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Organization and Development of Brain Stem Auditory Nuclei in the Chick: Ontogeny of Postsynaptic Responses

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ABSTRACT

The onset of responsiveness to eighth nerve stimulation was examined in n. magnocellularis and n. laminaris, (second- and third-order neurons) of the chick brainstem auditory system. Extracellular microelectrode mapping techniques were used to examine postsynaptic responses in *in vitro* brainstem preparations. Two specific questions were addressed. First, what is the earliest time at which postsynaptic action potentials can be evoked in n. magnocellularis and n. laminaris by eighth nerve stimulation? Second, does responsiveness to eighth nerve stimulation develop along a spatial gradient in n. magnocellularis and, if so, how does this gradient compare with other developmental events observed in the chick auditory system?

Postsynaptic responses in n. magnocellularis were first recorded at 11 days of incubation. Nucleus laminaris responses to direct stimulation of n. magnocellularis were also first recorded at 11 days, although n. laminaris responses to eighth nerve stimulation were not seen until 12 days of incubation.

A gradient of response development within n. magnocellularis was indicated by mapping of responsive sites on days 11–13. At 11 days, responses to eighth nerve stimulation were restricted to the most anteromedial portion of n. magnocellularis. Between 11 and 13 days, cells in increasingly more posterolateral portions of n. magnocellularis became responsive. This anteromedial-to-posterolateral gradient in n. magnocellularis is correlated with the basal-to-apical gradient of morphogenesis observed in the basilar papilla and morphogenetic gradients previously observed in n. magnocellularis and n. laminaris.

The avian brain stem auditory system is a useful preparation in which to investigate the influence of afferent connections and synaptic activity on the development of structural and functional properties of neurons. The organization of this system is relatively simple. The basilar papilla is tonotopically organized, with the base responding to high frequencies and successively apical regions maximally sensitive to progressively lower frequencies (Ryals and Rubel, '82; Vanzulli and Garcia-Austt, '63; Von Bèkèsy, '60). Eighth nerve fibers reflect this tonotopic organization in their projection from the periphery to nucleus magnocellularis (NM) (Boord, '69; Boord and Rasmussen, '63; Ramón y Cajal, '08); neurons responding to high frequencies are localized in the anteromedial portion of NM and those responding to lower frequencies are found in more posterolateral regions (Rubel and Parks, '75). NM neurons, in turn, project bilaterally to nucleus laminaris (NL) (Boord, '69; Parks and Rubel, '75; Ramón y Cajal, '08). NL is composed of a monolayer of cell bodies with bipolar dendrites; dorsal dendrites are innervated by axons from the ipsilateral NM and ventral dendrites receive projections from the contralateral NM. This bilateral innervation is tonotopically organized and individual NL cells are activated by similar frequencies presented to either ear (Rubel and Parks, '75).

Beginning with the classic contribution of Levi-Montalcini, both descriptive and experimental studies of this system have prompted speculation regarding possible re-

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lationships between developmental events and the transmission of impulse activity (Jhaveri and Morest, '82; Levi-Montalcini, '49; Parks, '79; Rubel et al., '76; Smith, '81). Speculation has been limited, however, by a lack of data concerning the stage at which the system is first capable of such transmission. The present study addresses that question. Using an isolated brainstem preparation the time at which electrical stimuli applied to the eighth nerve or NM first elicit postsynaptic extracellular responses in NM or NL is reported. By identifying the area of the nucleus magnocellularis in which such activity was elicited preliminary indications of a spatio-temporal gradient also emerged.

MATERIALS AND METHODS

Fertilized Hubbard X Hubbard chicken eggs were obtained from a commercial breeder and communally incubated at 37.5°C and 50–60% relative humidity. Sixty-eight 9 to 14 day old embryos were used in this study. (Hatching occurs at 21 days.) The methods of brainstem isolation and *in vitro* maintenance are described in detail elsewhere (Hackett et al., '82; Jackson, '79). Brainstems dissected under cold avian Tyrode's solution were maintained at room temperature while continuously superfused with oxygenated Tyrode's solution.

