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Formation of the Avian Nucleus Magnocellularis from the Auditory Anlage

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ABSTRACT

In the avian auditory system, the neural network for computing the localization of sound in space begins with bilateral innervation of nucleus laminaris (NL) by nucleus magnocellularis (NM) neurons. We used antibodies against the neural specific markers Hu C/D, neurofilament, and SV2 together with retrograde fluorescent dextran labeling from the contralateral hindbrain to identify NM neurons within the anlage and follow their development. NM neurons could be identified by retrograde labeling as early as embryonic day (E) 6. While the auditory anlage organized itself into NM and NL in a rostral-to-caudal fashion between E6 and E8, labeled NM neurons were visible throughout the extent of the anlage at E6. By observing the pattern of neuronal rearrangements together with the pattern of contralaterally projecting NM fibers, we could identify NL in the ventral anlage. Ipsilateral NM fibers contacted the developing NL at E8, well after NM collaterals had projected contralaterally. Furthermore, the formation of ipsilateral connections between NM and NL neurons appeared to coincide with the arrival of VIIIth nerve fibers in NM. By E10, immunoreactivity for SV2 was heavily concentrated in the dorsal and ventral neuropils of NL. Thus, extensive pathfinding and morphological rearrangement of central auditory nuclei occurs well before the arrival of cochlear afferents. Our results suggest that NM neurons may play a central role in formation of tonotopic connections in the auditory system. J. Comp. Neurol. 498:433–442, 2006.

Indexing terms: nucleus laminaris; cochlear nerve; SV2; Hu C/D; neurofilament; retrograde labeling; embryonic hindbrain

The auditory system has evolved to encode sound intensity, spectral pattern, and location by using topographic maps at all levels of the circuit. Development of the sound localization circuitry requires precision in pathfinding and target selection to connect cells properly along the tonotopic axis and orthogonal to that axis. In birds, auditory information from the basilar papilla, the avian homolog of the mammalian cochlea, enters the brainstem via the VIIIth cranial nerve to synapse on nucleus angularis (NA) and nucleus magnocellularis (NM; reviewed in Kubke and Carr, 2000; Ryugo and Parks, 2003; Rubel et al., 2004). Afferent synapses in NM and NA are arranged topographically according to frequency of sound, and these tonotopic projections are preserved at each ascending level of the auditory system. NM sends phase-locked information bilaterally to nucleus laminaris (NL), which acts as a coincidence detector (Parks and Rubel, 1975; Carr and Konishi, 1990; Carr and Boudreau, 1996).

Because of their essential role in localizing sound, their well-documented homogeneity and projection patterns,

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NM neurons represent a model cell population for studying neuronal specification, migration, pathfinding, and targeting in the developing nervous system. The structure of NM neurons is essential for their role in accurately processing the cues necessary for localization of sound in the avian auditory system. At maturity, each NM neuron extends one collateral to contact the dorsal dendrites and soma of ipsilateral NL neurons, whereas the other collateral crosses the midline to contact the ventral dendrites and soma of homotypic contralateral NL neurons. The pattern of terminal arbors on each side of the brain is anisotropic and precisely follows an isofrequency contour (Young and Rubel, 1983). Dendritic morphology in NM is also intricate because dendrites form only to be resorbed coincident with onset of synaptic activity with their afferent innervation (Hackett et al., 1982; Jackson et al., 1982; Jhaveri and Morest, 1982; Young and Rubel, 1986). At the same time, large synaptic calyces that cover 80–90% of the somal area of NM neurons develop as a means to facilitate fast and accurate firing responsible for high-fidelity, phase-locked frequency coding (Parks, 1981).

The auditory system develops early in the chick embryo (reviewed in Rubel and Fritzsch, 2002; Rubel et al., 2004). Neurons destined to become NM are generated in rhombomeres 5, 6, and 7 between embryonic days (E) 2 and 3, whereas its target, NL, is generated from cells slightly later in more lateral regions of rhombomeres 5 and 6 at E3–3.5 (Rubel et al., 1976; Marin and Pulles, 1995; Cramer et al., 2000a). Cochlear nerve axons penetrate NM in a middle-to-posterior-to anterior sequence between E6 (St 29) and E10 (St 36) (Molea and Rubel, 2003). Cells born in rhombomere 5 reside along the high-frequency (rostromedial) axis, whereas those from rhombomere 7 eventually code for low-frequency sounds by residing in the caudolateral region of nucleus magnocellularis (Cramer et al., 2000a).

Although much is known about the early generation and the later stages of maturation of these neurons, much less is known about the intervening time that comprises the migratory period. By E4.5, the auditory anlage, a transient region at the most lateral regions of the caudal brainstem, has formed (Book and Morest, 1990). Although auditory hindbrain neurons (nucleus angularis, magnocellularis, and laminaris neurons) and vestibular neurons of the tangential nucleus have been hypothesized to comprise the auditory anlage, the organization of the structure and how it gives rise to auditory nuclei have been difficult to elucidate. The auditory anlage may offer a venue for cellular interaction between NM and NL, but it is not known where prospective NM neurons are located in the anlage with respect to NL neurons, how these two populations segregate, and when synaptogenesis commences with respect to morphogenesis of the nuclei.

In the present study, we assessed the differentiation of auditory brainstem neurons by using markers of neuronal differentiation, Hu C/D, neurofilament, and SV2. We then identified the timing of development within the auditory anlage by retrogradely labeling NM neurons from the contralateral hindbrain and by labeling afferents via the VIIIth nerve. We show that NM neurons establish contralateral projections before formation of contacts with ipsilateral NL. Furthermore, NM projections form in the absence of synaptogenesis from cochlear afferents. These results suggest that NM neuronal identities are prespecified and guide the formation of auditory circuitry in the hindbrain.

MATERIALS AND METHODS

Fertile white leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from a local supplier (Oliver Merrill and Sons, Londonderry, NH) and stored at 18°C. On day 0 of incubation, they were placed into an incubator set at 38°C. Embryos were harvested on the indicated days and staged according to Hamburger and Hamilton (1951 reprinted 1992).

