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## Cilium Length: Influence on Neural Tonotopic Organization

Abstract. *Previous studies have suggested that variations in the length of the hair cell cilia contribute to auditory nerve fiber tuning and tonotopic organization. In the granite spiny lizard, cilium length and the tonotopic organization of nerve fibers are correlated: fiber characteristic frequency increases as cilium length decreases. This results in an increasing fiber characteristic frequency in both the apical and basal direction, a pattern not previously seen in any vertebrate.*

In the mammalian auditory system, acoustic stimulation causes the basilar membrane to vibrate. The membrane is mechanically tuned in that a particular frequency will cause maximum vibration at one location on the membrane, with the basal portion of the membrane responding best to high frequencies and the apical portion to low frequencies. Membrane motion is transduced into neural excitation by the hair cells, the cochlear sensory receptors, which are located in the organ of Corti and situated on the basilar membrane. Vibration of the basilar membrane causes displacement of the hair cell cilia. This results in changes in the hair cell receptor potentials and the activity of primary auditory nerve fibers. Individual fibers are tuned —that is, they are most sensitive to a certain stimulus frequency (characteristic frequency). Fibers of different characteristic frequencies (CF's) display a tonotopic organization: fibers with high CF's innervate the basal region of the cochlea, whereas those with low CF's innervate the apical region. In the mammal, tonotopic organization of the nerve is consistent with the mechanical tuning of the basilar membrane.

For the mammal, it is accepted that the mechanical tuning of the basilar membrane contributes to neural tuning and tonotopic organization. For many years, however, there has been a controversy as to the need for additional mechanisms. Recent data on hair cell receptor potentials and basilar membrane mechanics suggest that other mechanisms, located at the hair cells, may also contribute to fiber tuning and tonotopic organization (*1*, *2*). In addition, measurements of basilar membrane motion in the alligator lizard indicate that tuning of the basilar membrane does not contribute significantly to neural tonotopic organization; other mechanisms must be present in that animal (*3*, *4*).

Systematic variations in the length of hair cell cilia have been observed in several animals. This variation may contribute to neural tonotopic organization (*3*, *5*). In the chinchilla, cilium length systematically decreases in the basal direction along the cochlea (*5*). This has also been observed in two nonmammalian vertebrates—the chick (*6*) and the alligator lizard (*3*). In the chinchilla and the alligator lizard, where neural tonotopic organization is known, there is a correlation between decreasing cilium length and increasing CF. However, the causal effects of cilium length are difficult to separate from position on the basilar membrane.

The ear of the granite spiny lizard (*Scelopolus orcutti*, family Iguanidae) provides an excellent opportunity to investigate the relation between cilium

