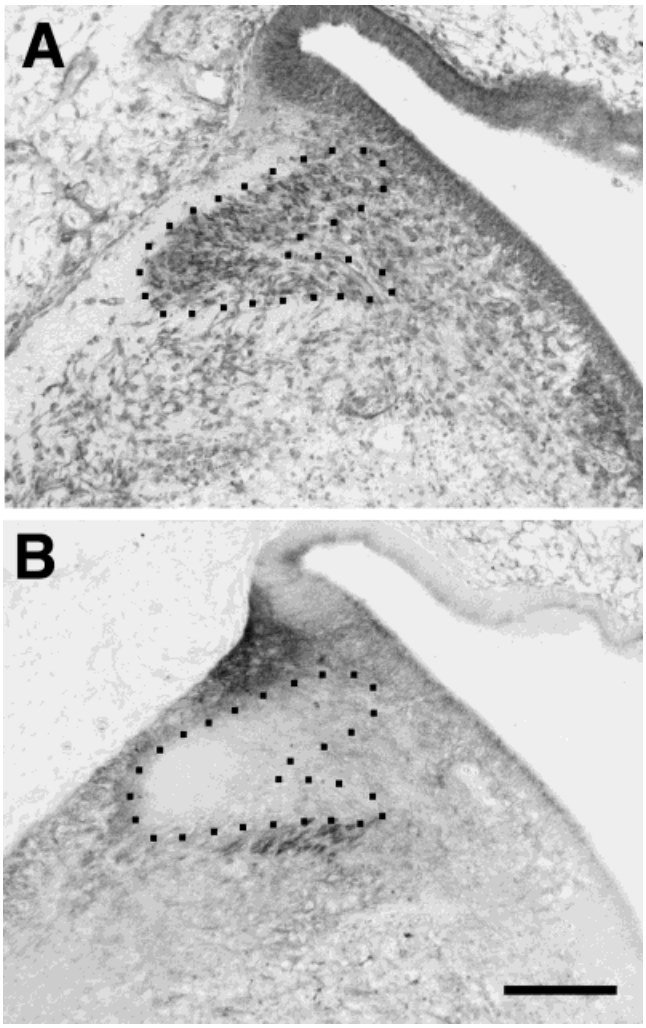


Fig. 3. EphA4 immunolabeling on embryonic day 8 coronal paraffin sections. **A:** Nissl section showing the outline (dotted line) of the auditory anlage near the dorsolateral edge of the brainstem. Lateral is to the left; dorsal is up. **B:** Adjacent section stained with an antibody to EphA4, showing that the anlage is largely devoid of labeling, with the exception of light neuropil labeling in the medial part of the anlage. Scale bar = 100 μm in B (applies to A,B).

ing in the axons entering NM from its lateral side and limited expression in the neuropil of NM (Fig. 4B). In NL, there is EphA4 staining in the dorsal and ventral neuropil, but staining intensity is markedly asymmetrical. EphA4 expression is much more intense in the dorsal neuropil than in the ventral neuropil. Other structures in the brainstem, including cells in the superior olivary nucleus (SON) as well as cerebellar fibers, also stain for EphA4. These fibers are ventral to NL and are intensely labeled; they can be seen continuing into the cerebellum in some sections.

At E10, the neurotrophin receptor TrkB is also expressed asymmetrically in the neuropil of NL, with the staining intensity stronger in the ventral neuropil. This pattern is the opposite of that seen with EphA4 staining. Figure 5 shows immunohistochemistry with EphA4 and TrkB on adjacent paraffin sections within an E10 brainstem. In these sections, it is clearly evident that the staining patterns are complementary (Fig. 5A,C); the higher power images (Fig. 5B,D) reveal the opposing polarized staining in the NL neuropil. TrkB immunohistochemistry stains NM and n. angularis (NA), whereas these nuclei show only pale labeling with EphA4 immunohistochemistry. Auditory fibers, cerebellar fibers, and NL cell bodies express EphA4 but not TrkB.

At E11 (data not shown), EphA4 staining in NL and NM is similar to that in the E10 embryo. Expression of TrkB at E11 is also similar to that found at E10.

