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Ontogeny of Tonotopic Organization of Brain Stem Auditory Nuclei in the Chicken: Implications for Development of the Place Principle

WILLIAM LIPPE AND EDWIN W RUBEL
Departments of Otolaryngology and Physiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

ABSTRACT

The morphological development of the cochlea begins in the base or midbasal region and spreads toward the apex. In adults, the base responds maximally to high-frequency sounds and lower frequencies are represented progressively toward the apex. This predicts that responses to sound should occur initially to high frequencies and gradually change to include lower frequencies. Paradoxically, animals respond first to relatively low frequencies and last to high frequencies.

We have previously proposed that this discrepancy results from an ontogenetic change in spatial coding of frequency along the cochlea (Rubel et al., '76). According to this model, only the basal end of the cochlea transduces sound early in development but it responds to low frequencies. During maturation the representation of low and midrange frequencies shifts apically and the base becomes responsive to high frequencies. This hypothesis predicts that the tonotopic organization within the central nervous system should change during development; neurons at any given location within an auditory nucleus should become maximally responsive to successively higher frequency sounds during development.

In the present study this prediction was tested by using microelectrode recording procedures to map the tonotopic organization of nucleus magnocellularis (NM) and nucleus laminaris (NL), first- and second-order auditory nuclei, in chickens at three ages: embryonic day 17, 1 day posthatch, and 2–4 weeks posthatch. The characteristic frequencies of neurons having the same anatomical location were quantitatively compared across ages.

The tonotopic order in NM and NL was similar at all ages; responses to high-frequency sounds were recorded anteromedially and lower frequencies were located progressively more caudolaterally. However, there was a striking quantitative change in tonotopic organization. Neurons at a given location in both nuclei became maximally responsive to progressively higher frequencies during development. The characteristic frequencies of neurons in embryos and newly hatched chicks averaged, respectively, 1.00 (± 0.06, S.E.M.) and 0.34 (± 0.04) octaves lower than their predicted adult values. All regions in both nuclei showed a statistically significant increase in characteristic frequency during development except the most posterolateral (low-frequency) sector. Too few neurons were recorded from this region to be able to reliably estimate characteristic frequency.

These results support the hypothesis that the spatial coding of frequency along the cochlea shifts during development. This has three implications: (1)

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William Lippe's present address is Department of Otorhinolaryngology, University of Oklahoma Health Sciences Center, P.O. Box 26307, South Pavilion, Oklahoma City, OK 73126. Address reprint requests there.

it helps explain the discrepancy between structural and functional development. (2) it suggests that all neurons will be maximally stimulated by low frequencies at some time during development. (3) it indicates that the values assigned to the place code are not fixed and immutable.

Key words: nucleus magnocellularis, nucleus laminaris, basilar membrane, spatial representation of frequency, spatial coding of frequency

The spatial coding of sound frequency has been one of the fundamental organizing principles in studies of the vertebrate auditory system. The spatial representation of frequency along the cochlea, known as the place principle, was first demonstrated in von Békésy's classic studies of basilar membrane motion in human and animal cadavers (von Békésy, '60). These studies showed that sound stimulation produces a traveling wave of motion along the cochlear partition, and that the maximum point of deflection of the traveling wave varies systematically as a function of sound frequency. High frequencies produce a maximum deflection at the base while low frequencies are represented progressively toward the apex. Electrophysiological studies have subsequently shown that a spatial representation of frequency is preserved at all levels of the central auditory system in tonotopic frequency maps, the orderly arrangement of neurons according to their characteristic frequency or frequency to which they are most sensitive (Rose et al., '59, '63; Tsuchitani and Boudreau, '66; Goldberg and Brown, '68; Aitkin et al., '70; Aitkin and Webster, '71; Reale and Imig, '80).

Recent attempts to explain inconsistencies between the structural and functional development of the auditory system have led to the suggestion that the spatial coding of frequency along the cochlea changes during development (Rubel et al., '76; Rubel, '78; Jackson et al., '82). In brief, the morphological maturation of the cochlea proceeds from the base or midbasal region toward the apex. This finding has been reported in virtually every species studied, including birds, and is apparent in the formation of afferent and efferent synapses, the differentiation of receptor and supporting cells, and the formation of intercellular spaces (Wada, '23; Änggard, '65; Kikuchi and Hilding, '65; Mikaelin and Ruben, '65; Pujol and Marty, '70; Bredberg, '68; Chandler, '84; Fermin and Cohen, '84). Because cochlear development proceeds from base to apex, animals would be expected to respond first to high-frequency sounds and later to low frequencies. Paradoxically, electrophysiological and behavioral responses occur first to low- or midrange-frequency sounds and gradually change to include high frequencies (see Gottlieb, '71; Rubel, '78; Ehret, '83; Romand, '83, for reviews of this literature). This low- to high-frequency gradient of functional development also characterizes the later maturation of physiological and behavioral thresholds (Ehret and Romand, '81; Gray and Rubel, '81, '85; Rebillard and Rubel, '81) and physiological response properties (Brugge et al., '78; Moore and Irvine, '79).

Several hypotheses have been proposed to explain this paradoxical dissociation between structural and functional development (Larsell et al., '44; Vanzulli and Garcia-Austt, '63; Pujol and Marty, '70; Saunders et al., '73), including the idea that the inner ear functions differently in the developing animal than in the adult. In particular, we have previously proposed the following scheme. The transduction of sound occurs first at the early developing basal or midbasal region of the cochlea. However, the basal end of the immature cochlea, unlike that in the adult, is maximally responsive to low- rather than to high-frequency sounds. During development the point of maximum sensitivity to low- and midrange-frequency sounds gradually shifts apically as the basal end becomes responsive to higher frequencies (Fig. 1; Rubel et al., '76; Rubel, '78).

One prediction made by this hypothesis is shown in Figure 1. A change in the spatial representation of frequency along the cochlea will be mirrored as a corresponding change of tonotopic organization within the central auditory system. During the period of development in which the spatial coding of low- and midrange-frequency sounds is shifting apically along the cochlea, the tonotopic organization of any nucleus within the central auditory system will be (1) orderly and qualitatively similar to that in the adult, but (2) shifted to lower frequencies. Neurons at any given location in a nucleus will be most sensitive to progressively higher frequencies as development proceeds.

The avian auditory system is a useful preparation for studying the development of tonotopic organization and testing these predictions. Birds, like humans, are precocial with respect to the development of hearing, and the onset of auditory function occurs embryonically (Vanzulli and Garcia-Austt, '63; Saunders et al., '73; Jackson and Rubel, '78; Jackson et al., '82). Nucleus magnocellularis (NM) and nucleus laminaris (NL), first- and second-order auditory nuclei, have a relatively simple geometry and pattern of afferent innervation which enables their tonotopic organization to be quantitatively described at different times during development (Rubel and Parks, '75). The basilar papilla (cochlea) is tonotopically organized with high-frequency sounds represented at the proximal end and lower frequencies represented progressively more distally (von Békésy, '60; Ryals and Rubel, '82). Neurons at the anteromedial pole of NM receive projections from the proximal end of the basilar papilla and respond to high-frequency sounds; neurons at progressively posterolateral locations in NM are innervated by more distal points along the papilla and respond to progressively lower frequencies (Boord and Rasmussen, '63; Rubel and Parks, '75). NL receives a frequency-matched, spatially segregated input via NM. The tonotopic organization of NL also extends along its anteromedial to posterolateral axis, reflecting a topographically organized input from NM (Parks and Rubel, '75; Rubel and Parks, '75).

In the present experiment microelectrode recording procedures were used to map the tonotopic organization of NM and NL at different times during development. The results show that the tonotopic organization of these nuclei shifts in an orderly way during ontogeny. Preliminary results of

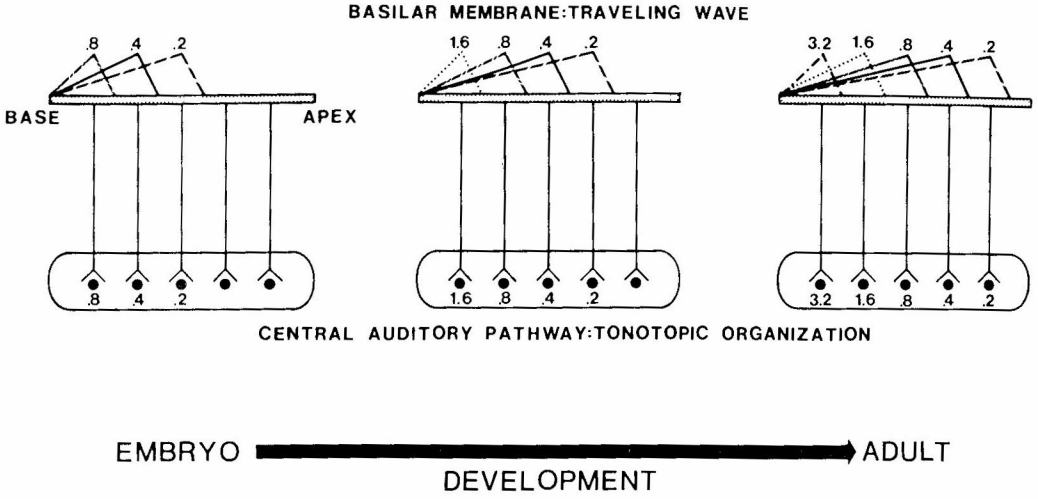

Fig. 1. Model of shifting place code discussed in text (from Rubel, '84). The basilar membrane projects topographically onto central auditory nuclei via the cochlear nerve. The change in tonotopic organization that would occur in central auditory nuclei as the maximum point of deflection of the traveling wave to low- and midrange-frequency sounds (indicated in kHz) shifts apically along the basilar membrane during development is schematically illustrated. The hypothesis of a shifting place code also predicts that the position of hair cell damage along the basilar membrane resulting from high-intensity, pure tone stimulation will occur more apically in animals exposed at later times during development (see Rubel and Ryals, '83).

this study have been reported previously (Lippe and Rubel, '83).