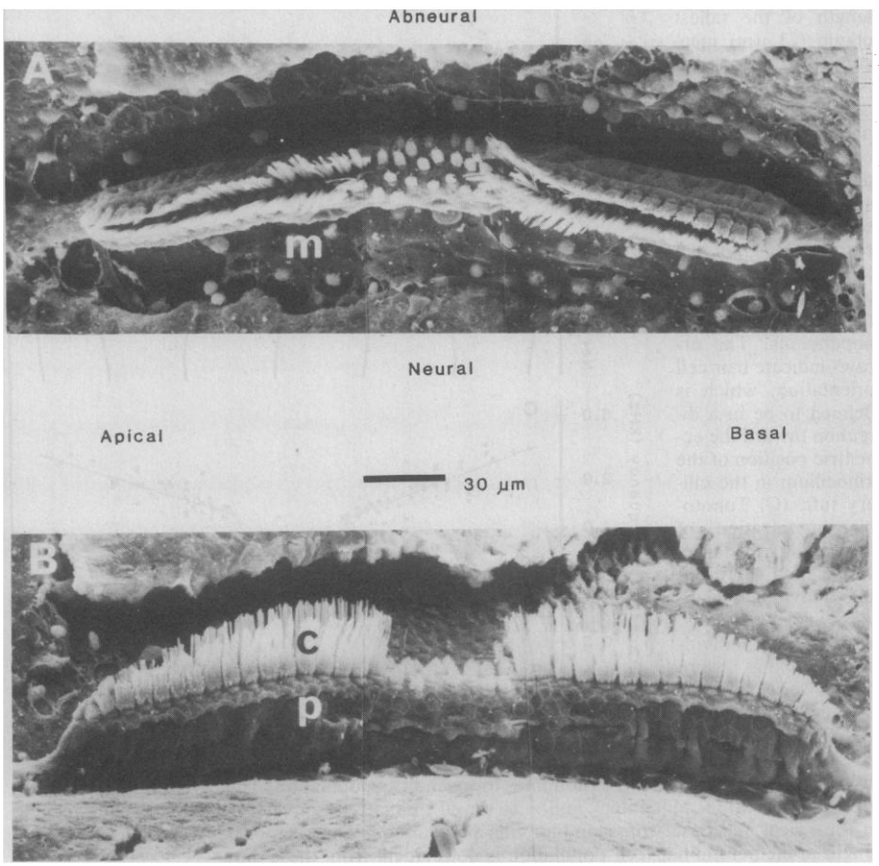


Fig. 1. Scanning electron micrographs of the right basilar papilla (*p*) of the granite spiny lizard. Note the three distinct hair cell populations in the top (A) and side (B) views. The central population has shorter cilia and a tectorial membrane (not visible). Both the apical and basal populations have longer freestanding cilia (*c*) which decrease systematically in length along the papilla. The basilar membrane (*m*) is evident in the top view (A). The membrane does not decrease systematically in width in the basal direction. Neural means toward the auditory nerve; abneural, away from the nerve.


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length and the tonotopic organization of primary fibers. In one hair cell population in the basilar papilla (the reptilian analog of the organ of Corti) cilium length decreases in the apical direction; in another population, cilium length decreases in the basal direction. By measuring cilium length and the tonotopic organization of the auditory nerve, we were able to determine if tonotopic organization correlates better with cilium length or with direction along the basilar membrane. We found that tonotopic organization does correlate well with cilium length, even though this results in a tonotopic organization that differs from the classic pattern (increasing frequency in the basal direction) observed in other vertebrates.

The basic morphological features of the lizard's ear and cilium length were determined through the use of the scanning electron microscope (SEM). We prepared the ear of the granite spiny lizard for SEM evaluation in the manner described by Miller (*7*), critically drying it with liquid Freon as described by Lim (*8*). This drying process leads to approximately 25 to 30 percent shrinkage by the specimen (*9*). Finally, the ears were sputter-coated with approximately 200 Å of gold/palladium in argon. We examined the specimens with a calibrated SEM (ETEC Autoscan).

For electrophysiological recording, the lizards were anesthetized with ethyl carbamate in 20 percent solution in physiological saline with a dose of 0.0125 ml per gram of body weight. The basic surgical procedure has been described (*10*). The cochlea is approached from the ventral side of the animal. The round window membrane and surrounding bone are removed to expose the auditory nerve. Using glass microelectrodes, we recorded spike activity from individual auditory nerve fibers close to the basilar papilla, where the fibers form a wide, thin sheet. The CF of each fiber was measured by manually adjusting the frequency and intensity of the acoustic stimuli (tone bursts, generated by a closed, calibrated acoustic system) while monitoring the spike activity of the fiber. Fiber CF was recorded as the electrode was systematically advanced in one direction across the nerve using a three-dimensional hydraulic system (Narishige, MO-103), which could accurately position the electrode relative to the nerve. The relation between fiber CF and the location of the microelectrode in the nerve indicated the tonotopic organization.

The basilar papilla of the granite spiny lizard has three distinct hair cell populations, with the middle population having the shortest cilia (Fig. 1). The tectorial membrane, which is present for the middle hair cell population, was removed during preparation and is not visible in the micrograph. The apical and basal hair cell populations are similar except that cilium length decreases in opposite directions along the basilar membrane. In the apical and basal populations there are two rows of hair cells with free-standing cilia. These cilia are not associated with any other structures but stand free in the cochlear fluid. The basic morphological features observed in the granite spiny lizard are consistent with findings in other lizards of the genus *Sceloporus* (*11*).

For all three populations we measured the length of the tallest cilium in each ciliary tuft (*12*). In the center population, cilium length varied between 4 and 11 μm with little systematic change along the papilla (Fig. 2). In the two free-standing cilia populations, cilium length systematically decreased with location along the basilar membrane and varied between 5 and 23 μm. In the apical population, cilium length decreased in the apical direction, whereas in the basal population, it decreased in the basal direction.