Stimulating and recording electrodes

Stainless steel bipolar electrodes were used to stimulate the eighth nerve and, occasionally, NM. Electrodes were insulated to their tips, which were 0.4 mm wide. Stimuli (30–60 μsec duration; 10–90 V) were delivered at a repetition rate of 0.3–1.0 Hz. Recording electrodes were filled with a solution of 4.0% horseradish peroxidase (HRP) in 1.0 M potassium acetate and then bevelled to a resistance of 25–40 MΩ at 30 Hz.

Eighth nerve and NM stimulation

The eighth nerve enters the dorsolateral aspect of the brainstem just posterior to the cerebellar peduncles. Subsequent to entry, eighth-nerve fibers fan out to innervate the various portions of NM. The stimulating electrode was placed on the nerve just at the point of entry into the brainstem (Hackett et al., '82). While recording from the nerve as close to the point of entry as possible, the position of the stimulating electrode was adjusted until a maximum afferent volley was achieved. (The eighth nerve afferent volley and other responses recorded from this system are characterized in a previous report [Hackett et al., '82].) This procedure helped assure that, in the case of each subject, as many eighth nerve axons were activated as possible. It did not, of course, guarantee that *all* afferent fibers were being stimulated. It was important, therefore, that eighth nerve axons destined for a particular portion of NM not be systematically understimulated. In an effort to minimize this possibility, stimulus intensity was adjusted during recording from NM in order to be sure that a maximum afferent volley was achieved at the site of *every* electrode penetration. During sampling of NM neuronal responses in these penetrations, the stimulus intensity was maintained at the level just adequate to yield the maximum afferent volley. The questions of adequate and uniform stimulation are considered further in the DISCUSSION.

In 21 preparations, the stimulating electrode was placed directly on the surface of the medulla over portions of NM and the contralateral NL was explored for responses. (Recording from the ipsilateral NL is difficult under these circumstances because the stimulating electrode overlies a large portion of the nucleus.) Direct stimulation of NM in this manner is possible because of the proximity of NM cells and fibers to the surface of the brainstem. This procedure allowed some assessment of the functional status of the NM innervation of NL at stages when eighth nerve stimulation was relatively ineffective in driving NM neurons.

Electrophysiological mapping of NM

In each preparation, an effort was made to explore as much of the anterior-posterior and medial-lateral dimensions of NM as possible. In all penetrations, the electrode was lowered very slowly while the eighth nerve was stimulated at a rate of about 0.3 Hz. This low stimulation rate was used because, at the younger ages (<13 days of incubation), most NM units would not follow rates of 1.0 Hz or more. The presence or absence of synaptically driven single-unit action potentials within each penetration and the electrode depth at which such responses occurred were noted. Action potentials were classified as postsynaptic on the basis of two criteria: (1) fatigue of the response at stimulus repetition rates that had no effect on the amplitude of the afferent volley; and (2) blockade of the response by application of low calcium (0.5 mM), high magnesium (1.5 mM) and manganese (2 mM) Tyrode solution (see Fig. 1). The first criteria was applied in all cases; the second criteria was applied to at least one response in each preparation. Postsynaptic responses recorded from outside the boundaries of NM, as determined by histological reconstruction of recording sites, were eliminated from subsequent data analyses.

The position of electrode penetrations with respect to one another was carefully noted on a scaled grid during recording. After the last penetration had been explored, the electrode was returned to the site of the first penetration from which postsynaptic responses were recorded. If, at this time, postsynaptic responses in that penetration were absent (NM action potentials) or diminished (eighth nerve afferent volley) the preparation was discarded. This provided a check against misinterpretation of sites as unresponsive due to general deterioration of the tissue during the recording session. About 5% of preparations were discarded on this basis. After this check of preparation viability, small extracellular HRP deposits were made at the positions of two of the penetrations. An average of 12 penetrations were made in any one brainstem.