METHODS

Subjects and surgical procedures

Electrophysiological mapping experiments were carried out in a total of 114 Hubbard × Hubbard chickens at three different times during development: 17 days of incubation (embryos: n = 67), 5–24 hours posthatch (hatchlings: n = 15), and 2–4 weeks posthatch ("adults": n = 32). In addition, previously published data on the tonotopic organization of NM and NL in 2–4-week posthatch White Leghorn and Red Cornish chickens have been included (Rubel and Parks, '75).

Fertile eggs were obtained from a commercial supplier and incubated in the laboratory in a forced-draft incubator at 37°C and 50–60% humidity. At 17 days of incubation (hatching = 21 days) embryos were prepared for recording by gently pulling their head through a small hole made in the shell overlying the air space. The head region was infiltrated with a local anesthetic (2% Procaine), 1 mg of gallamine triethiodide (Flaxedil, American Cyanamid) was injected into the dorsal neck muscle to eliminate spontaneous movements, and the tissue surrounding the right ear was dissected away so that the tympanic membrane could be visualized. The egg and embryo were placed inside a humidified and temperature-controlled (38°C) Plexiglass chamber, and the embryo's head was securely supported by inserting its beak into a cup filled with silicone impression rubber. Following a midline incision, the skin and connective tissue covering the dorsal cranium were retracted. The right cerebellar cortex was then exposed by removing a small piece of the overlying skull and dura.

Heart rate was monitored throughout the experiment with Grass platinum subdermal electrodes inserted through two small holes made in the shell to either side of the embryo (Jackson and Rubel, '78). When the embryo was in good condition its heart rate was 240–300 beats/minute. Any arrhythmia indicated that the physiological condition of the embryo was deteriorating. When this occurred, the experiment was terminated. Embryos typically remained in excellent physiological condition for 18–24 hours.

Adults and newborn hatchlings were prepared for electrophysiological recording as previously described (Rubel and Parks, '75). They were anesthetized with a combination of Chloropent (Fort Dodge Laboratories; 0.15 cc/100 g body weight, i.p.) and Ketalar (Parke-Davis; 8 mg/100 g body weight, i.m.) and 0.04 mg of atropine sulfate was injected subcutaneously to reduce respiratory congestion. Adults were also tracheotomized to further minimize respiratory problems. During the experiment a surgical depth of anesthesia was maintained with supplementary doses of Chloropent and Ketalar, core body temperature was thermostatically controlled at 37°C, and birds were regularly hydrated with subcutaneous injections of 5% dextrose in 0.9% saline.

The tissue surrounding both ears was removed and the dorsal cranium was exposed by making a midline incision and retracting the overlying skin and muscle. In adult birds, the head was supported by attaching bars to screws secured in the skull and immersing the beak in a cup of dental acrylic. In newborn hatchlings, the skull has not fully calcified and the head was supported with a bar glued to the beak and dorsal skull with cyanoacrylate glue. The occipital bone overlying the cerebellar cortex was removed. After ligating the middorsal sinus and reflecting back the dura, the entire cerebellum was aspirated. This exposes the floor of the fourth ventricle and allows the fibers of the cochlear nerve to be directly visualized on the dorsal surface of the medulla as they pass over NM. The exposed brain stem was covered with a pool of warm mineral oil.

Sound delivery and calibration

Sound stimuli were presented by using a calibrated, closed delivery system. Sound delivery tubes were positioned over the ear openings and sealed to the side of the head with hearing aid impression compound (Tru-Mold). A Bruel and Kjaer calibration earphone (model HT0003) coupled to the

delivery tube was used for sound presentation. The sound pressure level at the ear opening was measured with a calibrated probe tube inserted down the center of the delivery tube and connected to a General Radio half-inch electret microphone (model 1560-P42). This system was able to deliver 100 Hz–5.0 kHz sounds at a peak amplitude of 110–125 dB (SPL). Prior to the beginning of each experiment the system was calibrated with a General Radio Wave Analyzer (model 1900A).

Pure tones were generated with a Wavetek function generator (model 186), and white noise was produced with a Grason-Stadler noise generator (model 901B). Sound stimuli were led through an electronic switch and then to a manually controlled attenuator. During the experiment sound delivery could be independently controlled to either ear. Sound stimuli were either pure tones or white noise bursts of 50–100-msec duration with rise/fall times of 5 msec. Stimuli were presented to adults and hatchling chickens at rates of 1.0–5.0/second. Embryos were stimulated at 0.5–1.0/second because of the decreased response which occurs at higher rates of stimulus presentation.

Recording procedures

Recordings of single units and unit clusters were made with glass-insulated tungsten microelectrodes having exposed tips of 5–30 μm and impedances of 0.5–3.0 MΩ. The unit activity was amplified, high-pass filtered, viewed on an oscilloscope, and listened to with an audio monitor. The amplified unit activity was also led to a WPI window discriminator whose output was monitored on an oscilliscope and led to an Ortec Model 4620 signal processor for generating poststimulus time histograms.

Physiological recordings were carried out inside a double-walled sound-attenuated chamber (Industrial Acoustics Corporation). In embryos the electrode was lowered manually through the cerebellum while 90 dB (SPL) white noise bursts were presented. Entry of the electrode into the cochlear nerve overlying NM and NL was indicated by a sudden increase in spontaneous and sound-evoked activity. Stereotaxic coordinates could not be used for placement of the electrode because it was not possible to reliably position embryos in a standard orientation. However, after some experience it was possible to use surface landmarks and locate NM and NL within one or two electrode penetrations. In adults and hatchlings the electrode was visually positioned in the cochlear nerve overlying NM.

The subsequent movement of the electrode through NM and NL was controlled from outside the recording chamber by an electronically driven microdrive. The vertical movement of the microdrive was stopped when a unit or unit cluster was isolated; the depth of the electrode was then recorded and the characteristic frequency (CF) was determined. The characteristic frequency was judged by monitoring the evoked activity on both an oscilloscope and audio monitor and determining the frequency at which the lowest excitatory response threshold occurred. In approximately half the recordings the characteric frequency was also determined by computing poststimulus time histograms. In all cases the characteristic frequencies determined by the two methods agreed within 100–200 Hz. At the termination of each electrode penetration one or more small marking lesions were made at different depths along the electrode tract by passing an 8-μA anodal current for 10 seconds through the tip of the recording electrode. The microlesions were later used to identify recording points and calculate tissue shrinkage. Typically, only three to five electrode penetrations were made per bird with the adjacent penetrations spaced 200–300 μm apart. With a greater number of penetrations we have had considerable difficulty in accurately localizing electrode tracts.

Histological procedures

At the end of the experiment the bird was overdosed with Chloropent. Embryos were staged according to Hamburger and Hamilton ('51). The brain was blocked *in situ* at the same angle as the electrode penetrations. The head was immersed in Bouin's fixative for 12–24 hours, and the brain was embedded in paraffin. Serial 10 μm coronal sections were cut through the region of the brain stem that included NM and NL and stained with thionin.

Electrode tracts were identified by examining the stained sections under the light microscope. Each tract was drawn at a total magnification of 155×. Tissue shrinkage was calculated separately for each brain by measuring either the distance between two microlesions in the same electrode tract or the distance between adjacent electrode tracts. The calculated shrinkage and the records of recording depth from the experimental protocol were then used to plot the recording locations on the drawings of the electrode tracts. Although responses to sound were recorded from the regions surrounding NM and NL, only recordings which were located within NM or NL are included in the data presented below.

Data analysis

The methods for quantitatively representing and analyzing the tonotopic organization of NM and NL are similar to those previously used to study the functional organization and development of these regions (Rubel and Parks, '75; Smith and Rubel, '79; Jackson et al., '82). The position of each unit and cluster recorded in NM and NL was plotted on horizontal projections of these nuclei constructed from each brain. The distances of the medial and lateral edges of NM and NL from the midline of the brain were measured along a line orthogonal to the midline and plotted as a function of each section's anterior-posterior position. This constitutes a two-dimensional reconstruction or horizontal projection of the nuclei as viewed from a dorsal perspective. The distances of each unit cluster from the midline and anterior pole of the nucleus were measured and plotted on the horizontal projection. By setting the total medial-lateral and anterior-posterior extents of the nuclei equal to 100%, each unit cluster and its characteristic frequency was specified in terms of percentile posterior-anterior and lateral - medial positions. This method of normalization allows positional data to be combined from animals of the same age and compared between animals of different ages despite variations in the absolute size of the nuclei.

The absolute sizes of NL and NM change over the ages studied; however, their shapes and the number of neurons remain constant. Thus, group mapping data are presented here on mean composite horizontal projections of NM and NL (Fig. 2). The mean projections were constructed by averaging individual horizontal projections at every fifth percentile along their anterior-posterior dimensions from three Hubbard × Hubbard chickens 2–4 weeks posthatch.

