

# Timing and Topography of Nucleus Magnocellularis Innervation by the Cochlear Ganglion

DAVID MOLEA<sup>†</sup> AND EDWIN W RUBEL<sup>\*</sup>

Virginia Merrill Bloedel Hearing Research Center, Department of Physiology and Biophysics, Department of Otolaryngology-HNS, University of Washington, Seattle, Washington 98195-7923

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## ABSTRACT

This series of experiments examined the arrival and organization of cochlear nerve axons in the primary auditory brainstem nucleus, nucleus magnocellularis (NM), of the chick. DiI and DiD were injected into the cochlear nerve, cochlear ganglion, and basilar papilla (i.e., avian cochlea) in fixed tissue and labeled axons were studied in NM and its vicinity. Cochlear nerve axons first penetrate NM between stages 29 (E6) and 36 (E10). Axons penetrate NM in a middle-to-posterior-to-anterior developmental sequence; the anterior, high-frequency region of NM receives axons last. When cochlear nerve axons arrive in the NM, they are already organized in a topographic map related to the position of their cell bodies along the basilar papilla, foreshadowing the tonotopic mapping observed between NM and the basilar papilla later in development. Evidence of a topographic map was also observed in the other primary auditory brainstem nucleus, nucleus angularis. These results indicate that topographic mapping of position (and ultimately characteristic frequency) between the basilar papilla and NM is established as cochlear nerve axons arrive in the NM prior to the onset of synaptic activity. *J. Comp. Neurol.* 466:577–591, 2003. © 2003 Wiley-Liss, Inc.

**Indexing terms:** chicken; cochlea; cochlear nerve; cochlear nuclei; embryo; tonotopic map

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One of the characteristic features of the nervous system, especially sensory and motor systems, is the precise, topographic connections between levels. The development of these topographic connections has been a major focus of research, a large part of which has focused on connections within the visual system and on the role of activity in the formation and refinement of topographic maps (Sperry, 1963; Udin and Fawcett, 1988; Goodman and Shatz, 1993; Holt and Harris, 1993; Mize and Lo, 2000; Katz and Crowley, 2002). Research on the initial formation of topographic maps in other sensory systems suggests that the maps are already established prior to the onset of activity (Young and Rubel, 1986; Udin and Fawcett, 1988; Agmon et al., 1995; Holt and Harris, 1998; Wang et al., 1998; Friauf and Lohman, 1999). In the somatosensory system, for example, the topography of thalamocortical projections develops before the synaptic activity of these connections (Dawson and Killacky, 1985; Agmon et al., 1995). In the chicken auditory system, the topographic connections between the primary and secondary auditory brainstem nuclei, nucleus magnocellularis (NM), and nucleus laminaris (NL), respectively, are established prior to the formation of functional synapses (Jackson et al., 1982; Young and

Rubel, 1986). Even in the visual system, topographic connections appear to be established in the absence of activity as the retinotectal projection grows into the tectum (Sperry, 1963; Fraser and Perkel, 1989; Holt and Harris, 1993) and as geniculocortical connections form ocular dominance columns (Crowley and Katz, 1999, 2000; Crair et al., 2001; Katz and Crowley, 2002). Thus, the common pattern of development seems to be that the topography of connections is established as the axons invade their targets (Rubel and Cramer, 2002).

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<sup>†</sup>Dr. David Molea's current address is Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI.

<sup>\*</sup>Correspondence to: Edwin W Rubel, Virginia Merrill Bloedel Hearing Research Center, University of Washington, Box 357923, Seattle, WA 98195-7923. E-mail: rubel@u.washington.edu

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The topographic representation of frequency is well established at all levels of the auditory system, from the cochlea to the cortex (Tunturi, 1952; Woolsey, 1960; Rose et al., 1960, 1963; Manley, 1970, 1971; Aitkin and Webster, 1972; Merzenich and Brugge, 1973; Pritz, 1974a,b; Rubel and Parks, 1975; Zaretsky and Konishi, 1976; Echteler, 1985; Feng, 1986; Irvine, 1986; Carr, 1992; Carr and Code, 2000). A one-dimensional frequency map along the mammalian cochlea and avian basilar papilla (i.e., the avian cochlea) is transferred through the subsequent levels of the auditory system by precise topographic connections. Although a great deal of research has focused on the role of experience in the refinement and stabilization of connections in sensory systems, few data are available in any species on the development of the initial topography of projections at the first level of the auditory system, i.e., from the cochlea to the brainstem. Leake et al. (2002) recently reported that connections between the base of the cochlea and the cochlear nuclei are already established in the neonatal cat at or before birth, prior to the onset of spontaneous activity in the auditory nerve.

In the chicken, frequency is mapped along the length of the basilar papilla from low frequency (LF) at the distal end (apex) to high frequency (HF) at the proximal end (base; von Békésy, 1960; Rubel, 1978; Rubel and Ryals, 1983; Ryals and Rubel, 1985; Manley et al., 1987, 1989, 1991; Chen et al., 1994; Jones and Jones, 1995). This frequency map is reestablished in the primary auditory brainstem nuclei, NM and nucleus angularis (NA), by the topographic connections of VIIIth nerve axons. In NM, the frequency map is oriented from posterolateral (LF) to anteromedial (HF; Fig. 1; Rubel and Parks, 1975), whereas in NA the map is oriented from ventral (LF) to dorsal (HF, Fig. 1; Warchol and Dallos, 1990).

The purpose of this study was to determine when cochlear nerve axons first penetrate NM and if a tonotopic organization of the projection is already established when they arrive. By injecting lipophilic neuronal tracers (e.g., DiI) into the cochlear nerve or into discrete regions of the cochlear ganglion and basilar papilla, we determined when and where the central processes of cochlear ganglion cells penetrate the NM in chick embryos from stage 25 (E4.5) to stage 38 (E12). The results indicate that: 1) cochlear afferents penetrate the NM between stages 29 (E6) and 36 (E10); 2) a topographic pattern appears to be present when the axons arrive; and 3) the temporal pattern of axon penetration into the NM does not follow a "basal-to-apical" order.

## MATERIALS AND METHODS

Fertilized white leghorn chicken eggs (*Gallus domesticus*) were purchased from H&N International (Redmond, WA) and incubated at 38°C in a humidified, forced-draft incubator. Embryos were staged according to Hamburger and Hamilton (1951). Developmental age of the embryo will be referred to by both the stage and the corresponding age appropriate to the Hamburger and Hamilton staging, e.g., stage 30 (E6.5). A total of 155 embryos (stages 25 [E4.5] to 38 [E12]) were used in this investigation, 95 of which yielded 214 usable injections (summarized in Table 1). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Wash-

## Fixation and basilar papilla injections

The carbocyanine dyes used for injections, CM-DiI (C-7000, Molecular Probes, Eugene, OR) and DiD (D-7757, Molecular Probes) were prepared according to Krull and Kulesa (1998). Embryos were fixed by direct immersion in 4% paraformaldehyde/2% glutaraldehyde for 4 hours to 43 hours at 4°C, washed, and staged. The cranium and ventricular systems of older embryos were opened to allow fixative better access to the brain. The basilar papilla and cochlear ganglion were then exposed, taking care to leave the acoustic-vestibular ganglion (AVG) and its connections to the brainstem intact. CM-DiI or DiD were then injected into the basilar papilla, cochlear ganglion, or cochlear nerve as follows. Micropipettes were pulled on a Brown Flaming micropipette puller, broken to a tip diameter of ~20–30 µm, and filled with either 0.5 mg/ml CM-DiI or 0.5 mg/ml DiD. The dyes were injected through the basilar papilla into the cochlear ganglion or into the cochlear nerve using a micromanipulator and a Picospritzer (General Valve, East Hanover, NJ). All injections were monitored and documented using a Leica MZFLIII fluorescent dissecting microscope and a Sony three chip CCD color video camera connected to a Macintosh computer running NIH Image (v. 1.62). After one or more injections into the cochlear ganglion or cochlear nerve, the embryos were incubated in fixative at 37°C for a minimum of 9 days to allow diffusion of the dyes. The majority of embryos (94%) were incubated for more than 20 days. We found that 9 days, however, was sufficient to label cochlear nerve axons and terminals in both NM and NA in stage 37 (E11) embryos.

