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EphA4 signaling promotes axon segregation in the developing auditory system

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Abstract

Precision of synaptic connections within neural circuits is essential for the accurate processing of sensory information. Specificity is exemplified at cellular and subcellular levels in the chick auditory brainstem, where nucleus magnocellularis (NM) neurons project bilaterally to nucleus laminaris (NL). Dorsal dendrites of NL neurons receive input from ipsilateral, but not contralateral, branches of NM axons whereas ventral dendrites are innervated by contralateral NM axons. This organization is analogous to that of the mammalian medial superior olive (MSO) and represents an important component of the circuitry underlying sound localization. However, the molecular mechanisms that establish segregated inputs to individual regions of NL neurons have not been identified. During synapse formation in NL, the EphA4 receptor is expressed in dorsal, but not ventral NL, neuropil, suggesting a potential role in targeting synapses to appropriate termination zones. Here, we directly tested this role by ectopically expressing EphA4 and disrupting EphA4 signaling using in ovo electroporation. We found that both misexpression of EphA4 and disruption of EphA4 signaling resulted in an increase in the number of NM axons that grow aberrantly across NL cell bodies into inappropriate regions of NL neuropil. EphA4 signaling is thus essential for targeting axons to distinct subsets of dendrites. Moreover, loss of EphA4 function resulted in morphological abnormalities of NL suggestive of errors in cell migration. These results suggest that EphA4 has multiple roles in the formation of auditory brainstem nuclei and their projections.

Keywords: EphA4; Nucleus magnocellularis; Nucleus laminaris

Introduction

In the avian brainstem, auditory nerve axons synapse ipsilaterally on nucleus magnocellularis (NM) neurons. NM axons in turn contact nucleus laminaris (NL) cells, which are arranged as a sheet that is one cell thick and possess dorsal and ventral sets of dendrites (Jhaveri and Morest, 1982; Smith and Rubel, 1979). NL is the first nucleus to receive bilateral auditory inputs, with ipsilateral and contralateral NM axons segregated onto dorsal and ventral dendrites, respectively (Young and Rubel, 1983). The organization of these inputs facilitates neural computations that permit sound localization (Agmon-Snir et al., 1998; Carr and Konishi, 1990; Overholt et al., 1992; Schwarz, 1992; Sullivan and Konishi, 1986; Takahashi et al., 1984; Young and Rubel, 1983). These connections form early in development, with segregation of the ipsilateral and contralateral inputs present from the outset. In this study, we investigated the role of the Eph family protein EphA4 in the establishment of this pathway.

Signaling through Eph receptor tyrosine kinases (RTKs) and their ligands, the ephrins, serves an important role in the targeting of neuronal projections in several regions of the nervous system, including topographic maps in the visual system (Cheng et al., 1995; Drescher et al., 1995; Flanagan and Vanderhaeghen, 1998; Hindges et al., 2002; Mann et al., 2002b), hippocamposeptal projections (Yue et al., 2002a), and motor axon pathway selection (Eberhart et al., 2002; Helmbacher et al., 2000). These proteins are also necessary for compartmentalization of cell groups, most notably in the hindbrain (Mellitzer et al., 1999). Eph–ephrin interactions tend to be inhibitory for cell migration and axon outgrowth

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(Drescher et al., 1995; Krull, 1998; Xu and Wilkinson, 1997), although recent studies suggest that in some cases these interactions are attractive (Hindges et al., 2002; Holmberg et al., 2000; Mann et al., 2002b).

We previously examined the spatiotemporal pattern of expression of several members of the Eph family during development of the chick auditory brainstem nuclei (Cramer et al., 2000b, 2002). The EphA4 RTK was expressed in a particularly intriguing expression pattern. At ages when NM axons have arrived near NL and begin to form synaptic connections in NL (Jackson et al., 1982; Saunders et al., 1973; Young and Rubel, 1986), EphA4 is asymmetrically expressed in NL dendritic regions; during this limited embryonic period, expression in the dorsal dendrites far exceeds that in the ventral NL dendrites. These results led to the hypothesis that EphA4 is necessary to establish segregated projections from ipsilateral and contralateral NM to dorsal and ventral NM dendrites, respectively. In this study, we tested this hypothesis by misexpressing EphA4 and by blocking EphA4 signaling to determine whether EphA4 activity has a role in the formation of bilaterally segregated inputs to NL.

Methods

In ovo electroporation

Regions of the hindbrain that give rise to NL (Cramer et al., 2000a) were targeted for transfection. E2 embryos were windowed and a 30% solution of India Ink in sterile phosphate-buffered saline (PBS) was injected beneath the embryo. A small hole was made in the roof plate overlying the region of rhombomere 5 (r5). Purified plasmid DNA, reconstituted in Tris–EDTA and diluted to 2 μg/μl sterile water, was injected into the neural tube near r5 using a pulled glass micropipette attached to a Picospritzer. Five to 10 pulses of 50-ms duration at 10–15 psi were used. Electroporation was performed using a BTX 830 electroporator attached to etched tungsten electrodes. The positive electrode was placed near the lateral region of r5 and the negative electrode was placed just lateral to the embryo near r5 with 50–200 μm of separation between the electrodes. Electrodes did not contact the embryonic tissue directly. Voltage was applied using three trains of six pulses, each with 50-V amplitude and 20-ms duration. The eggshell was taped closed, and the egg was placed in a 37°C humid incubator for 8 days.