HRP iontophoresis and histological reconstruction

HRP was iontophoresed using positive 1 μamp, 200 msec pulses delivered at 1.0 Hz for 30–60 seconds. Tissue was fixed in a cold solution of 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) within 10 minutes after iontophoresis of HRP. Brainstems were fixed for 3 hours, put through two 10-minute washes of cold 0.1 M phosphate buffer, and allowed to equilibrate in a solution of cold phosphate buffer and 30% sucrose. Serial frozen sections were cut at 60μm in the coronal plane, reacted for HRP

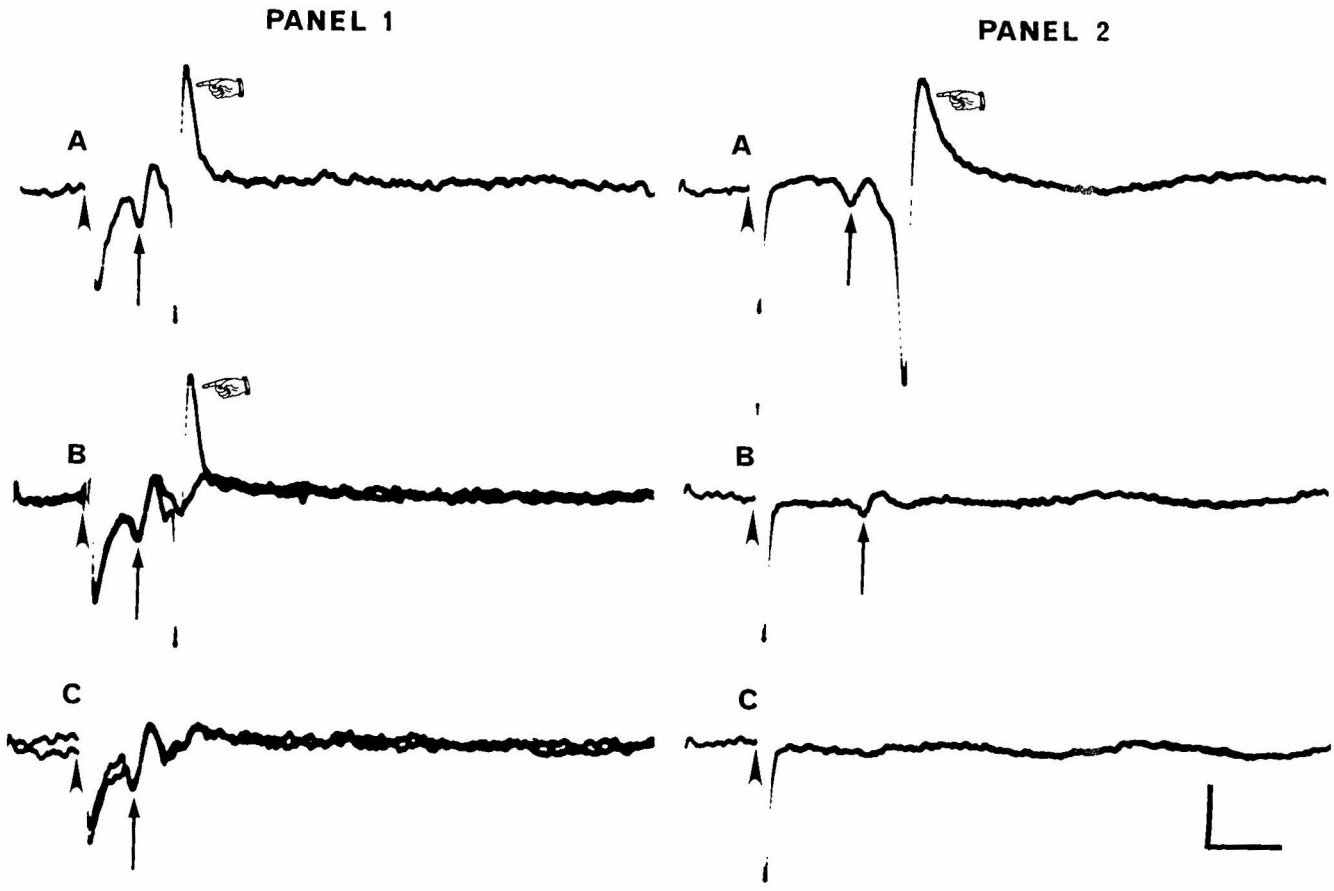

Fig. 1. Examples of field potentials and single cell responses and the effects of superfusion with low Ca, high Mg, and Mn Tyrode. Panel 1 shows the block of NM single-unit response to eighth nerve stimulation. In A, the afferent volley and NM action potential are shown. After 70 seconds of superfusion with the low Ca Tyrode, the action potential began to fail (B). After 110 seconds, the NM action potential could not be evoked (C). The low Ca superfusate, however, had no observable effect on the eighth-nerve afferent volley. The NM response recovered within 90 seconds after resumption of superfusion with normal Tyrode. In Panel 2, the low Ca Tyrode was used to block the response of an NL unit to eighth nerve stimulation. A of Panel 2 shows the NL cell's action potential and the small positive-negative field potential which represents the afferent volley from NM to NL. After 1 minute of superfusion with the low Ca Tyrode, the NL response had disappeared and the field potential was reduced in amplitude. At 90 seconds (C), neither the field potential nor the NL unit response could be recorded; both recovered within 2 minutes after resumption of superfusion with normal Tyrode. Calibration: 5 msec, 4 mV. Negative up. Arrowheads, arrows, and pointers indicate positions of shock artifact, afferent volley, and postsynaptic spike, respectively.

by the Hanker-Yates method (Hanker et al., '77), dehydrated, and coverslipped.

