J Comp Physiol (1982) 145:437–445

Journal of Comparative Physiology · A

The Tonotopic Organization of the Bullfrog Amphibian Papilla, an Auditory Organ Lacking a Basilar Membrane

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Accepted November 2, 1981

Summary. Intracellular dye-injection studies have revealed tonotopic organization of the bullfrog (*Rana catesbeiana*) amphibian papilla, an auditory organ lacking a basilar membrane or its equivalent. The best excitatory frequency (BEF) for auditory stimuli was identified in each of twenty-nine VIIIth-nerve afferent axons that subsequently were traced to their peripheral terminations at the sensory surface. Among those axons, the five with BEFs greater than 550 Hz all terminated in the caudalmost region of the papilla, the ten with the BEFs greater than 300 Hz and less than or equal to 550 Hz all terminated in the central region of the papilla, and the fourteen with BEFs equal to or less than 300 Hz all terminated in the rostralmost region of the papilla (Fig. 4). The tectorium is very much larger and presumably more massive under the low-frequency region of the papilla than it is under the high-frequency region (Fig. 1). Higher-frequency axons tended to innervate few (one to four) receptor cells, and low-frequency axons tended to innervate many (six or more). Higher-frequency axons often terminated in large claw-like structures that engulfed the basal portions of individual hair cells and in this way were morphologically similar to type I terminals in the inner ears of higher vertebrates.

Introduction

Among the eight inner-ear sensory surfaces of the frog, the one that generally is conceded to be unique to the amphibians is the amphibian papilla (de Burlet 1928; Geisler et al. 1964; Wever 1974). In all three amphibian orders, the amphibian papilla resides on the ceiling of a separate chamber, most of which is bounded by the thick labyrinthine wall (Fig. 1 is a sketch of the chamber in the ranid frogs). The chamber is open on its lateral side to the general endolymphatic space of the pars inferior, which it shares with the chambers of the sacculus, the lagena, and the basilar papilla. Part of the amphibian papillar chamber wall is formed by an extremely thin layer of epithelial cells that form a contact membrane between the endolymph of the chamber and the perilymph of the amphibian periotic canal (Harrison 1902; Lombard and Bolt 1979). This canal provides direct fluid coupling to the oval window, which in most of the recently derived anurans is connected directly to the sound-conducting apparatus in the middle ear (Lombard and Straughan 1974). The chamber ceiling, on which the papilla itself resides, is especially thick and also serves as the floor of the crus commune of the horizontal and posterior semicircular canals. The papillar branchlet of the VIIIth cranial nerve invades the ceiling to innervate the papilla, but the amphibian periotic system does not invade the ceiling at all (Lewis 1976; Lombard 1980). Thus the amphibian papilla is without an analog of the basilar membrane found in the auditory organs of reptiles, birds and mammals.

Hanging from the papilla is an acellular tectorium comprising two distinct layers: an apparently fibrous proximal layer immediately adjacent to the papillar surface and a more dense, apparently gel-like distal layer extensively invaded by a system of pores and tunnels (Retzius 1881; Wever 1973). The fibers of the proximal layer appear to be connected directly to the microvilli of the supporting cells of the sensory surface (Lewis 1976). The dense gel of the distal layer apparently is coupled directly to the ciliary arrays of most of the hair cells, the point of attachment in each case being a bulb at the distal end of the kinocilium (Lewis 1976). Over the more rostral, trian-

Abbreviations: *BEF* best excitatory frequency; *HRP* horseradish peroxidase

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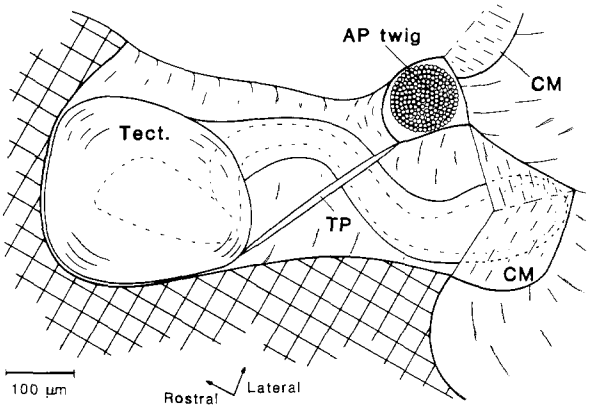

Fig. 1. Amphibian papillar chamber of a ranid frog, viewed from the ventral side (left ear). Cross-hatching represents the thick, transected labyrinthine wall. The sensory epithelium, indicated by the *dashed outline,* is covered by the tectorial membrane (*Tect.*) with its tectorial projection (*TP*). The amphibian papillar twig (*AP twig,* shown transected in the figure) curls around the ventral side of the papillar chamber, just rostral to the contact membrane (*CM*) that separates the chamber from the amphibian periotic canal. As it approaches the contact membrane, the sensory epithelium curves sharply upward, toward the viewer