After the incubation period, the basilar papilla with the cochlear ganglion attached and the brainstem were dissected. The basilar papilla and cochlear ganglion were examined using standard fluorescent or confocal microscopy to assess the effectiveness of the injection. If an injection failed to label ganglion cells and axons, the tissue was not used in our analysis. Brainstems were embedded in 4% low-melting agarose (Gibco BRL, Grand Island, NY) and serially sectioned using a Vibratome (Vibratome, St. Louis, MO) at 50 or 100 µm per section. All sections were then counterstained with 1% bisbenzimidazole, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and examined using a standard fluorescent microscope or a BioRad (Hercules, CA) MRC-1024 confocal laser scanning microscope running BioRad LaserSharp software, v. 2.1A.

For publication, digital images were transferred to Adobe PhotoShop (v. 4.0–5.5; San Jose, CA). All images are pseudocolored to show DiD injections in red and DiI injections in green.

## Determination of injection size and location

Images of the injection site in the basilar papilla and cochlear ganglion were taken at the time of the injection, and both the apical and basal ends of the basilar papilla were marked on the digital images for later length measurements using NIH Image. The measurements taken of the injection site are defined in Figure 1A. The injection location and size were determined by measuring the length of the basilar papilla ( $L_{BP}$ ) and the distances from the apex to the apical (DAB) and basal (DBB) boundaries of the injection. The distances were normalized to the length of the basilar papilla and expressed as a percentage of the distance from the apex. The apex was used as the

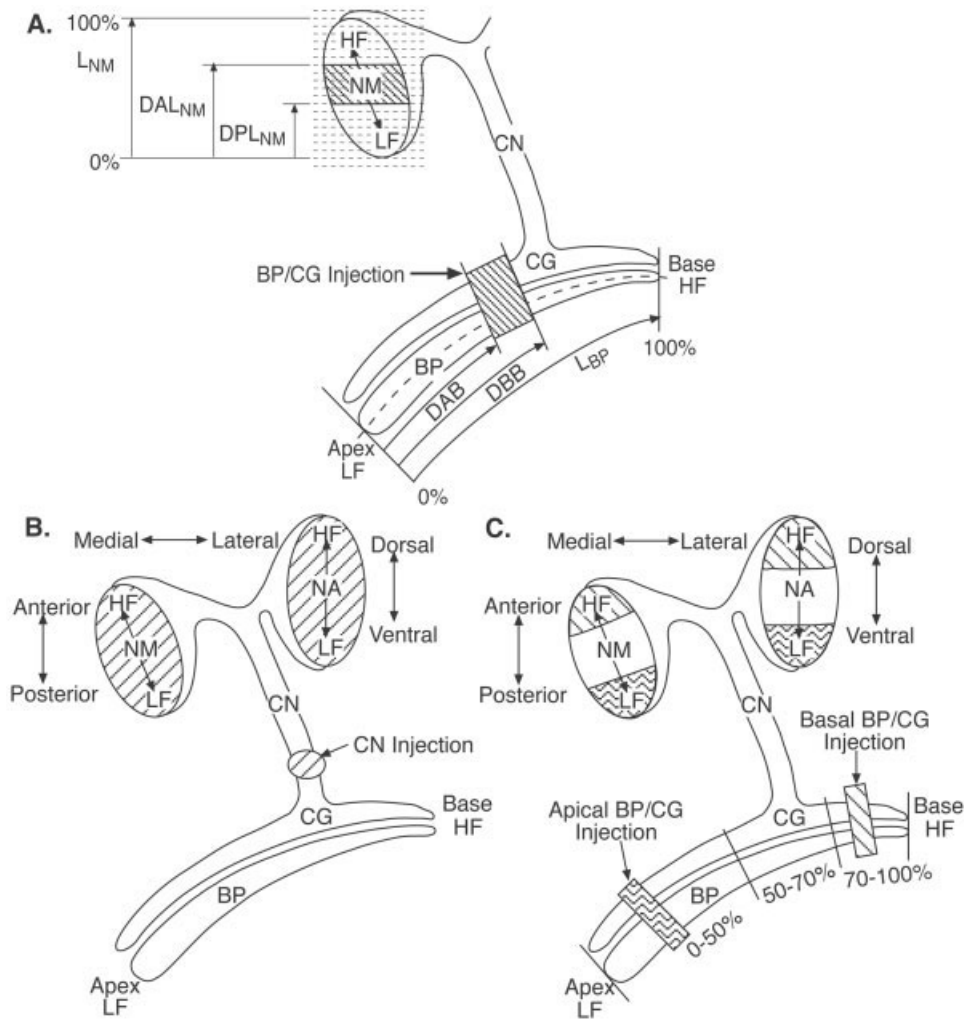


Fig. 1. Definition and measurement of injections and quantification of labeling in nucleus magno-cellularis. Diagrammatic representations of the projection from the basilar papilla (BP) via the cochlear ganglion (CG) and cochlear nerve (CN) to the primary auditory brainstem nuclei, nucleus magno-cellularis (NM), and nucleus angularis (NA). The tonotopic map is shown for both NM (posterior–anterior axis) and NA (dorsal–ventral axis). The division of the basilar papilla into apical (0–50%), middle (50–70%), and basal (70–100%) regions is shown in C. **A:** Parameters designated for quantification of basilar papilla/cochlear ganglion (BP/CG) injections and resultant labeling in NM. The BP/CG injection and resultant labeling are indicated by hatching. The length of the basilar papilla ( $L_{BP}$ ) and the distances to the apical (DAB) and basal (DBB) boundaries of the injection were measured from the apex of the basilar papilla along the longitudinal

midline of the basilar papilla (dashed line). Serial transverse brainstem sections were collected and numbered. The length of NM ( $L_{NM}$ ) and the distance of the anteriormost ( $DAL_{NM}$ ) and posteriormost ( $DPL_{NM}$ ) labeled axons penetrating NM were determined from the posterior boundary of NM. **B:** Cochlear nerve (CN) injections should label axons projecting to all regions of NM and NA as indicated by the hatching. **C:** Apical BP/CG injections are expected to label the posterior region of NM and ventral region of NA and basal BP/CG injections are expected to label the anterior region of NM and the dorsal region of NA. The area of labeling in NM reflects the labeling of fibers of passage passing through the injection site as well as the cochlear nerve axons innervating the injection site. HF, high frequency; LF, low frequency.

reference point because it is more easily visualized and is not undergoing rapid morphological changes during the period of development studied (Cotanche and Sulik, 1985).

**Determination of the size and location of labeling in NM**

The quantification procedure for assessment of the position of labeled axons in NM is also illustrated in Figure 1A. Sections containing NM were identified using autofluorescence or bisbenzimid staining and the posterior-to-anterior length of NM ( $L_{NM}$ ) was then cal-

culated. Sections containing labeled axons penetrating into NM were then identified and the distance from the posterior boundary of NM to the anteriormost ( $DAL_{NM}$ ) and posteriormost ( $DPL_{NM}$ ) label in NM calculated and normalized to the length of NM. These distances are reported as percent distance from the posterior boundary of NM.