Single and multiple linear regression analyses were used to quantitatively describe the relationship between best frequency and position in NL and NM. One-way analyses of variance and student t-tests were used to assess the

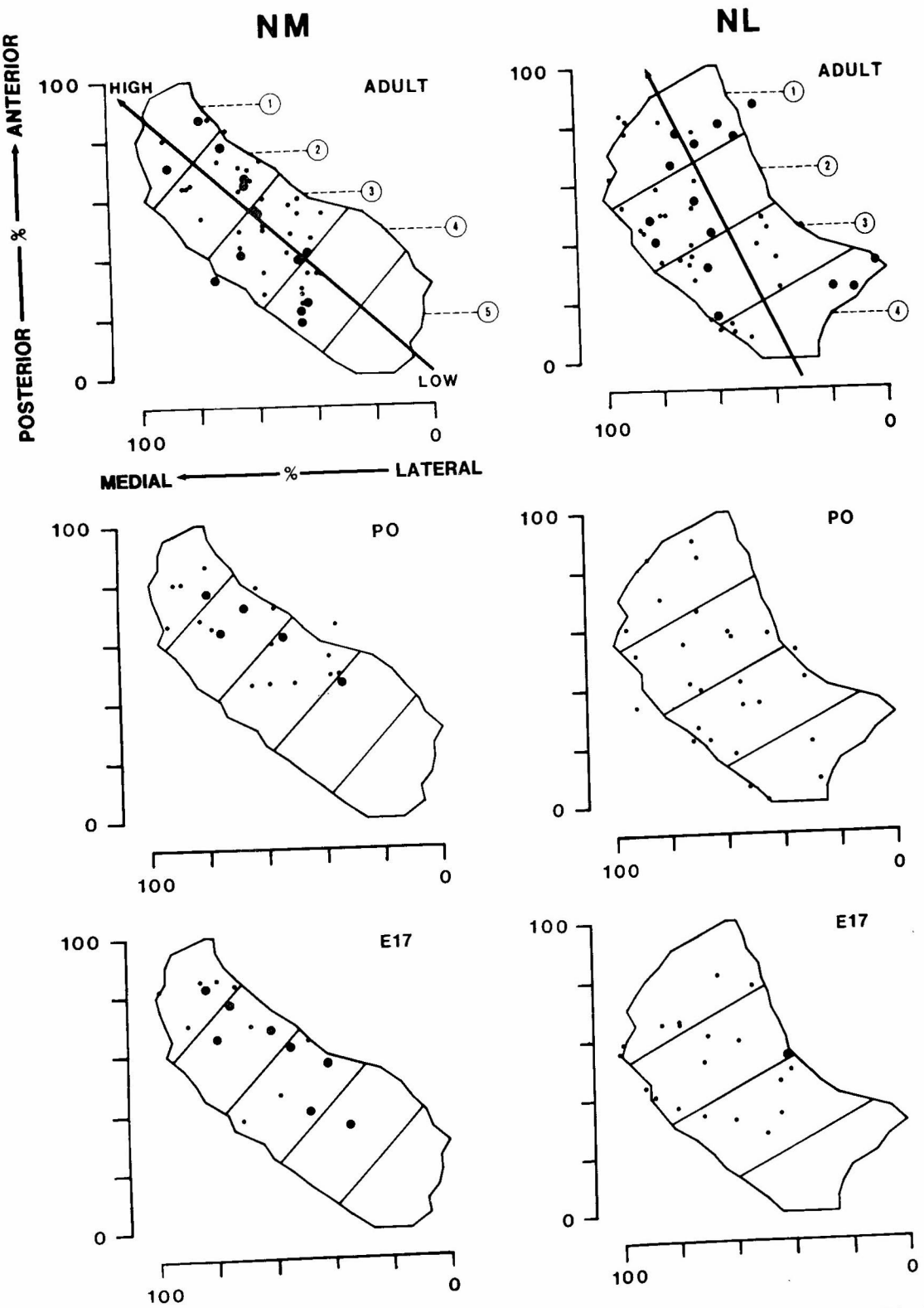

Fig. 2. Mean horizontal projections of nucleus magnocellularis (NM) and nucleus laminaris (NL) on the right side of the brain (see Methods). Dots indicate positions of the units and unit clusters which were used for quantitative analyses in adult chicks (top), young hatchlings (P0—middle), and 17-day embryos (E17—bottom). Large dots are locations where more than one unit or unit cluster was recorded. The arrows overlying the adult plots show the orientation of the frequency axes (tonotopic gradients) in NM and NL. The horizontal projections have been divided into four (NL) and five (NM) sectors perpendicular to the frequency axes for data analysis. See Figures 6 and 10 and Results for details.

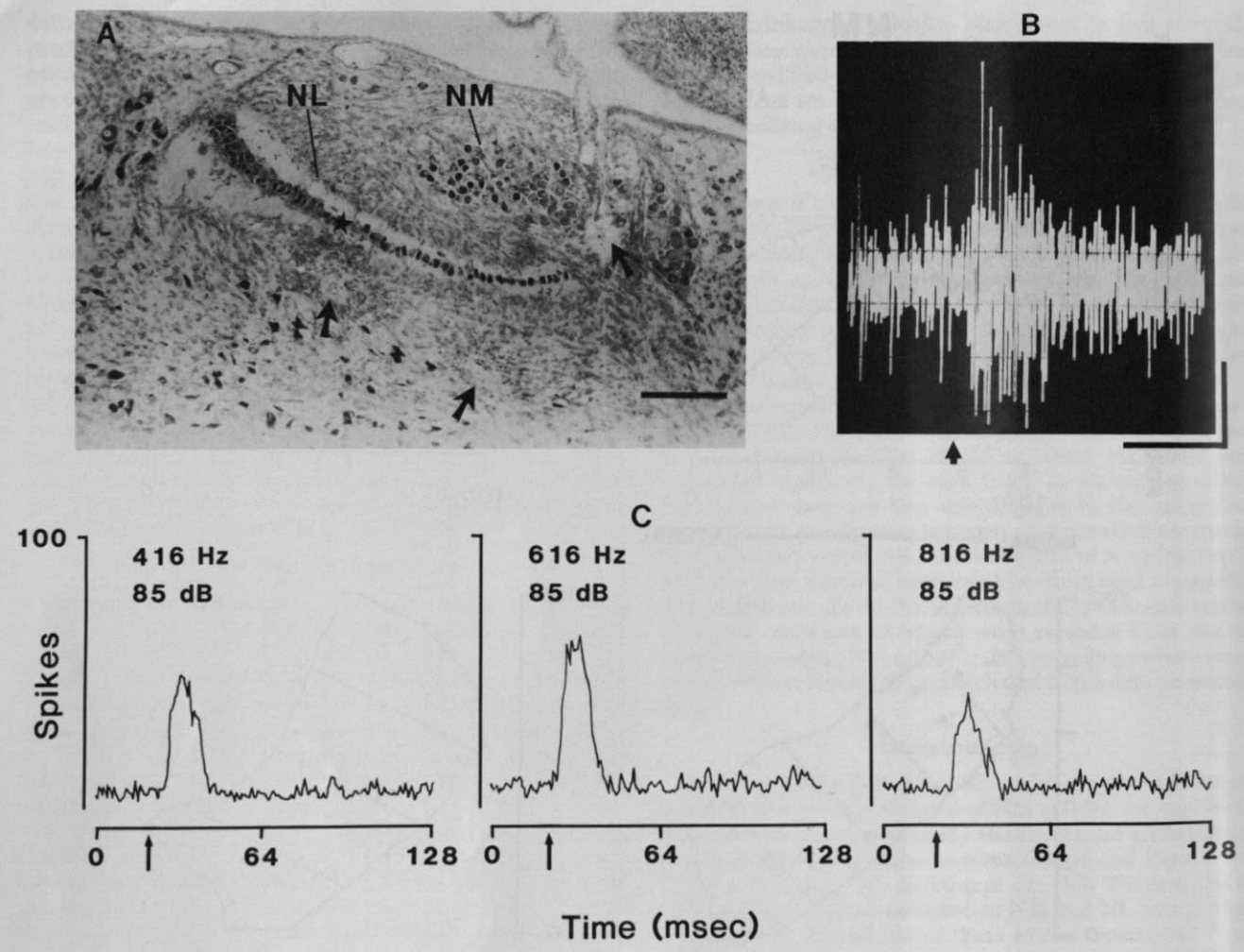

Fig. 3. A. Photomicrograph showing an electrode tract and marking lesion (large arrow) in nucleus magnocellularis (NM) of a 17-day embryo. The marking lesions in two other electrode tracts (not visible in this section) are indicated by the two smaller arrows below nucleus laminaris (NL). Calibration bar = 0.2 mm. B. Multiple unit activity recorded in nucleus laminaris (star in A) to a 616-Hz, 40-msec-duration tone burst. Ipsilateral stimulation; stimulus onset indicated by arrow. Calibration bars = 20 msec and 200 μV. C. Poststimulus time histograms of largest units shown in B to ipsilateral stimulation. The characteristic frequency of these units was 616 Hz and their threshold was 65 dB (SPL). Histograms summed over 64 repetitions. Stimulus duration was 40 msec; stimulus onset indicated by arrows.

statistical significance of changes in tonotopic organization at different stages of development.

RESULTS

The observations reported here are based on 80 electrode penetrations that passed through NM or NL in adults, 54 in newborn hatchlings, and 62 in embryos. These yielded 102, 107, and 150 units and unit clusters that were located within NM and NL in adults, hatchlings, and embryos, respectively. Many of these recordings were excluded from the quantitative analyses of tonotopic organization described below, either because their locations could not be determined accurately or the brains from which they were made were cut askew to our standard plane of sectioning. However, the same overall patterns and developmental differences in tonotopic organization were observed in these units and unit clusters and those included in the quantitative analyses. The locations of all units and unit clusters that were used for quantitative analysis are shown on planar projections of NM and NL in Figure 2.

Response properties

Responses to sound in embryos and hatchlings were qualitatively similar to those observed in adult birds in the present and previous studies (Stopp and Whitfield, '61; Konishi, '70; Rubel and Parks, '75; Sachs and Sinnott, '78). Units and unit clusters had well-tuned responses (Fig. 3). Neurons in NM showed excitatory responses to ipsilateral stimulation. The majority of recordings in NL were from multiple units and included both pre- and postsynaptic elements. The individual units which were isolated showed binaural, excitatory responses, indicating that they were NL neurons. Although their response properties were not analyzed quantitatively, they had approximately the same characteristic frequency to stimulation of either ear.

We did observe two obvious differences between recordings from embryos vs. hatchlings and adults. First, response thresholds were 20–40 dB higher in embryos than in hatchlings and adults. Second, the pattern of spontaneous activity recorded from NM and NL in embryos was

different than in hatchlings and adults (Fig. 4). Multiunit recordings in adults and hatchlings showed high levels of spontaneous activity without any obvious rhythm or pattern. In contrast, spontaneous activity in embryos occurred in bursts at regular intervals of 1.5–2.0 seconds. This rhythmic pattern of spontaneous discharge was observed in every embryo and was not found in recordings from nonauditory regions of the brain stem. The bursts of spontaneous activity did not occur synchronously with any obvious movements of the embryo, respiratory activity (pulmonary respiration in embryos begins on day 19), or the embryo's heart beat. The rhythmicity was an excellent indication of the embryo's condition. Large amplitude-evoked activity was recorded when rhythmic discharges were present. If the rhythmicity became poorly defined or disappeared, the amplitude of evoked activity also decreased and the embryo typically died within an hour.