Following histological processing, low-magnification camera lucida drawings were made of each serial section through NM and NL. The boundaries of both NM and NL were drawn from each section under phase-contrast optics. The distances from the midline to the medial and lateral edges of the nuclei were measured in every section and then plotted against the rostro-caudal position of the section to produce a planar projection of the nuclei (cf. Rubel and Parks, '75). This projection is equivalent to a two-dimensional view of each nucleus from a dorsal perspective. As noted above, two HRP deposits separated by a known distance were made at the time of recording. Tissue shrinkage was calculated on the basis of their relative position in the processed tissue.

Once the planar projection was constructed and tissue shrinkage was determined, the position of each recording site could be reconstructed and plotted on the planar projection. By setting the total anterior-posterior (A-P) and medial-lateral (M-L) extents of NM at 100%, the position of each recording site could be specified in terms of its percentage A-P and percentage M-L. This manner of expressing position constitutes a normalization procedure which allows positional data to be compared across subjects and ages irrespective of absolute differences in the dimensions of the nucleus (Rubel and Parks, '75; Smith and Rubel, '79; Smith, '81). Mean composite planar projections of NM at each day from 11 to 14 days are used to display the group mapping data (see Fig. 2). These average planar projections were compiled from the individual NM planar projections of all embryos which contributed mapping data at the specified age.

RESULTS

The procedures outlined above produced reliable results at all of the ages studied. Figure 1 shows typical potentials

recorded in NM and NL and the effects of low Ca^{++} superfusate (see MATERIALS AND METHODS) on these potentials.

Onset of responsiveness

Principal findings regarding the onset of responsiveness in this system are described chronologically. The number of viable preparations studied at each age is given in parentheses.