The auditory nerve fibers can be divided into three distinct populations on the basis of tonotopic organization (Fig. 2) and other physiological properties (*13*). Fibers in the middle of the nerve, which presumably innervate hair cells in the central population, have CF's between 250 and 900 Hz. We were not able to demonstrate a systematic tonotopic organization within this fiber population. The fibers along the apical and basal extremities of the nerve have higher CF's between 900 and 4300 Hz. Fibers in the basal edge have a tonotopic organization with increasing frequency in the basal direction. They presumably innervate hair cells in the basal free-standing cilia population. Fibers on the apical edge of the nerve have a tonotopic organization with increasing frequency in the apical direction. These fibers presumably innervate hair cells in the apical free-standing cilia population. Fibers in all three populations demonstrate an in-

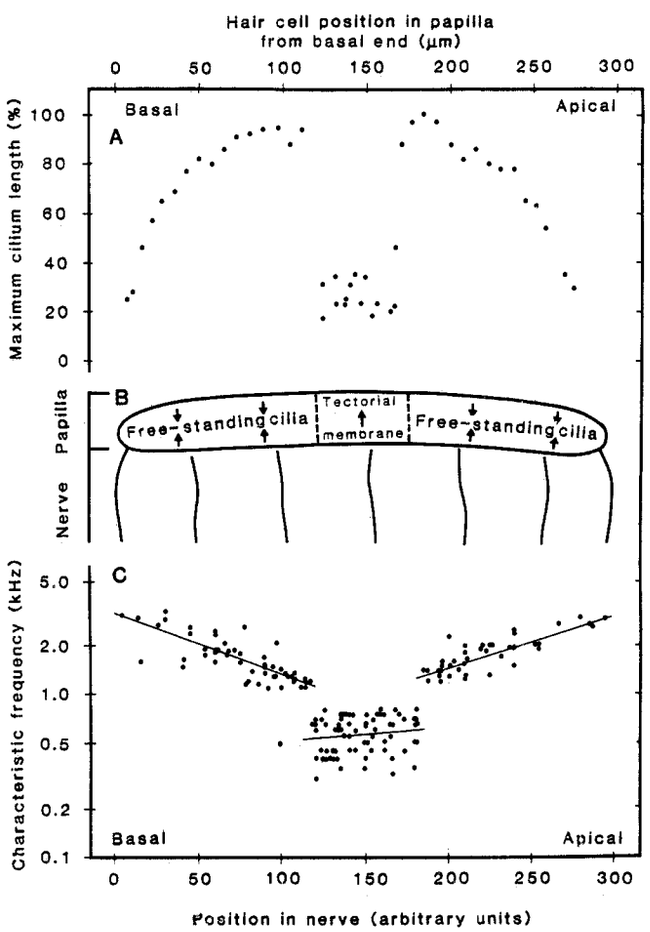


Fig. 2. Relation between cilium length and tonotopic organization in the granite spiny lizard. (A) Cilium length, plotted as a percentage of the length of the tallest cilium (23 μm) measured in that papilla, is shown as a function of position along the papilla for the left ear of one animal. These data are representative of what has been found in other ears. (B) Diagram of a top view of the papilla and nerve showing the three hair cell populations. The arrows indicate hair cell orientation, which is defined to be in a direction toward the eccentric position of the kinocilium in the ciliary tuft. (C) Tonotopic organization of the auditory nerve. These data represent 15 individual mappings in eight animals. Because the individual mappings were normalized so that they could be plotted together, position is given in arbitrary units. The solid lines are regression lines for each fiber population. The correlation coefficients are +.82 (apical), +.15 (center), and −.80 (basal). Fiber CF is inversely related to cilium length in the apical and basal free-standing cilia populations. The relation between CF and cilium length in the central hair cell population is not evident from these data.

creasing sharpness of tuning with increasing CF.

In this lizard, there is a strong relationship between free-standing cilium length and the tonotopic organization of the nerve. As cilium length decreases in the basal and apical populations, fiber CF increases, even though this results in increasing fiber CF in both the apical and basal direction. To our knowledge, this pattern of tonotopic organization has not been found in any other vertebrate.

Hair cells in the central population have the shortest cilia, and the corresponding fibers have the lowest CF. Since this population has a tectorial membrane rather than free-standing cilia, different mechanisms may be responsible for fiber tuning in the two types of hair cell populations in this papilla. Cilium length varies within the central population, but not systematically along the papilla. Cilium length seems to increase in an abneural direction across the papilla. It is not possible from these data to determine the relation between cilium length and fiber CF for hair cell populations with a tectorial membrane.