Tonotopic organization in adults

The tonotopic organization of NM and NL in adult Hubbard × Hubbard chickens observed in this study was similar to that previously described for mature White Leghorn and Red Cornish birds (Rubel and Parks, '75). The characteristic frequency of neurons progressively increases as a recording electrode moves from posterior to anterior or lateral to medial in both NM and NL. Throughout most of NM an electrode that passes dorsoventrally encounters neurons whose characteristic frequencies do not differ by more than 250 Hz. Nissl-stained sections show that these functional isofrequency columns are correlated with a columnar arrangement of cell bodies. The most caudolateral 20–30% of NM is an exception (Fig. 2). Neurons in this region are innervated by the macula lagena and do not respond to sound (Boord and Rasmussen, '63; Boord and Karten, '74; Rubel and Parks, '75).

To quantitatively describe the relationship between characteristic frequency and position within NM and NL, the data were analyzed using single linear regression analyses (Rubel and Parks, '75). The percentile positions of each unit and unit cluster along the posterior-anterior and lateral-medial dimensions of the brain were determined from its location on the planar projections of NM or NL (Fig. 2). Characteristic frequency was regressed separately on each positional dimension for both nuclei. Since no breed differences in tonotopic organization were found, the 47 unit clusters recorded from adult chickens here were combined with 93 clusters previously recorded from mature White Leghorn and Red Cornish birds (Rubel and Parks, '75). This provided a total of 140 unit clusters (NM = 70; NL = 70) in adult chickens for quantitative analysis. The least-squares regression lines ± 1 standard error of estimate are shown in Figure 5.

These analyses show that there is a highly significant relationship between characteristic frequency and both posterior-anterior and lateral-medial position in each nucleus. The correlation between posterior-anterior position and characteristic frequency was 0.83 ($P < .001$) in NM and 0.87 ($P < .001$) in NL. The correlations for lateral-medial position were 0.81 ($P < .001$) in NM and 0.60 ($P < .001$) in NL. Thus, either positional dimension considered alone accounts for between 36–75% of the variance of characteristic frequency in NM and NL.

Because characteristic frequency correlates with both positional dimensions, a better prediction of characteristic frequency can be obtained with a multiple regression of

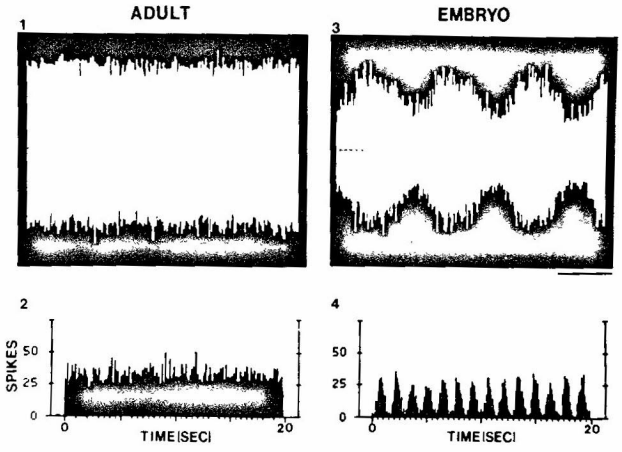

Fig. 4. Recordings of spontaneous multiple unit activity from nucleus magnocellularis in a 2-week-old chicken (adult) and a 17-day embryo. The top panels (1, 3) are tracings of multiple unit activity from an oscilloscope screen. The lower panels (2, 4) are histograms of the number of spikes which occurred during a single 20-second period of the spontaneous activity shown in the top panels. They are records of the largest units in the multiple-unit recordings. Note that spontaneous multiple-unit activity in adults occurs without any apparent rhythm whereas in embryos it occurs in bursts at regular intervals. Bar = 1 second.

characteristic frequency on posterior-anterior and lateral-medial positions. These analyses show that position accounts for 81% of the variance in characteristic frequency in NM ($r = .90$, $P < .001$) and 87% in NL ($r = .94$, $P < .001$). Thus, characteristic frequency can be predicted accurately as a function of a neuron's Cartesian coordinate position in both NM and NL.

The multiple regression analyses also show that the tonotopic gradients in NM and NL extend along the long axis of their planar projections (Fig. 6). Units with high characteristic frequencies are located anteromedially and neurons responding to low frequencies are located progressively posterolaterally. Neurons which are located along a given line oriented orthogonal to the posterolateral-anteromedial axis of the planar projections are predicted to have the same characteristic frequency. Thus, the tonotopic organization of NM and NL is represented most accurately by plotting characteristic frequency as a function of percent distance along the tonotopic (posterolateral-anteromedial) axes (Fig. 6C).

Comparison of tonotopic organization in adults, hatchlings, and embryos

Representative surface maps and electrode penetrations that passed through approximately the same regions of NM and NL in adults, hatchlings, and embryos are shown in Figure 7. The tonotopic organization of NM and NL in adults, hatchlings, and embryos is compared quantitatively in Figure 8. The relationships between characteristic frequency and posterior-anterior and lateral-medial positions in adults are shown by the linear regression lines ± 1 standard error of estimate in each panel (replotted from Fig. 5). The relationships between best frequency and position in hatchlings and embryos are indicated by the scatter plots. These consist of 59 unit clusters (NM = 28; NL = 21) recorded from seven embryos and 53 clusters (NM = 26; NL = 27) recorded from seven hatchlings.

Three findings are apparent from Figure 8. First, the highest-frequency sounds to which neurons were tuned in-

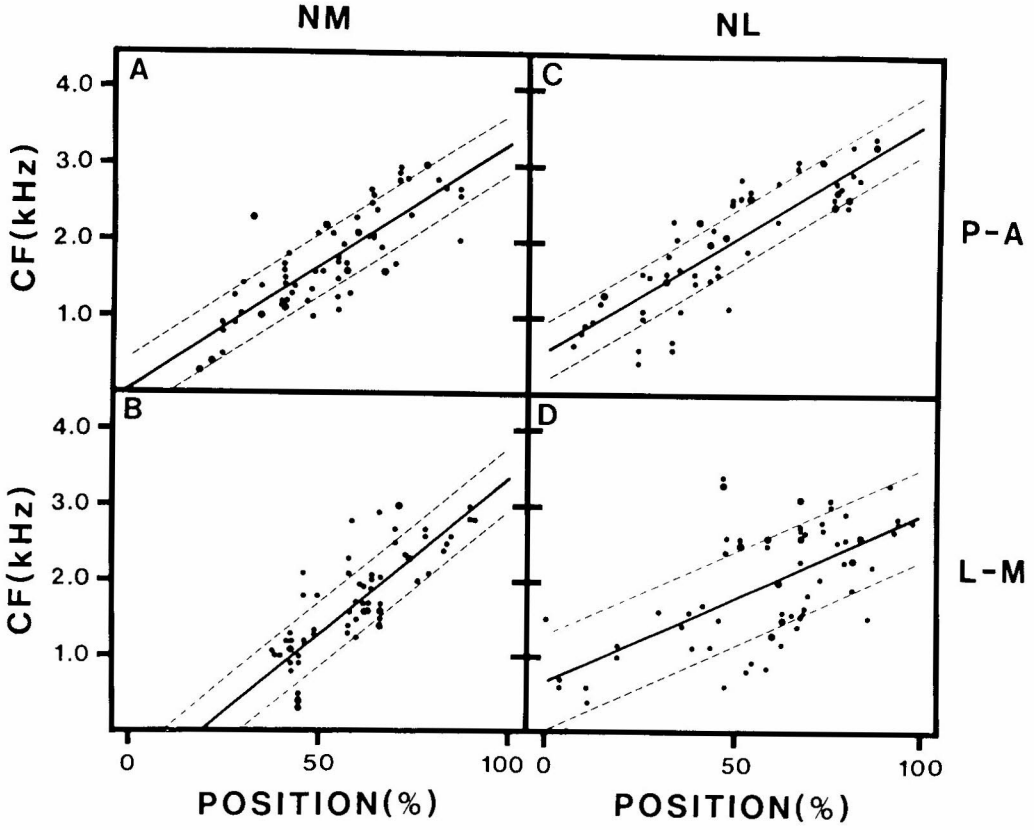

Fig. 5. Scatter plots and linear regressions ± 1 standard error of estimate (solid and dashed lines, respectively) relating the characteristic frequency (CF) of units to percent posterior-to-anterior and lateral-to-medial position of the electrode tip in nucleus magnocellularis (NM) and nucleus laminaris (NL) of adult chickens. Large dots indicate locations where more than one unit or unit cluster having the same CF was recorded. A. Regression of CF on posterior-anterior position in NM: CF (Hz) = 32(%p-a) - 5; standard error of estimate = 401 Hz; 70 neurons. B. Regression of CF on lateral-medial position in NM: CF = 41(%l-m) - 770; standard error of estimate = 425 Hz; 70 neurons. C. Regression of CF on posterior-anterior position in NL: CF = 30(%p-a) + 582; standard error of estimate = 399 Hz; 70 neurons. D. Regression of CF on lateral-medial position in NL: CF = 22(%l-m) + 691; standard error of estimate = 640 Hz; 70 neurons. Data combined from the present study and Rubel and Parks ('75).

creased progressively during development. The range of characteristic frequencies observed increased from 285–2,017 Hz in embryos, to 300–2,820 Hz in hatchlings, to 300–4,100 Hz in adults. This systematic increase in high-frequency sensitivity during development does not reflect a bias between groups in the regions sampled since recordings were made from the anteromedial (high-frequency) region of NM or NL at all ages. Second, a well-defined tonotopic organization is present in both embryos and hatchlings, and it is qualitatively similar to that in adults. Progressively higher characteristic frequencies are observed as a recording electrode is moved from lateral to medial or from posterior to anterior in both NM and NL. Furthermore, in embryos and hatchlings, as in adults, dorsoventrally oriented electrode penetrations through NM encounter neurons with approximately the same characteristic frequencies. Finally, there is a striking quantitative difference in tonotopic organization between adults and both embryos and hatchlings. At every position in embryos, the characteristic frequencies observed were lower than in adults. This was true along both dimensions in each nucleus. In newly hatched chicks the characteristic frequencies observed at each position in both NM and NL were intermediate between those in embryos and adults.