Day 9. No response could be elicited from the system at this stage (n = 7)

Day 10. The eighth nerve afferent volley was first recorded from the surface of the brain stem above NM. Also, the NM afferent volley (see Fig. 1, Panel 2) was recorded in the region of NL following direct stimulation of the contralateral NM. Addition of 0.5% Lidocaine to the superfusate blocked both responses rapidly and completely, eliminating the possibility that these potentials were stimulus-related artifacts. No postsynaptic responses were recorded in NM following eighth nerve stimulation or in NL following either eighth nerve or direct NM stimulation. (n = 9)

Day 11. Postsynaptic action potentials were first recorded in NM. Responsive units seemed to be confined to the most anteromedial portions of NM. No response to eighth nerve stimulation was recorded in NL. However, single-unit NL responses could be elicited by direct stimulation of the contralateral NM (n = 14)

Day 12. NM unit responses to eighth nerve stimulation were more frequently encountered and appeared to be distributed over a larger extent of the nucleus. The first NL responses to eighth nerve stimulation were also recorded at this stage. High stimulus-intensity, relative to the threshold level for evoking NM responses, was required to evoke NL action potentials, and responses were intermittent even at low stimulus repetition rates (0.3 Hz). The former characteristic suggests that, at least at this developmental stage, converging excitatory inputs from several NM neurons are required to drive NL cells (also see Hackett et al., '82). (n = 13)

Day 13. NM unit responses were recorded from all parts of the nucleus. NL showed a marked elevation in responsiveness to eighth nerve stimulation as compared with 12-day levels. (n = 10)

Gradient of response development in NM

As reported above, the first NM responses to eighth nerve stimulation were recorded at 11 days of incubation and these responses appeared to be limited to the anteromedial portion of NM. At later stages of development, the nucleus was more uniformly responsive. The spatial gradient in the development of NM innervation by eighth nerve afferents implied by these qualitative observations was more thoroughly assessed using the mapping techniques described above (see MATERIALS AND METHODS). Maps of recording sites in NM were generated for 24 individual subjects, aged 11–14 days. Each recording site was classified as responsive or unresponsive on the basis of whether one or more synaptically-evoked action potentials had been recorded from NM neurons at that locus. Data were grouped according to subject age. The four group-data plots, one for each day from 11–14 days, are presented in Figure 2. All recording locations, including unresponsive (open circles) and active sites (filled circles) are shown in relation to the average planar projection of NM at the specified age.

The data presented in Figure 2 further suggest that the spatial distribution of responsive units in NM changes over the period from 11 to 14 days. At 11 days, responses were recorded only at the anteromedial pole of NM. By 13–14 days, responsive units were found throughout the nucleus. In addition, the overall proportion of responsive sites increased dramatically from day 11 (11%) to day 12 (41%), and again from day 12 to day 13 (79%); the proportion of responsive sites was essentially unchanged between day 13 and day 14 (86%).

To determine if this spatial gradient of functional development is consistent with the tonotopic organization of NM the following analysis was undertaken. A line representing the tonotopic organization (i.e., perpendicular to the isofrequency planes), as determined by Rubel and Parks ('75), was drawn across each of the four average planar projections. Each projection was then divided into four equal sectors perpendicular to this frequency axis (see Fig. 2: 14 DAYS). Because the tonotopic gradient reflects the orderly topographic projection from the basilar papilla to NM, the four sectors illustrated in Figure 2 comprise projections from increasingly more apical portions of the basilar papilla as one moves from Sector 1 (anteromedial) to Sector 4 (posterolateral).

The proportion of responsive sites found in each sector on each of the 4 days is presented in Table 1. The earliest responses to eighth nerve stimulation were recorded in the most anteromedial sectors and, over time, there was an increase in the relative responsiveness of more posterolateral sectors.

DISCUSSION

Onset of responsiveness

The interpretation of negative results is an obvious matter for concern in developmental studies of this type. In the present case, two observations suggest that the failure to record postsynaptic responses from either NM or NL at and before 10 days of incubation does indeed reflect an immaturity of innervation. First, a robust eighth nerve afferent volley was routinely evoked at 10 days with the same stimulating electrodes and stimulus parameters used with older subjects. Simiarly, direct stimulation of NM at 10 days produced an afferent volley which could be recorded in the region of the contralateral NL. These observations indicate that both eighth nerve and NM axons were electrically excitable in preparations of this age and that effective stimulation of those axons was achieved. Of course a major limitation of this study is that only extracellular action potentials were routinely recorded. Thus any discussion of these observations must be in terms of "impulse transmission through these nuclei." Conclusions regarding synaptogensis *per se* must await intracellular analyses across these ages.