By reporting injection position as percent distance from the apex (LF region) and NM labeling as percent distance from the posterior boundary of NM (LF region), we are able to relate the positions to the frequency maps observed

TABLE 1. Summary of Cochlear Nerve (CN) and Basilar Papilla/Cochlear Ganglion (BP/CG) Injections, and Number of Injections Labeling Axons in the Hindbrain, NM, and NA

CN injections									
Stage	Animals	Injections	Hindbrain <sup>1</sup>	%	NM <sup>2</sup>	%	Checked <sup>o</sup>	NA <sup>3</sup>	%
25	1	1	1	100%	0	0%			
26	2	3	3	100%	0	0%			
27									
28									
29	2	3	3	100%	0	0%			
30	1	1	1	100%	0	0%			
31	2	4	4	100%	2	50%			
32	5	5	4	80%	3	60%	2	2	100%
33	7	7	7	100%	6	86%	4	4	100%
34	2	4	4	100%	4	100%	4	4	100%
35	3	3	3	100%	3	100%	3	3	100%
36	3	3	3	100%	3	100%	1	1	100%
37	3	3	3	100%	3	100%	3	3	100%
38									
BP/CG injections									
Stage	Animals	Injections	Hindbrain <sup>1</sup>	%	NM <sup>2</sup>	%	Checked <sup>o</sup>	NA <sup>3</sup>	%
25									
26									
27									
28				50%	0	0%			
29	7	19	14	74%	3	16%			
30	7	13	6	46%	0	0%			
31									
32	5	12	11	92%	5	42%	5	4	80%
33	11	19	16	84%	10	53%	10	8	80%
34	8	13	10	77%	8	62%	10	9	90%
35	12	28	22	79%	16	57%	22	21	95%
36	8	21	21	100%	19	90%	20	20	100%
37	14	34	34	100%	34	100%	30	30	100%
38	6	13	13	100%	13	100%	13	13	100%

<sup>1</sup>Number of injections labeling axons that penetrated the hindbrain.

<sup>2</sup>Number of injections labeling axons penetrating NM or NA, respectively.

<sup>3</sup>Number of injections specifically examined for labeled axons in NA.

later in development and in mature animals (Rubel and Parks, 1975; Lippe and Rubel, 1985).

## RESULTS

Two types of injections were used in this study. In order to label as many cochlear nerve axons as possible from either the base or apex of the basilar papilla, we injected dye into the cochlear nerve as it arises from the basilar papilla and cochlear ganglion (Fig. 1B). In some cases, we also injected dye along the entire length of the basilar papilla and cochlear ganglion. These injections are referred to as CN injections. In order to label a subset of cells and axons related to a particular region of the basilar papilla and cochlear ganglion, dye was injected into discrete bands transverse to the future frequency (apical-to-basal) axis of the basilar papilla and cochlear ganglion (Fig. 1C). These injections are referred to as BP/CG injections because both the basilar papilla and cochlear ganglia are injected. At the ages examined in this study, the cochlear ganglion lies adjacent to the basilar papilla along its entire length so that the length along the basilar papilla corresponds to the length along the cochlear ganglion. A summary of all useable CN and BP/CG injections is shown in Table 1.

Both CN and BP/CG injections labeled afferent axons innervating the basilar papilla, vestibular axons innervating the lagenar macula, and efferent axons projecting from the brainstem and innervating either the basilar papilla or lagenar macula. Previous studies in birds (Boord and Karten, 1974; Code, 1995; Kaiser and Manley,

1996), as well as our own injections into the lagena ( $n = 2$ ; data not shown), indicate that the axons innervating the lagenar macula do not innervate the primary cochlear nuclei, NM and NA, and will not be discussed further in our results. Labeled efferent axons were also observed and follow a separate path through the vestibular part of the AVG and VIIIth nerve root into the brainstem. This path is the same as previously described by Boord (1961) for the cochlear and lagenar efferents in caiman and pigeon. Since others have studied the efferent projection in detail (Boord, 1961; Fritzsche et al., 1993; Simon and Lumsden, 1993; Code, 1995; Fritzsche, 1996; Kaiser and Manley, 1996; Manley and Köppl, 1998), we did not examine it except to see if any efferents sent collateral axons to NM or NA. We did not observe any labeled efferents projecting to NM or NA as a result of our injections and conclude that NM and NA do not receive collaterals from any inner ear efferent projections during the time examined in this study.

### Cochlear nerve axons arrive in NM between stage 29 (E6) and stage 34 (E8)

None of the six injections which labeled VIIIth nerve axons in the hindbrain in stage 25–28 (E4.5–5.5) embryos labeled axons projecting into the region where we would expect to find the anlage of NM and NA. Figure 2 shows axons labeled by a CN injection in a stage 25 (E4.5) embryo. Some of these axons penetrated into the mantle zone of the hindbrain, projecting primarily along a radial trajectory towards the fourth ventricle, and not towards the

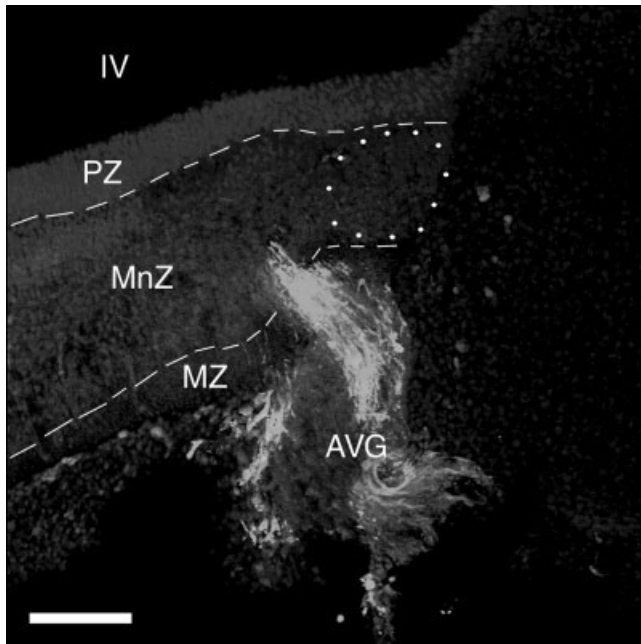


Fig. 2. DiI-labeled axons from a CN injection penetrating the brainstem at the lateral extent of the marginal zone (MZ) in a stage 25 (E4.5) embryo. Note that the axons do not project towards the dorsolateral corner of the brainstem where the auditory anlage develops (dotted outline). Dorsal is up and medial is to the left. AVG, acoustic-vestibular ganglion; IV, fourth ventricle; MnZ, mantle zone; PZ, proliferative zone. Scale bar = 100  $\mu$ m.

dorsolateral corner of the hindbrain where the auditory anlage forms (dotted outline; Cramer et al., 2000a).

Cochlear nerve axons begin to penetrate NM at around stage 29–30 (E6–6.5), the first age when the auditory anlage can be identified in the dorsolateral corner of the hindbrain using standard histological stains (Knowlton 1967; Young and Rubel, 1986; Book and Morest, 1990), bisbenzimidazole, or autofluorescence. Only three of the 24 CN or BP/CG injections which labeled axons in the hindbrain at stage 29–30 (E6–6.5) labeled axons that penetrated NM. Figure 3 shows the results from two BP/CG injections into a stage 29 (E6) embryo. The injections are shown in the inset at the bottom; the apical injection covered 0–50% and the basal injection covered 64–100% of the basilar papilla. Figure 3A–C shows transverse sections through the auditory anlage (dotted outlines) from the anterior (71%, A), middle (57%, B), and posterior (42%, C) levels, respectively. Dorsal is up and medial is to the left. Figure 2B shows that the BP/CG injections labeled axons that penetrated into the middle region (50–64%) of NM just posterior to the VIIIth nerve root. Figures 2A and 2C show that although the injections labeled axons anterior and posterior to the section in Figure 2B, these axons were located ventral and lateral and did not penetrate into the auditory anlage. No labeled axons were observed dorsal to the anlage, where cochlear nerve axons typically penetrate the anterior region of NM at later ages. In addition to cochlear nerve axons, the dense labeling ventral to the auditory anlage probably contains vestibular axons, possibly lagena in origin.

