Characteristics of Auditory Stereocilia in the Apical Turn of the Echolocating Bats by Scanning Electron Microscopy

Jinyong Kim[1], Yongwook Jung[1,2],*

[1]Laborotory of Electron Microscopy, [2]Department of Anatomy, Dongguk University College of Medicine, Gyeongju 780-714, Korea

*Correspondence to:
Jung Y,
Tel: +82-54-770-2404
Fax: +82-54-770-2447
E-mail: jungyw@dongguk.ac.kr

The auditory system of the Korean greater horseshoe bat (*Rhinolophus ferrumequinum korai, RFK*) is adapted to its own echolocation signal, which consist of constant frequency (CF) element and frequency modulated (FM) element. In contrast, the Japanese long-fingered bat (*Miniopterus schreibersii fuliginosus, MSF*) emits FM signals. In the present study, the characteristics of stereocilia in *RFK* (a CF/FM bat) and *MSF* (a FM bat) were studied in the apical turn of the cochlea where the lower frequencies are transduced. Stereocilia lengths and numbers were quantitatively measured in *RFK* by scanning electron microscopy and compared with those of *MSF*. Each inner hair cells (IHCs) of *RFK* possessed three rows of stereocilia, whereas *MSF* possessed five rows of stereocilia. Gradients in stereocilia lengths and numbers of stereocilia of the IHCs of *RFK* were found to be less pronounced and fewer, respectively, than those of *MSF*. Each outer hair cells (OHCs) possessed three rows of stereocilia in both species. OHCs stereocilia in *RFK* that distinguished it from *MSF* were a shorter length and a greater number of stereocilia. These features suggest that the apical cochleas of *RFK* are adapted for the processing of higher frequency echolocation calls rather than that of *MSF*.

Key Words: Echolocating bats, Scanning electron microscopy, Stereocilia

INTRODUCTION

As insectivorous microchiropteran bats, Korean greater horseshoe bats use echolocation to orient within the environment and prey on flying insects (Chung et al., 2010). *Rhinolophus ferrumequinum* sp. widely inhabits Europe, China, and Korea. Recently populations have been declining in Europe, whereas their number is growing in the Korean peninsula (Csorba et al., 2003). However, few research studies have been conducted on the Korean greater horseshoe bats in the Republic of Korea.

Echolocation is a type of biological sonar that is used by several types of echolocating animals. These animals emit calls into the environment, listen for returning echoes, and use these to locate and identify objects. The echolocation calls of the Korean greater horseshoe bat (*R. ferrumequinum korai, RFK*) consist of long constant frequency (CF) element, which is invariably terminated by a brief downward frequency modulated (FM) element (49 kHz) (Chung et al., 2010). The CF component is used to detect wing-beating insects in dense foliage, and its frequency is species-specific (*R. ferrumequinum*, 83 kHz; *Rhinolophus rouxi*, 78 kHz) (Vater & Lenoir, 1992). In contrast, the Japanese long-fingered bat, *Miniopterus schreibersii fuliginosus* (*MSF*), while foraging on the wing, emits sequences of brief, downward-FM signals (80~20 kHz) with a low dominant frequency of 44 kHz when approaching and catching prey (Jones & Teeling, 2006; Liang et al., 2011).

The mammalian cochlea performs a frequency analysis of acoustic signals containing low to high frequency tones by systematically processing these signals using hair cells located in the apical to basal region of the cochlea, respectively. The

basis of this frequency map is primarily attributed to the natural vibrating frequency of the basilar membrane along the cochlear duct (Bekesy, 1960). Other factors, however, including the properties of the tectorial membrane, the lengths and numbers of hair cell stereocilia (Steel, 1983) and the stiffness variations of stereocilia (Strelioff & Flock, 1984) may also play a role in determining the frequency selectivity.

In a recent study, it was proposed that the micromechanical properties of hair cell stereocilia play important roles in the detection of frequency changes and in electro-mechanical transduction (Lim, 1986). The lengths and numbers of auditory stereocilia have been studied in echolocating bats in the apical, middle, and basal turns (Yao et al., 2007). In addition, relations between stereocilia lengths and quantities and hearing at different hair cell locations have been studied in the bird cochlea (Tilney & Saunders, 1983). However, the precise roles played by stereocilia length and number are not fully understood as they vary by cochlea segment and between species.

Thus, in the present study, we examined the characteristics of stereocilia at the fifth half-turn (8.1~8.5 mm from the basal end) of cochlea in two different types of echolocating bats by scanning electron microscopy (SEM). Bruns & Schmieszek (1980) demonstrated that the apical region from 8 mm to the apex represented frequencies lower than 40 kHz in the greater horseshoe bat (*R. ferrumequinum*). Apical turns have received less research attention than the basal turns of CF/FM and FM bats, which process high frequency echolocation signals. Furthermore, no investigation has been conducted on the stereocilia of the apical turn of *RFK* (a CF/FM bat) or of *MSF* (a FM bat), nor has any comparative study been undertaken on the auditory stereocilia of CF/FM and FM bats.