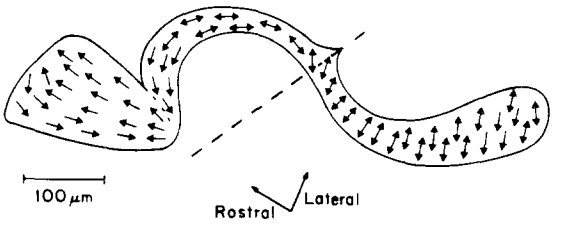

Fig. 2. Outline of the bullfrog amphibian papillar sensory epithelium (left ear), with *arrows* depicting the morphological polarizations of the hair cells. The rostralmost, triangular-shaped region comprises two areas, within each of which the hair cell polarization is more or less uniform, as indicated by the *one-headed arrows.* Over most of the more caudal, S-shaped region, hair cells of opposite polarization are mixed, as indicated by the *two-headed arrows.* The sharp corner, midway along the lateral margin of the sensory epithelium, lies immediately over the tectorial projection, the position of which is indicated by the *dashed line*

gular-shaped region of the papilla, the distal layer of the tectorium is thick, occupying virtually all of the adjacent chamber volume not already taken up by the thin, proximal fibrous layer. Over the more caudal, S-shaped region, the tectorium is thin, and over most of its length is gradually tapered with the caudalmost end being thinnest. In this region the tectorium occupies less than one-third of the adjacent chamber volume. Most of the remaining chamber volume is filled with endolymph. The exception is a small volume occupied by a very thin, diaphragm-like projection from the tectorium, extending from ceiling to floor and from wall to wall in the chamber. This projection is attached around its entire periphery to the microvilli of the epithelial cells lining the chamber wall; but probing it with an eyelash, one can find a vertical slit with overlapping edges. Therefore, the projection appears to form an imperfect barrier between two subvolumes of the papillar chamber. The contact membrane borders one of these subvolumes; the opening to the pars inferior borders the other. The tectorial projection thus appears to be directly in the path of endolymph motion that presumably would be induced by acoustical signals impinging on the chamber through the amphibian periotic canal. For that reason, Wever (1973) called the projection a 'sensing membrane.' In the most derived anurans, adjacent to the tectorial projection there is a transition in the axis of morphological polarization (defined to be the axis of bilateral symmetry of the cell surface, passing through the center of the cell surface and through the kinocilium) of the hair cells (see Fig. 2).

The adult bullfrog amphibian papilla is innervated by approximately 1,500 myelinated axons from the posterior branch of the VIIIth nerve. Almost all of those axons exhibit diameters (including myelin sheath) less than 5 μm (Dunn 1978). Efferent synapses are more abundant on hair cells in the rostral regions of the papilla than on those in the more caudal regions (Flock and Flock 1966). Recording from afferent axons in the bullfrog VIIIth nerve, previous investigators (Frishkopf and Goldstein 1963; Frishkopf and Geisler 1966; Liff et al. 1968; Feng et al. 1975) found that auditory response was sharply tuned, with best excitatory frequencies (BEFs) ranging from less than 100 Hz to approximately 1,500 Hz. On the basis of their locations in the nerve, the axons with BEFs below 1,000 Hz were presumed to originate at the amphibian papilla, while those with BEFs above 1,000 Hz were presumed to originate at a second putatively auditory organ, the basilar papilla. Feng et al. (1975) identified two classes of afferent axons from the amphibian papilla itself. One class, exhibiting BEFs from less than 100 Hz to approximately 700 Hz, was found to be susceptible to two-tone inhibition. The other class, with BEFs from approximately 400 Hz to approximately 900 Hz, was not inhibitable. The amphibian-papillar origin of both classes was determined in an experiment in which the posterior branch of the VIIIth nerve was transected in such a manner as to leave only the amphibian-papillar twig intact.

Spatially separate origins of the two classes *within* the amphibian papilla was suggested by the results of comparative morphological and physiological studies involving four anuran species (the bullfrog [*Rana*

catesbeiana], the American toad [*Bufo americanus*], the green toad [*Bufo debilis*], and Couch's spadefoot toad [*Scaphiopus couchi*]). The lower-frequency population of amphibian-papillar afferent axons exists relatively undiminished in all four species; the population of higher-frequency, noninhibitable axons is relatively large in the bullfrog and the American toad, markedly diminished in the green toad, and totally absent in Couch's spadefoot toad (Feng et al. 1975; Capranica and Moffat 1974, 1975). Examination of the amphibian-papillar morphology in these species (Lewis 1977) revealed pronounced differences in the lengths of the posterior-most portion of the papillar sensory epithelium (i.e., the region with transverse hair-cell polarization, which we have given the name 'caudal extension'). In Couch's spadefoot toad the caudal extension was absent; in the green toad it was very short; and in the American toad and the bullfrog it was long. Thus the length of the caudal extension of the amphibian papilla was closely correlated with the size of the population of higher-frequency, noninhibitable axons originating at the amphibian papilla.

These results suggested not only the possibility of spatial localization of the mechanisms underlying two-tone inhibition, but also a spatial sorting of frequency (i.e., tonotopy) within the amphibian papilla. Because of its lack of an analog of the basilar membrane, the amphibian papilla often has been assumed to lack tonotopy. However, the spatially graded tuning required for tonotopy could be provided, in part, by the tectorium, with its rostrocaudal gradation of bulk (and therefore, presumably, mass). One might suspect that the more massive rostral portions of the tectorium would be tuned to lower frequencies, and the less massive caudal portions would be tuned to higher frequencies. These are precisely the associations (caudal = higher frequencies; rostral = lower frequencies) suggested by the comparative studies.