Cochlear nerve axons penetrated throughout NM over a two-day period from stage 29 (E6) to stage 34 (E8). Figure

4A shows that the percentage of CN injections (unfilled bars) that labeled axons penetrating into the parenchyma of NM increased from stage 29 (E6) to stage 34 (E8), when 100% of these injections labeled axons somewhere in NM. Figure 4A also shows that the percentage of BP/CG injections (filled bars) labeling axons penetrating into NM increases as well, but does not reach 100% until stage 37 (E11). Since BP/CG injections label axons projecting from a discrete region of the basilar papilla and cochlear ganglion, this result suggests that there may be regional differences as to when cochlear nerve axons penetrate NM.

### Cochlear nerve axons arrive in NM in a middle-to-posterior-to-anterior sequence

Cochlear nerve axons penetrated the middle region of NM first, then the posterior region, and finally the anterior region of NM (i.e., in a middle-to-posterior-to-anterior temporal-spatial sequence). Figure 4B shows the cumulative spatial distribution of labeled axons penetrating the auditory anlage or NM from all injections at a particular stage. The labeled axons observed in the neuropil of NM at stage 29 (E6) were seen in the middle region of NM around the level of the cochlear nerve root. By stage 32 (E7.5), labeled axons were observed penetrating all but the anterior 12% of NM. Labeled axons were not observed to penetrate the most anterior regions of NM until stage 34 (E8), and only one of the 10 embryos injected at stage 34 (E8) showed labeled axons in the most anterior 10% of NM. The arrival of axons in the anterior region was reliably seen at stage 36 (E10) and later.

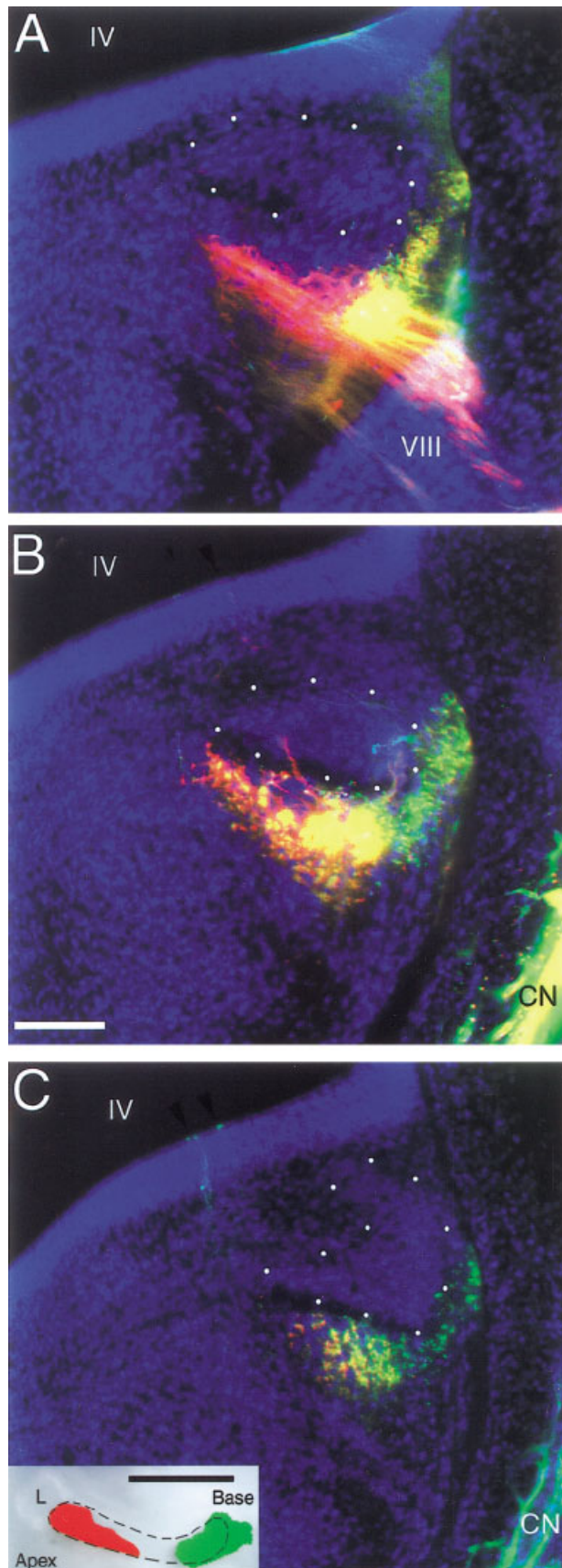
Figure 4C shows the percentage of BP/CG injections that labeled axons penetrating into the parenchyma of NM, characterized by the location of the basal (HF) boundary of the injection in the basilar papilla. Based on the tonotopic organization of NM, this boundary indicates the maximum rostral position of labeled axons in NM that would be expected in the mature system. (For example, an injection with its basal boundary at the 50% position of the basilar papilla should have labeled axons restricted to the posterior 50% of NM.) Figure 4C shows that the percentage of injections into the basal region of the basilar papilla and cochlear ganglion that labeled axons penetrating into the parenchyma of NM (70–100%, hatched bars) lagged considerably behind the percentage of apical (0–50%, unfilled bars) and middle (50–70%, filled bars) injections that labeled axons penetrating into NM. Furthermore, about 40% of basal injections into stage 35 (E8–9) and younger embryos did not even penetrate the hindbrain, even though they labeled cochlear ganglion cells and their axons in the cochlear nerve.

### Topography of cochlear nerve axons in NM

To examine whether topography exists in the connections between the basilar papilla/cochlear ganglion and NM when cochlear nerve axons first penetrate NM, we determined the posterior-to-anterior position and extent of NM penetrated by axons labeled by BP/CG injections. This could be done for all but the largest injections, i.e., those used to determine when any axons arrive in NM (above). In addition, in 25 cases dual BP/CG injections were made into the same basilar papilla to assess the topography of the projection in the same animal.

Our results indicate that the initial apical-to-basal projection from the basilar papilla to NM is topographically





organized along the posterior-to-anterior axis. Apical (LF) regions of the basilar papilla and cochlear ganglion project to more posterior (LF) regions of NM than do basal (HF) regions. This mapping is most clearly seen when two dyes are injected into the same basilar papilla. Figures 5 and 6 show the results from two BP/CG injections into each basilar papilla of the same stage 37 (E11) embryo. The insets in Figure 5 show the location of each injection; anterior is up in the insets. DiD (red) was injected into the basal (HF) region of the papilla on the left but into the apical (LF) region of the papilla on the right and DiI (green) was injected into the apical (LF) region of the papilla on the left but into the basal (HF) region on the right. Figure 5 shows a low-power confocal montage of a transverse section of the hindbrain through the anterior (80%) level of NM (dotted outlines); dorsal is up. The axons penetrating NM (arrows) were labeled by the basal BP/CG injections. Figure 6 shows a series of high-power confocal images of transverse sections through the left (dotted outline) and right NM of the same case as shown in Figure 5. Figure 5A–D shows a sequential series of sections, in 10% increments, from anterior (70%) to posterior (40%) levels of NM. Figure 5E shows a section from the 20% level of NM. The apical injections (green on the left and red on the right) mapped to a more posterior region of NM than the basal injections (red on the left and green on the right). Note that the labeled axons are also progressively more medially located in NM as one moves from anterior-to-posterior through the sections. This indicates that the labeled axons innervate an anterolateral-to-posteromedial band resembling isofrequency planes, which are at an angle to the posterior-anterior axis (Rubel and Parks, 1975).

Cochlear nerve axons are topographically arranged within the cochlear nerve as well. Figure 7 shows the results from two BP/CG injections into each basilar papilla of another stage 37 (E11) embryo. The injection locations are shown in the inset: anterior is up in the inset. In Figure 7, a low-power confocal montage of a transverse section through the cochlear nerve roots and NM (dotted outlines) shows that the axons labeled by each injection passed through separate regions of the cochlear nerve. Basal axons are located medially while apical axons are located more laterally.