One purpose of this study was to establish the temporal relationship between the present measure of functional development and previously described morphogenetic events occurring in this system. Our results suggest that the capacity for synaptic transmission of impulses develops during a period of major structural change (Jhaveri, '78; Rubel et al., '76; Smith, '81). Two such changes, cell death and dendritic growth, merit discussion.

A developmental event that seems to correspond in both time and space to the onset of effective transmission is cell death. Rubel et al. ('76) have shown that the period of

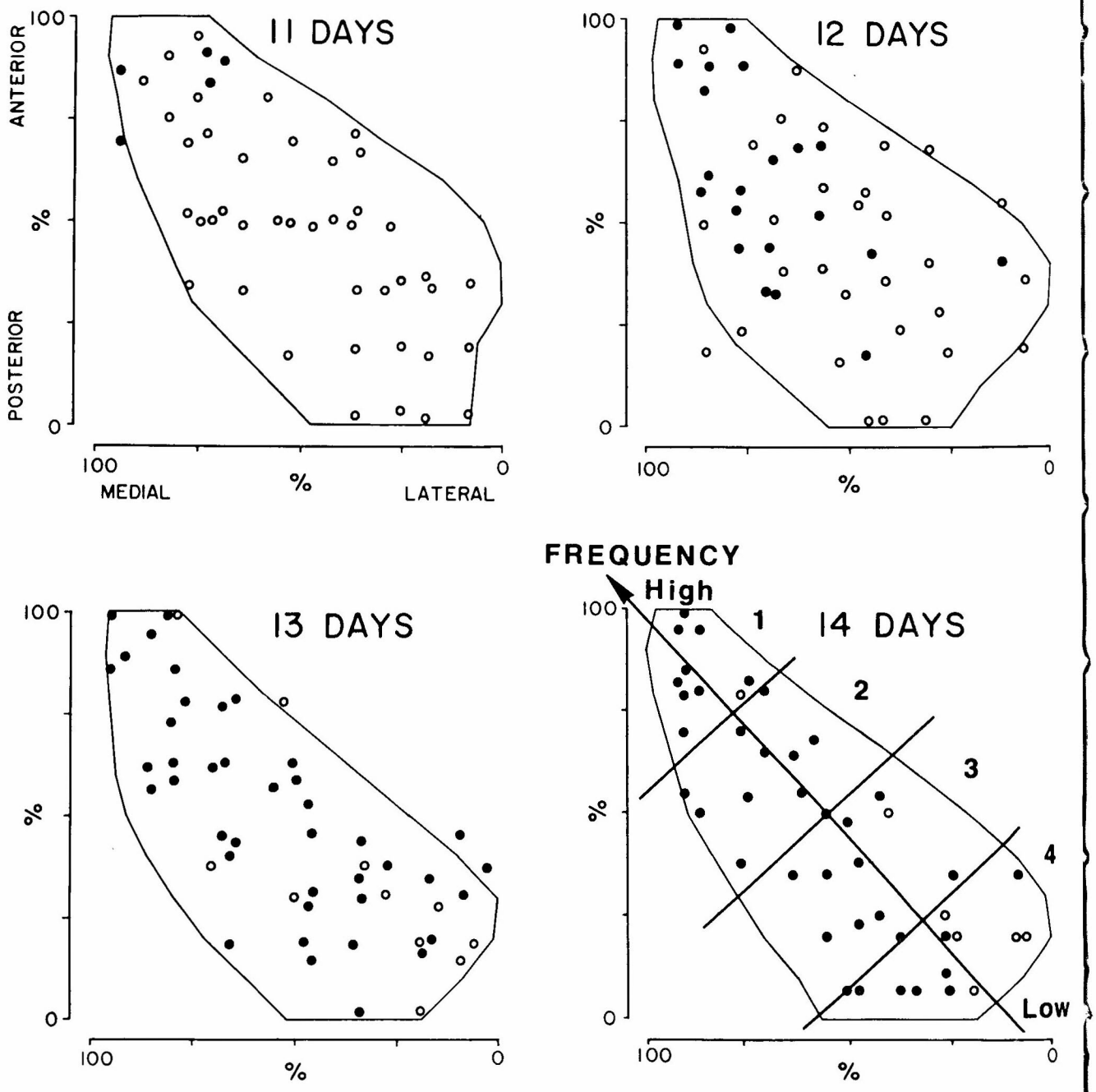

Fig. 2. Responsive and unresponsive sites in NM at 11, 12, 13, and 14 days. Filled circles represent sites in NM at which one or more postsynaptic responses to eighth-nerve stimulation were found; open circles represent unresponsive sites. Data are plotted over planar projections of NM derived by averaging the NM projections from all subjects of the specified age. All sites shown were within their individual NM boundaries although an occasional point lies outside the averaged projection. Overlying the 14-day plot are the arrow showing the orientation of the frequency axis (Rubel and Parks, '75) and sector divisions used in analyzing these data.