In this paper, we present the results of a direct test of amphibian papillar tonotopy in the bullfrog. The BEFs were identified in 39 amphibian papillar afferent axons, and those afferents then were filled with dye and traced to their origins in the papillar sensory epithelium. In addition to providing convincing evidence of tonotopy in the amphibian papilla, our results provide details of the peripheral arborization patterns of the identified papillar afferents; and taken together with similar results for 27 basilar papillar fibers, they also provide convincing corroboration of the division of sensitivity between the two papillae.

Materials and Methods

A shipment of freshly caught bullfrogs was sent to us every ten days by our supplier in the San Joaquin Valley. After their arrival, the frogs were kept in a large aquatic holding system with flowing water providing a flushing time constant of approximately 8 h. During the course of our experiments, two surgical routines were employed. In our earlier work, at least 24 h before it was to be used in an experiment, a frog was completely anesthetized by immersion in a 0.4% solution of ethyl m-aminobenzoate, methanesulfonic acid salt (MS 222) for approximately 10 to 20 min, and surgery then was carried out. During surgery, the frog was covered with gauze dampened with a more dilute (approximately 0.04%) MS 222 solution to maintain anesthesia and to facilitate cutaneous respiration. Surgery consisted of removal of a small patch of skin on the roof of the mouth, drilling through the underlying bone and cartilage with a dental drill to expose the dura directly over the VIIIth nerve in the cranial cavity (the otic capsule remaining intact), removal of a small piece of dura and very careful removal of part of the sheath over the nerve. The lateralmost sheath material, containing the major arteries to the inner ear, was left undisturbed in order to insure preservation of circulation. The hole in the roof of the mouth was plugged with sterile Gelfoam, and the frog was placed in a moist, isolated aquarium tank to recover. During recovery, the frog was covered with gauze moistened with tap water. On recovery from anesthesia, the frog was observed for signs of damage to the inner-ear circulation (i.e., listing or other signs of vestibular malfunction). Approximately 1 h before it was to be used in an experiment, the frog was given an intramuscular injection of tubocurarine (approximately 9 μg per gram body weight) dissolved in amphibian Ringer solution. From this point on, great care was taken to avoid any physical trauma whatsoever to the immobilized animal. No further surgery was carried out, and special care was taken to avoid contact with the dura, which is especially sensitive. The animal was placed on its back on a rigid, gauze-covered tray and covered with gauze moistened with tap water. The lower jaw was retracted gently and both jaws were held firmly with wire frames cushioned with damp gauze and connected to the tray. The Gelfoam was removed and the circulation to the inner ear was examined. The animal then was ready for identification and dye-marking of axons.

In our most recent work, approximately 1 h before surgery the frog was given an intramuscular injection of sodium pentobarbital (approximately 50 μg/g body weight) in a standard commercial solution for injection (40% propylene glycol, 10% alcohol). The same surgical procedures were followed through the removal of the nerve sheath. Then the animal was placed directly on the gauze-covered tray and readied immediately for identification and dye-marking of axons.

Our microelectrodes were pulled from thin-walled glass capillary tubing with a fiber welded to one edge (standard 'Omega' glass, W-P Instruments Inc. type TW150F-4). The tips were filled by capillary action with a 5% (by weight) solution of the fluorescent dye Lucifer Yellow CH (Stewart 1978) in distilled water; and the shanks were filled with a 5% (by weight) solution of Procion Red in distilled water. The electrode was inserted into a plastic holder containing a 0.5 M KCl solution and a mixture of fine silver powder and finely ground AgCl, compressed into a pellet and attached to a silver wire. The indifferent electrode comprised a plastic petri dish filled with an agar gel made from 0.5 M KCl, with a second Ag/AgCl pellet embedded in the agar. The moist surface of the agar was in direct contact with the surface of the animal. The electrode was advanced into the fluid of the cranial cavity, directly over the VIIIth nerve, and its impedance was determined as well as the dc potential of the entire loop (two electrodes plus frog). If the impedance was between 50 and 100 MΩ, the experiment proceeded; if the impedance was outside that range, the electrode usually was replaced. The dc potential typically was less than 20 mV. If it was greater than approximately 40 mV, the electrode circuit was checked for problems. Before the electrode was advanced into the nerve, a backing voltage of +40 mV was applied across the tip (added to the dc voltage existing in the loop).

In our earlier work we used an open-field source (Sennheiser HD 424) to apply acoustical stimuli, and a calibrated microphone (Sennheiser MKH 104) to monitor the intensity. The source was placed approximately 5 cm from the tympanum of the stimulated ear, centered on an axis passing through the two ears of the animal. In our recent work we used a closed-field system, comprising a Telephonics TDH 49P driver and a Brüel and Kjaer 4166 condenser microphone mounted together in a brass housing designed by Capranica (see Capranica and Moffat 1975); and the protocol of Capranica's laboratory was followed in the use of the closed-field source. Prior to an experiment, the output port of the source was placed next to the tympanum of the frog and sealed around the perimeter of the tympanum with silicone grease. Care was taken to see that no direct contact was made between the wall of the port and the surface of the tympanum, and that no grease adhered to the tympanum itself. At the end of an experiment, before the closed-field system was moved, its frequency response was calibrated. The absolute calibration of the microphone was determined with a Brüel and Kjaer 4230 sound level calibrator.