Plasmids

The pMES plasmid vector was used to introduce DNA for transfection via in ovo electroporation (Swartz et al., 2001). This construct contains a chick β-actin promoter, a CMV-IE enhancer, and an EGFP reporter with an internal ribosome entry site (Swartz et al., 2001). Experimental embryos received full-length EphA4 cloned into pMES (Eberhart et al., 2002) or a kinase-inactive dominant-negative EphA4 (Ethell et al., 2001) removed from the pcDNA3 vector by cleavage at flanking *Eco*RI sites and cloned into pMES at the *Eco*RI site within the polylinker region. The kinase-inactive form of EphA4 results in greatly reduced phosphorylation of wild-type EphA4, indicating that it acts as a dominant negative to disrupt EphA4 signaling (Yue et al., 2002a). Control embryos received pMES alone.

Verification of transfection

Transfection was assessed by examination of EGFP fluorescence in dissected brainstems using a fluorescence stereomicroscope and in sectioned material. In addition, we performed immunohistochemistry on some transfected embryos using methods described previously to detect EphA4 expression following electroporation (Cramer et al., 2000b; Eberhart et al., 2000). Tissue was embedded in paraffin or agarose, sectioned, and labeled using a rabbit polyclonal antibody that recognizes the C terminal region of EphA4 (Soans et al., 1994). EphA4 immunolabeling was visualized using a Vector ABC kit or a fluorescent secondary antibody conjugated to Alexa 594 (Molecular Probes, Eugene, OR).

In vitro axon labeling

The method used to determine the trajectories of NM axons in embryos after manipulation of EphA4 signaling was adapted from Young and Rubel (1983, 1986) and is depicted in Fig. 1. E10 chick embryos were removed from the egg and the brainstem was quickly dissected in Tyrode's solution (8.12 g/l NaCl, 0.22 g/l KCl, 1.43 g/l $NaHCO_3$, 0.2 g/l $MgCl_2$, 0.333 g/l $CaCl_2$, and 22 g/l dextrose) infused with 95% O_2/5% CO_2. Rhodamine dextran amine, MW = 3000 (Molecular Probes), was made at 6.25% in a solution containing 0.4% Triton X-100 in PBS. A pulled glass micropipette, broken to about 10 μm, was filled with dye solution and attached by fine tubing to a Picospritzer. The dye was pressure injected using one or two 50-ms pulses at 10 psi into the dorsal midline of the brainstem to label only crossed NM–NL fibers but not ipsilaterally projecting fibers. In some cases, rhodamine dextran was injected into NM on only one side of the brain. The tissue was then immersed in Tyrode's solution continuously perfused with 95% O_2/5% CO_2 for 4–8 h, then fixed for 2–6 h in 4% paraformaldehyde at 4°C. The tissue was rinsed in PBS, cryoprotected in 30% sucrose for several hours, then embedded in OCT medium. Cryostat sections were cut in the coronal plane at 12–14 μm, and sections were coverslipped using Glycergel mounting medium. Alternate sections were counterstained using bisbenzimide to label cell nuclei and assist in the identification of NM and NL. Because of the curve of the brain at the level of the brainstem, what we have termed coronal is synonymous with transverse within this region; this plane of section shows the ipsilateral and

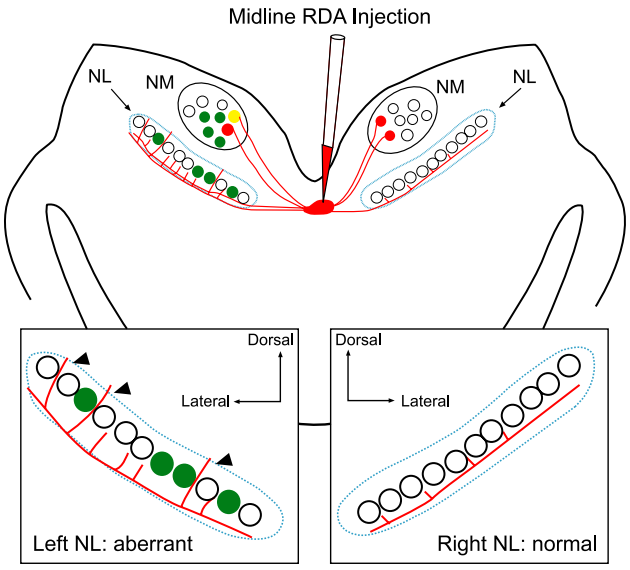

Fig. 1. Schematic diagram illustrating the organization of projections in the auditory brainstem and the method for tracing axonal projections following electroporation. This section is coronal, which is regionally transverse in this area of the brainstem. Green indicates expression of EGFP in an embryo transfected on the left side only. Red indicates the rhodamine dextran injection site at the midline of the brainstem, through which only contralateral NM axons project. Blue dashed lines indicate the boundaries of the NL neuropil dorsally and ventrally. Insets show patterns that would be observed if axonal trajectories are aberrant (left) or normal (right); these drawings summarize the patterns observed in subsequent figures.

contralateral projection areas most clearly. The axes of this plane are shown schematically in Fig. 1.

Data analysis

Embryos were included in the study only if EGFP labeling was extensive throughout NL and if axonal tracing cleanly labeled NM axons that crossed the midline to the contralateral NL. For each section in which NL could be identified, axons that extended beyond the line of NL cell bodies and into inappropriate regions of NL were counted. Axons were counted only when they could be followed without interruption. The mean number of aberrant axons per section was determined for each embryo. Analysis of variance was used to compare mean values for animals treated with EGFP alone, full-length EphA4/EGFP, kiEphA4/EGFP, and EphA4/EGFP in NM but not NL. Pairwise *t* tests were used to evaluate the significance of differences between groups.