Immunohistochemistry

Embryos were removed from the egg and quickly decapitated. The hindbrain was dissected in cold phosphate-buffered saline (PBS; 0.02 M KCl, 1.37 M NaCl, 0.1 M Na_2HPO_4, 0.02 M KH_2PO_4, pH 7.4), immersed in Zamboni's fixative (4% paraformaldehyde, 15% picric acid in 0.2 M sodium phosphate buffer, pH 7.4) for 1 hour, rinsed in PBS, and equilibrated in 30% sucrose overnight at 4°C. The tissue was then embedded and frozen in mounting medium (3-50100; Richard Allen Scientific, Kalamazoo, MI) and sectioned by using a Microm HM 560 cryostat. Alternate serial 30-μm transverse sections containing the auditory brainstem nuclei were collected on positively charged slides (Superfrost Plus, Fisher) for immunohistochemistry. Sections were postfixed in Zamboni's fixative for 20 minutes followed by washes of PBS containing 0.5% Triton X-100 (PBST) for 10 minutes. A blocking solution containing 5% fetal calf serum in PBST was applied for 1 hour at room temperature. Primary antibodies were diluted and incubated in blocking solution overnight at 4°C. Mouse monoclonal anti-human Hu C/D (A-21271; Molecular Probes, Eugene, OR; originally isolated by M. Marusich in the University of Oregon Monoclonal Antibody Facility as mAb 16A11) was used at a dilution of 1:250. In the chick, 16A11 is specific for HuC/D, an antigen specifically expressed by cells undergoing neuronal differentiation (Wakamatsu and Weston, 1997). Rabbit anti-neurofilament, 150 kD (Chemicon International Inc., Temecula, CA AB1981), was raised in rabbits against purified neurofilament polypeptide and was used at 1:500. We have shown that this antibody selectively labels neurons in the chick (Lee et al., 2002). Mouse anti-SV2 (Feany et al., 1992), an ammonium sulfate-concentrated culture supernatant prepared in the Nishi Lab from culture medium collected from hybridomas purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) was used at 1:20, with the titer of the antibody solution determined by serial dilution after each new lot is prepared. This anti-SV2 labels synaptic vesicles in the chick (Horch and Sargent, 1995). Goat anti-mouse Cy3 and goat anti-rabbit Alexa 488 (Molecular Probes) were used in dilutions of 1:750. Slides were rinsed three times for 10 minutes each in PBST before and after secondary antibody application and coverslipped with PermaFluor Aqueous Mounting Medium (Thermo Electron Corporation, Pittsburgh, PA) containing 0.5 μg/ml Hoechst DNA dye (Sigma, St. Louis, MO). Specificity of antibody staining for each set of sections examined was determined by either omitting the primary antibody (for the mouse monoclonals) or substituting the appropriate dilution of a non-immune rabbit serum.

Retrograde labeling

To label NM neurons retrogradely, E5, E6, E7, and E8 chick embryos (n = 4 at E5 and E7; n = 5 at E6 and E8) were removed from the egg, and the brainstem was quickly dissected in ice-cold chick Ringer's solution (0.13 M NaCl, 0.004 M KCl, 0.001 M KH_2CO_4, 0.012 M $NaHCO_3$, 0.001 M $MgCl_2$, 0.002 M $CaCl_2$, 0.0001% phenol red, and 2 g/l dextrose infused with 95% O_2/5% CO_2) infused with 95% O_2/5% CO_2. Crystals of AlexaFluor 488 dextran conjugate, MW = 10,000 (Molecular Probes) were applied by using an insect pin to the right hindbrain. The tissue was then immersed in chick Ringer's solution, continuously perfused with 95% O_2/5% CO_2 for 10–15 hours and then fixed for 1 hour in Zamboni's fixative. The tissue was then processed for Hu C/D immunohistochemistry as described above. Double-labeling experiments in which the ipsilateral VIIIth nerve was labeled in conjunction with the contralateral auditory anlage were performed on E6 and E8 hindbrains (n = 5 per age group). Crystals of AlexaFluor 488 dextran conjugate, MW = 10,000 (Molecular Probes), were applied to the right auditory anlage, whereas crystals of rhodamine-conjugated dextran, MW = 10,000 (Molecular Probes), were applied to the nerve root of the vestibulocochlear (VIIIth) nerve. Tissue was processed as before.

Confocal microscopy

Thirty-micrometer sections containing the auditory hindbrain nuclei were imaged by using a Nikon Eclipse E800 microscope equipped with a Nikon C1 confocal with 20× Plan Apo (NA 0.785) or 60× Plan Apo (NA 1.4) oil objective lens, EZ-C1 software, and UV, Argon, and He/Ne lasers exciting at 408, 488, and 543 nm and emitting at 404, 500–530, and 555–615, respectively. Digital images were captured and exported to Adobe Photoshop (v6.0) for presentation. All images were prepared without postcollection manipulation unless noted in the figure legend.

RESULTS

Developmental patterning of the auditory anlage

To track the development of cells within the anlage, we monitored the expression of neuronal specific proteins: Hu C/D (red in Fig. 1), a ribonucleic acid (RNA)-binding protein that is expressed in the cell body shortly after neuronal differentiation commences (Lee et al., 2002), neurofilament (green in Figs. 1–3), a cytoskeletal protein that is highly expressed in axons but excluded from the perisynaptic region, and SV2 (red in Figs. 2, 3), an integral membrane protein expressed in synaptic vesicles (Whitehead et al., 1982; Feaney et al., 1992; Nealen, 2005). Serial transverse sections from fixed hindbrains of chicken embryos between E4.5 and E14 were processed for immunohistochemical analysis.

The auditory anlage is visible as early as E5 (St 26) as a confluence of cells at the dorsolateral edge of the brainstem at the level of the VIIIth nerve. Nearly all cells within the auditory anlage have commenced neuronal differentiation as evidenced by staining for Hu C/D (Fig. 1); however, they show little neurofilament (Figs. 1 and 2A,B) or SV2 staining (Fig. 2B). More prevalent are neurofilament-positive fibers from the VIIIth nerve, which fill a cell-free region below the developing anlage (Fig. 1).