## E12–15

By E12, EphA4 staining in NL neuropil is strong and clearly symmetrical on the two sides of the sheet of NL somata. An example is shown in Figure 6. In addition, NM

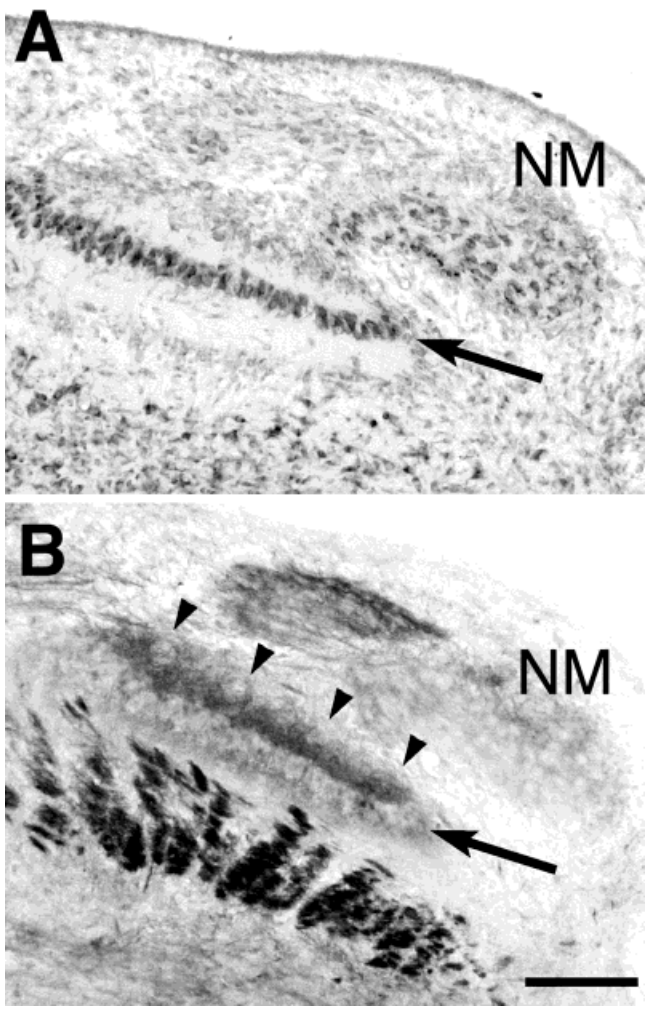


Fig. 4. EphA4 immunohistochemistry in coronal paraffin sections of an embryonic day 10 brainstem. **A:** Nissl-stained section showing the nuclei magnocellularis (NM) and laminaris (NL). Arrow indicates the line of NL cell bodies. Lateral is to the left; dorsal is up. **B:** EphA4 immunolabeling in an adjacent paraffin section. In NL, cell bodies are pale, whereas the neuropil around NL is asymmetrically labeled, with the dorsal neuropil (arrowheads) more intensely and extensively labeled than the ventral neuropil. Fibers around NM cells are labeled, but NM cell bodies do not appear labeled. Ventral to NL, cerebellar projection fibers are intensely labeled. Scale bar = 100 μm in B (applies to A,B).

and NA are not labeled. The VIIIth nerve is still lightly stained at some brainstem levels.

At E14–15, sagittal and coronal sections stained with EphA4 immunohistochemistry reveal NL staining that remains symmetric across the dorsal and ventral regions of the neuropil. Both neuropil areas and the NL cell bodies are intensely stained (Fig. 7A,C). Labeling is more extensive in posterolateral regions of the nucleus, corresponding with the gradient of dendritic arbor size that emerges at E15 (Smith and Rubel, 1979; Smith, 1981). There is a slight variation in the intensity of label related to the tonotopic axis of the nucleus. Posterolateral regions of the nucleus, corresponding to low frequency tuned cells, are slightly more intensely labeled than anteromedial regions, which respond best to higher frequencies. NM is unlabeled.

### E18 through posthatch

At E18, staining in the neuropil around NL is reduced in intensity and in dorsoventral extent. At this age, NM cell bodies have moderate levels of expression. E18 sections are shown in Figure 7B,D. At P4, no immunolabeling with antibody to EphA4 is evident in either NM or NL (data not shown).

## DISCUSSION

The results presented in this study reveal that the EphA4 receptor is expressed in distinctive patterns at several different embryonic stages in the chick hindbrain. Early in embryogenesis, high expression is limited to rhombomeres 3 and 5, and within these rhombomeres labeling is refined to alternating longitudinal stripes at E5–6. The significance of the labeling pattern at this age and the potential function of the receptor are not known. One possibility is that the expression pattern is related to migratory pathways of cells forming the nuclei of the brainstem. Eph/Ephrin interactions seem to be inhibitory for cell migration during dispersal of neural crest derived cells (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997). In addition, in early development, interactions between EphA4 in rhombomeres 3 and 5 and ephrin B2 in even rhombomeres seem to limit migration across rhombomere boundaries (Xu et al., 1995, 1996, 1999; Xu and Wilkinson, 1997; Mellitzer et al., 1999). The action of these receptors and their ligands on cell-cell interactions regulating cell migration, thus, constitutes an important role in early development, during which patterns of cell migration provide a framework for subsequent differentiation and formation of synaptic connections. EphA4 expression is required for the formation of mesodermal somites (Durbin et al., 1998) and may be involved in setting up compartments in the cerebral cortex (Donoghue and Rakic, 1999) and the cerebellum (Lin and Cepko, 1998; Rogers et al., 1999; Karam et al., 2000). In the early formation of NM and NL, both nuclei have precursors in rhombomere 5, but these precursors are located in adjacent but distinct mediolateral positions within the rhombomere (Cramer et al., 2000). Thus, one interesting possibility is that the stripes of expression at E5 are related to the migratory pathways of cells that will make up the auditory anlage, and subsequently, the mature nuclei. It is not known whether similar migratory pathways are followed by cells derived from rhombomere 3. However, because the stripes are in register between the two rhombomeres, it is possible that a general scheme of migratory routes is set by EphA4 expression.