Results

Segregated NM–NL projections in control-transfected embryos

Control embryos were transfected with plasmids containing EGFP alone to ascertain that electroporation did not result in disrupted segregation. Following incubation to E10, when the auditory brainstem nuclei can be identified and axons are normally in their appropriate locations within NL, EGFP labeling was examined within the auditory nuclei and brainstem tissue was labeled so that the trajectories and terminations of NM axons could be identified within NL.

Control embryos transfected with plasmids containing EGFP alone showed normal segregation between ipsilateral and contralateral NM–NL projections. Axons were labeled using injection of RDA at the midline so that only contralateral projections would be labeled, as ipsilateral projections do not traverse through this region (Fig. 1). At E10, EGFP transfection was extensive through the auditory brainstem (Fig. 2a) and labeling was evident in many NL cell bodies and in NM neurons, including their axonal projections (Fig. 2b). NL cells at E10 are surrounded by a neuropil zone free of neuronal and glial cell bodies. In control embryos, contralateral NM axons could be followed to the ventral region of the NL neuropil (Fig. 2c). Axons occasionally extended into the cell body layer of NL, but rarely exceeded the cell body zone to enter the dorsal neuropil, as in the right side of Fig. 1. The relationship between axonal trajectories and the line of cell bodies in NL was examined using bisbenzimide labeling (Fig. 2d). Axons that extended into inappropriate territory in bisbenzimide-counterstained sections were counted throughout the rostrocaudal extent of NL in sections where NL could be identified by its laminar structure and cell-free zone (n = 28 sections). Twelve embryos were transfected with EGFP alone in this control group; of these six had sufficient axonal label to evaluate axonal trajectories. The mean number of NM axons (±standard deviation) that terminated in inappropriate regions of NL was 1.5 ± 1.2 axons per section or about 40.6 axons throughout NL.

Targeting errors in embryos transfected with EphA4

In ovo electroporation was used to introduce either full-length EphA4 for misexpression experiments or kinase inactive EphA4 (kiEphA4) to disrupt EphA4 signaling into the developing auditory brainstem at E2, when the embryo is accessible and when the positions of the precursors for the auditory brainstem nuclei are known (Cramer et al., 2000a). Plasmids (described in Methods) also encoded an EGFP reporter. To evaluate the role of EphA4 in guidance of NM axons, we examined the projection patterns of individual NM axons in NL from embryos transfected with full-length EphA4. We verified that EGFP expression was correlated with EphA4 immunolabeling (Figs. 3a, b) and that EphA4 expression was abnormal after ectopic expression. In these experiments, transfected brainstem tissue was sectioned and immunolabeled with an antibody specific for EphA4. Fig. 3a shows a transfected NL neuron with EGFP expression as well as

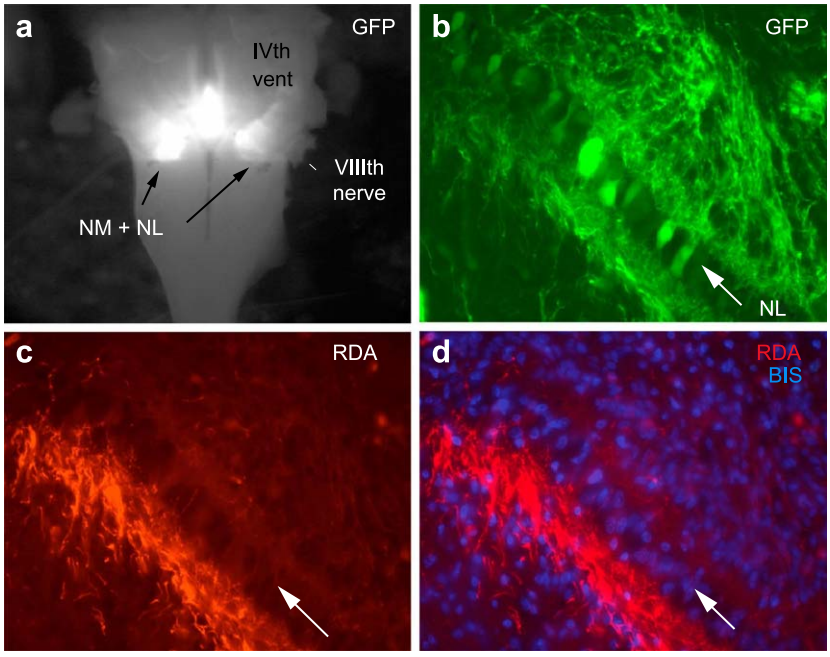

Fig. 2. Axons projecting from NM to NL are restricted to appropriate regions of the neuropil in control-transfected embryos. (a) An embryo at E10 following transfection with EGFP at E2. Bright areas demonstrate sustained GFP expression in NM and NL. (b) The same embryo was cryosectioned at 12 μm in the coronal plane. EGFP is expressed in the auditory brainstem, with expression prominent in NL cell bodies and neuropil as well as axons originating in NM. Arrow indicates line of NL neuronal cell bodies. (c) Pattern of labeling following placement of rhodamine dextran amine (RDA) into the midline in the same section as that shown in (a). Axons arising in contralateral NM are restricted to a region ventral to the line of cell bodies in NL. (d) RDA-labeled axons shown together with bisbenzimide (BIS) fluorescence to show the relationship between axon terminations and nuclei NL cells.

