

Development of Auditory Neuronal Responses in Avian Embryos (auditory-vocal behavior/single-unit recording/tonotopic organization/change in threshold)

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ABSTRACT Neurons in the cochlear nuclei of duck embryos become responsive to tonal stimuli in an orderly spatial and temporal sequence. Cells responding to lower frequencies are recorded before those responding to higher frequencies. Auditory neurons are spatially arranged according to their characteristic frequencies, indicating a point-by-point projection of the basilar membrane. Areas of the nuclei where the apical segment of the basilar membrane projects become responsive to sound before regions representing more basal segments. These progressive changes with age are accompanied by a gradual rise both in neuronal sensitivity and in frequency range of maximum sensitivity. The development of auditory neural responses is timed to prepare the embryo for the onset of prenatal mother-young communication.

In precocial birds, chicks start walking and feeding for themselves shortly after hatching. Among these birds a considerable amount of vocal-auditory interaction takes place between parents and chicks in eggs before hatching. In guillemots (*Uria aalge*), chicks in eggs can learn to recognize individual characteristics of the calls of their parents (1). Incubator-hatched day-old domestic mallard ducklings approach a loudspeaker emitting the assembly call of wild mallard (*Anas platyrhynchos*) in preference to a speaker producing the assembly call of wood duck (*Aix sponsa*) or domestic chicken (*Gallus domesticus*) (2). This ability of ducklings to discriminate between alien signals and that of their own species appears as early as 5 days before hatching (2). This report presents the preliminary results of neurophysiological and histological studies made in an attempt to discover the neuro-embryological correlates of the behavioral observation mentioned above.

METHODS

Eggs of white Peking ducks (*Anas platyrhynchos*) were purchased from C & R Duck Farm, Westhampton, Long Island, N.Y. They were incubated at 38° and relative humidity of 55% in a forced-air incubator. In order to standardize the developmental age, the eggs were refrigerated before incubation (3). This procedure was later omitted since incubated and candled eggs obtained from the duck farm exhibited much smaller individual differences in age than those refrigerated. 141 Embryos, 14 newly-hatched ducklings, 7 ducklings of older ages, and 1 adult male duck were used.

Single-unit recordings were made in a small plastic incubator containing a rack for the egg, earphone-microphone monitor assembly, and beak and head holders. The heads of embryos were lifted from the egg through a window made on the shell. The beak was fixed on a metal plate with dental cement, and the weight of the head was also supported by two metal prongs on the back of the neck. A calibration earphone made by Brüel and Kjaer was used as a sound source. A brass adaptor led sound from the earphone to the ear opening

in a closed system. Since the embryonic head is soft, the opening of the adaptor was gently pushed against the ear opening and all gaps were sealed with high-vacuum silicon grease in order to prevent leakage of sound, which makes sound calibration unreliable. Sound pressure levels near the ear opening were measured with a probe tube attached to a calibrated 12-mm Brüel and Kjaer condenser microphone in conjunction with a General Radio 1900 wave analyzer. The adaptor and probe tube were calibrated.

Embryos, in stages before onset of pulmonary respiration, were immobilized with about 0.025 mg of Flaxedil (10 mg/ml) injected into the neck muscle. Embryos respiring through the lungs were anesthetized with 0.10 ml of Equithesin. Recordings were made from the cochlear nucleus, which consists of two parts in birds, nucleus magnocellularis and nucleus angularis. Since the stereotaxic placement of the electrode is unreliable in soft embryos, the cochlear nuclei were exposed by removal of the cerebellum. A large part of the nucleus magnocellularis and a portion of the nucleus angularis are visible on the floor of the fourth ventricle. A tungsten micro-electrode was placed with the aid of a microdrive assembly on desired points in the nuclei under a Zeiss Operating Microscope. The response of an auditory neuron is a function of sound intensity and frequency (4). Each neuron responds to a band of frequencies. The frequency to which a neuron is most sensitive is called the characteristic frequency of that neuron. The instrumentation for single-unit recording and methods of sound generation and "tuning" of auditory neurons have been described (4). Characteristic frequencies were measured to the nearest 10 Hz. Variation in the developmental age, size of embryo, and swelling and shrinking in the operated part of the brain made it impossible to construct composite maps of distribution of characteristic frequencies based on data obtained from many individuals. Therefore, an attempt was made to map the cochlea nuclei of each embryo as thoroughly as possible. For this purpose, the electrode was placed first near a conspicuous landmark such as the aqueduct of Sylvius, and it was then moved on a grid plan in both the rostrocaudal and mediolateral directions. At each point of penetration the characteristic frequencies of neurons were determined and recorded in the vertical sequence in which they occurred. In addition to visual placement of the electrode in the nuclei, lesions were made with the recording electrode in several reference points, and the electrode tracks were identified histologically.