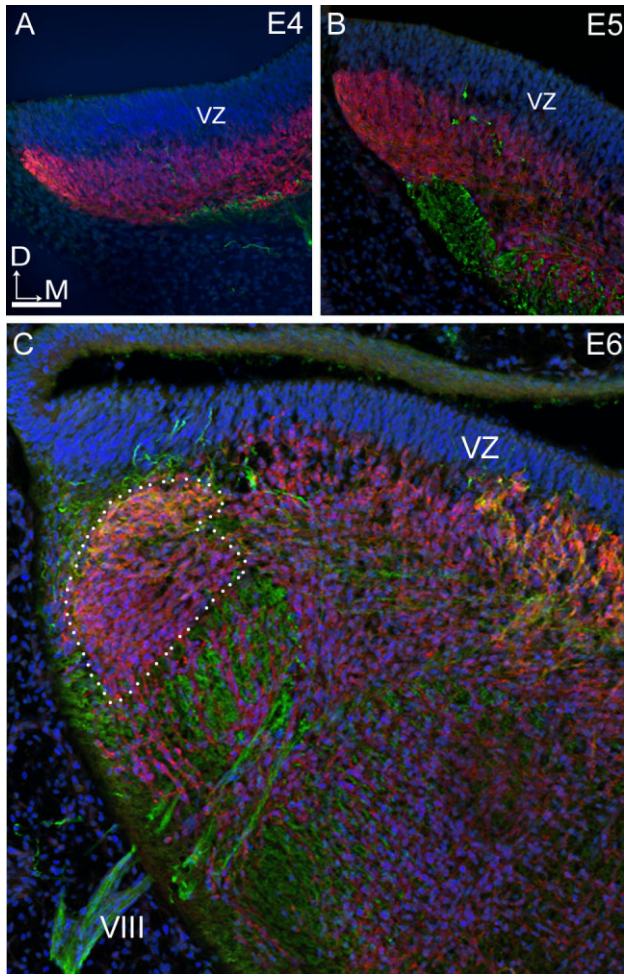

Fig. 1. Early formation of the auditory anlage in the hindbrain. Sections of the hindbrain were stained with antibodies against neuron-specific RNA-binding protein, Hu C/D (red), neurofilament (green) and with Hoechst dye (blue) to label all cellular nuclei. The left half of each transverse section is shown. **A:** At E4 (St 25), virtually all of the cells below the ventricular zone (VZ) are labeled with Hu, indicating that they have commenced neuronal differentiation, but the auditory anlage is not visible. **B:** At E5 (St 28), the presence of the ventral neuropil delineates the forming auditory anlage. **C:** By E6, the anlage is clearly identifiable at E6 because the neurons are surrounded by a cell-free region (white dotted line). The orientation of the section is indicated by the arrows above the calibration bar (D: dorsal; M: medial). Calibration bar = 100 mm for E4 and E5; 25 mm for E6.

By E6 (St 29), the auditory anlage is visible as a "C"-shaped structure, with neurofilament-positive fibers projecting from the anlage toward the midline (Fig. 2C). There is little SV2 within the anlage, but SV2 is more abundant below the anlage in the neuropil where cochlear afferent fibers are first visible along the lateral boundary of the anlage (Fig. 2C). Over the next 2 days, the anlage undergoes considerable morphological change as an invaginating cell-free region further defines the "C" shape. Neurofilament staining increases after E7 and axons from the midline are visible beneath the cell-free region ventral to the anlage (Fig. 2C,D). SV2 staining increases dramatically in intensity during this time as well. Between E6 and E8, the hindbrain grows dramatically in size, and there is