EphA4 protein, which is expressed in both dorsal and ventral neuropil in this cell. Fig. 3b shows a field of transfected brainstem cells, illustrating that EphA4 is expressed in EGFP-labeled cells. The distribution of these proteins is slightly different, with EGFP more concentrated in the cell body and EphA4, a transmembrane protein, localized to the plasma membrane of the somata as well as extensively throughout neuronal processes. These data demonstrate that EGFP expression reliably reports misexpression of EphA4. Transfection was observed extensively in NL neurons (Fig. 3c) as well as in NM neurons and axons (Figs. 3c, d).

Embryos transfected to misexpress EphA4 (n = 38) were evaluated for axonal trajectories when NL morphology was intact (see below) and when rhodamine labeling successfully filled axons from contralateral NM. In embryos included in the axonal analysis (n = 10), many more axons traversed the line of cell bodies in NL compared to control embryos described above; these appeared similar to the left NL in Fig. 1. The mean number of misrouted axons was 5.1 ± 2.9, or 142.8 throughout NL. Individual axons were counted in relationship to the position of cell bodies in NL (Figs. 3e–g). In addition, in some cases, clusters of NL axons were observed within gaps in NL cell bodies (Fig. 3e, asterisk). The growth of axons across the line of NL and the presence of terminal arbors in the inappropriate compartment of NL neuropil suggest that these aberrantly growing NM axons terminate on inappropriate dendrites of NL cells.

While most embryos were transfected bilaterally within the auditory brainstem nuclei, a subset of embryos was focally transfected only on the left and showed EGFP expression only in the left NM and NL. In these cases, the contralateral side served as a control, and axonal segregation patterns were evaluated both on the transfected side and on the untransfected control side. Fig. 3f shows axonal labeling in one such embryo on the transfected side, while the contralateral, untransfected side is shown in Fig. 3g. Contralateral axons are disorganized and readily enter into the dorsal, ipsilateral recipient zone of the NL neuropil (Fig. 3f), while in the untransfected side axonal projections terminate in the ventral region appropriate for contralateral axons (Fig. 3g). The mean number of misrouted axons on the transfected side was 6.5 ± 1.2 (SEM; n = 4), while the mean number of misrouted axons on the control side was 3.3 ± 1.6; this difference was statistically significant ($P < 0.005$, paired t test). Thus, misexpression of EphA4 results in targeting errors on the transfected side.

Misexpression of EphA4 produces errors only when NL cells are transfected

The effects of EphA4 gain-of-function on NM axon targeting could be explained by changes in the distribu-