Equalizers were not employed with either the closed-field source or the open-field source, and both exhibited intensity variation with frequency. In the range 300 Hz to 1,200 Hz, the intensity of the closed-field source was constant to within ± 1.5 dB; from 80 Hz to 300 Hz the intensity increased smoothly with frequency at 6 dB/octave. Measured at the position of the tympanum, the intensity from the open-field source was constant to within ± 4.2 dB between 200 Hz and 1,200 Hz, falling off smoothly at approximately 6 dB/octave below 200 Hz.

During the search for auditory axons, the stimulus frequency was swept rapidly from approximately 80 Hz to 2,000 Hz. Once an auditory axon was penetrated, its response band was determined crudely from a slower frequency-sweep, then its stimulus-response properties were examined carefully with series of tone bursts approximately 10 Hz apart. Each series passed at least twice over the entire response band, once with successively increasing frequencies and once with successively decreasing frequencies. Each tone burst had a duration of approximately 0.5 s and rise and fall times of approximately 50 ms. The tone burst interval was approximately 1.0 s. The intensity of each successive tone-burst series was reduced by 5 dB until it was apparent the stimulus intensity was close to threshold. At that time the intensity differences were reduced to 1 or 2 dB. The presence or absence of response (i.e., increased axon spike rate) to a particular tone burst was estimated visually with an oscilloscope and/or aurally with an audio spike monitor. In our earliest experiments, these estimates were made on-line. In more recent experiments (including all of the closed-field experiments) they were made from tape-recorded data and were corroborated by computer analysis.

Once the physiological data were obtained for a penetrated auditory axon, Lucifer Yellow was injected into it iontophoretically either by passage of 0.5 s, negative 3 to 10 nA current pulses at the rate of 1/s, or (more recently) by a negative 3 nA (peak to peak) sinusoid superimposed on a negative 1.5 nA dc current. After dye injection, the backing voltage was applied again and the electrode was withdrawn from the nerve, into the fluid of the cranial cavity. At this point, its impedance was checked; and if the electrode had not been broken the animal immediately was prepared for histological observations. Because unintentional dye injection may occur if an electrode breaks, the electrode noise was monitored constantly for signs of breakage. If such signs occurred, the electrode was withdrawn and its impedance checked. Whenever an electrode was determined to have broken, the experiment either was aborted completely or a second pass was attempted at a new location unambiguously distinguishable from the first. In most cases, dye injection was attempted into one axon only per ear.

After completion of the dye injection procedure, the animal was decapitated and the otic capsule was opened carefully with a pair of fine rongeurs. The head then was immersed in a 10% formalin solution in phosphate buffer, in accordance with the protocol published by Stewart (1978). After 48 h of fixation, the cranium and otic capsule were opened completely, the VIIIth nerve was severed at the brain, and the entire membranous labyrinth was removed with the VIIIth nerve attached. The nerve and labyrinth then were dissected, with the anterior branch of the nerve remaining with the pars superior and the posterior branch remaining with the pars inferior. The amphibian papilla then was dissected free from the rest of the pars inferior to facilitate viewing, and the various pieces were dehydrated in a graded ethanol/water series and then cleared in methyl salicylate. The pieces were placed separately in pools of methyl salicylate in depression slides, covered with 0.15 mm cover slips and viewed under a fluorescence microscope. The latter was a Zeiss Universal Laboratory microscope with ultraviolet epi-illumination. The illuminating light was filtered with a 4,348.5 Å band pass filter (109 Å 50% bandwidth), and the observed light was passed through a low pass barrier filter with a 5,200 Å cutoff. The objectives that we found most useful were the Zeiss Neofluar 16/0.4 lens for low-magnification exploration and the Zeiss Planapo 40/1.0 oil immersion lens (with variable numerical aperture) for high-resolution viewing. We also found that closing the aperture of the illuminator, often to its smallest diameter, allowed us to trace very weakly filled axons which otherwise were lost in the background autofluorescence.

Results

As we advanced the electrode into the VIIIth nerve, we observed sudden negative shifts in the dc potential, accompanied by positive-going spikes. We took this combination of events to be a signal of penetration of the sheath surrounding an axon. Advancing the electrode a little farther often produced increases in the amplitudes of the negative dc potential and the spikes, which we took to be signs of increased proximity to the axon at the core of the sheath. Occasionally, the dc and spike potentials appeared to increase in several discrete steps, perhaps indicating penetration of successive layers of sheath. The maximum obtainable spike amplitude varied markedly from axon to axon, ranging from less than 1 mV to more than 60 mV. Occasionally an axon would exhibit very large spikes at first, with the amplitude of the spikes then gradually diminishing. When this happened, the spike would exhibit two peaks as the amplitude passed through approximately the 8–10 mV range. Below that level, the later of the two peaks disappeared, leaving only the single, earlier peak. We took this sequence of events to reflect the onset of conduction blockage at the site of penetration, presumably resulting from injury to the axon. We believe that the large, single-peaked spike passed the point of penetration unimpeded and that the double peak reflected marginal conduction past that point, with the earlier peak corresponding to the spike approaching the electrode and the later peak corresponding to the spike moving on toward the CNS after delayed, passive conduction past the electrode. The small, residual single peak

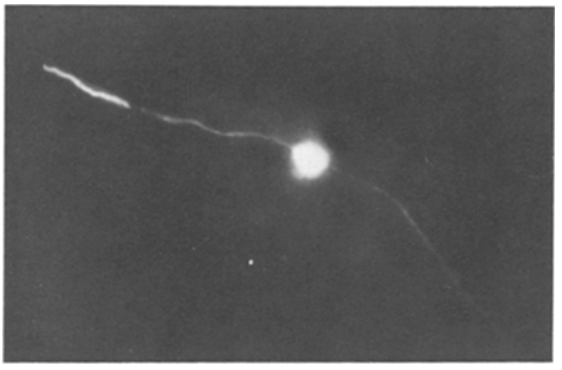

Fig. 3. Dye-filled axon (with myelin sheath) and cell body of an auditory neuron of the bullfrog

then would correspond to a spike approaching the electrode but not getting past it. Most of our dye-injection attempts were made on axons having spike amplitudes in the last class (i.e., less than 8–10 mV). Generally, however, we attempted dye injection only on axons exhibiting spike amplitudes greater than 2 mV.