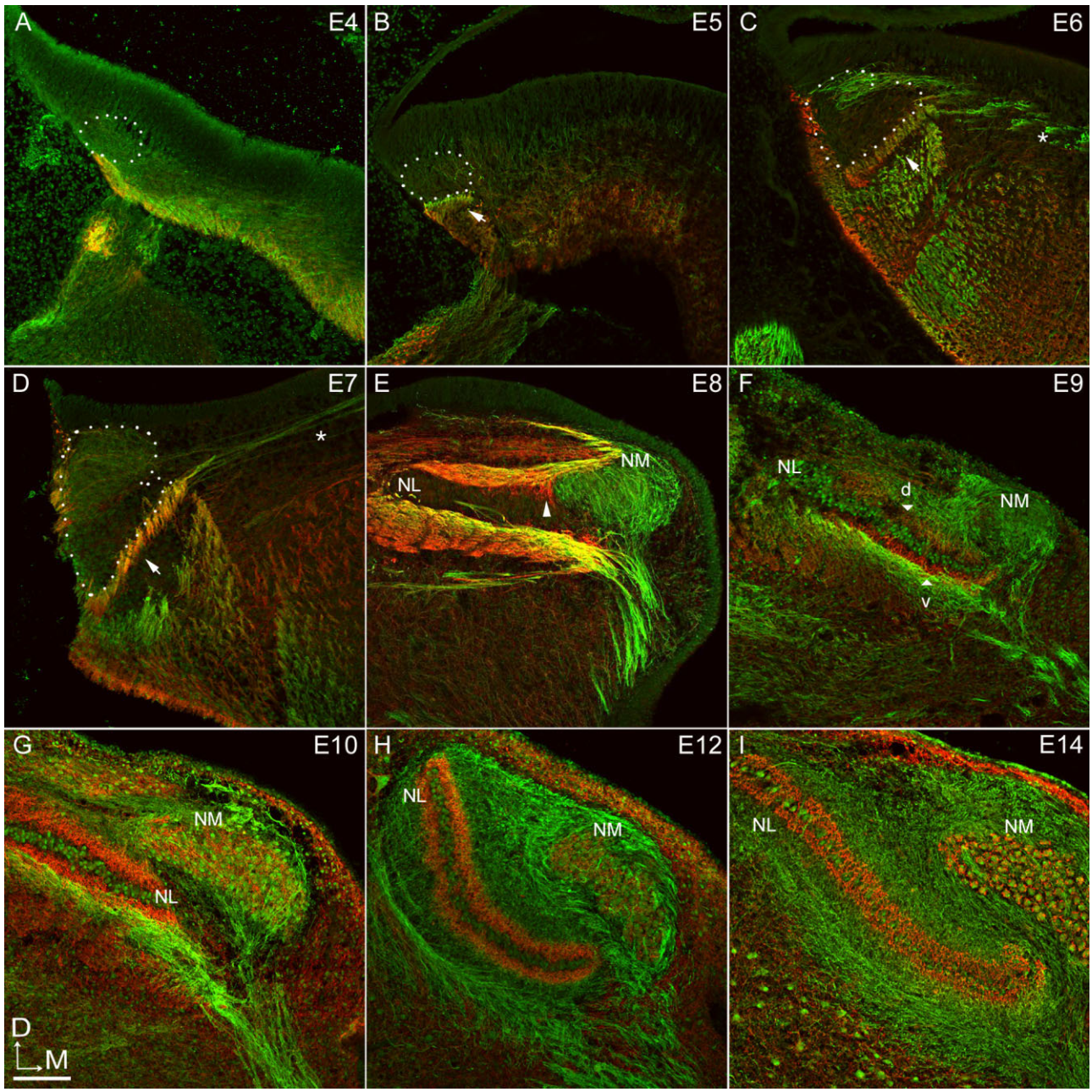

Fig. 2. Maturation of the auditory anlage. Antibodies against the axonal protein, neurofilament (green), and the synaptic vesicle protein, SV2 (red) reveal the development of NM and NL from the auditory anlage in transverse sections of the growing brainstem. **A:** At E4 (St 26), the lateral edge of the brainstem that will give rise to the auditory anlage (dotted circle) is largely devoid of specific neuronal markers. Arrow indicates site where the VIIIth nerve is penetrating. **B:** By E5 (St 28), the neuropil (arrow) is visible ventral to the anlage (dotted circle). **C:** At E6 (St29), neurofilament-positive axons from neurons in the dorsal auditory anlage (outlined with dotted line) are clearly visible. SV2 staining intermixed with neurofilament can be seen in the neuropil region (arrow). **D:** Maturation of the auditory anlage (outlined with dotted line) progresses with distinct labeling of contralaterally projecting axons at E7 (St31; asterisk) and a greater increase in SV2 immunoreactivity (arrow), indicating synaptogenesis in the neuropil ventral to the anlage. **E:**. Nucleus magnocellularis (NM) and nucleus laminaris (NL) are clearly distinct at E8 (St 34). Dramatic increases in the amount of neurofilament and SV2 staining from NM to NL (arrowhead) are evident. **F:** By E9 (St 34), NL somata are neurofilament positive, and an asymmetry in the intensity of SV2 staining is seen in the dorsal (d) vs. ventral (v) neuropil of NL (arrowheads). **G,H,I:** The further synaptic development and axonal refinement in E10, E12, and E14, respectively. The orientation of all sections is indicated by the arrows above the calibration bar (D: dorsal; M: medial). Calibration bar = 100 μm for all images.

an apparent lateral to medial displacement of the anlage (compare Fig. 2D with 2E). Between E8 and E9, segregation of cells into NM and NL becomes visible in a rostral to caudal fashion. The rostral sections of the E8 hindbrain contain a nucleus that is clearly the nascent NM (Fig. 2E); neurofilament staining is well established, whereas SV2

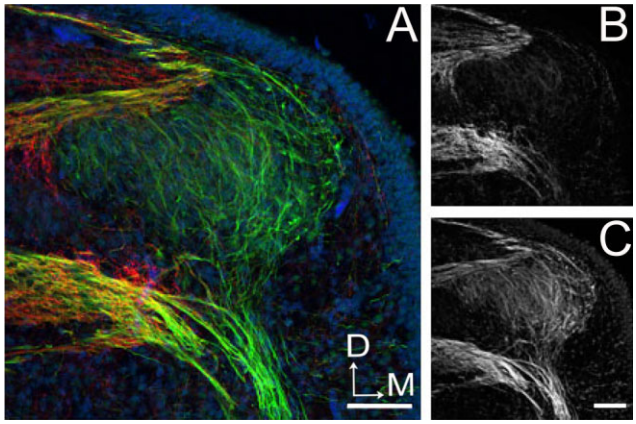

Fig. 3. Synaptogenesis between NM and NL, as seen with SV2 immunoreactivity, proceeds before afferent synapses form on NM neurons. At E8, NM is devoid of SV2 staining, whereas trafficking of SV2 within axons to NL and punctate staining in the neuropil of NL is established. **A:** Overlay of staining observed for SV2 (red), neurofilament (green), and cellular nuclei (blue). **B:** Red channel alone shows the paucity of SV2 in NM (asterisk). **C:** Green channel alone shows the extensive neurofilament staining of NM axons. The orientation of the section is indicated by the arrows above the calibration bar (D: dorsal; M: medial). Calibration bar = 50 μm for all panels.

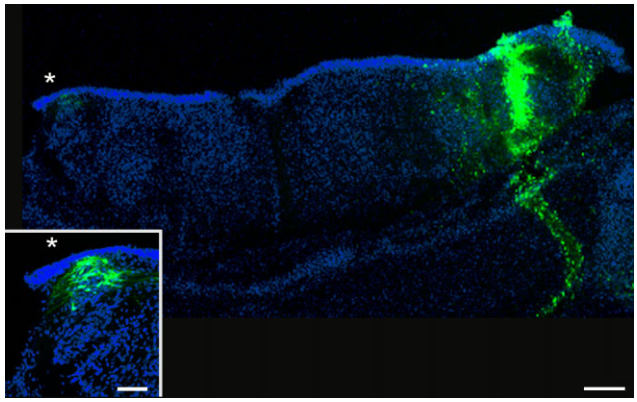

Fig. 4. Dextran used to label NM neurons that have crossed the midline is confined to the hemisection to which it was applied. Dextran was applied to the left side of a brainstem isolated at E6 (St 29) and incubated overnight in oxygenated chicken Ringer's solution to allow transport. A low-power view shows the entire extent of the transverse section. Dextran permeates the lateral injected side but does not penetrate the contralateral side except for neuronal cell bodies (asterisk) that were filled by retrogradely transported dye. In the inset at higher magnification, the labeled cell bodies and processes can be clearly seen. Calibration bars = 100 μm.

staining is absent within NM. In addition, ventrally coursing fibers from the contralateral NM, combined with the projection from the ipsilateral NM, and a concentration of SV2 staining serve to delineate an unmistakable NL (Fig. 2E). In contrast, the anlage has not clearly segregated into NM and NL in the most caudal sections of the E8 hindbrain, which have an appearance reminiscent of sections from E7 (Fig. 2D). When serial sections from the most caudal portions to the most rostral portions of this region are viewed at E8, the developmental sequence of NM and NL segregation is most readily apparent.