In this lizard, the particular structures associated with the hair cells—tectorial membrane and free-standing cilia—seem more important for determining fiber CF than location on the basilar membrane. Within the free-standing cilia populations, cilium length is inversely related to fiber CF, even when cilium length decreases in the apical direction. These results support the hypothesis that the length of hair cell cilia influences auditory nerve fiber tuning.

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12. There are three sources of error in determining cilium length. (i) In general, the specimen will shrink as it is prepared. Since we do not know how much the cilia shrink, our measurements of cilium length may be an underestimate of the actual length in the live animal. (ii) Some error may occur in properly orienting the cilia for measurement; however, errors in orientation as great as 25° result in measurement errors of less than 10 percent. (iii) It is difficult to measure cilium length if the cilia are not straight. We found that the cilia were straight and well organized immediately after sample preparation. With time, however, the cilia curl. Measurements were made while the cilia were relatively straight. Because of these possible errors, we have plotted cilium length (Fig. 2A) as a relative percentage of the maximum length measured in that papilla.
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## Bodian's Silver Method Stains Neurofilament Polypeptides

Abstract. *Bodian's silver method was used to stain polypeptides of rat spinal cord or peripheral nerve separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The bands corresponding to the three polypeptide subunits of the neurofilaments were intensely impregnated. Two other polypeptides were stained inconsistently and less intensely. The tubulin band was stained weakly or not at all; other polypeptides, including glial fibrillary acidic protein, actin, and vimentin, remained unstained. This novel application of Bodian's method provides indirect proof that neurofilaments are the neuronal subcellular structure stained by the technique.*

Reduced silver staining techniques have been used to study nervous tissue since the beginning of the century. In 1936 Bodian (*1*) introduced a new staining procedure that is based on the combination of silver proteinate and metallic copper and is highly selective for axons. This procedure has been widely used by neuroanatomists to trace axonal pathways and by neuropathologists to study abnormal axons and to visualize pathological changes in the nerve cell characterized by an accumulation of abnormal fibrillary structures. These structures, called neurofibrillary tangles, are the hallmark of senile and presenile dementia (Alzheimer's disease) (*2*). When Bodian's staining method is applied to routine histology sections, axons, some dendrites, and fibrillary structures in large neurons are stained black (*1, 3*). Thin collagen fibers of vessels, probably type III collagen or reticulin fibers, are also often stained (*4*).

The subcellular structures stained by Bodian's method and the chemical reaction involved have not been determined. Potter (*5*) correlated the ultrastructure of axons, dendrites, and synaptic endings with the intensity of the staining by various reduced silver techniques and concluded that Bodian's method stains cell structures rich in neurofilaments. Electron microscopic studies (*6, 6a*) suggested that silver granules are selectively aligned along neurofilaments in axons and presynaptic endings. However, the poor preservation of the tissue and the unsatisfactory reproducibility make this approach difficult (*6a, 7*).

The relative specificity of the Bodian silver stain is probably due to the formation of insoluble silver nuclei on specific cell structures. During the formation of silver nuclei (a process similar to the formation of the latent image in photography), the metal is reduced at the staining sites (*8*). The reducing groups responsible for the silver impregnation in axons have not been identified, although carbonyl and sulfhydryl groups are probably involved (*9*).

During an immunocytochemical study of a toxic neuropathy in which antibodies to the 68,000-dalton subunit of the neurofilament were used (*10*), we observed a marked similarity in staining between immunoperoxidase and Bodian's silver method (Fig. 1). We used Bodian's method to stain polypeptides of whole spinal cord and peripheral nerve separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*11*). Strips about 1 cm wide were cut from Coomassie-stained gels, immersed in distilled water for 1 hour to remove the fixative, and placed in a horizontal sliding freezing microtome (Lipshaw Manufacturing Corp.). Sections approximately 70 μm thick were cut, mounted under water on gelatin-coated slides (*12, 13*), and stained (*1, 14*).

The bands of the three polypeptide components of the neurofilament were intensely stained (Fig. 2). Two additional bands of approximately 92,000 and 74,000 daltons were stained inconsistently and less intensely. Tubulin was stained weakly or not at all. The weak and inconsistent staining of the tubulin


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