Advancing the electrode into the nerve from the ventral side, we consistently penetrated vestibular axons first. These almost always were followed by auditory axons with BEFs greater than 1,000 Hz. Then came an abrupt jump to auditory axons with low BEFs (100 to 300 Hz); and from that point on the BEFs of the auditory axons gradually increased until they again approached 1,000 Hz. Interspersed among the penetrations of auditory axons were infrequent penetrations of non-auditory (presumably vestibular) axons. Occasionally this pattern was broken by penetration of very low frequency axons immediately after the vestibular axons, followed by penetration of the axons with BEFs greater than 1,000 Hz and so on.

Once our methods were well established, our dye-filling success rate (number of successful dye injections/number of attempted injections) was approximately 35% for the auditory axons. Almost all of the attempted injections produced a small blister of dye in the myelin sheath, easily visible under the fluorescence microscope. In most cases, the entire corresponding segment of sheath also exhibited fluorescence (Fig. 3); and, in approximately one out of three cases, the axon itself had been filled with dye, in which case it exhibited distinctive fluorescence and could be traced beyond the sheath segment and on to the periphery. When an electrode had broken in the nerve the results were quite obvious. A large spot of fluorescence, not associated with a single sheath, was found; and, occasionally, several axons were filled from that dye spill, although more commonly none were filled. In the few cases in which a deliberate injection was attempted in a nerve in which an electrode already had been broken, the results of that attempt were discarded unless they clearly and unambiguously could be sorted from the consequences of the broken electrode. In most cases this problem did not arise because a single electrode had been used to make a single pass into the nerve, a single injection had been attempted, and the electrode had been withdrawn intact.

Afferent axons were distinguished from efferents by the presence of a dye-filled cell body for each of the former in one of the two VIIIth nerve ganglia (Fig. 3). To date we have identified only one (accidentally) filled efferent axon. It was traced to a region close to the basilar papilla and presumably innervated one or more blood vessels (Dunn 1978). Altogether, 72 auditory afferent axons have been identified, filled and traced. Among those, 39 were found to originate at the amphibian papilla, 27 at the basilar papilla, and 6 at the saccular macula. All 27 basilar-papilla axons exhibited BEFs greater than 1,000 Hz, and all 39 amphibian-papillar axons exhibited BEFs less than 1,000 Hz. BEFs for the saccular axons ranged from 60 Hz to 160 Hz.

At the points at which they were filled, the amphibian-papillar axons typically exhibited sheath diameters ranging from 2 to 4 μm and core axon diameters ranging from less than 1 μm to approximately 2 μm. Once an axon had been traced to the amphibian papilla, the autofluorescence of the hair cells in that organ allowed us to determine very easily the position of the axon with respect to the sensory surface. In the basal region of the hair cell we often found several especially bright spots of autofluorescence that might have been associated with synaptic structures. As the dye-filled axons fanned out from the end of the amphibian-papillar twig of the VIIIth nerve, they coursed well above the sensory epithelial layer, in the thick labyrinthine wall forming the ceiling of the papillar chamber. Ten of the 39 axons could not be traced to their point of emergence into the epithelial layer. Twenty-nine of the axons, however, could be traced to a point at which they projected abruptly downward, deep into the epithelial layer. Of those, 20 were sufficiently well filled with dye to allow detailed tracing of their peripheral arborizations. In all of these axons, and in numerous axons filled accidentally by spilled dye, the arborizations were confined to the immediate vicinity of the point of emergence of the parent axon into the epithelial layer.

All 39 amphibian-papillar axons that were identified and traced showed the same general pattern: those with BEFs less than 300 Hz projected to or toward the rostralmost regions of the papilla; those with BEFs between 300 and 600 Hz projected to or