Maturation of projections within nucleus magnocellularis and laminaris

Although the reorganization of the auditory anlage into NM and NL is completed throughout the hindbrain by E10, maturation of the nuclei continues. From E8 through E14, neurons form a laminar sheet in NL (Fig. 2E–I) (Rubel et. al., 1976). By E9, the classic pattern of looping ipsilateral NM fibers that contact the dorsal neuropil of NL emerges (see green neurofilament immunoreactivity emerging from NM and contacting dorsal neuropil [d] in Fig. 2F). At E9, SV2 staining within the ventral neuropil of NL is consistently more intense than in the dorsal neuropil (Fig. 2F). By E10, both NM and NL appear more developed as SV2 staining becomes highly visible and more punctate within both nuclei and the intensity of neurofilament and SV2 staining equalizes between the dorsal and ventral neuropils of NL (Fig. 2G–I).

Synapses on NM neurons, as defined by SV2 staining, develop gradually from E9 to E14. At E8, SV2 staining can be seen in fibers near the developing NL (Fig. 2E, arrowhead), but NM is devoid of SV2 (Fig. 3). The first faint clusters of SV2 immunoreactive varicosities in NM are visible beginning at E9 (Fig. 2F), increasing in expression and localization near the cell bodies by E12 (Fig. 2H). By E14, calycal synapses are recognizable as SV2 staining surrounds the somata of NM neurons (Fig. 2I).

Retrograde labeling

We placed fluorescently conjugated dextran in half of the hindbrain at various ages to identify the cell bodies of NM neurons whose axons had crossed the midline. Retrogradely labeled cell bodies within the auditory anlage are observed as early as St 29 (E6; Figs. 4 and 5). Labeled neurons are not observed in embryos at St 28 (E5.5) even if the label was placed directly at the midline. Because no visible neurofilament-positive structures are seen projecting toward the midline at St 28 (Fig. 2B,C), it is likely that NM neurons extend axons that travel over 500 μm between St 28 and St 29, a time period corresponding to approximately 24 hours. At St 29 (E6), retrogradely labeled neurons are primarily located within the dorsomedial aspect of the auditory anlage and are labeled throughout its rostrocaudal extent (Fig. 5A–C), although there are more labeled neurons in rostral sections. At this age, only a few ipsilateral projections of labeled NM neurons are apparent, and they are localized to the dorsolateral cell-free region above the auditory anlage (Fig. 5A). By E8, the rostral-to-caudal segregation of retrogradely labeled NM neurons is apparent (compare Fig. 5D with 5F). In addition, anterogradely labeled NM axons visible at the ventrolateral aspect of the anlage suggest the location of the putative NL (Fig. 5D,E), but the same fibers have yet to reach NL in the most caudal portions of the hindbrain at E8 (Fig. 5F). It is of interest that it appears that only the neurons located in the most dorsomedial aspect of the anlage have rounded cell bodies with ipsilateral fibers emanating from them (Fig. 6A,C). Cells located elsewhere throughout the anlage have a bipolar shape with only small projections visible (Fig. 6B). This bipolar morphology is reminiscent of migrating cells, suggesting that these cells are moving toward the dorsomedial anlage before extending their ipsilateral collateral. At E8, the rostral portion of the anlage segregates into nuclei characteristic of NM and NL (Fig. 5). NM is well isolated, and