Basal injections label more anterior regions of NM while apical injections label more posterior regions. Figure 8 shows the results from all 25 dual BP/CG injections. The left column shows the location and extent of apical (gray) and basal (black) injections (the unfilled bars represent the apex-to-base length of the basilar papilla and under-

Fig. 3. DiI- and DiD-labeling the brainstem of in a stage 29 (E6) embryo receiving two BP/CG injections on the right side. An anterior (71%, A), a middle (57%, B), and a posterior (42%, C) section through the auditory anlage (dotted outlines) are shown. Dorsal is up and medial is to the left. The two BP/CG injections are shown in the inset in C: DiI (green) was injected into the base and DiD (red) was injected into apex. A few axons can be seen penetrating into the auditory anlage in the middle section (B), but most of the labeled axons reside just ventral and lateral to the auditory anlage in all three sections. Both injections labeled vestibular axons that enter a dense fiber tract just ventral to the auditory anlage. CN, cochlear nerve; IV, fourth ventricle; L, lagena; VIII, VIIIth nerve root. Scale bar in B = 100  $\mu$ m and applies to all panels; inset scale bar = 500  $\mu$ m.

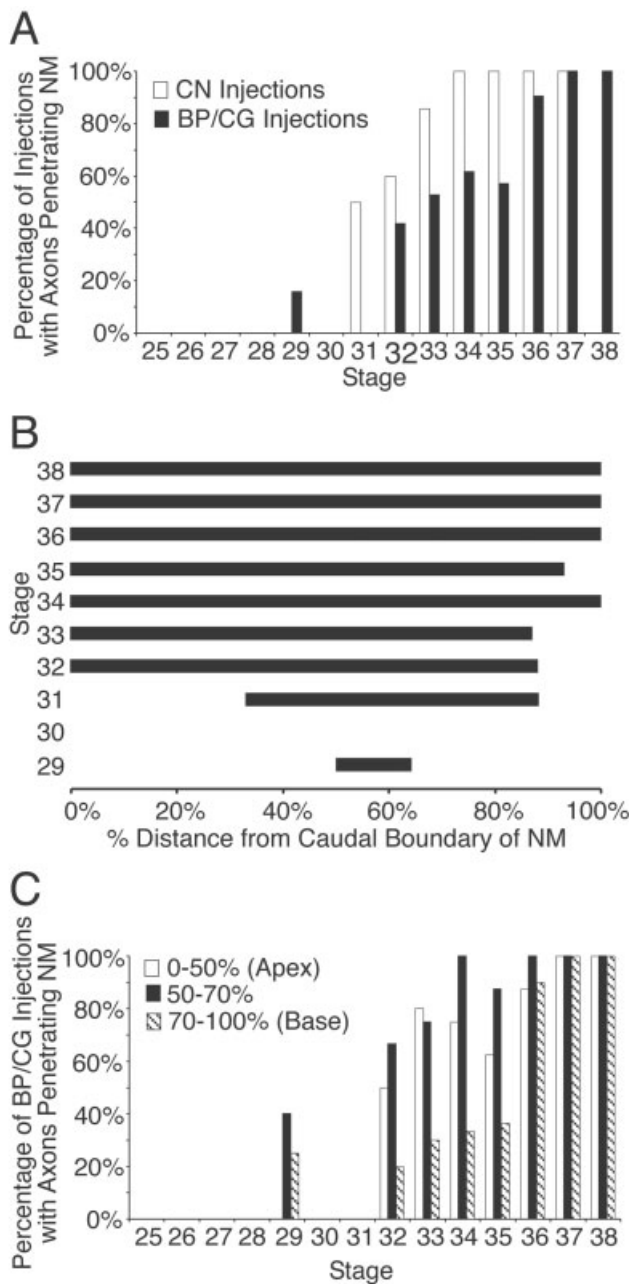


Fig. 4. **A:** Percentage of CN and BP/CG injections labeling axons that penetrated NM at different stages. The percentage of injections labeling axons that penetrated NM increased with age. The delay between the CN and the BP/CG injections was due to the later arrival of axons from the basal tip of the basilar papilla. **B:** Cumulative spatial distribution of labeled axons penetrating the auditory anlage or NM by stage. Each black bar represents the region of NM penetrated by labeled axons from at least one CN or BP/CG injection at that stage. Injections into only one of the 10 embryos at stage 34 (E8) labeled axons at the anterior tip of NM (above 90%). Note the middle-to-posterior-to-anterior gradient of penetrating axons between stages 29 (E6) and 36 (E10). None of the 14 CN and BP/CG injections at stage 30 (E6.5) labeled axons penetrating NM (See Table 1). **C:** Percentage of BP/CG injections labeling axons that penetrated NM at different stages grouped by the position of the basal injection boundary. Note that basal injections did not reliably label axons that penetrate NM until around stage 36 (E10).

lying cochlear ganglion). The middle and right columns show the location of the most posterior and most anterior labeled axons, respectively, from each injection (the unfilled bars represent the posterior-to-anterior length of NM). All injections labeled axons throughout a continuous region of NM. In 22 of 25 cases (88%), axons labeled by basal injections were located at the same or more anterior positions in NM than axons labeled by apical injections. In 23 of 25 cases (92%), axons labeled by apical injections were located at the same or more posterior positions in NM than axons labeled by basal injections.

Linear regression analysis confirms that, by stage 34 (E8), a topographic mapping exists between the length of the basilar papilla and the posterior-to-anterior axis of NM. Figure 9 shows scatter plots for three comparisons of position between BP/CG injections and the position of labeled axons in NM (basal injection boundary versus most anterior labeled axons in NM (Fig. 9A), mean injection position versus mean position of label in NM (Fig. 9B), and apical injection boundary versus most posterior labeled axons in NM (Fig. 9C)) for injections at stages 29–33 (E6–9), stages 34–36 (E8–10), and stages 37–38 (E11–12). As shown in Figure 9D, the correlations are statistically significant for all three comparisons at stages 34–36 (E8–10) and stages 37–38 (E11–12). The lower correlation coefficient for stage 34–36 (E8–10) apical injection boundaries (Fig. 9C) is probably due to the labeling of fibers of passage from the apical regions of the basilar papilla and cochlear ganglion. None of the comparisons of injection at stage 29–33 (E6–8) were significant (Fig. 9D), probably because cochlear nerve axons do not arrive in all regions of NM until stage 34 (E8).

**Arrival of cochlear nerve axons in NA**

Due to the difficulty of identifying NA at the ages examined in this study, we did not examine the penetration of cochlear nerve axons in the same detail as in NM. Cochlear nerve axons penetrate NA by stage 37 (E11) and are already organized in a dorsoventral topography similar to the future frequency axis (Warchol and Dallos, 1990). As early as stage 32 (E7.5), labeled cochlear nerve axons were observed penetrating into an anterolateral region of the auditory anlage where NA can later be identified. By stage 37 (E11), labeled cochlear nerve axons are segregated along the dorsoventral axis of NA. Figures 5 and 7 show two examples of the dorsoventral organization of labeled axons in stage 37 (E11) embryos.