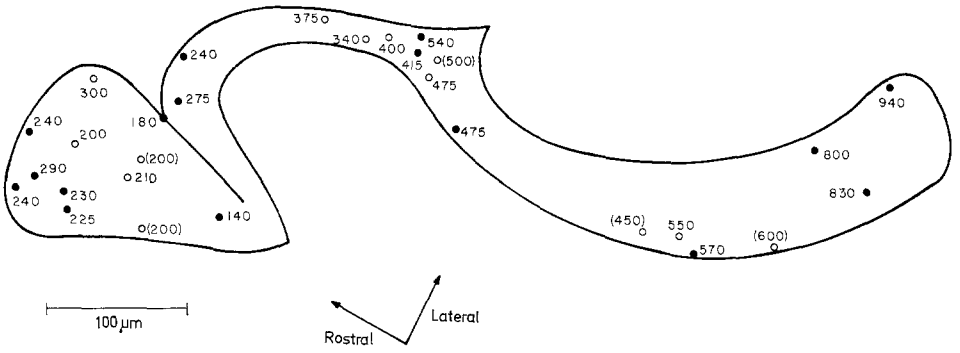

Fig. 4. Outline of the sensory epithelium of the bullfrog amphibian papilla (left ear), showing the sites of termination and the best excitatory frequencies (BEFs) of dye-filled auditory afferent axons. The caudal portion of the sensory epithelium, which ordinarily would curve sharply upward toward the viewer, has been flattened in the diagram. BEFs shown in parentheses are approximate (see text), while those shown without parentheses are accurate to within 10 to 20 Hz. *Open circles* represent axons characterized by open-field acoustical stimuli; *filled circles* represent axons characterized by closed-field stimuli. Each afferent axon entered the sensory epithelium at the point marked and contacted one or more hair cells in the immediate vicinity. Data from 29 ears, both left and right, have been combined in this map

toward the middle regions of the papilla; and those with BEFs between 600 and 1,000 Hz projected to or toward the caudalmost regions of the papilla. No axon was found to branch before reaching its terminal arborization (therefore, no axon was found to project to or toward more than one of the three major regions of the papilla). The general relationship observed between BEF and papillar projection is apparent in Fig. 4, which shows the points of emergence into the sensory epithelium of the 29 traceable axons.

The size and shape of the amphibian-papillar sensory surface varied slightly from animal to animal. The sensory-surface outline in Fig. 4 represents a composite idealization. The projections of the dye-filled fibers were assigned locations in this composite on the bases of proportional distances between key landmarks. In the rostral-most, triangular-shaped region, each projection was located on the basis of its relative position between the caudalmost apex and the rostralmost margin, as well as on the basis of its relative position between the medial and lateral margins. In the more caudal, S-shaped region, each projection was located on the basis of its relative position between the rostral or caudal end of the region and the sharp corner on the lateral margin (which corresponds to the position of the tectorial projection), as well as on the basis of its relative position across the width of the region. Thus, for example, the 830 Hz axon terminated at a point approximately 0.17 of the distance from the caudal end of the sensory surface to the sharp lateral corner (measured along the midline of the surface), and approximately 0.27 of the distance from medial to lateral margin (measured along a line approximately normal to the two margins).

In spite of the presence, generally, of spontaneous spike activity, most of the axons represented in Fig. 4 exhibited sharp transitions (easily sensed with aural and visual clues) between sound intensities and frequencies that elicited spike-rate increase and those that did not. BEF was determined within 10 to 20 Hz. For each of the other axons (those for which the estimated BEFs are enclosed in parentheses), the high level of spontaneous activity combined with lack of sharp tuning forced us to estimate the BEF at a sound intensity somewhat above the lowest transition (threshold) level; and we arbitrarily placed the BEF at the approximate center of the frequency band over which clear responses were observed. The worst case was the unit with the 450 Hz estimated BEF (in parentheses). The response-band edges in that case were approximately 350 Hz and 550 Hz. Among the axons represented in Fig. 4 and exhibiting sharp transitions, the transition (threshold) level at BEF ranged from between 30 and 40 dB SPL to between 80 and 90 dB SPL.

The number of hair cells innervated by the individual afferent axons varied from one to approximately fifteen. Five out of seven arborizations traceable in the rostralmost, triangular-shaped region innervated six or more hair cells, while only two out of thirteen arborizations traceable in the more caudal, S-shaped region innervated that many. In fact eight of the thirteen arborizations in the S-shaped region innervated three hair cells or fewer. One of the axons traced to the triangular region innervated only one hair cell, and that cell had been filled with dye. This was the only instance of dye-coupling observed in the amphibian papilla; three cases of single-hair-cell innervation in the S-shaped region were not accompanied by dye coupling. Two cases of dye coupling were observed in the basilar papilla, each corresponding to an axon innervating one hair cell.