Fig. 6. The relationship of characteristic frequency (CF) to location of the electrode tip along the tonotopic axes in nucleus magnocellularis (NM) and nucleus laminaris (NL) of adult chickens. A. The multiple linear regression equation predicting CF as a joint function of percent lateral-to-medial (L-M) and posterior-to-anterior (P-A) positions defines a plane of CF (pincushioned) which is located above the horizontal projection of the nucleus (stippled). This is illustrated for NM. The direction of maximum increasing slope of the regression plane is indicated by the upper arrow. The projection of this arrow downward defines the orientation of the frequency axis or direction of increasing frequency on the horizontal projection of the nucleus (lower arrow). It points from posterolateral (low frequencies) to anteromedial (high frequencies). Neurons located along lines perpendicular to the frequency axis on the horizontal projection have the same CF. The angle between the frequency axis and the lateral-medial axis on the horizontal projection (indicated by curved arrow) is calculated from the multiple linear regression equation which relates CF to percent lateral-medial and posterior-anterior positions. This angle is equal to arctangent (% posterior-anterior regression coefficient/% lateral-medial regression coefficient). B. Horizontal projection of NM with direction of tonotopic axis indicated by arrow. Distance along the tonotopic axis has been normalized to 100%. C. Scatter plots and single linear regressions ± 1 standard error of estimate (solid and dashed lines, respectively) relating CF to percent distance along the tonotopic gradients in NM (upper panel) and NL (lower panel). Large dots indicate locations at which more than one unit or unit cluster having the same CF was recorded. The multiple linear regressions which relate CF to percent posterior-anterior and percent lateral-medial positions are as follows: NM; CF(Hz) = 20(%p-a) + 23(%l-m) - 765; standard error of estimates = 319 Hz. NL; CF = 26(%p-a) + 13(%l-m) - 50; standard error of estimate = 293 Hz. A, anterior; P, posterior; M, medial; L, lateral.

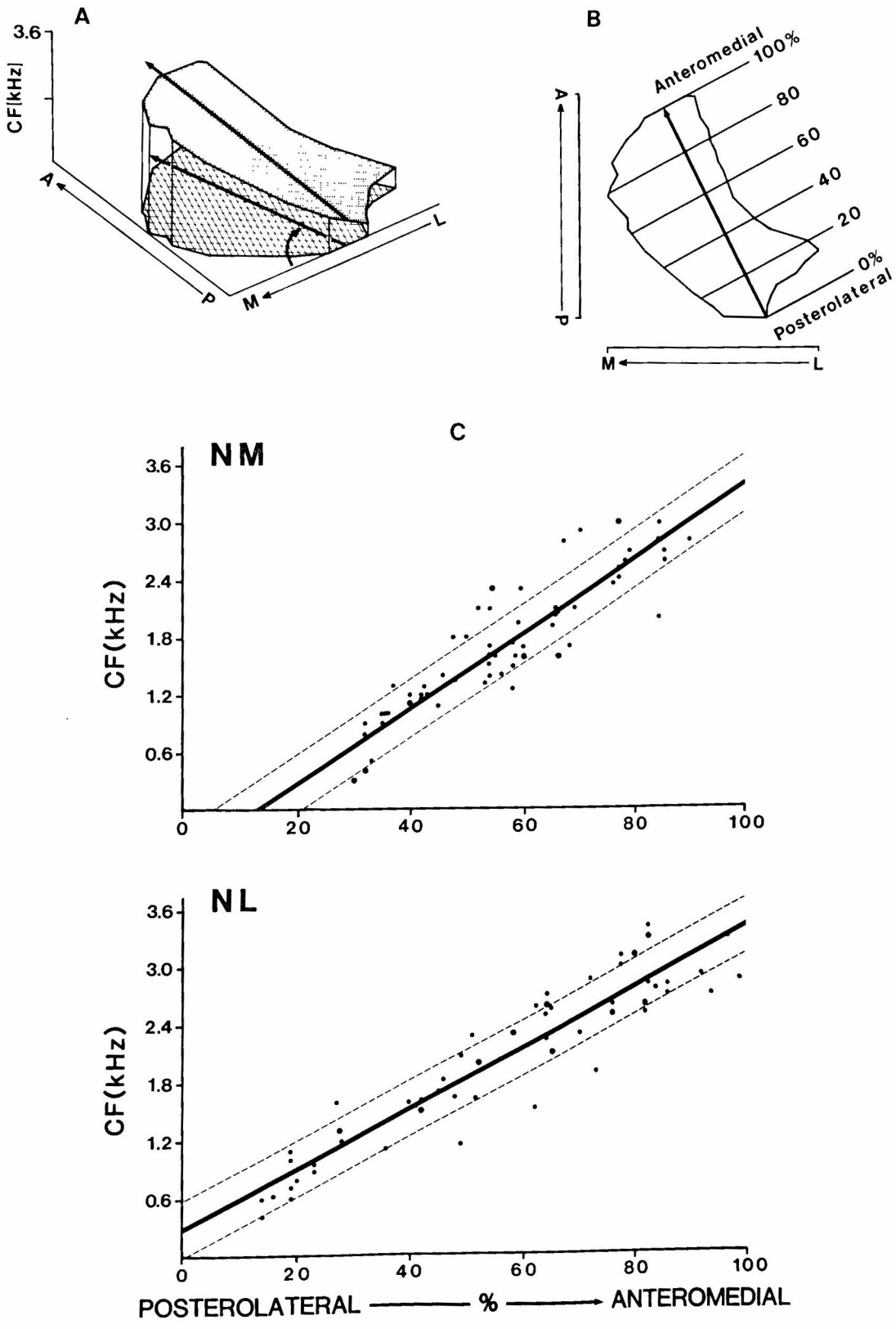
A
3.6
CF[kHz]
A
P
M
L
B
Anteromedial
100%
80
60
40
20
0%
Posterolateral
A
P
M
L
C
NM
3.6
3.0
2.4
1.8
1.2
0.6
CF(kHz)
0
20
40
60
80
100
NL
3.6
3.0
2.4
1.8
1.2
0.6
CF(kHz)
0
20
40
60
80
100
POSTEROLATERAL
%
ANTEROMEDIAL