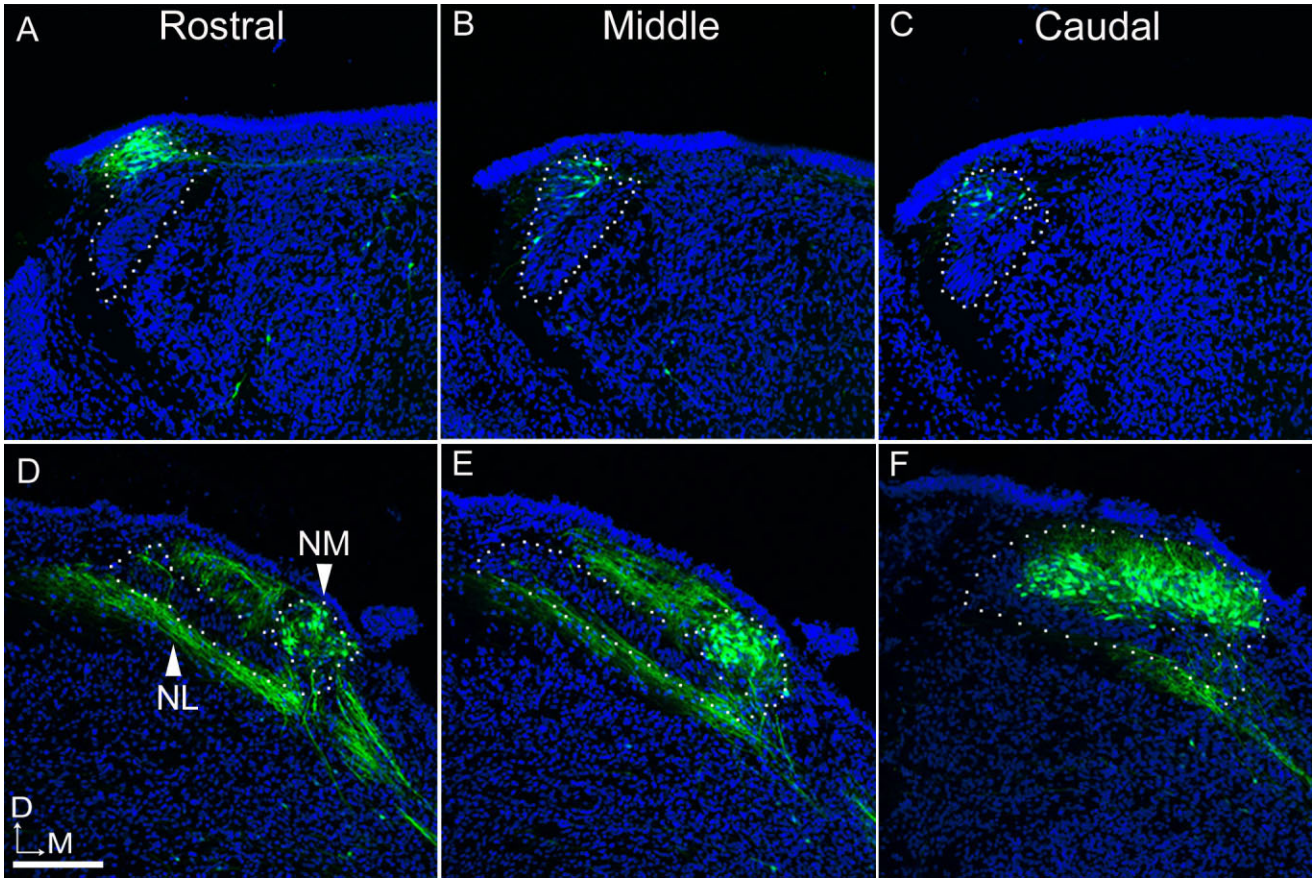

Fig. 5. Rostral-to-caudal progression of development of NM neurons. Neurons are retrogradely labeled with Alexa-488 Dextran (green), and all nuclei in the sections have been visualized with Hoechst dye (blue). **A–C:** E6 (St 29) embryos. **D,E:** E8 (St 34) embryos. At E6, the labeled neurons are located laterally near the rhombic lip, and contralateral axons are labeled, but few ipsilateral collaterals are present (A). By E8, the rostral-to-caudal segregation of labeled neurons into a more medially located nucleus is apparent in rostral and middle sections (D,E), whereas the caudal E8 neurons are still more diffusely dispersed (**F**). At E8, prominent ipsilateral collaterals are observed in addition to the contralateral fibers; these fibers coalesce in a region likely to be the developing NL. Calibration bars = 150 μm.

NL is bracketed by labeled fibers from both ipsi- and contralateral projecting NM neurons (Fig. 5, arrows).

Afferent and efferent connections within the anlage

To identify the location of VIIIth nerve fibers with respect to NM neurons within the auditory anlage, the cochlear nerve root was labeled just before entry into the brainstem with rhodamine conjugated dextran. In the same brains, Alexa 488-conjugated dextran was applied to the contralateral brainstem to label definitively the NM neurons. Between E6 (St 29) and E8 (St 34), cochlear afferents have entered the brainstem and coalesced at the ventral cell-free region of the auditory anlage (Fig. 7). In two cases at E6, we observed rhodamine-labeled fibers extending from the entry point at the lateral edge of the brainstem and looping medially around the rostral anlage to halt just external to the auditory anlage (data not shown). At E8, cochlear afferents were localized to the region of NM but were few in number. Some afferents traveled through nascent NL to enter NM from below, whereas most fibers followed a tangential path to contact NM neurons after traveling laterally around the auditory anlage (Fig. 7). Contact with NM neurons was present but sparse, which is corroborated by the paucity of SV2 staining we observed within NM at this age (compare with Fig. 3) as well as previous reports of VIIIth nerve contact (Jackson and Parks, 1982; Pettigrew et al., 1988; Molea and Rubel, 2003). Thus, although afferent VIIIth nerve fibers are traversing near the auditory anlage, NM neurons are developing without afferent input at the early stages of the auditory anlage.

DISCUSSION

The present study examined the early organization and differentiation of NM neurons within the maturing auditory anlage by using immunohistochemistry, retrograde labeling of NM axons, and orthograde labeling of the Vlllth nerve axons. At the earliest time that we could identify the auditory anlage, St 29 (E6), a few NM neurons from throughout the rostral to caudal extent of the anlage had begun to reach their contralateral targets in NL. This appears to be well before afferent connections from the VIIIth nerve have reached NM. Because NM contacts NL as many as 2 days before contacts from incoming cochlear

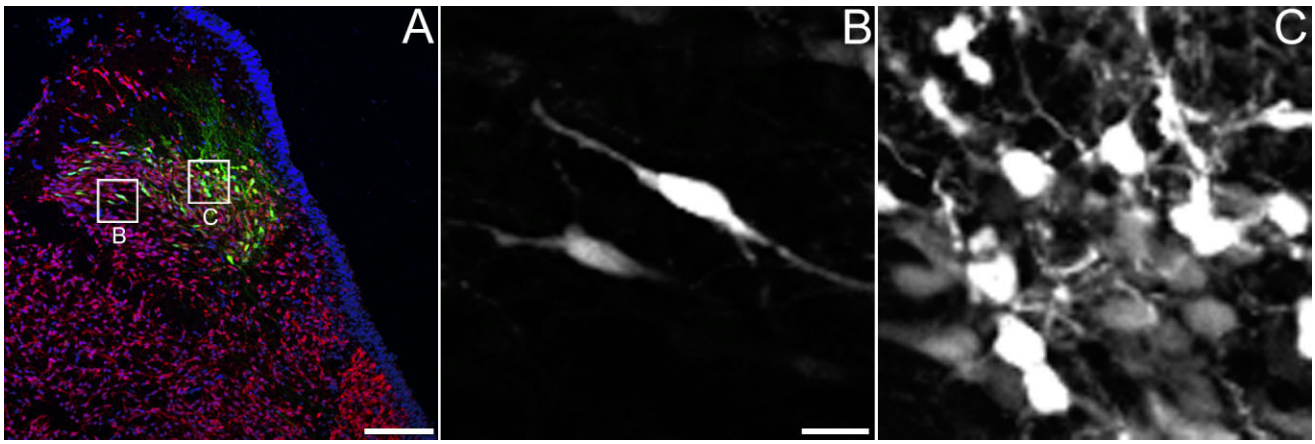

Fig. 6. Morphology of lateral NM neurons suggests that they migrate dorsomedially within the auditory anlage. **A:** Caudal E8 brainstem section stained with Hu C/D (red), Hoechst (blue) and labeled in the contralateral auditory anlage with Alexa-488-dextran (green) to identify NM neurons within the auditory anlage. **B:** Enlargement of box B in A showing elongated profile of neurons at the lateral portion of the anlage, suggesting a migratory phenotype. **C:** Enlargement of box C in A showing that NM neurons within the dorsomedial portion of the anlage are round and more static in appearance. Calibration bars = 100 μm in A; 20 μm in B and C.