A second very intriguing pattern of EphA4 expression was observed at E10 and E11. At this time, the dorsal neuropil of NL is much more intensely labeled than the ventral neuropil. The dorsal neuropil contains dorsal dendrites from the NL cells, along with axonal terminations from ipsilateral NM, but not from contralateral NM. At E10 and E11, axons have arrived and the first synaptic connections are forming (Jackson et al., 1982; Young and Rubel, 1986). Although axons arrive at both the dorsal and ventral regions of NL by E9, the dynamics of terminal arbor formation are very different for these regions between E9 and E14. From E8–9 on, the contralateral pro-

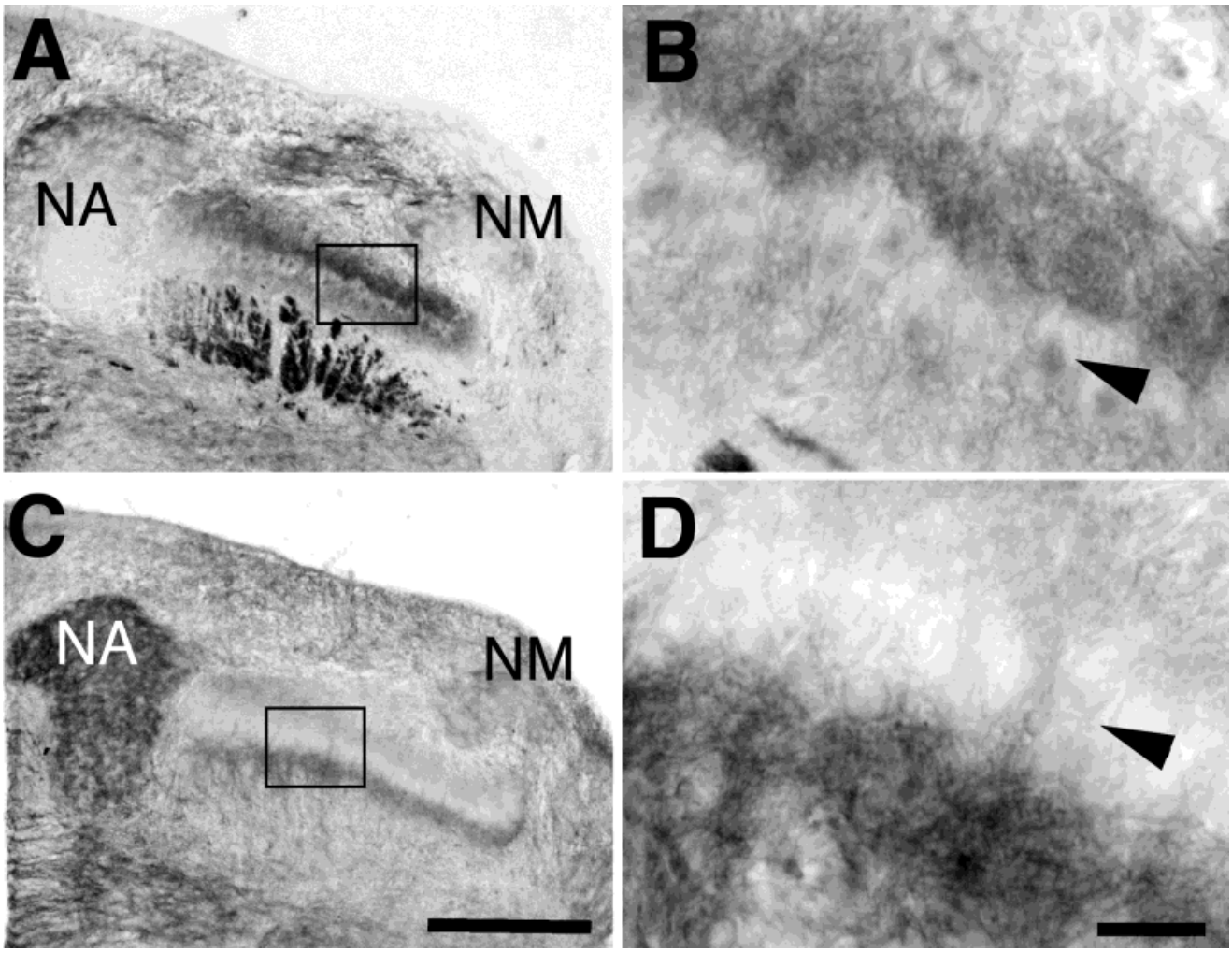


Fig. 5. Comparison of EphA4 and TrkB immunohistochemistry in adjacent sections at embryonic day (E) 10. **A:** EphA4 immunohistochemistry at E10, in a coronal section with dorsal side up and lateral to the left. Staining is pale in and around the nuclei magnocellularis (NM) and laminaris (NL). NL neuropil is labeled, with substantially more intense label on the dorsal side. **B:** Higher magnification of the area outlined in A. Arrowhead indicates region of cell bodies in NL, where some nuclei appear labeled. **C:** An adjacent section stained with an antibody directed against TrkB. The labeling pattern is complementary to that seen with EphA4 antibodies. Labeling is seen in NM and NA, and in the ventral dendrites of NL cells. **D:** Higher magnification of the area outlined in C. Arrowhead indicates region of cell bodies in NL. Scale bars = 200 μm in C (applies to A,C); 20 μm in D (applies to B,D).