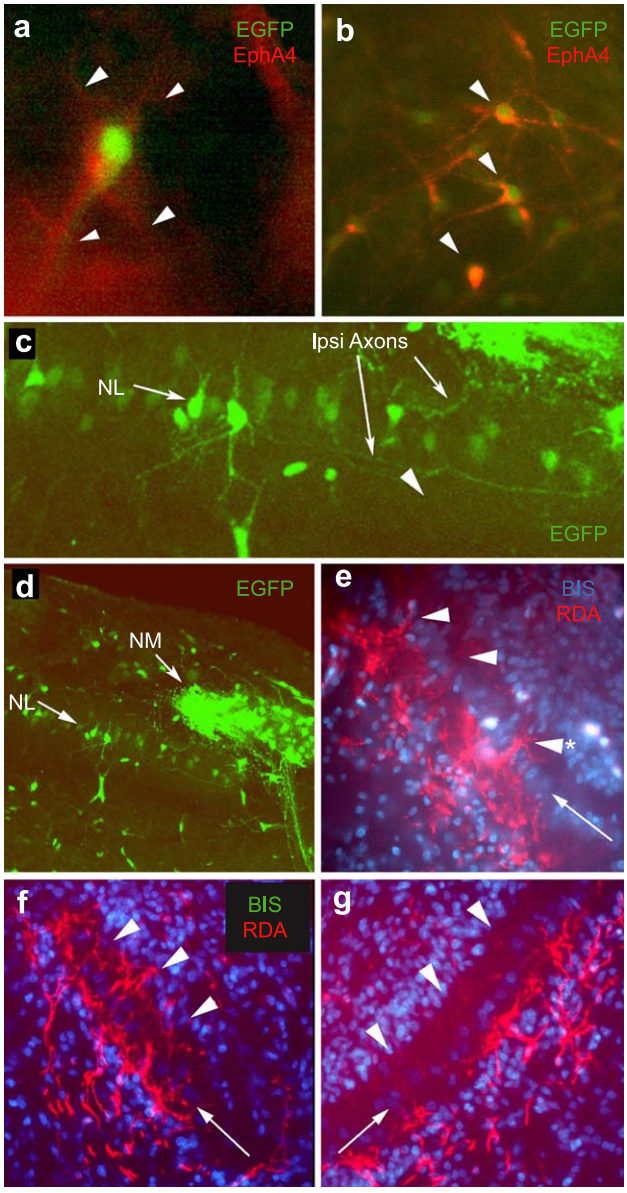

Fig. 3. Overexpression of EphA4 in the auditory brainstem results in errors in the projections of NM axons in NL. Panels (a) and (b) show that EGFP-expressing neurons also express EphA4. (a) A transfected NL neuron with EphA4 immunoreactivity. Expression is observed in both dorsal and ventral dendrites of this neuron (arrowheads). (b) A field of transfected neurons within the brainstem. EphA4 immunolabeling coincides with EGFP expression. Arrowheads indicate cell bodies of transfected neurons. (c) Extensive transfection is observed within NL neurons. This field also shows ipsilateral NM axons with EGFP expression. (d) Transfection is seen in both NM and NL, with abundant NM cell body and axonal transfection. (e) Misexpression of EphA4 results in disrupted axonal targeting. Merged image with RDA labeling and bisbenzimide showing axons in relation to cell body line in NL. Axons appear disorganized and extend into the dorsal neuropil region of NL. NM axons grow across the cell body line into inappropriate regions of NL (arrowheads). Bundles of axons grow past the line of NL cell bodies (asterisk). (f and g) Two sides of a section through a brainstem that was transfected only on the left side. Arrow indicates line of cell bodies in NL. Arrowheads indicate the dorsal border of the dorsal neuropil region. On the transfected side (f), labeled contralateral NM axons are seen in this dorsal region; the control, untransfected, side (g) contains labeled contralateral NM axons restricted to the appropriate ventral neuropil region.

tion of EphA4 in NL neurons or by misexpression of EphA4 in NM axons, which do not normally express any EphA4 (Cramer et al., 2000b). To evaluate the latter possibility, we examined the axonal projection patterns of embryos transfected in NM but not in NL. Fig. 4a shows a section with transfected neurons in NM but not in NL. The pattern of RDA labeling from the midline is shown together with EGFP in Fig. 4b. Importantly, targeting errors were not seen. The mean number of mistargeted axons per section was 0.59 ± 0.07 (SEM; n = 3). Thus, ectopic expression of EphA4 resulted in a significant increase in the number of targeting errors when NM and NL were transfected but not when NM alone was transfected. These results suggest that alterations in EphA4 signaling within NL neurons are important for targeting of NM axons, suggesting that the action of this protein is non-cell autonomous.

Another way to evaluate the issue of cellular specificity is to examine the spatial relationship between targeting errors and transfected or non-transfected neurons. Axons that made targeting errors were seen in proximity to transfected NL neurons, even when few NL neurons misexpressed EphA4. Figs. 4c, d show an example of a section with a single NL neuron expressing EGFP (large arrowhead). An RDA-labeled axon projects aberrantly to the inappropriate region of the neuropil (small arrowhead). Most of these incorrect axon projections were seen within a cell diameter of a transfected NL neuron, again suggesting that these NM axons respond to cues from transfected NL neurons.

Targeting errors in embryos transfected with kiEphA4

To evaluate whether targeting of NM axons requires signaling through the EphA4 RTK, we used a loss-of-function approach. In this group of embryos, electroporation was used to introduce a plasmid containing a kinase-inactive form of the protein (kiEphA4). Kinase-inactive constructs have a dominant negative action and reduce phosphorylation in response to ligand (Ethell et al., 2001; Yue et al., 2002b). When embryos expressing kiEphA4 in the auditory brainstem nuclei were examined at E10, large clusters of axons were seen traversing NL cell bodies (Figs. 5a–c). A total of nine embryos were successfully transfected with kiEphA4; axons could be counted in five of these embryos. The mean number of individual axons that were misrouted (±standard deviation) was 6.6 ± 2.1, or 185 axons throughout NL. The disruption of EphA4 signaling with kiEphA4 and misexpression of EphA4 both produced significant increases in the number of misrouted axons ($P < 0.005$; ANOVA). Transfection with kiEphA4 and EphA4 produced significantly greater errors than either control transfection or transfection with EphA4 in NM only (Fig. 5d). These results suggest that misexpression of EphA4 and kiEphA4 produces errors in targeting of NM axons to regions within NL neurons.

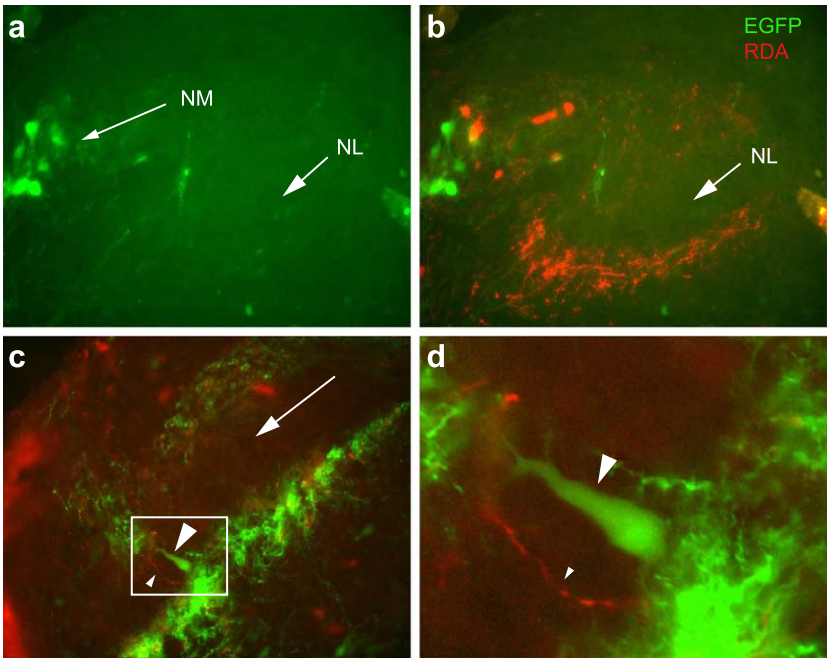

Fig. 4. Targeting errors are not seen when NM but not NL is transfected with EphA4. (a) EGFP shows that cells in NM but not in NL are transfected. (b) RDA labeling from the midline of the brainstem, shown together with EGFP, does not reveal an increase in targeting errors. No errors are seen in this section. Arrow indicated the location of the line of neuronal cell bodies in NL. Targeting errors tend to occur near transfected NL neurons. (c) A single transfected neuron is observed in this section (large arrowhead). An RDA-labeled axon (small arrowhead) traverses the line of cell bodies (indicated by the arrow) into the inappropriate (dorsal) region of the NL neuropil. An EGFP-labeled axon is also seen in the field, but this axon could not be followed to the dorsal region of the NL neuropil. (d) An enlarged view of the highlighted area from (c).