**DISCUSSION**

This study examined the arrival and early organization of cochlear nerve axons in the primary auditory brainstem nucleus, nucleus magno-cellularis (NM). Cochlear nerve axons penetrate NM between stages 29 and 36 (E6–E10) in a middle-to-posterior-to-anterior, temporal-spatial sequence, beginning as early as stage 29 (E6). The arrival and penetration of NM by basal (HF) cochlear nerve axons appears to be delayed in comparison to axons innervating the middle and apical parts of the cochlea. Cochlear nerve axons are organized in a topographic map in the nerve and when they arrive in NM, foreshadowing the tonotopic map observed later in development. Evidence of a dorsoventral topographic map, similar to the mature tonotopic map, was also observed in the other primary auditory brain-

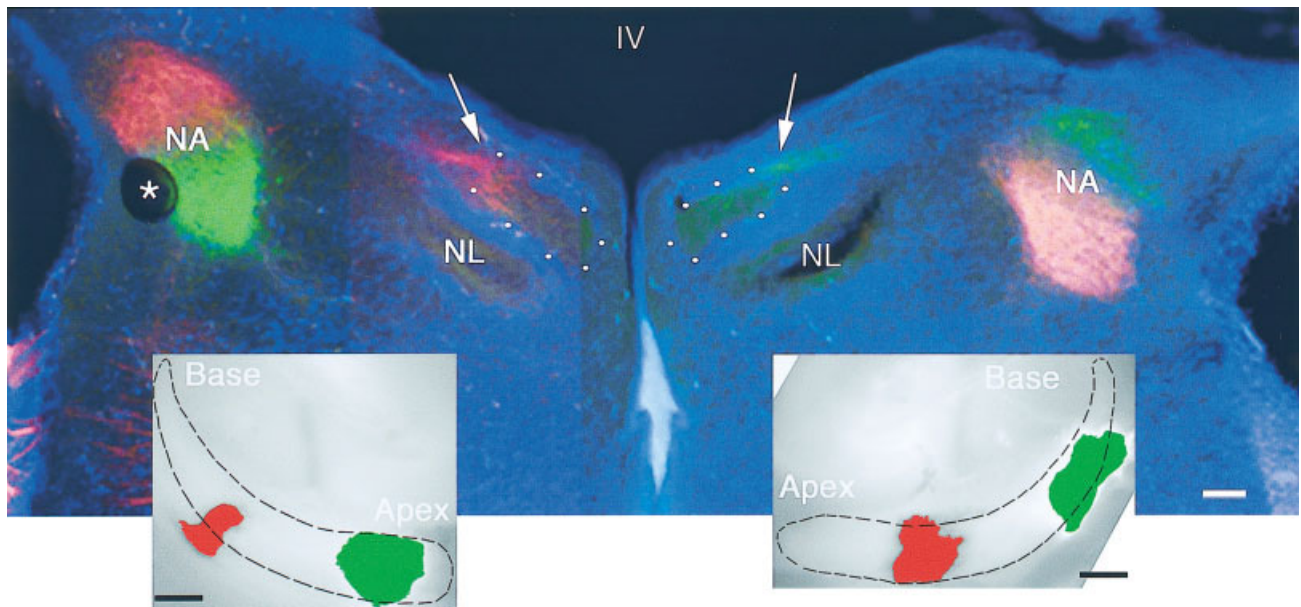


Fig. 5. Labeling from dual injections into both basilar papillas of a stage 37 (E11) embryo. Dorsal is up in the brainstem and anterior is up in the insets showing the dual BP/CG injections into the left and right basilar papillas (dashed outlines). DiI-labeling is shown in green and DiD-labeling is shown in red. This low-power, confocal montage of a single brainstem section shows DiI-labeled (right) and DiD-labeled (left) axons (arrows) in the anterior part of NM (80% level, dotted

outlines). The DiI- and DiD-labeling in NA segregated along a dorsal-ventral axis so that apical injections labeled the ventral region of NA (green on the left and red on the right) while basal injections labeled the dorsal region of NA (red on the left and green on the right). IV, fourth ventricle; NL, nucleus laminaris; \*air bubble. Scale bar for the brainstem = 100  $\mu$ m; inset scale bars = 500  $\mu$ m.

stem nucleus, nucleus angularis (NA), as the axons penetrate that nucleus.

#### Arrival of cochlear nerve axons

Our results did not verify previous reports (Knowlton, 1967; Book and Morest, 1990) that identified a relationship between NM cells and cochlear nerve axons prior to stage 29 (E6). Knowlton (1967) claimed that NM could be identified as early as stage 26 (E4.5–5) “on the basis of the relationships certain groups of neuroblasts sustain to acoustic root fibers.” Between stages 25 and 27 (E4.5–E5), Book and Morest (1990) traced cochlear nerve fibers into what they called the acoustic-vestibular anlage (see Fig. 1 in Book and Morest, 1990). Since our injections into the cochlear nerve and cochlear ganglion between stages 25 and 28 (E4.5–5.5) did not label axons projecting towards where the auditory anlage will form (Cramer et al., 2000a), the nerve fibers observed in these earlier studies may have been vestibular in origin. While we cannot eliminate the possibility that some cochlear nerve axons contact future NM cells in the process of migration on the basis of negative evidence, if this were true, we would have expected to see labeled cochlear nerve axons penetrating the auditory anlage as soon as it can be identified. Only 12.5% of the injections, in only 2 of the 17 animals injected at stages 29–30 (E6–6.5), labeled axons penetrating into the auditory anlage. Prior to stage 29 (E6), none of the six cases in which labeled axons entered the hindbrain labeled axons in the region of the prospective auditory anlage. Hence, in most cases, cochlear nerve axons have not made contact with NM precursor cells even at stage 29 (E6).

#### Penetration unrelated to other developmental gradients in NM

The middle-to-posterior-to-anterior penetration of cochlear nerve axons into NM does not coincide with any of the known developmental gradients in NM. A number of developmental processes in NM, including cell death (Rubel et al., 1976), proliferation and elimination of dendritic processes (Parks and Jackson, 1984), and calretinin expression (Parks et al., 1997; Kubke et al., 1999), occur along an anterior-to-posterior gradient. These processes, however, occur after E10 (stage 36), when cochlear nerve axons are already present in all regions of the nucleus. Additionally, these processes occur even if the cochlear nerve is absent (Parks, 1979; Parks and Jackson, 1984; Rubel and Parks, 1988; Lippe et al., 1992; Parks et al., 1997), suggesting that the arrival and targeting of cochlear nerve axons are directed by different signals than are other processes that have been studied in NM.

Cochlear nerve axons innervating NM also appear to arrive well before synaptogenesis. NM cells and cochlear nerve axons are in proximity to each other as early as stage 29 (E6) and, by stage 36 (E10), cochlear nerve axons are present in all regions of NM. Electrophysiological studies (Jackson et al., 1982; Pettigrew et al., 1988) determined that synaptic transmission between the cochlear nerve and NM is not seen until stage 36 (E10), up to 4 days after the first cochlear nerve axons penetrate the nucleus. Jackson et al. (1982) also mapped the locations of synaptic transmission at different ages and suggested that functional synaptogenesis