jecting NM axons course below the NL cells along an isofrequency line and send short collaterals dorsally into the ventral neuropil. In contrast, the ipsilateral (dorsal) input from NM grows initially to a single topographically appropriate location in NL and then collateral branches later emerge to form an array of collaterals orthogonal to the tonotopic axis (along an isofrequency line), forming the mature projection (Young and Rubel, 1983, 1986). Interestingly, this transformation occurs between E9 and E11, the precise time of the asymmetrical expression of EphA4 in the NL neuropil regions (Young and Rubel, 1986). This pattern of axonal growth is reminiscent of cultured chick retinal ganglion cells (Davenport et al., 1999) in the presence of ephrin, in which ephrin-induced growth cone collapse causes collateral branches to form from the main trunk of the growing axons. The difference in the patterns of axonal growth between dorsal and ventral surfaces of NL, thus, may be related to patterns of EphA4 expression.

The expression of EphA4 in the brainstem changes again at E12, when staining in the neuropil on both sides of the NL cell bodies is intense and symmetric. No label is observed in NM. This pattern of label persists in the auditory brainstem through E15. This period of development coincides with the period of developmental cell death in NL (Rubel et al., 1976; Solum et al., 1997), the emergence of GABAergic terminals in NM and NL (Code et al., 1989), and the development of a gradient in dendritic arbor size in NL (Smith and Rubel, 1979; Smith, 1981). After approximately E15, posterolateral (low frequency) NL dendrites have large, complex arbors, whereas anteromedial (high frequency) NL dendrites have smaller, simpler arbors. Thus, EphA4 expression in NL neuropil at E12–E15 may be involved in mediating these later developmental events in the ontogeny of the auditory brainstem.

At E12, it is highly likely that EphA4 is located on NL dendrites, as staining is evident in NL cell bodies, whereas