The temperature of the embryo was maintained at 38° by a Yellow Spring Instrument Co., model 73 telethermometer. Humidity within the incubator box was maintained high enough to prevent the embryo from drying. The incubator box and its accessories were placed in a walk-in sound-insulated room. The electrode was driven by a hydraulic microdrive

DAY	19-20	23	27
Longest vertical tonotopic sequence	2 7 0 1 2 0	1 4 4 0 1 1 6 0 7 1 0 6 3 0 4 9 0	2 9 1 0 2 5 9 0 2 3 5 0 1 8 0 0 9 0 0 6 4 0
Highest Characteristic Freq.(Hz)	6 4 0	3 8 1 0	3 9 1 0

FIG. 2. Progression in vertical tonotopic organization with age.

arranged. Lowest-frequency units are located most rostrally and higher-frequency units occur more caudally and medially (toward the midline of medulla) (Fig. 1a). By day 21, the highest characteristic frequency has risen to about 2300 Hz. Also, more units can be recorded in each electrode penetration at this time. There is an indication of an orderly arrangement of frequencies during each penetration; lower-frequency units occur beneath higher-frequency ones (Fig. 1b), so that by this age three directions of tonotopic organization can be recognized. Day-22 embryos show a further expansion of areas yielding responding cells and a clearer tonotopic organization (Fig. 1c).

This pattern of development seems to continue until day 27, day 28 being the time of hatching. In Fig. 2, the longest vertical sequences of neurons together with the highest characteristic frequencies obtained for different age classes are listed. There is a trend toward longer sequences with increasing developmental ages. The highest frequency in any of the tonotopic sequences in Fig. 2 is lower than the highest frequency listed for the same age class. This result is due to the fact that neurons with the highest characteristic frequency occur near the medial and posterior margin of the nucleus where there is no vertical tonotopic arrangement. So far, no distinction has been made between the two parts of the cochlear nucleus, since the area where the tonotopic organization was mapped corresponds to the zone of overlap between the two nuclei. Systematic probing of the nonoverlapping anterior two-thirds of the nucleus magnocellularis indicated no responses until day 22-23, when neurons responding to frequencies higher than 2300 Hz suddenly appear there overnight. Except for the above fact, sampling of neurons in the cochlear nuclei of adult ducks indicated that the basic pattern of distribution of characteristic frequencies established by day 22 does not differ from the adult pattern.

Differential responses to tonal frequencies are but one aspect of auditory capability. During development, there is a gradual lowering of neuronal thresholds with age (Fig. 3). Units recorded from day 19-20 embryos were all quite insensitive; the lowest threshold being about 60 dB (reference 0.0002 dynes/cm²). By day 23, the lowest threshold reached about 44 dB. Another parameter that changes with age is the frequency range in which the embryo is most sensitive. This range is about 300-400 Hz for day 19-20 embryos, 500-800 Hz for day 23 embryos, and 1000 Hz for a newly hatched duckling. This pattern of development seems to continue until, at most, the second day after hatching. By this time the auditory capability of the duckling is similar to that of the adult duck (6). Since the data for newly hatched and 2-day-old ducklings are based on only two individuals, the results contain some uncertainty. For other age classes, the results were considered