Targeting errors occur near NL neurons misexpressing EphA4

We quantified the rate at which mistargeted axons grow near transfected neurons in four embryos expressing a dominant-negative EphA4 construct. This construct contains a deletion in the cytoplasmic domain and produces a significant increase in axon targeting errors. Embryos with 50% or less of NL cells transfected were included. We found that 71.6 ± 14.9% (SD; n = 4 embryos) of mistargeted axons were within one cell diameter of a transfected NL neuron. Figs. 6a, b show errors in a section with several transfected NL neurons; arrowheads indicate aberrant axons growing near transfected cells. Figs. 6c, d show a section with a single transfected NL neuron; here the only axon growing into the inappropriate part of NL is near the transfected neuron. We also examined axons in control transfected embryos. While these had very few aberrant projections, we evaluated whether these errors were found near EGFP-transfected NL neurons. In contrast to embryos misexpressing EphA4, in two control transfected embryos with about 50% of NL cells transfected, only 36.7 ± 4.7% of mistargeted axons coursed near transfected NL neurons (data not shown). These results suggest that in our experiments, errors in NM axon targeting specifically result from misexpression of EphA4 in NL neurons.

Role for EphA4 in NL morphology

Our quantitative analyses of the experimental cases were very conservative and are likely to be an underestimate of the actual degree of terminal arbor misrouting. In this analysis, we did not include the large fascicles of axon branches passing through the cell body lamina of NL because individual axons in these fascicles could not be resolved. Notably, large fascicles of misrouted axons coursing through the cell body lamina of NL are not seen in normal embryos or hatchlings (Young and Rubel, 1983; Young and Rubel, 1986) or in control-transfected embryos.

To evaluate the effects of experimental changes in EphA4 signaling on the overall morphology of NM and NL, the morphology of the nuclei was examined using bisbenzimide labeling. None of the EGFP-transfected control embryos exhibited gross morphological abnormalities or changes in NL morphology (Fig. 7a). However, misexpression of full-length EphA4 resulted in gross abnormalities in 11% of transfected embryos (Fig. 7b), and disruption of EphA4 signaling with kiEphA4 resulted in abnormalities in 55% of transfected embryos (Figs. 7c, d). Extensive abnormalities of the hindbrain included reduced brainstem size, reduced extent of the IVth ventricle, and changes in shape of the auditory region of the brainstem. In NL, the abnormal morphology included a disorganized

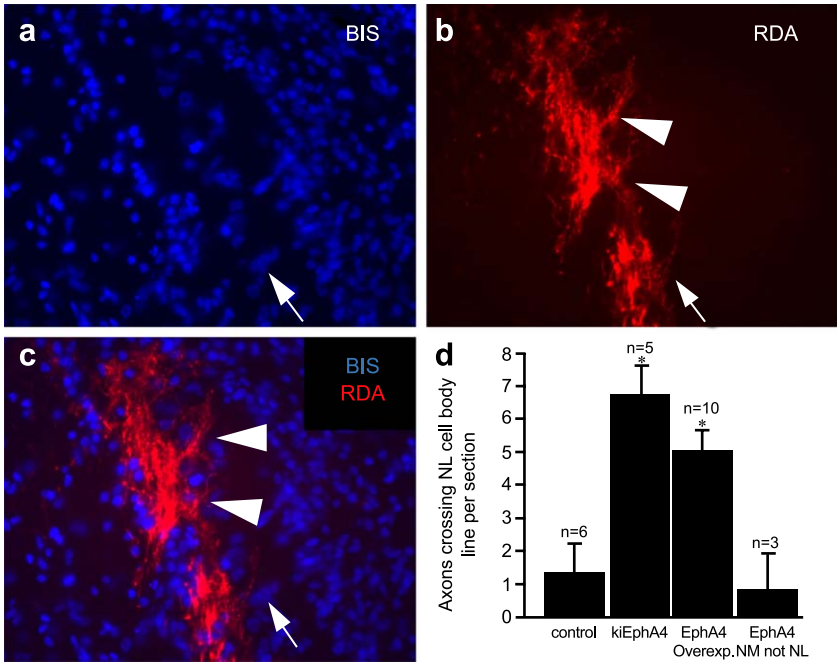

Fig. 5. Expression of kiEphA4 results in an increase in the number of axons that grow past NL cell bodies into the inappropriate compartment of NL neuropil. (a) Bisbenzimide labeling to indicate the region of NL cell bodies (arrow). (b) Same section as (a). RDA labeling reveals extensive mistargeting of axons and several large fascicles. (c) Merged image demonstrates the relationship between labeled NM axons and growth across NL. The fascicles are predominantly found in NL regions with gaps in the line of cell bodies. (d) Histogram showing the number of axons per section that grow across the NL cell body lamina into the opposite neuropil region under each experimental condition. Error bars denote SEM. ANOVA indicates that the number of mistargeted axons varies with misexpression condition ($P < 0.0005$). Pairwise t tests show that EphA4 and kiEphA4 produce significant increases in the number of axons per section that grow across the NL cell body lamina ($\pm$SEM) into the inappropriate neuropil region in comparison to control transfection with EGFP. Transfection with EphA4 in NM but not NL does not result in an increased number of targeting errors. * Denotes significantly different from controls ($P < 0.005$ for kiEphA4, $P < 0.05$ for EphA4 overexpression).