Figure 6

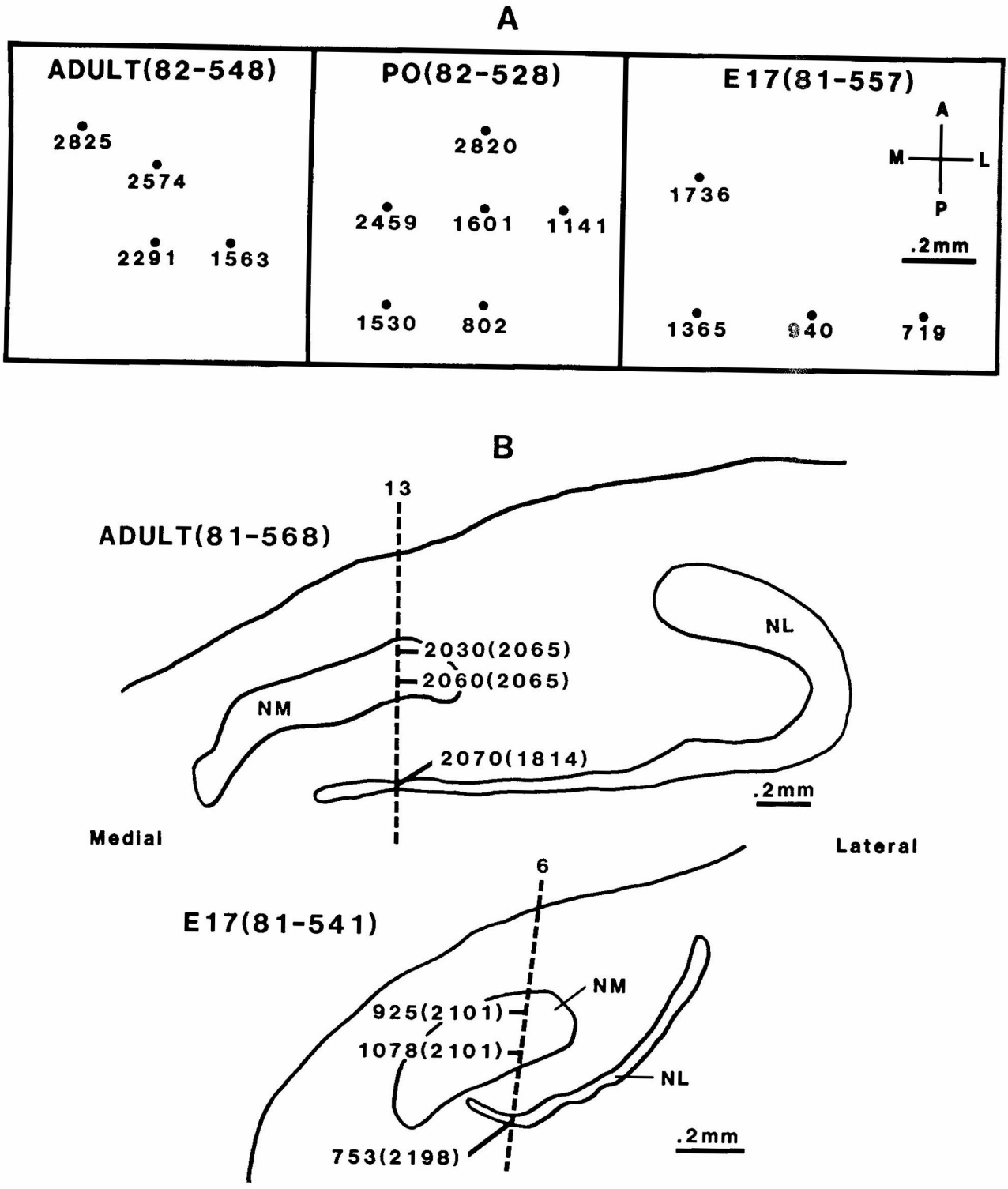

Fig. 7. A. Representative surface maps from an adult, 17-day embryo (E17), and young hatchling (P0) showing the tonotopic organization when the electrode tip was located within nucleus magnocellularis and/or nucleus laminaris. The median characteristic frequency (in Hz) recorded in each electrode penetration is shown. Note that higher characteristic frequencies are recorded as the recording electrode is moved anteriorally or medially at all ages. A, anterior; P, posterior; M, medial; L, lateral. B. Reconstructions of representative electrode penetrations which passed through approximately the same locations in nucleus magnocellularis (NM) and nucleus laminaris (NL) in an adult and a 17-day embryo. The observed characteristic frequency and the frequency predicted by the adult multiple linear regression equations (in parentheses) are shown for each unit or unit cluster recorded in NM and NL. Note that the characteristic frequency of each unit in the embryo is lower than the predicted frequency; observed and predicted frequencies are approximately equal in the adult. Percentile posterior-to-anterior and lateral-to-medial locations of units in NM: Adult, 65%, 64%; E17, 69%, 62%. Percentile posterior-to-anterior and lateral-to-medial locations of units in NL: Adult, 34%, 73%; E17, 41%, 88%.

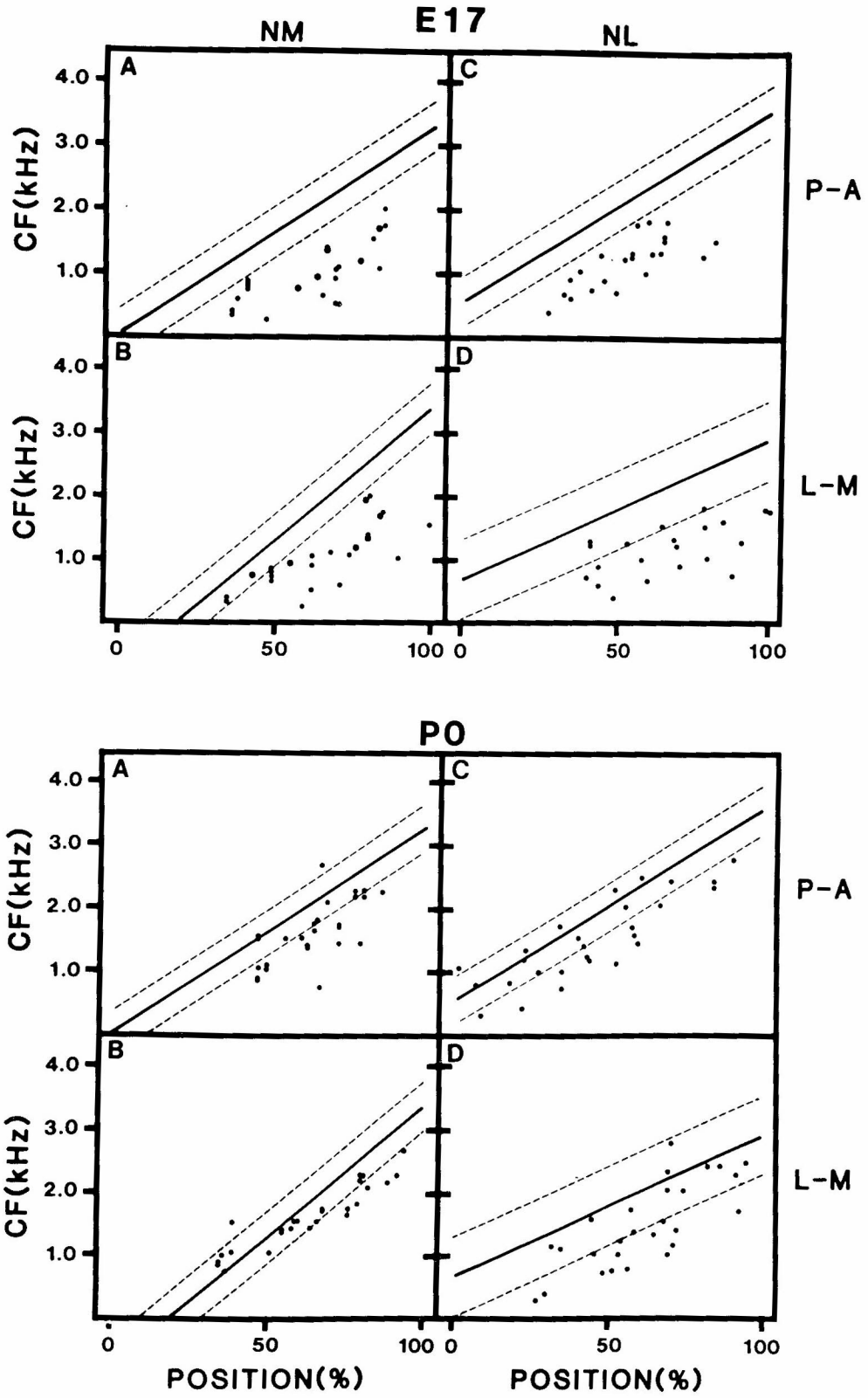

Fig. 8. Comparison of tonotopic organization between adults and 17-day embryos (E17, upper panel) and adults and young hatchlings (P0, lower panel) in nucleus magnocellularis (NM) and nucleus laminaris (NL). The relationship between position of the electrode tip and characteristic frequency (CF) in adults is shown by the regression lines ± 1 standard error of estimate (replotted from Fig. 5). The relationship between position and CF in embryos and young hatchlings is shown by the scatter plots. P-A, posterior-to-anterior. L-M, lateral-to-medial.

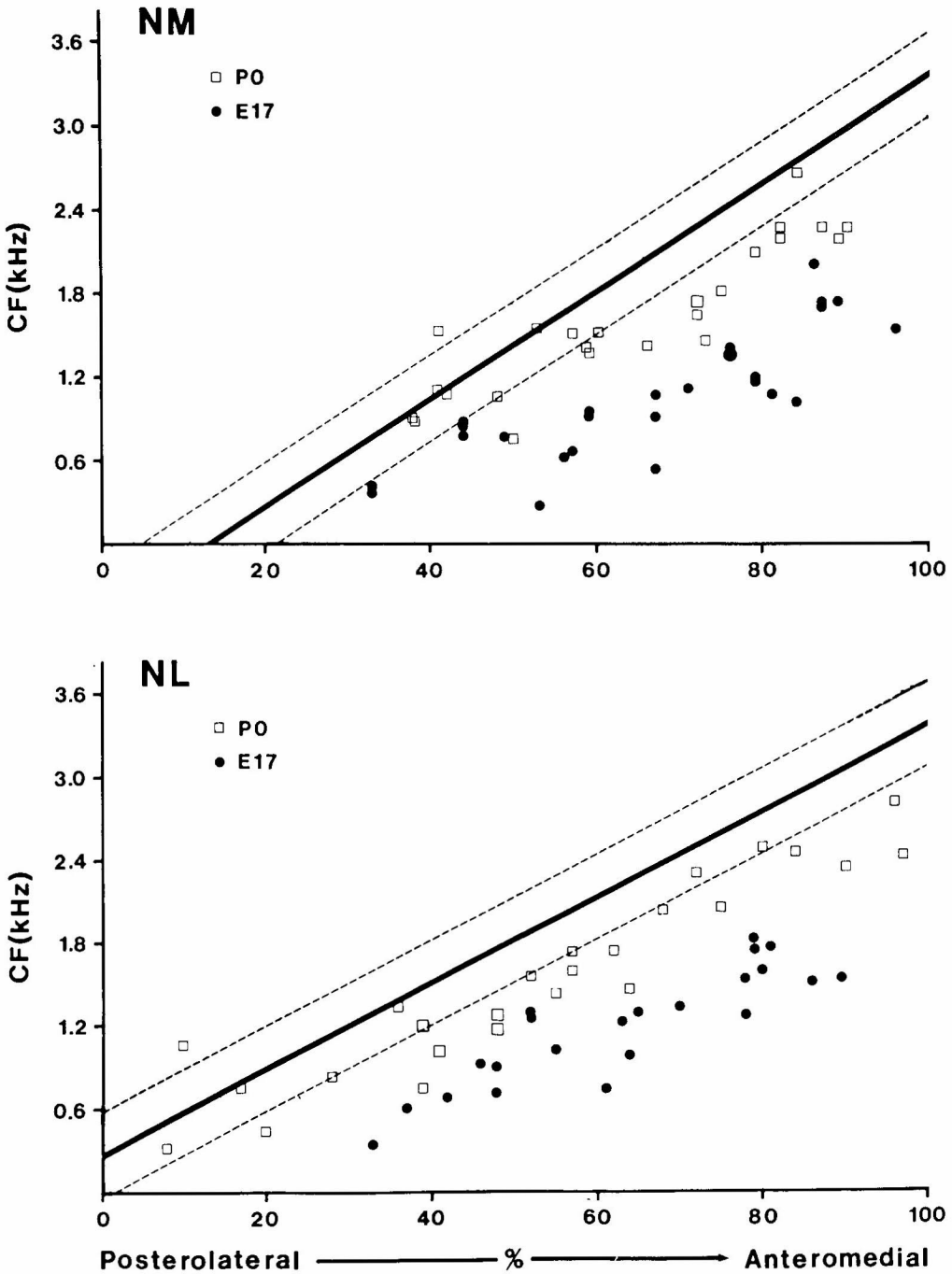

Fig. 9. Comparison of tonotopic organization between adults, young hatchlings (PO), and 17-day embryos (E17) in nucleus magnocellularis (NM, upper panel) and nucleus laminaris (NL, lower panel). The relationship between position of the electrode tip along the tonotopic gradient and characteristic frequency (CF) in adults is shown by the regression lines ± 1 standard error of estimate (replotted from Fig. 6) The relationship between CF and position in embryos and young hatchlings is shown by the scatter plots. Note that the data points from E17 units (dots) are considerably below the regression lines; data points from PO units (open squares) are intermediate.