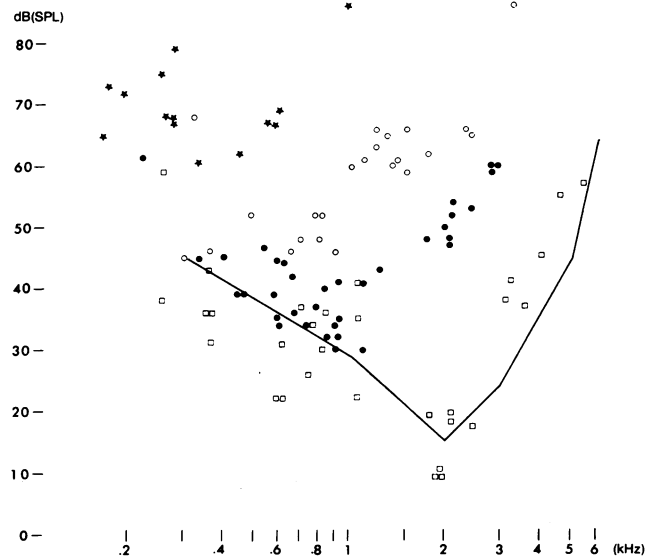


FIG. 3. Thresholds of auditory neurons at their characteristic frequencies. *Day 20; O, day 23; ●, immediately after hatching; □, 2 days after hatching; solid line, behavioral audiogram of an adult male mallard duck (from Trainer, 1946). Ordinate, thresholds in dB (0.0002 dyne/cm²); abscissa, sound frequencies in kHz.

representative when at least three individuals produced similar data.

Preliminary histological examinations of the embryonic cochlear nuclei indicated the presence of all basic adult structural features, such as the large ovoid cells characteristic of the magnocellular nucleus, as early as day 14. Also, the areas of the nuclei in early embryos that did not contain responding auditory cells did not differ from the responding areas in gross histological appearances. Cross-sections of the cochlea of day 17 and day 18 embryos contained all basic adult histological characteristics. At these ages, well-differentiated hair cells were clearly recognizable throughout the length of the basilar membrane.

The female mallard duck produces broody calls as her eggs approach the day of hatching (7). She leads her young to the nearest water as soon as they can follow her. During this exodus she gives assembly calls. The calls consist of a series of brief notes about 100 msec in duration. Notes in both of these calls contain two or more harmonically related frequencies, namely 750 Hz, 1125 Hz, 1875 Hz, and 2250 Hz, which are harmonics of 375 Hz. The broody and assembly calls seem to

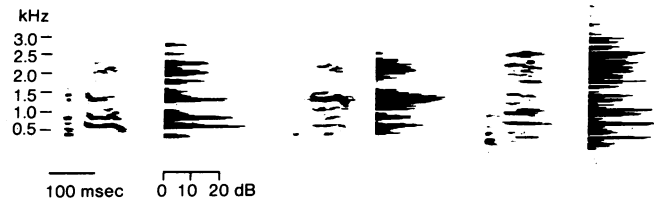


FIG. 4. Narrow-band sound spectrograms of mallard maternal assembly calls. Frequency-time and frequency-amplitude spectrograms of each note are presented side by side. The first and second notes were consecutive, and the third note was separately delivered by a single bird. Ordinate, frequencies in kHz; abscissa, time in msec for frequency-time display and relative amplitudes in dB for frequency-amplitude display. Original recordings by Caswell.

differ from each other in the distribution of spectral energy. The sound spectrographic analyses of calls recorded from a wild female mallard sitting on a nest show a major concentration of energy at 750 Hz and another less-prominent peak at 1125 Hz. The spectrogram of a broody call presented by Hess (7) indicates the same pattern of energy distribution. Some assembly call notes may resemble the broody call note, such as the first note in Fig. 4. However, the distribution of spectral energy in assembly notes seems to vary even among those recorded from a single bird (Fig. 4). Although spectral energy is concentrated at those harmonic frequencies mentioned above, the relative amplitudes of different components vary even between notes delivered consecutively, like the first and second notes in Fig. 4. Notes in both the broody and assembly calls often begin with a low-pitched impulsive noise, which is not included in the frequency-amplitude spectrograms in Fig. 4. According to the recordings made by Caswell, notes without the introductory noise may be interspersed among those with the noise. The presence or absence of the noise component as well as the variation in the distribution of spectral energy may well be correlated with the intensity of calling.