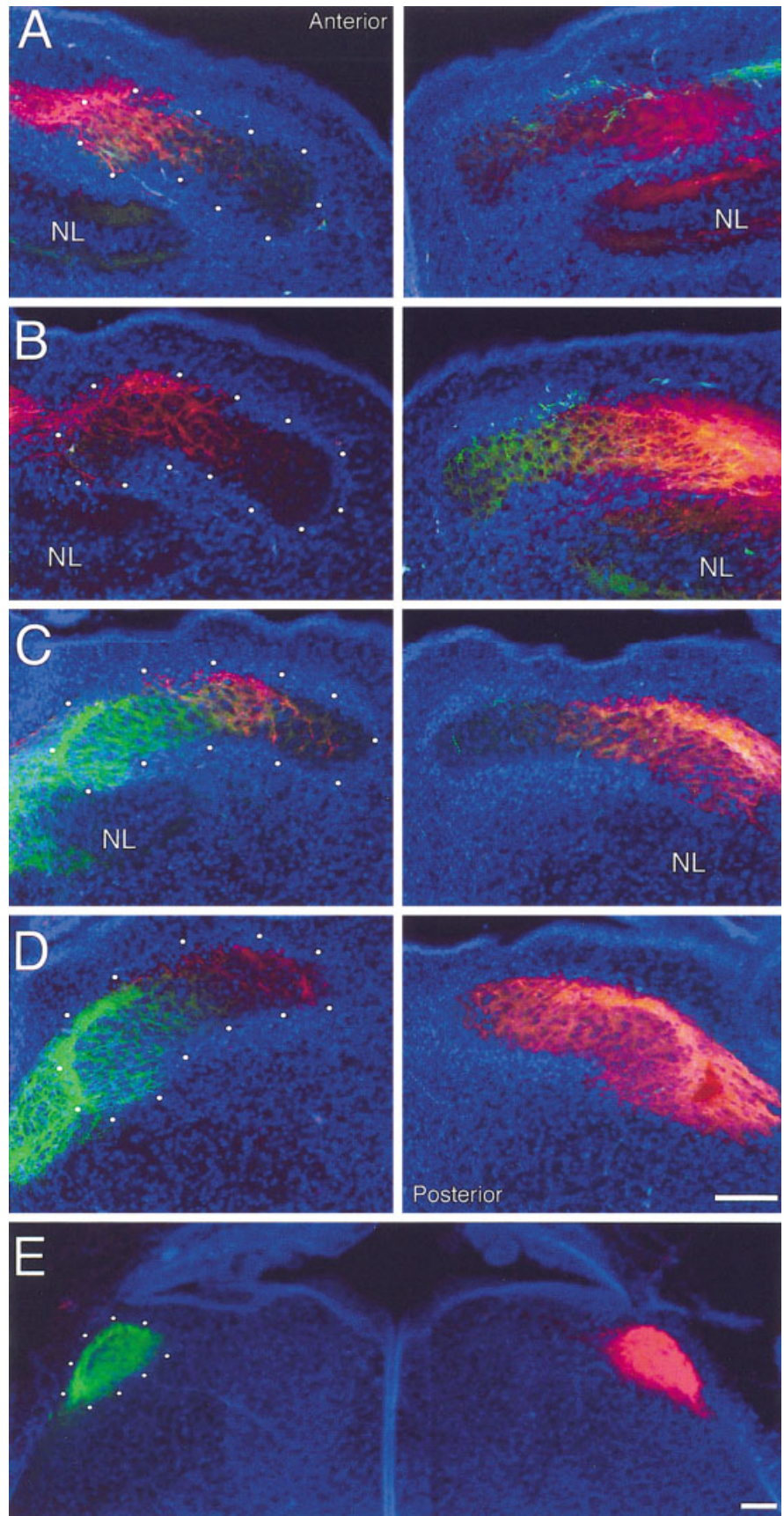


Fig. 6. High-power, confocal images showing the anterior-to-posterior transition of labeling in NM from basal and apical injections in the same animal shown in Figure 5. The BP/CG injections are shown in insets in Figure 5. DiI-labeling is shown in green and DiD-labeling is shown in red. Each panel shows a transverse section through NM on the left (dotted outlines) and right sides of the same brainstem section. Dorsal is up and medial is towards the middle of the figure. **A–D**: 10% increments in order from anterior (70% level, A) to posterior (40% level, D). Note how the label shifts from lateral to medial as you move posteriorly in NM. NL, nucleus laminaris. **E**: From the 20% level of NM, shows that the axons labeling the most posterior regions of NM originate from the apical injections. Scale bar in D = 100 μm and applies to panels A–D. Scale bar in E = 100 μm.



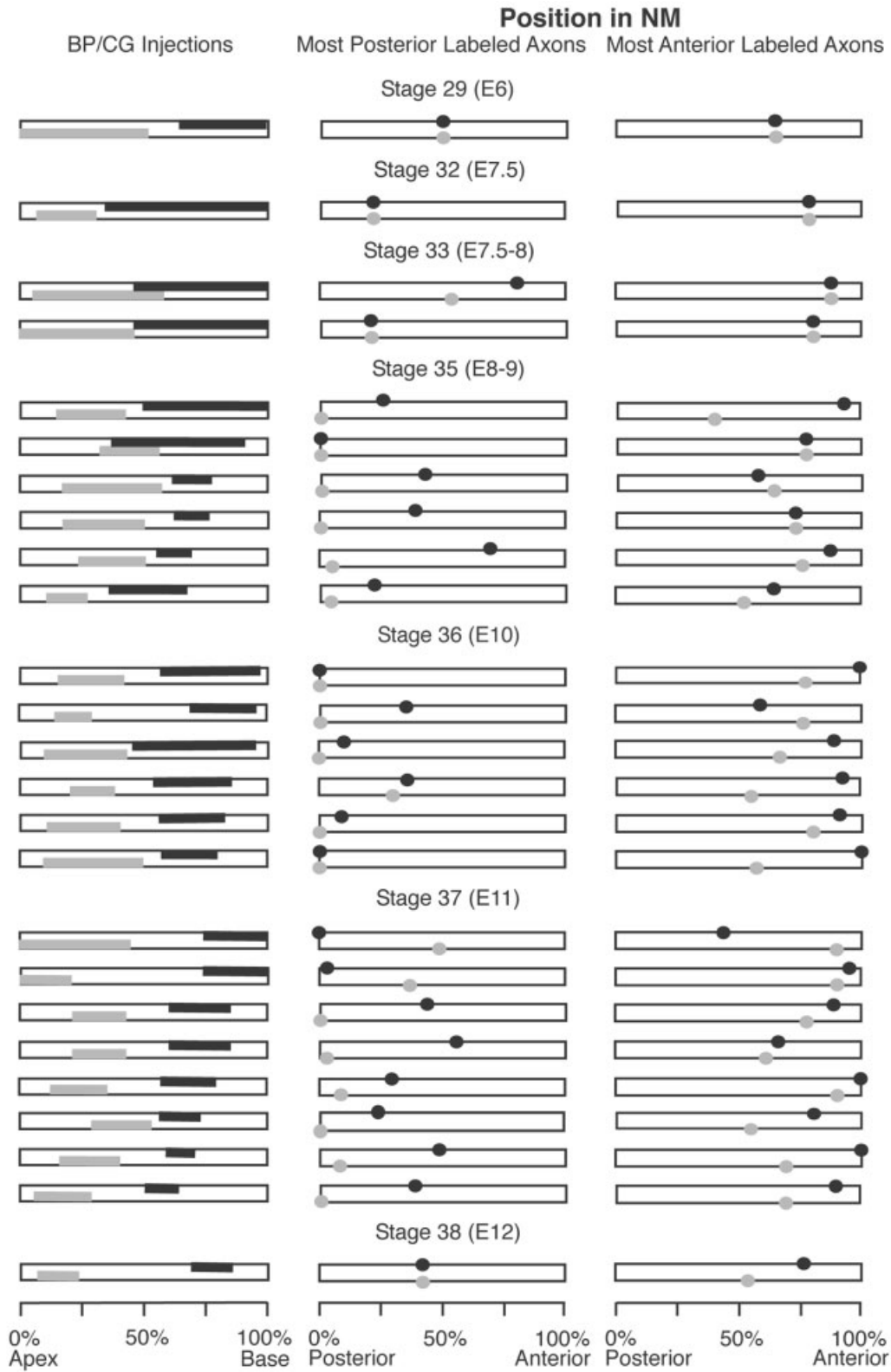


Fig. 8. Dual BP/CG injections into the same basilar papilla and the resultant label in NM by stage. The open rectangles represent the apical-to-base length of the basilar papilla (left column) or the posterior-to-anterior length of NM (middle and right columns). The left column shows the location and size of the apical (gray) and basal

(black) BP/CG injections into the basilar papilla. The middle column shows the location in NM of the most posterior axons labeled by apical (gray dot) and basal (black dot) injections. The right column shows the location in NM of the most anterior axons labeled by apical (gray dot) and basal (black dot) injections. All 25 dual injections are shown.