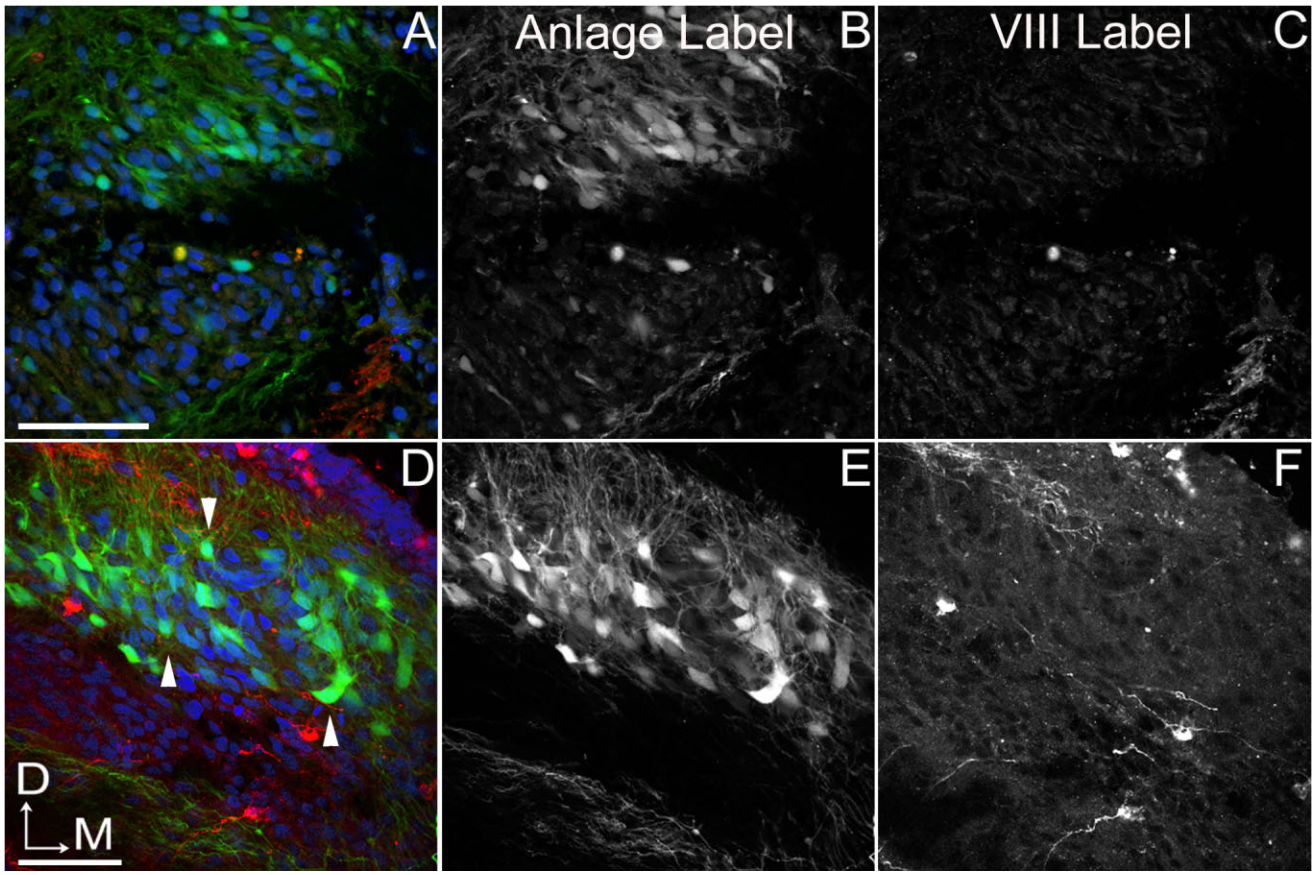

Fig. 7. Cochlear afferents begin to contact NM neurons at E8. Embryonic brainstems from E8 embryos were dissected and double labeled with Alexa-488-dextran (green) to identify NM neurons and with Rhodamine-dextran applied to the cochlear nerve stump to identify afferents (red). Arrowheads point to areas where afferents contact NM. Black and white images show the neurons (middle) and the afferents (right) in isolation. Calibration bar = 50 μm.

afferents, we suggest that NM plays a central role in the formation of tonotopic connections, perhaps by specifying tonotopic identity in NL while guiding appropriate tonotopic afferents to synapse within NM (Young and Rubel, 1986; Book and Morest, 1990; Molea and Rubel, 2003).

The identity of NM neurons at E6 in the anlage was inferred from the pattern of retrograde labeling from the contralateral hindbrain. NM axons cross the midline; hence, neuronal cell bodies are retrogradely labeled by dye that is placed in the contralateral hindbrain. The pattern of segregation of labeled neurons between E6 and E8 indicates that virtually all labeled neurons residing in the anlage form a nucleus characteristic in position and size to NM, rather than migrating to positions more characteristic of nucleus angularis or one of the vestibular nuclei. In addition, labeled axons crossing the midline project to the ventral portion of the anlage, which eventually develops into NL, the nucleus with which NM neurons synapse. The location of nucleus angularis (NA) neurons remains obscure in the auditory anlage. We saw no evidence of a population of neurons within the anlage that would be positionally and temporally correlated with the appearance of NA. Rubel et al. (1976) show that NA neurons are generated after NM and NL, and Book and Morest (1990) suggest that NA neurons migrate into the auditory anlage later than NM and NL neurons. We would have expected to see a population of neurons segregating as NA in the most rostral and dorsolateral portion of the auditory anlage, but by E8, retrogradely labeled NM neurons fill that entire region. This raises the possibilities that NA neurons migrate out of the anlage before E6; they are dispersed within the anlage so that no obvious pattern emerges, or they do not originate from the anlage.