DISCUSSION

According to Boord and Rasmussen (8), who used nerve degeneration techniques, the avian inner-ear basilar membrane is projected onto the cochlear nucleus. The primary auditory fibers maintain the spatial pattern of innervation on the basilar membrane as they course from the cochlea to the cochlear nucleus in the medulla. In the nucleus angularis, fibers innervating the apical third of the basilar membrane terminate in the most ventral and rostral region, those from the middle third of the membrane are situated in the area more caudal and dorsal to the terminal zone of apical fibers, and those of the basal third project to the most caudal and dorsal region. Each auditory fiber divides into two branches as it enters the medulla, one connecting with the nucleus angularis and the other with the nucleus magnocellularis where basal, middle, and apical fibers terminate, respectively, in rostromedial, middle, and caudolateral areas.

The neurophysiological counterpart of the orderly pattern of projection mentioned above is the tonotopic organization. The characteristic frequency of a second-order auditory neuron is correlated with the site of innervation on the basilar membrane by the connecting primary fiber. High, intermediate, and low frequencies cause the maximum amplitude of vibration, respectively, in the basal, middle, and apical segments of the avian basilar membrane (9). In agreement with this observation, the areas in the avian cochlear nuclei receiving basal, middle, and apical fibers contain, respectively, high, intermediate, and low characteristic frequencies. The spatial sequence in which characteristic frequencies occur indicates a point-by-point projection of the basilar membrane (4). The single-unit results reported above show an orderly *spatial* and *temporal* sequence in which the tonotopic organization is established. Neurons in the projection areas of the apical segment become responsive to tonal stimuli before those in the regions representing the middle and basal segments. About day 19 or 20, low-frequency neurons can be recorded in the rostromedial area of nucleus angularis where apical fibers terminate. The cells in the areas immediately caudal, dorsal,

and medial to the above region are the next to become responsive to tonal stimuli of higher frequencies. Further development follows the same pattern, culminating in the formation of the adult tonotopic organization. This developmental phenomenon does not seem to be correlated with any gross histological changes in the inner ear and cochlear nuclei. Similarly, the morphological differentiation of the basilar membrane, hair cells, ganglion cells, and the cochlear nuclei of the medulla is completed by day 12 or 13 in chick embryos (10, 11). This is the stage at which cochlear microphonics is first recorded (12). While the onset of auditory neural responses may be correlated with histological events, there does not seem to be any gross histological change associated with the progressive development of frequency sensitivity later on.

The progressive development of the tonotopic organization with age is accompanied by a gradual rise both in neuronal sensitivity and in the frequency range of maximum sensitivity. Measurement of cochlear microphonics indicated a gradual rise in the frequency range of maximum sensitivity in chick embryos and in some mammals after birth (12, 13). There are several possible causes for the above phenomena. Changes in the mechanical properties of the basilar membrane, gradual development of transducer function in the hair cells, progressive activation of synapses between primary auditory fibers and hair cells or of those between primary fibers and cells in the cochlear nucleus, impedance changes in the mechanical system of the middle ear, etc.

Duck embryos break into the air sac around day 24, when they begin to vocalize as pulmonary respiration starts. They perforate a hole on the egg shell (pipping) around day 26. Hess (7), working with wild mallard ducks, observed vocal-auditory interactions between mother and chicks before hatching. These interactions seem to synchronize hatching of the eggs. The mallard maternal calls contain mostly low frequencies that are well within the range of characteristic frequencies of day-23 embryos. These birds are most sensitive to 500–800 Hz. There is a strong energy component around 750 Hz in the broody call. Thus, the development of auditory neural responses is timed to prepare the embryo for the onset of prenatal mother-young communication.

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