normal morphogenetic cell death in both NM and NL begins at about 11 days and is largely over by 13 days. Further, cell death in both nuclei occurs along an anteromedial-to-posterolateral gradient similar to that observed in this study. The correlation between the time that postsynaptic responses were first recorded from NL and the onset of cell death in NM is compatible with the generally accepted notion that normal cell death is related to the failure of some neurons to form or maintain synaptic contacts with target cells (Jacobson, '78). A further question, however, concerns the role of *afferent* synaptic contacts in maintaining their target neurons during this period. Early otocyst removal prevents the formation of the inner ear and eighth nerve and leads to marked hypoplasia and hypotrophy of NM (Levi-Montalcini, '49; Parks, '79). Although eighth nerve fibers can normally be seen entering

TABLE 1. Sector by Sector Breakdown of Response Proportions in NM

Age (days)	Sector 1	Sector 2	Sector 3	Sector 4
11	0.45 (5/11)	0.000 (0/16)	0.000 (0/12)	0.000 (0/8)
12	0.78 (7/9)	0.46 (11/24)	0.250 (3/12)	0.000 (0/6)
13	0.91 (10/11)	0.87 (13/15)	0.81 (13/16)	0.50 (5/10)
14	0.90 (9/10)	1.00 (10/10)	0.93 (11/12)	0.62 (8/13)

NM as early as 6–7 days of incubation, degenerative consequences of otocyst removal are not observed until 11–13 days. The fact that we were first able to record NM responses to eighth nerve stimulation at 11 days is consistent with Levi-Montalcini's suggestion that the sustaining influence of eighth nerve axons on NM neurons is synaptically mediated. It is interesting to note that evoked responses to intense acoustic stimuli are also first recorded from the brainstem at 11–12 days of incubation (Saunders et al., '73).