The systematic changes in tonotopic organization that occur during development can be seen more clearly in Figure 9. Characteristic frequency is shown as a function of percent distance along the tonotopic axis (see Fig. 6) from posterolateral (low frequencies) to anteromedial (high frequencies). It can be seen that the characteristic frequencies observed at each location in NM and NL increased progressively during development. In the NM of embryos and hatchlings, characteristic frequencies averaged, respectively, 1.03 (±0.43) and 0.30 (±0.24) octaves lower than their predicted adult values. In NL, characteristic frequencies averaged 0.97 (±0.33) octaves lower than their predicted adult values in embryos and 0.36 (±0.35) octaves lower in hatchlings. A systematic increase in characteristic frequency during development was observed at all positions along the tonotopic axis except the most caudolateral por-

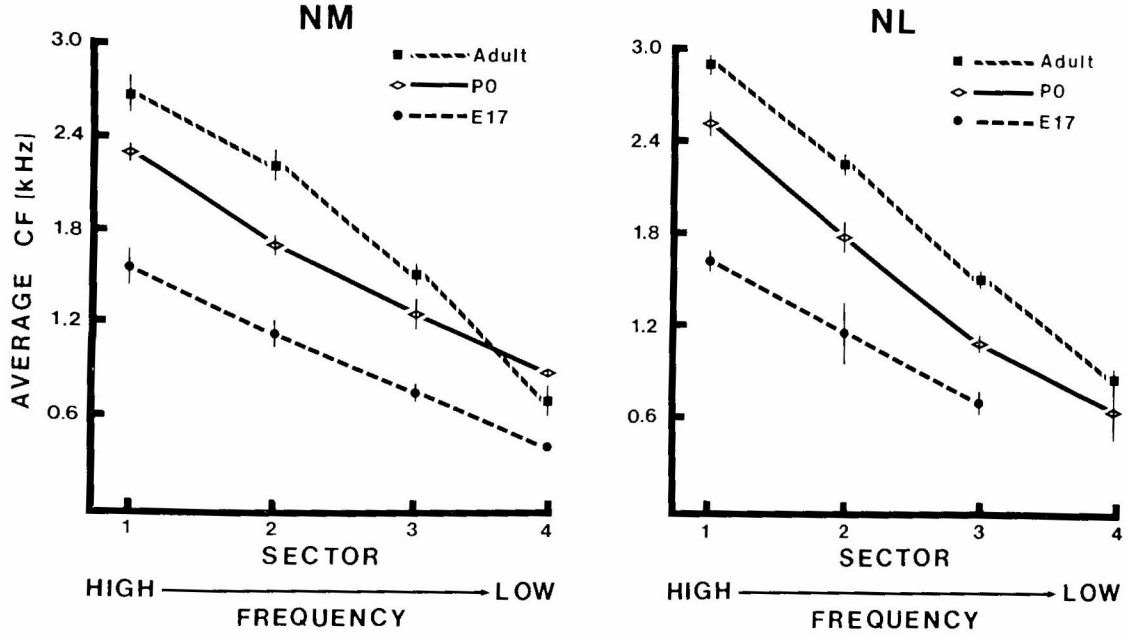

Fig. 10. Average characteristic frequencies (CF) ± 1 standard error of estimate in sectors 1–4 of nucleus magnocellularis (NM, left panel) and nucleus laminaris (NL, right panel) in adults, young hatchlings (P0), and 17-day embryos (E17). See text for details. Number of units in sectors 1, 2, 3, and 4 of NM: Adults = 6, 20, 31, 13: PO = 6, 9, 9, 2; E17 = 7, 9, 10, 2. Number of units in sectors 1, 2, 3, and 4 of NL: Adults = 22, 21, 17, 10; PO = 5, 9, 9, 4; E17 = 7, 8, 6, 0.

tions of NM and NL. These are relatively low-frequency regions and respond to sounds below approximately 1,000 Hz in adults. In the present experiment these regions were not well sampled. This is discussed further below.

For analytical purposes NL and NM were divided into four and five sectors, respectively, perpendicular to their tonotopic axes (Fig. 2). The most caudolateral sector in NM (sector 5) is innervated by the macula lagena and does not respond to sound. The remaining four sectors in NM and NL make up regions that receive input from progressively more apical regions along the basilar papilla as one moves from sector 1 (anteromedial) to sector 4 (caudolateral). The average characteristic frequency observed in each of these four sectors was calculated separately for adults, hatchlings, and embryos. These values (±1 S.E.M.) are plotted in Figure 10.

The average characteristic frequency decreased systematically from anteromedial (sector 1) to posterolateral (sector 4) at all ages in each nucleus. This reflects the similar pattern of tonotopic organization at all ages. In addition, the average characteristic frequency within each sector increased progressively during development, the only exceptions being in the most posterolateral regions of both nuclei. One-way analyses of variance showed a significant age effect within the first three sectors of each nucleus (Table 1). One-tailed t-tests were used to compare the average characteristic frequency of adults to both hatchlings and embryos within each of these three sectors. This is in accord with the directional hypothesis that the same location in either nucleus will be tuned to lower-frequency sounds in both hatchlings and embryos than in adults (Fig. 1). This prediction was confirmed (Table 1).

One-way analyses of variance failed to show a significant effect of age in the most posterolateral region (sector 4) of either NM or NL (Table 1). However, our sample sizes in these areas were too small to make reliable estimates. Thus, we cannot determine from our present observations whether the tonotopic organization of the posterolateral (low-frequency) regions of NM and NL also changes during development.

Figure 10 suggests that there was an equivalent change in the characteristic frequency of neurons regardless of position within NM or NL. To evaluate this, the difference between the observed and predicted characteristic frequencies, in octaves, was calculated for each unit in embryos and hatchlings. The average differences for sectors 1–3 were compared in embryos and hatchlings for each nucleus (Table 2). One-way analyses of variance failed to show a significant effect of sector. The only exception was in the NL of embryos where the changes in octaves in the third sector were significantly greater than in sectors 1 and 2 ($P < .05$; Newman-Keuls test for post hoc comparisons).

DISCUSSION

Two main observations concerning the development of tonotopic organization in avian brain stem auditory nuclei have been made. First, the gradients of tonotopic organization in NM and NL do not change between 17 days of embryonic development and 4 weeks after hatching. At each age responses to high-frequency sounds were recorded rostromedially while lower frequencies were found at more caudolateral locations. The similarity in tonotopic organization at all ages is consistent with previous studies which showed that the tonotopic order in both the inferior colliculus of the neonatal cat (Aitkin and Moore, '75) and the cochlear nuclei of the embryonic duck (Konishi, '73) does not change during the development of hearing. Secondly, a striking quantitative change in tonotopic organization oc-

TABLE 1. Statistical Comparisons Between Ages of the Average Characteristic Frequencies Within Each Sector of NM and NL

Sector	Ages[1] compared	NM F or t[2]	NM df	NM P	NL F or t[2]	NL df	NL P
1	A-PO-E	21.1	2/16	<.0001	63.1	2/31	<.0001
	A-PO	2.14	10	<.05	2.74	25	<.01
	A-E	5.51	11	<.0005	10.81	27	<.0005
2	A-PO-E	22.6	2/35	<.0001	38.0	2/35	<.0001
	A-PO	2.92	27	<.005	3.26	28	<.005
	A-E	6.00	27	<.0005	8.04	27	<.0005
3	A-PO-E	20.0	2/47	<.0001	30.9	2/29	<.0001
	A-PO	1.92	38	<.05	4.40	24	<.0005
	A-E	6.10	39	<.0005	7.18	21	<.0005
4	A-PO-E[3]	1.19	2/14	N.S.	0.056	1/12	N.S.

[1]A, adults; PO, hatchlings, E, embryos.
[2]One-way analyses of variance were used for A-PO-E comparisons; one-tailed t-tests were used for A-PO and A-E comparisons.
[3]Only adults and hatchlings were compared in the analysis of variance of sector 4 in NL. No neurons were recorded from this sector in the NL of embryos.

TABLE 2. Average Difference (in Octaves)[1] Between Observed and Predicted Characteristic Frequencies in Sectors 1–3 of NM and NL

Nucleus	Age	Sector[2] 1	2	3	F	df	P
NM	Hatchling	−0.30 (.06)	−0.43 (.04)	−0.23 (.12)	1.56	2/21	N.S.
NM	Embryo	−1.09 (.13)	−1.09 (.12)	−1.05 (.19)	0.26	2/23	N.S.
NL	Hatchling	−0.029 (.06)	−0.33 (.05)	−0.50 (.09)	2.33	2/20	N.S.
NL	Embryo	−0.79 (.07)	−0.93 (.11)	−1.25 (.14)	4.36	2/18	<.05[3]

[1]Difference (in octaves) for each unit = $\log_2$ (observed CF/predicted CF). The predicted CF was calculated from the adult multiple linear regression equations.
[2]Values shown are means (standard error of mean).
[3]The Newman-Keuls test for post hoc comparisons (Winer, '71) showed that sector 3 was significantly different from sectors 1 and 2 ($P < .05$; two-tailed). Sectors 1 and 2 were not significantly different from each other ($P > .05$).

curs. The neurons at a given location in NM and NL became maximally responsive or tuned to progressively higher frequencies during development. The characteristic frequencies recorded in 17-day embryos averaged one octave lower than those in adults while by 1 day after hatching this difference had decreased to one-third octave. Both of these findings are consistent with the hypothesis that the spatial representation of low- and midrange-frequency sounds shifts apically along the cochlear partition during development. This hypothesis is also supported by the recent observation that hair cell damage resulting from a specific high intensity, pure tone occurs at more distal loci along the papilla in birds exposed at later times during development (Rubel and Ryals, '83).