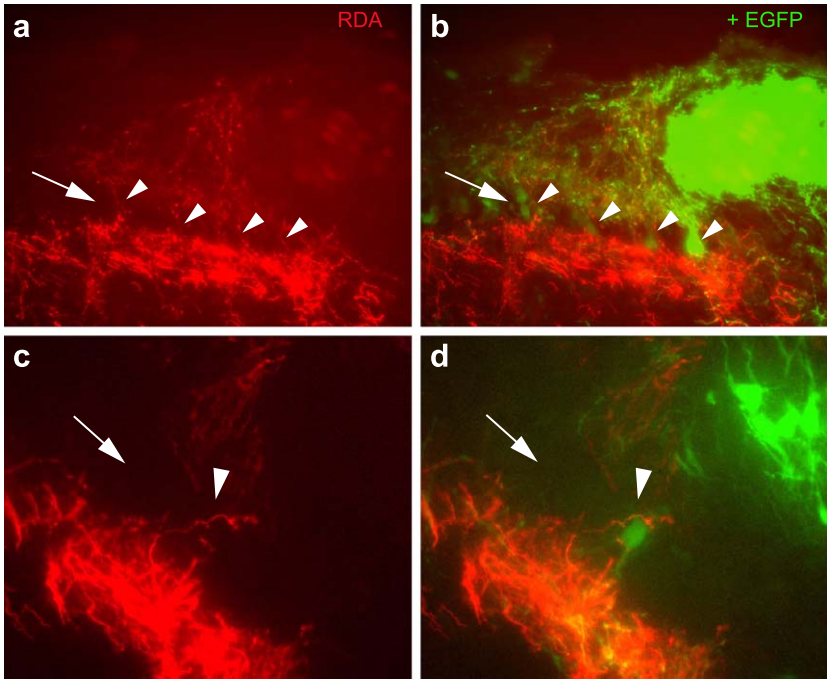

Fig. 6. Axon targeting errors tend to occur within a soma diameter of an NL neuron misexpressing EphA4. (a) Axon targeting errors (arrowheads) in an embryo expressing a dominant negative form of EphA4 lacking a region of the cytoplasmic domain. (b) The same field showing expression of EGFP. Targeting errors are near transfected NL neurons (arrowheads). (c) A different example showing a single mistargeted axon (arrowhead). (d) The same field showing that this axon courses adjacent to an NL neuron misexpressing EphA4/EGFP.

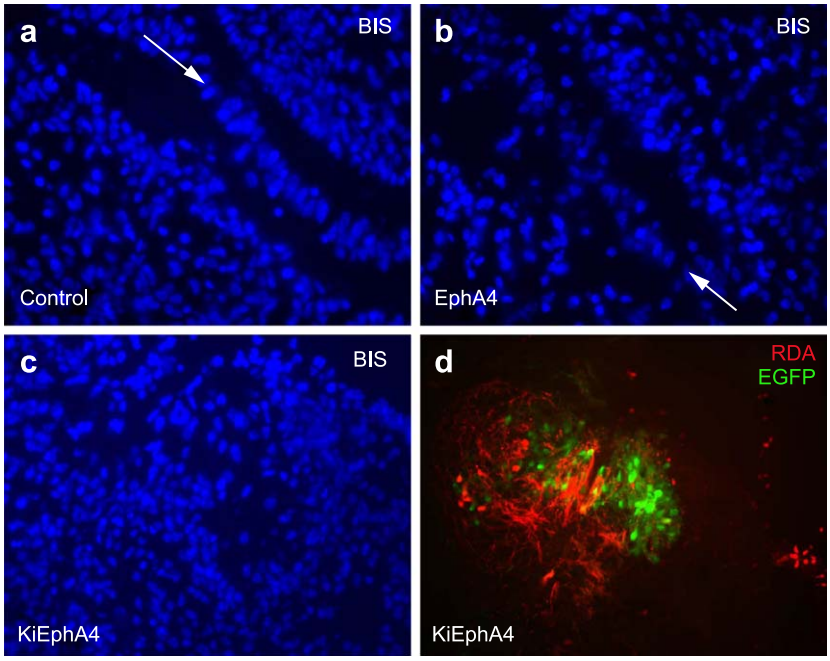

Fig. 7. Morphological changes in NL are seen after misexpression or loss-of-function of EphA4. (a) Bisbenzimide labeling in a control EGFP-transfected E10 embryo. Cell bodies within NL (arrow) are arranged in a sheet and cells appear tightly packed, and a cell-free space is clearly delineated dorsally and ventrally. (b) In some EphA4-misexpressing embryos, NL cells appeared disorganized, more sparsely packed (arrow), and the margins of the cell-free zone are less evident. (c) A kiEphA4-treated embryo with severe disruption of auditory nuclei morphology. NL cannot be identified within this bisbenzimide-labeled section. (d) The same section as (c) showing the location of EGFP-labeled cells in the region of the auditory nuclei and the disorganization of RDA-labeled axons (red) from the contralateral side.

array of NL cells (Fig. 7b) or, in the most pronounced cases, NL could not be identified and there was no identifiable cell-free zone (Fig. 7c). In these cases, it was not possible to quantify the number of axons that terminated in inappropriate zones of NL neuropil, but large groups of axons clustered within the region of the auditory brainstem nuclei (Fig. 7d); these axons occasionally extended beyond the dorsolateral region of the brainstem. These defects suggest that EphA4 has a role in the migration of NL cells from the auditory anlage or in the organized clustering of NL cell bodies within the lamina.