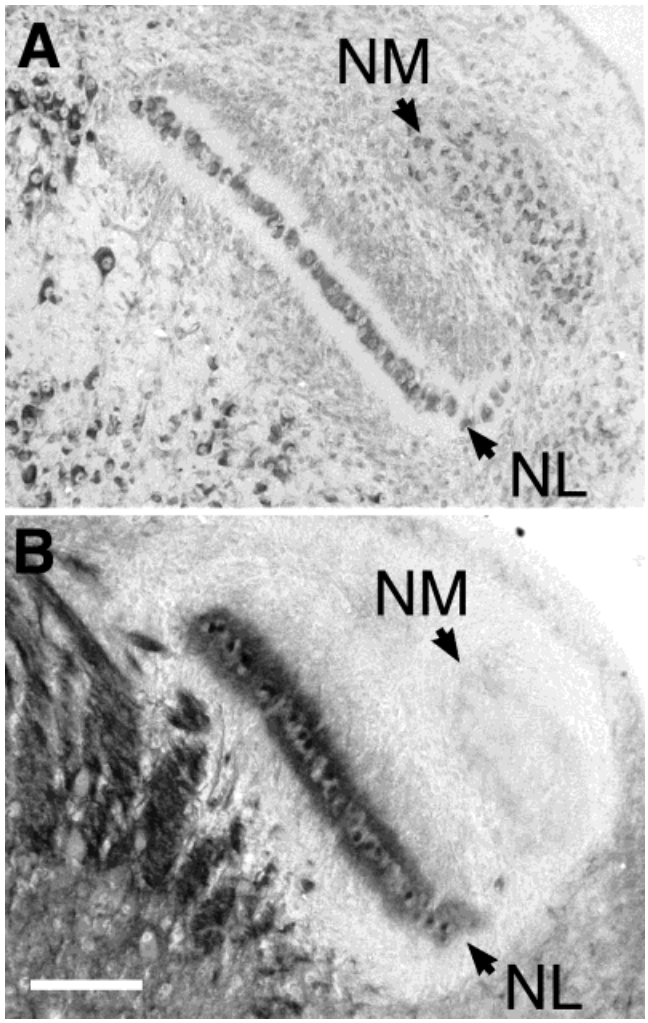


Fig. 6. EphA4 immunohistochemistry at embryonic day 12. **A:** Nissl-stained section showing the nuclei magnocellularis (NM) and laminaris (NL). Lateral is to the left; dorsal is up. **B:** EphA4 labeling in a section adjacent to that shown in A. No labeling is evident in NM. In NL, labeling is present in both neuropil and cell bodies. Staining in the neuropil around NL is symmetric, with intense labeling in both dorsal and ventral regions. Scale bar = 100 μm in B (applies to A,B).

there is no staining in NM, the other principal source of components to the neuropil around NL. At E10–11, EphA4 labeling in NM is pale, and cell body labeling in NL is also pale, so that it remains possible that neuropil labeling in dorsal NL neuropil arises from ipsilaterally projecting NM axons. Because the labeling in NM is pale, and because of the stronger evidence at E12, it is most likely that EphA4 is expressed in NL cell dendrites at both ages. In addition, the pattern of expression of EphA4 follows the changes in dendritic arbor size, so that at E15 there is a gradient in the extent of labeling with posterolateral regions most extensively labeled (see Fig. 7). This pattern further supports the expression of EphA4 in NL dendrites. However, this hypothesis is yet to be confirmed. In addition, it will be of interest to identify the ephrin ligands and their localization during brainstem development.

Ephrins have been shown to have a chemorepulsive effect on growing axons that express corresponding Eph receptors (Drescher et al., 1995; Nakamoto et al., 1996; Gale and Yancopoulos, 1997; Imondi et al., 2000). In NL, it seems that the receptor is expressed on the dendrites and in the cell bodies rather than on the growing axons. This distribution suggests the possibility that EphA4 is involved in regulating the dendritic morphology of NL cells. In addition, EphA4 expression in NL may serve to limit cell migration into the area to maintain the unique laminar morphology of the nucleus, which is surrounded by a cell-free neuropil region. The expression of EphA4 on dendrites may also be related to regulating axonal growth from NM, because EphA4 can effect phosphorylation responses in some ephrin ligands (Holland et al., 1996; Brückner et al., 1997; Mellitzer et al., 1999). The differential distribution of EphA4 at E10–11, thus, may selectively influence the growth and branching of NM axons. Eph receptors and ligands bind to PDZ proteins within the synapse, and they tend to be localized in synaptic regions (Torres et al., 1998). Thus, it is possible that EphA4 plays an additional role in the functional maturation of NM-NL synapses within the neuropil. These potential roles for EphA4 within the dendrites and cell bodies of NL are speculative, and further experimentation will be required to assess their validity.

During the period of asymmetric EphA4 staining around NL (E10–11), another receptor tyrosine kinase, TrkB, stains in a nearly complementary pattern. Interactions between TrkB and its ligand, the neurotrophin BDNF, have been shown to be chemoattractive. EphA4 and TrkB could have complementary roles in the formation of connections between NM and NL. After E18, EphA4 expression in the neuropil around NL is lost. In contrast, the asymmetric expression of TrkB label persists into late developmental stages (Cochran et al., 1999). Future experiments examining the distribution of ligands for these receptors, as well as loss or gain of function experiments, will be required to elucidate the roles of EphA4 and TrkB in the ontogeny of the auditory brainstem. On the basis of the distinct expression of these proteins, it is possible that interplay between these signaling systems serves to guide the development of the highly precise connections between NM and NL.

The present data begin to explore a potential role for Eph/ephrin signaling in the topologic specificity of connections in the auditory system. In the brainstem, the pattern of staining suggests a role in binaural segregation of axonal terminals. Here, EphA4 expression may be associated with forming specific patterns of connections distinct from topographic projections, such as in the striatal matrix/striosome pathways (Janis et al., 1999). However, in the avian auditory brainstem nuclei, expression of EphA4 (and TrkB) is restricted to regions within cells and not to different populations of cells. Moreover, the axons projecting to EphA4-expressing regions arise from the same cells as those projecting to TrkB expressing regions; the only difference between them is whether they are ipsilateral or contralateral to NL. Together, these proteins present a possible set of mechanisms by which binaural segregation can develop precisely in the avian brain.