Figure 5 illustrates the apparent variety of ter-

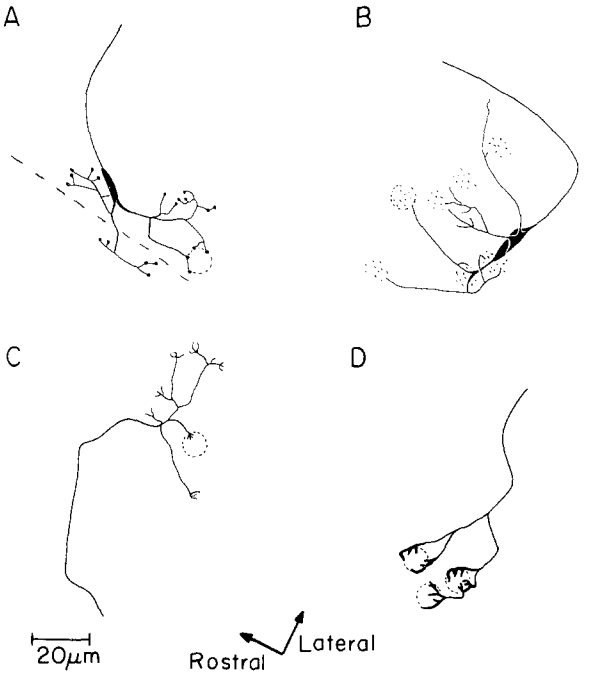

Fig. 5 A–D. Terminal arborization patterns of four of the axons depicted in Fig. 4. *Dashed circles:* hair cells. Orientations are the same as in Fig. 4. The calibration bar is uncompensated for tissue shrinkage which we estimate to be approximately 20%. **A** Fiber with BEF of 180 Hz (closed field stimuli) which contacted at least 8 hair cells. The straight *dashed line* represents the gap between the triangular region of the papilla (*lower left*) and the S-shaped region. **B** Fiber with BEF of 570 Hz (closed field stimuli) which contacted 8 to 10 hair cells on the medial edge of the S-shaped region of the papilla. **C** Fiber with BEF of 200 Hz (open field stimuli) which contacted 6 or 7 hair cells in the triangular region. This is the most rostral of the three 200 Hz fibers in Fig. 4. **D** Fiber with BEF of 475 Hz (open field stimuli) which contacted 3 hair cells in the S-shaped region

minal structures observed in the amphibian papilla. In some cases (Figs. 5A, B) the parent axon terminated in a dye-filled swelling, from which two or more branches arose. In other cases (Figs. 5C, D) no such swelling was observed. Each terminal branchlet ended either in a small dye-filled knob directly abutting a hair cell (Fig. 5A), in an array of discrete spots of dye scattered over the basal surface of a hair cell (Fig. 5B), in a compact arborization of very fine filaments filled with dye and spread over a small part of the basal surface of a hair cell (Fig. 5C), or in a claw-like structure with stout, dye-filled branchlets engulfing nearly the entire basal surface of a hair cell (Fig. 5D). Terminal arborizations in the S-shaped region very often exhibited one claw-like structure (associated with one hair cell) with two or three extremely fine branchlets each projecting to a neighboring hair cell and ending in a compact arborization of fine filaments (similar to the terminal structures in Fig. 5C).

So far, no direct correspondence has become evident between the number of hair cells innervated by an afferent axon and the auditory threshold exhibited by that axon. However, the data available to date are too sparse to allow sorting of the various other factors (e.g., BEF, types of hair cells innervated, location on papilla) that also might affect threshold.

Discussion

Dye-tracing of auditory axons to their peripheral origins is not new. It already has been done by Furukawa (1978) in 18 of the large-diameter saccular axons of the goldfish. In that study he employed the dye Procion Yellow. We already had attempted and failed to fill the much-smaller axons of the bullfrog VIIIth nerve with that dye; and, indeed, Furukawa was unable to fill the small saccular axons of the goldfish. For the larger bullfrog VIIIth-nerve axons, a member of our laboratory (R.A. Baird) found that horseradish peroxidase (HRP) also could be used for filling and tracing. However, HRP proved to be very difficult to inject into small axons, and tracing of filled axons in whole-mounts proved to be considerably more difficult than it was for the fluorescent dye, Lucifer Yellow.

Although the open-field and closed-field datum points in Fig. 4 appear to conform to the same general pattern of tonotopy, one must be cautious in aggregating these data. During each experiment the mouth of the frog was wide open; and the frog eustachian canal, with its extraordinarily large diameter, probably offers little resistance to air flow. Therefore, the two sides of the tympanum were nearly equally accessible to the open-field stimulus, with a path-length difference of approximately 2 cm for the airborne sound. As the stimulus frequency was decreased, this path length difference represented decreasing phase difference and, therefore, decreasing difference in sound pressure level across the tympanum. The attenuation (at least 40 dB) through the wall of the closed-field housing prevented the analogous effect for closed-field stimuli. No attempts were made to relate experimentally the BEFs estimated with the two stimulus modes.

Taken separately or together, the open-field and closed-field data provide strong evidence for the proposed tonotopy in the bullfrog amphibian papilla. Among the 29 axons identified in Fig. 4, the five with BEFs greater than 550 Hz all terminated in the caudalmost region of the papilla, the ten with BEFs greater than 300 Hz and less than or equal to 550 Hz all terminated in the central region of the papilla, and the 14 with BEFs equal to or less than 300 Hz all terminated in the rostralmost region of the papilla. The probability of this outcome, given a random distribution of frequency sensitivity over the papilla, is extremely small (e.g., on the order of 10^{-13} if the areas of the three regions were taken to be 0.25, 0.5,

and 0.25 respectively of the total papillar area and if the axon terminations were distributed with uniform density over the papillar area). Within the three areas definite trends are apparent. In the caudal, S-shaped region there is a definite tendency for BEF to increase as one moves caudally; and in the rostral, triangular region there is an apparent tendency for BEFs to increase as one moves rostrally. The small local deviations from strict tonotopy in Fig. 4 may be the result of aggregation of data not only from open-field and closed-field experiments but also from 29 frogs. On the other hand, they may very well reflect accurately the situation in an individual frog. In either case, they do not detract from the weight of the evidence for general tonotopy in the amphibian papilla.