The disorganization of NL cells induced by EphA4 misexpression may be involved in the misrouting of NM axons in some cases; aberrantly growing axons clustered in regions where NL cells left gaps in the line of cell bodies. However, morphological abnormalities in NL cellular organization cannot account for all of the misrouting of NM axons, as errors of axon trajectories were commonly observed in gain-of-function or loss-of-function cases in which NL cells had a normal morphology.

Discussion

In this study, we have tested the hypothesis that EphA4 has a role in restricting axonal projections to distinct regions of NL neurons. We found that misexpression of EphA4 and expression of a kinase-inactive form of EphA4 result in a highly statistically significant 5-fold increase in the number of axons that appear to terminate in inappropriate regions of NL cells compared to control-transfected embryos. The numerical extent of this misrouting is a conservative estimate, as only axons that could be resolved throughout their trajectory are included. Large clusters of axons with aberrant trajectories (Figs. 2b and 3b, c) were often observed in the experimental tissue, but never observed in control-transfected or normal tissue. The axons in these clusters could not always be resolved and followed individually and thus were counted as fewer axons than they likely contained. In addition, electroporation resulted in the transfection of many, but never all, auditory neurons and did not involve all regions of NL in any of the brains examined. The results of this study support a role for EphA4 in the formation of segregated projections from NM axons to NL, indicating that Eph receptor signaling directs axons to appropriate subcellular compartments, or appropriate dendrites, within their target cells.

The effects of misexpression with EphA4 were similar to those observed after disruption of EphA4 signaling with kiEphA4. Several different mechanisms could account for this similarity. One possibility is that the asymmetry of EphA4 activity in NL cells is necessary for segregation; both perturbations decrease the difference in EphA4 activity between the dorsal and ventral dendrites. A second possibility is that misexpression of EphA4 within NM axons (which do not normally express EphA4) may promote

misrouting of these axons. The mechanisms for this misrouting may depend on interactions between the misexpressed EphA4 and ephrin-B2 within the axons; this binding might make axons less responsive to other Eph receptors in the target. Our data do not support this second possibility; when NM, but not NL, was transfected, we did not see a significant increase in NM axon targeting errors compared to controls. However, the possibility remains that misexpression within NM axons has an effect when NL is also transfected; experiments limiting transfection to NL will be required to evaluate this possibility rigorously.

A third possibility is that the effects on NM axons occur strictly through an attractive, reverse signaling mechanism (Holland et al., 1996). In this case, both constructs produce additional extracellular EphA4 moieties, and these proteins would increase the signal to ephrin-B2 on NM axons. Because misexpression results in an increase in axon growth, this mechanism would require that EphA4-ephrin-B2 signaling be attractive rather than repulsive. Our data are consistent with a reverse signaling mechanism because misexpression within the target NL neurons produces a change in NM axon trajectories; moreover, aberrantly growing axons are usually seen near transfected NL neurons. However, other Eph family members are expressed in the NL neuropil and NM axons (Cramer et al., 2002); these may operate using forward signaling. Thus, while the present study demonstrates a role for EphA4 in binaural segregation, it is likely that this role is complex and depends on distinct types of signaling among several members of this family.

Interactions between Eph receptors and their ligands have an important role in guiding axons to appropriate regions within a target. For example, growth cones from thalamic axons collapse and form synaptic terminations specifically in response to a signal from cortical layer 4 neurons (Bolz et al., 1992; Molnar and Blakemore, 1991). This axonal guidance is mediated by ephrin-A5 (Mann et al., 2002a). In addition, ephrin-A3 regulates targeting of entorhinal cortical neurons to the appropriate layer of the dentate gyrus (Stein et al., 1999). In these examples, axons are targeted to distinct cell layers. In this study, we have examined mechanisms for axon targeting within a pathway whose layers are formed by subsets of dendrites. The role of these proteins in axonal targeting may thus extend to the subcellular localization of synaptic connections.

Patterns of connections in the auditory brainstem are extraordinarily precise and form the basis for perception of auditory stimuli based on small differences in timing. While deafferentiation studies have resulted in disrupted binaural segregation in NL (Rubel et al., 1981) and medial superior olive (MSO) (Kitzes et al., 1995) as well as alterations in the organization of inhibition in MSO (Kapfer et al., 2002), no previous perturbation has disrupted segregation in the initial development of cochlear nucleus projections to NL or MSO. The molecules that establish this robust precision have not previously been characterized. The results presented here demonstrate an important role for EphA4 in directing axons to appropriate regions within targets during the formation of neural circuits in the brainstem. Moreover, these results demonstrate the functional significance of Eph receptor expression in auditory brainstem pathways. Because Eph family proteins have a role in the formation of visual system pathways, these proteins may be important for the formation of neural circuits across several sensory modalities.

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