The changes in tonotopic organization observed here occurred in regions of NM and NL that, in adults, are tuned to frequencies higher than approximately 1,000 Hz. These changes would be correlated with a shift in the place code over approximately the proximal 40% of the papilla (von Békésy, '60; Ryals and Rubel, '82). We do not know if a shift in tonotopic organization also occurs in the low-frequency (caudolateral) regions of NM and NL because too few recordings were made from these areas. These regions are of particular interest because the model of a shifting frequency code predicts that the apical end of the cochlea and the brain stem regions to which it projects should be selectively unresponsive to sound during the very early development of hearing (see Fig. 1). The few units that were recorded from the caudolateral region of NM in embryos responded to sound. Thus, if only the basal end of the papilla transduces sound at some time during development this occurs earlier than E17.

Our results suggest that the tonotopic organization of NM and NL changes very little beyond 3–4 weeks after hatching. The tonotopic organization shifted by an average of 0.71 octaves in the 6 days between E17 and 1 day after hatching but only 0.22 octaves during the 4 weeks after hatching. Thus, any continued change in tonotopic organization (cf. Rubel and Ryals, '83) would likely be relatively minor and not detectable with the quantitative mapping methods used here. In agreement with this we found no evidence of a continued frequency shift in two 30–40-day-old birds which were mapped (data not presented here).

In order to place these results into a proper developmental perspective, two points should be emphasized. First, the changes in tonotopic organization described here are occurring during the mid- to late period of hearing development in birds. Physiological and behavioral responses to sound occur first on days 11–12 and 14 of embryonic development, respectively (Vanzulli and Garcia-Austt, '63; Saunders et al., '73; Jackson and Rubel, '78). By E17, the earliest age studied here, neurophysiological thresholds have decreased to within approximately 30 dB of adult thresholds, and the basilar papilla and its afferent innervation are well developed (Saunders et al., '73; Hirokawa, '78; Cohen and Fermin, '78; Rebillard and Rubel, '81; Fermin and Cohen, '84). One day after hatching behavioral and neurophysiological thresholds have reached adultlike levels for all but the highest frequencies (Saunders et al., '73; Kerr et al., '79; Rebillard and Rubel, '81; Gray and Rubel, '81, '85). Cochlear growth (Tilney, '84) and changes in middle ear admittance (Saunders et al., '83) continue for several months after hatching, but by 1 week posthatch, thresholds for all frequencies have reached mature levels (Saunders et al., '73; Rebillard and Rubel, '81; Gray and Rubel, '81, '85).

Second, several observations in mammals are also consistent with the hypothesis that spatial coding of frequency along the cochlea changes during development. Regions of the gerbil cochlear nucleus and cat auditory cortex that respond to high-frequency sounds in adults appear to be activated by lower frequencies during development (Pujol and Marty, '68; Ryan et al., '82). In addition, the cutoff frequency of the cochlear microphonic in gerbils increases during development (Harris and Dallos, '84). Thus, the process of a shifting frequency code appears to be general across species and not specific to birds.

Interpretations of the change in tonotopic organization

We believe that the systematic shift in tonotopic organization observed here is best explained by a change in the spatial encoding of frequency along the papilla. However, a number of assumptions and alternative explanations need to be considered.

The most likely alternative is that the properties of the immature middle ear (e.g., cartilagenous ossicles incompletely connected to each other, a highly compliant tympanic membrane, mesenchyme in the middle ear cavity, etc.) limit its ability to effectively transmit high-frequency energy to the cochlea (Finck et al., '72; Saunders et al., '73; Saunders '83; Brugge et al., '78). Thus, neurons in the developing and mature auditory systems might be tuned alike. However, because the immature conductive apparatus acts as a low-pass filter, some point on the low-frequency slope of a neuron's tuning curve would appear to be its best frequency. Several observations argue against this expla-

nation. First, this predicts that the relative amount of shift to lower best frequencies should be greatest for high-frequency neurons (those located in the anteromedial regions of NM and NL) and progressively less for lower-frequency units (those located more posterolaterally). However, this did not occur (see Table 2). Second, the tonotopic organization is still shifting 1 day after hatching although by this time the middle ear is cleared of mesenchyme and the sensitivity to all but the highest frequencies is no longer changing (Saunders et al., '73; Rebillard and Rubel, '81). Third, the increase in middle ear admittance which occurs during development in both hamsters and chicks is not greater for high- than for low-frequency sounds (Relkin et al., '79; Relkin and Saunders, '80; Saunders et al., '83). Finally, the complementary results of Rubel and Ryals ('83) provide no evidence for a selective attenuation of high-frequency sound transmission.

The change in tonotopic organization cannot be accounted for by a systematic shift in the relative location of neurons in NM and NL; the morphogenetic processes which might result in such a shift have ended by E17 (Rubel et al., '76). It is also unlikely that changes in tonotopicity were due to a deterioration in the physiological state of the preparation. This was of particular concern with the fragile embryos because of reports that trauma can cause a shifting of tuning toward lower frequencies (Robertson and Johnstone, '79; Schermuly and Klinke, '82; Heusden and Smoorenburg, '83). In embryos that were obviously in poor physiological condition (arrhythmic heart beat, reduced amplitude of spontaneous and evoked activity, absence of spontaneous rhythmic discharges), responses frequently occurred only to low-frequency, high-intensity sounds. However, this was never observed in healthy preparations. If more subtle changes in physiological state occurred slowly over time, one might expect that the amount of difference in frequency between embryos and adults would be correlated with time into the experiment. This was not found ($r = .27$, $P > .1$, $n = 25$ embryonic neurons). Additionally, in one preparation we recorded from the same location for over 8 hours with no change in tuning.

One final alternative explanation for the shift in tonotopic organization is that the representation of frequency along the basilar papilla remains fixed but the mapping of connections between NM and the papilla systematically shifts during development. Such a "sliding" or systematic remapping of connections takes place during the development of retinotectal connections in amphibians and fish (Gaze et al., '72, '74; Longley, '78; Reh and Constantine-Patton, '84; Easter and Stuermer, '84). Furthermore, it is well documented that changes in neuronal connections occur during development in both the central and peripheral nervous systems, including the auditory pathway (Pujol et al., '78, '79; Ivy et al., '79; Purves and Lichtman, '80; Innocenti, '81; Jackson and Parks, '82; Feng and Brugge, '83). Although we consider this unlikely, none of our present results exclude this possibility. However, a change in connections could not account for either the developmental shift in position of hair cell damage found by Rubel and Ryals ('83) or the progressive increase in the cutoff frequency of the cochlear microphonic reported by Harris and Dallos ('84).

Possible mechanisms underlying a shift in the place code

The spatial coding of frequency along the cochlear partition has classically been attributed to the mechanical properties of the basilar membrane (von Békésy, '60; Dallos, '81). Recent experiments in nonmammalian species have shown an important function of hair cell stereocilia in frequency analysis and tonotopic organization (Weiss et al., '76; Turner et al., '81; Holton and Hudspeth, '83). Circumstantial evidence suggests that stereocilia may contribute to frequency analysis in mammals and birds as well (Strelioff and Flock, '84; Lim, '80; Tilney et al., '82, '83). Thus, it seems likely that a shift in the cochlear frequency map is due to maturational changes in the mechanical properties of the basilar membrane and/or stereocilia.

Developmental changes in the cellular structure of the basilar membrane and organ of Corti that would affect their responses to sound have been described by several investigators (Kikuchi and Hilding, '65; Pujol and Marty, '70; Kraus and Aulbach-Kraus, '81; Chandler, '84; Fermin and Cohen, '84; see Romand, '83, for a recent review of this literature). The effect of these structural changes on the transduction properties of the cochlear partition and tuning is not known. However, it seems reasonable that the increase in stiffness and decrease in mass that several of these changes imply might shift the resonance at any point along the cochlear partition to progressively higher-frequency sounds during development (Kraus and Aulbach-Kraus, '81; Harris and Dallos, '84).

While a change in mechanics is the most parsimonious explanation for a shift in the place code, other candidates can be proposed. For example, the synaptology of the outer hair cells is changing well after the onset of cochlear function (Pujol et al., '78). In addition, the contribution of active influences to the development of cochlear response properties is not known (Kim, '80).

Implications

A development shift in the spatial frequency code along the basilar membrane helps explain the paradoxical dissociation between cochlear maturation and functional development. It also implies that the same neuron will be maximally stimulated by different-frequency sounds (progressively higher) during development. In this context, several studies have shown that in the auditory system, as in other sensory systems, the normal development of physiological response properties (Clopton and Winfield, '76; Clopton and Silverman, '77, '78; Silverman and Clopton, '77), neural structure (Coleman and O'Connor, '79; Webster and Webster, '79; Feng and Rogowski, '80; Conlee and Parks, '81; Gray et al., '82; Smith et al., '83), and perceptual behavior (Tees, '67; Gottlieb, '76; Clements and Kelly, '78; Kerr et al., '79; Knudsen et al., '82) depends upon external stimulation. During the early development of hearing *in utero*, the fetus's acoustic environment is dominated by relatively low-frequency sounds (Grimwade et al., '71; Armitage et al., '80). Thus, a change in spatial coding along the cochlea may allow normally occurring low-frequency sounds to provide stimulation necessary for normal development to *all* neuronal (cochleotopic) regions in central auditory nuclei. These sounds would first activate basal (presumptive high-frequency) projection regions and, progressively later in development, more apical or low-frequency regions.

Finally, a change in the spatial coding of frequency during development indicates that the place code is not fixed and immutable. This raises the intriguing possibility that the relationship between frequency and place along the cochlea may be altered or changed under other conditions as well. There is circumstantial evidence that the place

code is altered by trauma resulting from exposure to high-intensity sound (Ades et al., '74) and aminoglycoside antibiotics (Robertson and Johnstone, '79). It may also change during the normal process of aging (Willott, '82). Such possibilities, as well as the implications of a changing place code for the development of hearing and language acquisition in humans, need to be critically examined.

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