The presence of tonotopy in the absence of a basilar membrane or its analog is interesting inasmuch as it is the basilar membrane that is thought to endow the mammalian cochlea with its tonotopy. Interesting for similar reasons is the tonotopy that recently has been found in the basilar papillae of two lizard species (*Gerrhonotus multicarinatus* and *Sceloporus orcutti*). Although the lizard basilar papilla possesses a basilar membrane apparently analogous to the structure of the same name in the mammalian cochlea, the gradations of that membrane appear to be insufficient to provide sharp mechanical tuning through the ranges of frequency over which individual lizards are known to be sensitive (Wever 1965). Indeed, recent experiments by Peake and Ling (1980) confirmed that the basilar membrane in *G. multicarinatus* is very broadly tuned throughout, exhibiting no sharp tuning or tonotopy. Nevertheless, the individual afferents from the basilar papilla of that animal exhibit sharp tuning; and the papillar branch of the VIIIth nerve exhibits pronounced tonotopic organization, directly reflecting a corresponding organization over the papillar surface (Weiss et al. 1976, 1978). Pronounced VIIIth-nerve tonotopy recently has been reported in *S. orcutti* as well (Turner and Nielsen 1980; Turner et al. 1981).

There are several parallels between the basilar papillae of the two lizard species and the amphibian papilla of the bullfrog. Like the bullfrog amphibian papilla, the basilar papillae of *G. multicarinatus* and *S. orcutti* possess tectoria that are conspicuously more bulky over the low-frequency regions of the sensory epithelium than they are over the high-frequency regions. In fact the tectoria appear to be absent or nearly so over the high-frequency regions of the two lizard papillae. As does the bullfrog amphibian papilla, the *G. multicarinatus* basilar papilla exhibits denser efferent innervation at its low-frequency end than it does at its high-frequency end (Weiss et al. 1976). Like the bullfrog amphibian papilla, the *G. multicarinatus* basilar papilla exhibits two-tone inhibition in its low-frequency axons and no two-tone inhibition in its high-frequency axons; and, like the bullfrog amphibian papilla (Lewis 1976), the two lizard basilar papillae exhibit pronounced gradations in the morphology of their hair cells (Mulroy 1974; Turner et al. 1981).

The lizard and frog papillae are quite different, however, in the relative directions of their hair-cell-morphology gradients and their BEF gradients. While the shapes and lengths of the ciliary arrays vary along an axis that apparently is normal to the predominant axis of frequency-sensitivity variation in the bullfrog amphibian papilla, the two axes apparently coincide in the lizards. Thus, while variation in tuning might be related to variation in ciliary-array morphology in the two lizards, array morphology apparently is not the predominant factor in the bullfrog amphibian papilla. The axis of the gradient of tectorial thickness, on the other hand, does coincide with the axis of the gradient of frequency sensitivity in the bullfrog amphibian papilla, with the thickest portion of the tectorium being adjacent to the lowest-frequency region of the papilla and the thinnest portion of the tectorium being adjacent to the highest-frequency region of the papilla. If we assume that mass is directly related to thickness in the tectorium, then these are precisely the associations one would expect if the tectorium were serving as part of a distributed acoustical filter. Interestingly, the high-frequency end of the amphibian papilla is closest to the contact membrane, the putative entrance for acoustic energy into the papillar chamber. One therefore might propose a traveling-wave model for the amphibian papilla similar to that commonly applied to the cochlea (Békésy 1960), invoking a spatially-distributed, predominantly graded-mass element in place of the spatially-distributed, predominantly graded-elastic element of conventional models of the cochlea.

Based on the limited data available to date (20 completely traceable amphibian papillar axons with identified BEFs), there seems to be a strong tendency for the higher-frequency axons to innervate few (one to four) hair cells and the lower-frequency axons to innervate many (six or more). This tendency also was observed among unidentified fibers filled unintentionally from broken electrodes. Those that projected to the lower-frequency regions of the papilla terminated on many hair cells; those that projected to the higher-frequency regions terminated on few. One attribute of any distributed mechanical system in a viscous fluid is greater spatial spread at lower frequencies owing to boundary-layer effects (Landau and Lifshitz 1959). The variation in number of hair cells innervated could correspond in some way to this spatial spreading (Bialek and Schweitzer, submitted).

Aside from the general shape of the cell body,

the principal distinction between the type I and type II hair cells of higher vertebrates is the shape of the axon terminals associated with them (Wersäll 1956). Previously, only type II hair cells have been reported from amphibians (Wersäll et al. 1965). The claw-like termination (Fig. 5D) that we found on a large proportion of the higher-frequency axons, however, is similar to the calyx terminal associated with type I hair cells and thoroughly different from the clusters of small knobs associated with type II hair cells.

In addition to BEF, the afferent axons from the bullfrog amphibian papilla exhibit several degrees of freedom in their stimulus-response relationships, including threshold intensity, degree of adaptation (Megela and Capranica 1979), rate or time constant of adaptation, sharpness of tuning, and presence or absence of two-tone inhibition. These may correspond to such structural degrees of freedom as ciliary-array morphology, hair-cell polarization, and axon-terminal morphology. As we refine the frequency map of Fig. 4, we are identifying as many of these structural and functional degrees of freedom as we can, hoping to find evidence of any structure-function correspondences that may exist.

Acknowledgements. We wish to thank Dr. R.R. Capranica, Dr. A.L. Megela, and Mr. G.D. Harned, who collaborated in the filling of 7 of the units shown in Fig. 4 while the authors were visiting Dr. Capranica's laboratory at Cornell. This work was supported by the National Institutes of Health: Grant NS12359 from the National Institute of Neurological and Communicative Disorders and Stroke, and by Systems and Integrative Biology Training Grant T32-GM07379-03 from the National Institute of General Medical Sciences.

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