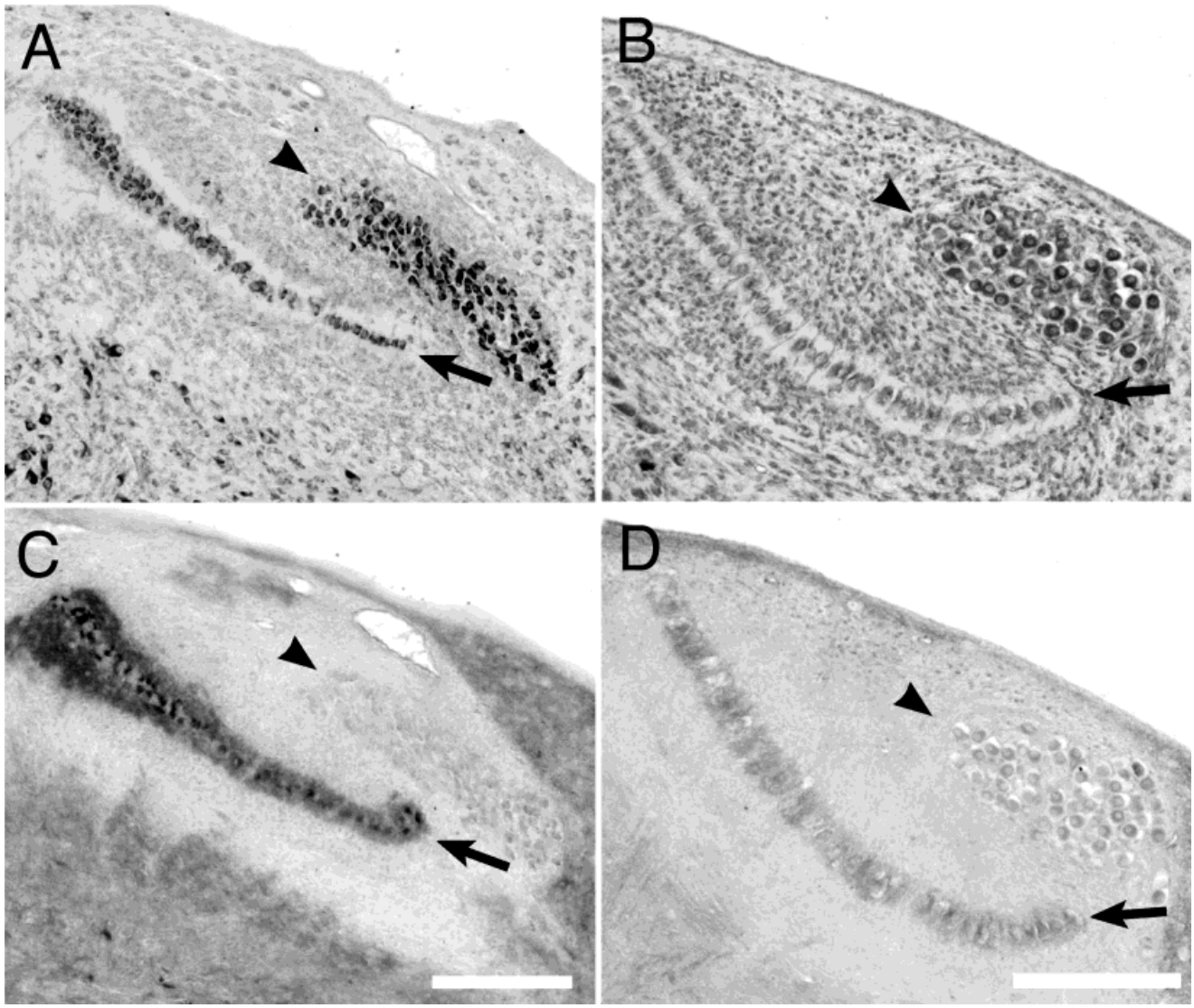


Fig. 7. EphA4 immunohistochemistry at embryonic day (E) 15 and E18. **A:** Nissl-stained section through nucleus magnocellularis (NM; arrowhead) and nucleus laminaris (NL; arrow) in a brainstem from an E15 embryo. **B:** Nissl section through NM and NL from an E18 brainstem. **C:** EphA4 immunohistochemistry at E15, in a section adjacent to that shown in A. Black arrowhead indicates NM, in which labeling is absent. Black arrow indicates the presence of labeling in NL cell bodies and symmetrically in the neuropil around NL. **D:** EphA4 immunohistochemistry in an E18 brainstem section adjacent to that shown in B. NM cells (arrowhead) are faintly labeled. NL cell bodies (arrow) and a limited extent of the neuropil are also labeled. In all panels, lateral is to the left and dorsal is up. Scale bars = 200 μm in C (applies to A,C); D (applies to B,D).

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## LITERATURE CITED

Brückner K, Pasquale EB, Klein R. 1997. Tyrosine phosphorylation of transmembrane ligands for Eph receptors. Science 275:1640–1643.