Our observations suggest that reorganization within the anlage is an active migratory process. Initially, retrogradely labeled NM neurons are distributed throughout the auditory anlage between E6 and E8. By imaging sequential serial sections of labeled embryos from the most caudal (and hence most immature auditory hindbrain) to the most rostral (mature hindbrain), we could discern an apparent coalescing of labeled cell bodies from the lateral to the dorsomedial region of the anlage to form NM. In addition, the morphology of the labeled cell bodies in the most lateral regions of the anlage is elongated, reminiscent of migratory cells. Thus, the migration of neuronal somata to form the mature nucleus occurs independently of the projection of either ipsilateral or contralateral axonal collaterals and independent of the Vlllth nerve axons (Levi-Montalcini, 1949; Parks, 1979). Such mechanisms have been observed during the development of the central nervous system. For example, cerebellar granule cells synapse on Purkinje cell dendrites before migrating from the external to the internal granule layer (Sotelo, 2004). In addition, neurons that comprise the deep cerebellar nuclei also interrupt migration from the ventricular zone to extend a contralaterally projecting axon before resuming migration to the mature cerebellar location. This does not rule out the possibility that nascent NM and NL cell bodies could be relocated by the addition of new cells on the lateral margins of the hindbrain (Book and Morest, 1990).

Accurate pathfinding of NM neurons and the morphological organization of cells into NM and NL appear to occur in the absence of synaptogenesis. SV2 protein is an integral component of the synaptic vesicle (Feany et al., 1992). At the light microscope level, this antigen marks an accumulation of synaptic vesicles and is an early marker of synaptic terminals (Yang et al., 2002; Nealen, 2005). SV2 staining of NM contacts on NL is well developed before any evidence of SV2 immunoreactivity in NM and before labeled cochlear afferents contact NM. The pattern of SV2 staining visible in NL at E8 is similar to the pattern seen in the neural retina where SV2 was diffusely located initially, but then quickly localized to the terminal region of photoreceptors (Yang et al., 2002). The timing of arrival of VIIIth nerve fibers adjacent to NM neurons raise the possibility that initial targeting of both NM collaterals may be required before synapse initiation is begun in NM. Perhaps the receipt of retrograde signals from both ipsilateral and contralateral collaterals provides the signal for VIIIth nerve synapse initiation. The pattern and organization of neurofilament and SV2 staining during these stages of development closely follow reports based on single-cell fills and Golgi preparations (Jackson and Parks, 1982; Young and Rubel, 1986; Book and Morest, 1990). The development of SV2 immunoreactivity that we observed corresponds well with previously described functional synaptic connections between afferents and NM neurons at E10–E11, after numerous connections with NL have been made (Jackson et al., 1982).

What directs targeting of the NM axon collaterals? As NM fibers approach their target, various proteins may play a role in axon outgrowth and synaptogenesis. One family, the Eph receptors and their ligands, ephrins, show broad expression in the auditory brainstem nuclei during development. Eph receptors and ephrins play critical roles in pathfinding and target selection in many systems. One member, EphB2 prevents NM axons from crossing the midline (Cramer et al, 2006), and is expressed at low levels at E4. Ephrin-B1 and EphB2 show common expression in the axons surrounding the auditory anlage and at the midline between E7 and E8 (the earliest age examined), whereas Ephrin-B2 is localized to cell bodies within the anlage (Cramer et al., 2002). At E10, when the cells have ceased migration and preliminary targeting is well underway, the pattern of ephrin expression has generally increased in the nuclei (but decreased at the midline). It is intriguing that there are two proteins that are differentially expressed in NL at E10. At E10, EphA4 is heavily expressed in the dorsal neuropil of NL (Cramer et al., 2000b; Cramer et al., 2002). In contrast, TrkB and ephrin-2 are prominently expressed in the ventral neuropil (Cochran et al., 1999). These proteins may be integral for NL dendritic maturation, which is required for proper coincidence detection. Perhaps other pathfinding molecules, such as netrin, robo, slit, or extracellular matrix cues, play a more prominent role at the earliest stages of NM development and pathfinding (Long et al., 2004). Alternatively, extracellular matrix cues may direct the initial targeting, as observed in the sorting of olfactory axons into the olfactory bulb and olfactory tract (Henion et al., 2005).

Our results indicate that morphological development of synapses between NM and NL precedes contacts between cochlear afferents and NM. Previous published reports provide electrophysiological evidence for this same conclusion (Jackson et al. 1982), show that otocyst removal does not cause significant changes in spatiotemporal patterns of dendritic development in NM and NL (Smith, 1981; Parks and Jackson, 1984), and show that the tonotopic "identities" of NM neurons are independent of cochlear nerve innervation (Lippe et al., 1992). Taken together, these results suggest strongly that NM serves as an early central organizer of synaptic connections in the avian

auditory system. NM neurons are born early (Rubel et al., 1976) and are specified in rhombomeres 5–7 of the developing hindbrain, whereas NL neurons are generated primarily from rhombomere 5, with a small contribution from rhombomere 6 (Cramer et al., 2000a; Marin and Puelles, 1995). Thus, positional identity can be encoded in NM through cues received in the rhombomeres. The early projection of NM axons to the contralateral NL in the absence of contact from cochlear afferents could specify tonotopic identity of NL. It has been shown that this projection is highly topographic from the earliest age it can be studied (Young and Rubel, 1986). Similarly, ingrowing afferents would seek positional cues already located on NM neurons. Thus, the auditory anlage represents an important period during NM development in which primary targeting of NM collaterals occurs during the coincident migration of the soma and the target NL neuron. Innervation of NM and NL appears to be dependent on the combination of contralateral collateral and ipsilateral collateral targeting before synapses are established within both NM and NL. This finding suggests that the initial organization of the auditory brainstem relies on the targeting of NM contralateral axons to NL but does not preclude further refinement of these connections through neuronal activity once afferent innervation has been established. Future studies that disrupt extension and targeting of the contralateral NM collateral would illuminate the possible role of NM as a central organizer of the avian auditory pathways.

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