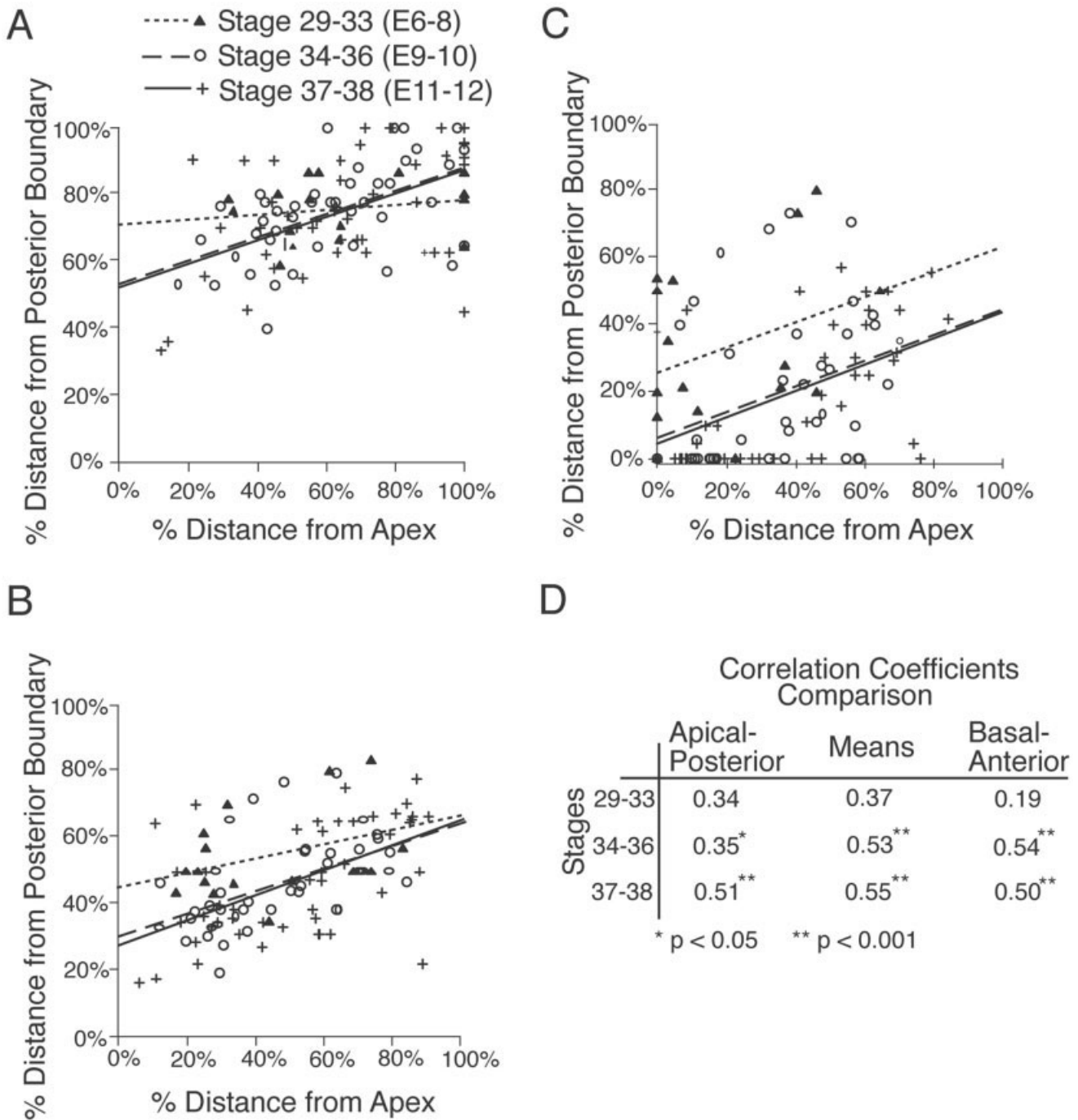


Fig. 9. Scatter plots and regression analysis of injection position versus the position of labeled axons in NM. Data points for each BP/CG injection and best-fit linear regression lines are shown for all BP/CG injections at stages 29–33 (E6–8; , [short dash lines]), 34–36 (E8–10; , [long dash lines]), and 37–38 (E11–12; [solid line]) in A, B, and C. **A:** Comparison of basal injection boundaries and the positions

of the most anterior labeled axons in NM (apical-posterior). **B:** Comparison of mean injection positions and mean positions of label axons in NM (means). **C:** Comparison of apical injection boundaries and positions of the most posterior labeled axons in NM (basal-anterior). **D:** Correlations coefficients (r) for each comparison in A, B, and C.

the basilar papilla and cochlear ganglion is longer and matures earlier than the basal end (Rebillard and Pujol, 1983; Cotanch and Sulik, 1984, 1985; Katayama and Corwin, 1989; Cohen and Cotanche, 1992; Goodyear et al.,

1995). A third limitation results from the fact that cells at the basal end of the cochlear ganglion and basilar papilla do not complete terminal mitosis until around stage 31 (E7; D’Amico-Martel, 1982; Katayama and Corwin, 1989).

Hence, many of the axons of the cochlear ganglion cells projecting to this region and the anterior (HF) region of NM are not present until stage 31–32 (E7–7.5). In addition, the axons innervating this region are the last to penetrate NM. A fourth limitation is that we determined the position of label in NM only along the posterior-to-anterior axis, whereas the actual tonotopic axis is rotated about 30° towards the midline (Rubel and Parks, 1975; Lippe and Rubel, 1985; Young and Rubel, 1986). As a result, our methods overestimate the extent of NM penetrated by labeled axons by at least 15%. These limitations, taken together, suggest that the correlation between injection position and the position of labeled axons in NM is probably much stronger than indicated by our results.

One popular question in developmental neurobiology concerns the role that activity-dependent processes play in the establishment, stability, and refinement of topographic maps (reviewed in Friauf and Lohman, 1999; Katz and Crowley, 2002; Rubel and Cramer, 2002; Rubel and Fritsch, 2002). As noted above, our methods do not permit us to make any conclusions about the precision or refinement of the initial map between the basilar papilla and NM beyond stating with assurance that some degree of organization exists as early as the axons can be seen penetrating the neuropil of NM. When combined with the results from other systems (retinotectal projections in goldfish (Stuermer and Raymond, 1989), *Xenopus* (Holt and Harris, 1983; Holt, 1984; Sakaguchi and Murphey, 1985; O'Rourke and Fraser, 1986), and chicken (Ichijo, 1999; Yates et al., 2001); the retinocollicular projection in ferrets (Chalupa and Snider, 1998); barrel cortex in mouse (Agmon et al., 1995); ocular dominance columns in cats (Crowley and Katz, 2000; Crair et al., 2001); chick auditory system (Young and Rubel, 1986)) considerable evidence exists to support the hypothesis that the topography of projections develops as axons grow into their targets and is independent of neuronal activity.

Evidence from other studies (reviewed in Rubel and Fritsch, 2002) also suggests that the initial topographic maps in the developing auditory system are quite precise as soon as they can be investigated. For example, Young and Rubel (1986) showed that NM axons projecting to NL grow into narrow and discrete rectangular areas corresponding to the isofrequency planes. A recent study in the cat (Leake et al., 2002) showed that the precision of the topographic map between the cochlea and cochlear nuclei is very precise in the neonatal kitten (cf. Rubel and Cramer, 2002). Injections into small, discrete regions of the basilar papilla at stages 37–38 and in the posthatch chick are needed to determine the precision of the initial topographic map and whether any refinement occurs later in development.

Gradients of Ephrins and Eph receptors are known to be involved in establishing the topographic map of the retinotectal system (O'Leary and Wilkinson, 1999; Thanos and Mey, 2001; Yates et al., 2001). Since Ephrins and Eph receptors are expressed in the cochlear nerve and NM during the period when cochlear nerve axons are first penetrating NM (Cramer et al., 2000b, 2002), they are likely to be involved in the formation of the topographic projection between the basilar papilla and NM. The expression of Ephrins and Eph receptors within NM remains to be determined but, as in the retinotectal system, we would expect some Ephrins or Eph receptors to be expressed in gradients corresponding to the frequency axis

of NM between stage 29 and 36 (E6–E10). Once the precision of the initial topographic map is determined, the role Ephrins and Eph receptors play in the formation of the initial mapping can also be tested by misexpressing them in the brainstem auditory nuclei or cochlear ganglion cells and determining whether the topographic mapping is disrupted.

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