Carr CE, Konishi M. 1990. A circuit for detection of interaural time differences in the brain stem of the barn owl. J Neurosci 10:3227–3246.

Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. 1995. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell 82:371–381.

Cochran SL, Stone JS, Bermingham-McDonogh O, Akers SR, Lefcort F, Rubel EW. 1999. Ontogenetic expression of trk neurotrophin receptors in the chick auditory system. J Comp Neurol 413:271–288.

Code RA, Burd GD, Rubel EW. 1989. Development of GABA immunoreactivity in brainstem auditory nuclei of the chick: ontogeny of gradients in terminal staining. J Comp Neurol 284:504–518.

Cramer KS, Fraser SE, Rubel EW. 2000. Embryonic origins of auditory brainstem nuclei in the chick hindbrain. Dev Biol 224:138–151.

Davenport RW, Thies E, Cohen ML. 1999. Neuronal growth cone collapse

triggers lateral extensions along trailing axons. Nat Neurosci 2:254–259.

Deitch JS, Rubel EW. 1984. Afferent influences on brain stem auditory nuclei of the chicken: time course and specificity of dendritic atrophy following deafferentation. J Comp Neurol 229:66–79.

Donoghue MJ, Rakic P. 1999. Molecular evidence for the early specification of presumptive functional domains in the embryonic primate cerebral cortex. J Neurosci 19:5967–5979.

Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, Bonhoeffer F. 1995. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell 82:359–370.

Durbin L, Brennan C, Shiomi K, Cooke J, Barrios A, Shanmugalingam S, Guthrie B, Lindberg R, Holder N. 1998. Eph signaling is required for segmentation and differentiation of the somites. Genes Dev 12:3096–3109.

Eph Nomenclature Committee. 1997. Unified nomenclature for Eph family receptors and their ligands, the ephrins. Cell 90:403–404.

Feldheim DA, Vanderhaeghen P, Hansen MJ, Frisén J, Lu Q, Barbacid M, Flanagan JG. 1998. Topographic guidance labels in a sensory projection to the forebrain. Neuron 21:1303–1313.

Flanagan JG, Vanderhaeghen P. 1998. The ephrins and Eph receptors in neural development. Annu Rev Neurosci 21:309–345.

Friedman GC, O'Leary DD. 1996. Eph receptor tyrosine kinases and their ligands in neural development. Curr Opin Neurobiol 6:127–133.

Gale NW, Yancopoulos GD. 1997. Ephrins and their receptors: a repulsive topic? Cell Tissue Res 290:227–241.

Gao PP, Zhang JH, Yokoyama M, Racey B, Dreyfus CF, Black IB, Zhou R. 1996. Regulation of topographic projection in the brain: Elf-1 in the hippocamposeptal system. Proc Natl Acad Sci USA 93:11161–11166.

Henkemeyer M, Marengere LE, McGlade J, Olivier JP, Conlon RA, Holmyard DP, Letwin K, Pawson T. 1994. Immunolocalization of the Nuk receptor tyrosine kinase suggests roles in segmental patterning of the brain and axonogenesis. Oncogene 9:1001–1014.

Henkemeyer M, Orioli D, Henderson JT, Saxton TM, Roder J, Pawson T, Klein R. 1996. Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell 86:35–46.

Holder N, Klein R. 1999. Eph receptors and ephrins: effectors of morphogenesis. Development 126:2033–2044.

Holland SJ, Gale NW, Mbamalu G, Yancopoulos GD, Henkemeyer M, Pawson T. 1996. Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. Nature 383:722–725.

Imondi R, Wideman C, Kaprielian Z. 2000. Complementary expression of transmembrane ephrins and their receptors in the mouse spinal cord: a possible role in constraining the orientation of longitudinally projecting axons. Development 127:1397–1410.

Jackson H, Hackett JT, Rubel EW. 1982. Organization and development of brain stem auditory nuclei in the chick: ontogeny of postsynaptic responses. J Comp Neurol 210:80–86.

Janis LS, Cassidy RM, Kromer LF. 1999. Ephrin-A binding and EphA receptor expression delineate the matrix compartment of the striatum. J Neurosci 19:4962–4971.

Karam SD, Burrows RC, Logan C, Koblar S, Pasquale EB, Bothwell M. 2000. Eph receptors and ephrins in the developing chick cerebellum: relationship to sagittal patterning and granule cell migration. J Neurosci (in press).

Krull CE, Lansford R, Gale NW, Collazo A, Marcelle C, Yancopoulos GD, Fraser SF, Bronner-Fraser M. 1997. Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. Curr Biol 7:571–580.

Küry P, Gale N, Connor R, Pasquale E, Guthrie S. 2000. Eph receptors and ephrin expression in cranial motor neurons and the branchial arches of the chick embryo. Mol Cell Neurosci 15:123–140.