There is a close temporal correspondence between the formation of effective eighth nerve-NM transmission as defined in the present study, and the growth of NM somatic processes, which begin to appear between 10 and 11 days of incubation (Jhaveri and Morest, '82). A similar correspondence was found by Landmesser and Pilar (Landmesser and Pilar, '72) in their study of the chick ciliary ganglion. In both NM and the ciliary ganglion, these somatic processes gradually disappear. As they do, synaptic contacts are found increasingly closer to the cell soma. Eventually, the target cells present a largely adendritic appearance, with calyx-type synaptic endings contacting the soma. It is tempting to use such temporal correlations to speculate about the possible "trophic" influences involved or about the function of the immature somatic processes. For example, this sequence of events suggests that the function of these transient somatic processes may be to increase the change or number of synaptic contacts (Landmesser and Pilar, '72). The subsequent relationship between these processes and the development of eighth nerve innervation is considered by Jackson and Parks ('82). It should be emphasized, however, that any conclusions must await more detailed and experimental analyses; correlations of synaptic ontogeny with both regressive and progressive changes in postsynaptic cells are readily found. For example, the *loss* or *absorption* of processes is sometimes temporally or spatially related to the development of postsynaptic responses (e.g., Jhaveri and Morest, '82; Landmesser and Pilar, '72). On the other hand, in this and other systems, the *growth* of the postsynaptic cells (Rubel et al., '76; Parks, '79) or of specific processes of postsynaptic cells (Rakic, '72; Jacoby and Kimmel, '82; Smith, '81) can be temporally correlated with structural or functional signs of innervation.

Gradient in development of transmission

The most surprising result of the present study was the suggestion that the capacity for impulse transmission through the eighth nerve and NM develops along a spatial gradient from anteromedial to posterolateral in NM. These results may reflect a gradient in the functional development of eighth nerve-NM synapses, a developmental gradient in the excitability of eighth nerve axons, or a gradient in excitability of NM cells alone. We favor the first explanation for several reasons: (1) As previously noted (MATERIALS AND METHODS), the intensity of electrical stimulation was adjusted during the recording sessions in order to maximize the eighth-nerve afferent volley recorded at the site of *each* electrode penetration. (2) No consistent variation in the intensity of stimulation required to achieve this maximum afferent volley in various portions of NM was noted within any age group. (3) In subjects older than 9 days, an eighth nerve afferent volley was recorded at *every* sampled site within NM regardless of whether any postsynaptic responses were evoked. (4) A robust eighth nerve volley was recorded throughout NM at 10 days, although no extracellular synaptically-evoked action potentials were recorded at that age. (5) A variety of studies on other systems have shown that the ability of afferent axons to generate and propagate action potentials in response to direct electrical stimulation develops well before the time that impulse activity in those axons is synaptically transmitted to target cells (Bennett et al., '74; Dennis and Miledi, '74; Purpura et al., '68; Robbins and Yonezawa, '71). For these reasons, then, we expect that the functional development of the synaptic apparatus coupling the eighth nerve to NM cells is a major factor responsible for the present results. Confirmation of this position will, of course, require intracellular analysis of NM cells as a function of age and position.

The anteromedial-to-posterolateral gradient described here for the development of NM responsiveness to eighth-nerve stimulation is also reflected in a variety of morphogenetic events within this system. These include cell death, an increase in nuclear volume, and development of NL dendrites (Rubel et al., '76; Smith, '81). Qualitative observations suggest that cochlear development also proceeds along a corresponding basal-to-apical gradient (Cohen and Fermin, '78; Hirokawa, '78) which is similar to that repeatedly observed in the mammalian cochlea (e.g., Pujol and Abonnenc, '77; Ruben, '67; reviewed by Rubel, '78). The data reported here provide the first clear evidence that at least one aspect of auditory system functional development follows the gradient of structural maturation which has been described in the cochlea, in NM, and in NL.

Because the mature cochlea is tonotopically organized with the basal portion responding primarily to high frequencies the morphological data suggest a gradient of functional development from high to low frequencies. The consistent observation in both birds and mammals, however, is that electrophysiological and behavioral responsiveness develops first to relatively low frequencies and later to progressively higher frequencies (see Gottlieb, '71; Rubel, '78). In light of repeated descriptions of a similar gradient of structural development in both the brainstem nuclei and the receptor, and the temporal concurrence shown here, it seems reasonable to suspect that early responses of the immature auditory system to low frequencies are generated in the basal portion of the cochlea and the anteromedial portion of brainstem nuclei. This reasoning is consistent with the suggestion that the incongruity between previous observations of structural and functional development reflects a maturational change in the transduction properties of the sensory epithelium (Rubel, '78).

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