Lefcort F, Clary DO, Rusoff AC, Reichardt LF. 1996. Inhibition of the NT-3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. J Neurosci 16:3704–3713.

Lin JC, Cepko CL. 1998. Granule cell raphes and parasagittal domains of Purkinje cells: complementary patterns in the developing chick cerebellum. J Neurosci 18:9342–9353.

Marín F, Puelles L. 1995. Morphological fate of rhombomeres in quail/chick chimeras: a segmental analysis of hindbrain nuclei. Eur J Neurosci 7:1714–1738.

Mellitzer G, Xu Q, Wilkinson DG. 1999. Eph receptors and ephrins restrict cell intermingling and communication. Nature 400:77–81.

Nakamoto M, Cheng HJ, Friedman GC, McLaughlin T, Hansen MJ, Yoon CH, O'Leary DD, Flanagan JG. 1996. Topographically specific effects of ELF-1 on retinal axon guidance in vitro and retinal axon mapping in vivo. Cell 86:755–766.

Nieto MA, Gilardi-Hebenstreit P, Charnay P, Wilkinson DG. 1992. A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. Development 116:1137–1150.

Overholt EM, Rubel EW, Hyson RL. 1992. A circuit for coding interaural time differences in the chick brainstem. J Neurosci 12:1698–1708.

Rogers JH, Ciossek T, Menzel P, Pasquale EB. 1999. Eph receptors and ephrins demarcate cerebellar lobules before and during their formation. Mech Dev 87:119–128.

Rubel EW, Parks TN. 1988. Organization and development of the avian brain-stem auditory system. In: Edelman GM, Gall WE, Cowan WM, editors. Auditory function: the neurobiological basis of hearing. New York: John Wiley & Sons. p 3–92.

Rubel EW, Smith DJ, Miller LC. 1976. Organization and development of brain stem auditory nuclei of the chicken: ontogeny of n. magnocellularis and n. laminaris. J Comp Neurol 166:469–490.

Smith A, Robinson V, Patel K, Wilkinson DG. 1997. The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. Curr Biol 7:561–570.

Smith DJ, Rubel EW. 1979. Organization and development of brain stem auditory nuclei of the chicken: dendritic gradients in nucleus laminaris. J Comp Neurol 186:213–239.

Smith ZD. 1981. Organization and development of brain stem auditory nuclei of the chicken: dendritic development in n. laminaris. J Comp Neurol 203:309–333.

Soans C, Holash JA, Pasquale EB. 1994. Characterization of the expression of the Cek8 receptor-type tyrosine kinase during development and in tumor cell lines. Oncogene 9:3353–3361.

Solum D, Hughes D, Major MS, Parks TN. 1997. Prevention of normally occurring and deafferentation-induced neuronal death in chick brainstem auditory neurons by periodic blockade of AMPA/kainate receptors. J Neurosci 17:4744–4751.

Theil T, Frain M, Gilardi-Hebenstreit P, Flenniken A, Charnay P, Wilkinson DG. 1998. Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. Development 125:443–452.

Torres R, Firestein BL, Dong H, Staudinger J, Olson EN, Huganir RL, Bredt DS, Gale NW, Yancopoulos GD. 1998. PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. Neuron 21:1453–1463.

Wang HU, Anderson DJ. 1997. Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. Neuron 18:383–396.

Winslow JW, Moran P, Valverde J, Shih A, Yuan JQ, Wong SC, Tsai SP, Goddard A, Henzel WJ, Hefti F, et al. 1995. Cloning of AL-1, a ligand for an Eph-related tyrosine kinase receptor involved in axon bundle formation. Neuron 14:973–981.

Xu Q, Wilkinson DG. 1997. Eph-related receptors and their ligands: mediators of contact dependent cell interactions. J Mol Med 75:576–586.

Xu Q, Alldus G, Holder N, Wilkinson DG. 1995. Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. Development 121:4005–4016.

Xu Q, Alldus G, Macdonald R, Wilkinson DG, Holder N. 1996. Function of the Eph-related kinase rtk1 in patterning of the zebrafish forebrain. Nature 381:319–322.

Xu Q, Mellitzer G, Robinson V, Wilkinson DG. 1999. In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. Nature 399:267–271.

Young SR, Rubel EW. 1983. Frequency-specific projections of individual neurons in chick brainstem auditory nuclei. J Neurosci 3:1373–1378.

Young SR, Rubel EW. 1986. Embryogenesis of arborization pattern and topography of individual axons in n. laminaris of the chicken brain stem. J Comp Neurol 254:425–459.

Zhang JH, Cerretti DP, Yu T, Flanagan JG, Zhou R. 1996. Detection of ligands in regions anatomically connected to neurons expressing the Eph receptor Bsk: potential roles in neuron-target interaction. J Neurosci 16:7182–7192.