MATERIALS AND METHODS

Animals
Animals were captured in an abandoned mine in the Tongyeong area (Gyeongsangnam-do, South Korea) between March and April 2013. Experimental procedures were reviewed and approved by the Animal Care and Use Committee of Dongguk University, and animal care and use were in accord with the guidelines issued by the National Institute of Health (Bethesda, MD, USA). Three cochleae from both species were used in the study.

Scanning Electron Microscopy
Bats were decapitated under ether anesthesia and cochleae were quickly removed from temporal bones and perfused in a fixative containing 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2). Bony otic capsules were removed using a forceps under a dissecting microscope to expose labyrinths. Specimens were fixed in the same fixative at 4°C, rinsed in 0.1 mol/L sodium cacodylate buffer, postfixed in OsO_4 for 2 hours, dehydrated in an increasing alcohol series (50%~100%), and soaked overnight in isoamyl-acetate. Cochleae were then critical-point dried using liquid CO_2 (HCP-2; Hitachi, Japan) and gold sputtered (E-100; Hitachi). Specimens were examined using a field emission SEM (S-4200; Hitachi). Each fifty inner hair cells (IHCs) and outer hair cells (OHCs) from *RFK* and *MSF* were examined at the fifth half-turn (8.1~8.5 mm from the basal end) of cochleas (a distance of between 69%~66% (Fig. 1A, rectangle) and 69%~71% (Fig. 1B, rectangle), respectively, from the basal end.

Statistical Analysis
To provide accurate measures of stereocilia length (from root to apex), prone stereocilia in SEM photographs were erected and stereocilia length values were obtained using SEM software (USB20 Image Software; Korea Research Institute of Standards and Science, Korea). Photomicrographs of IHCs stereocilia at different positions along sensory epithelium were evaluated to determine numbers per row, which provides a good measure of numbers of stereocilia per cell, despite vague borders between hair cells in SEM images. Micrographs
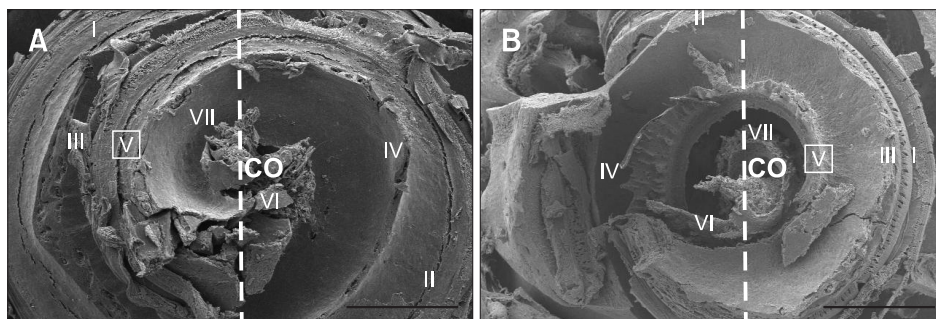

Fig. 1. Scanning electron micrographs of the cochleas of *RFK* (a CF/FM echolocater) (A) and *MSF* (a FM echolocater) (B). Individual half-turns (1st to 7th half turns) are numbered starting from basal ends. The vertical dotted lines divide cochleas into 7 half-turns. The stereocilia of inner hair cells and outer hair cells of *RFK* and *MSF* were examined at the fifth half-turn (rectangle). CO, cochleas; *RFK, Rhinolophus ferrumequinum korai*; CF, constant frequency; FM, frequency modulated; *MSF, Miniopterus schreibersii fuliginosus*. Scale bars=500 μm.

were taken at ×3,000 and enlarged to provide an effective magnification of at least ×20,000. Numbers of IHCs stereocilia per row were counted using the axis of the helicotrema at the 12 o'clock position, which was used as a reference point. Initially, stereocilia were counted 0.5 mm to the left and right of the reference point, and then 1 mm to the left and right around the same reference point. Numbers of OHCs stereocilia were determined by counting the total numbers of stereocilia bundles. The opening angles of stereocilia bundles of OHCs were measured using a protractor. Data were analyzed using Excel software (Microsoft Corp., USA), and results are expressed as mean±standard deviation. Groups were compared using unpaired t-test. p-values of <0.05 were considered significant.

RESULTS

The mean diameters of cochleas from *RFK* and *MSF* were 2.2 and 2.0 mm, respectively, total cochlear lengths were 12.3 and 11.8 mm and cochleas had 3.5 and 3.4 turns, respectively. *RFK* had a larger cochlear diameter and length than *MSF*. The cochlea in *RFK* and *MSF* were found to possess three and a half turns or 7 half-turns by bisecting cochleas vertically, as shown in Fig. 1.

Scanning electron micrographs of IHCs and OHCs at the apical turn of the organ of Corti of *RFK* and *MSF* are shown in Fig. 2. At the apical turn in both species, there were three rows of OHCs and one row of IHCs, which concurs with the description of *R. rouxi* (a related bat species) by Vater & Lenoir (1992). IHCs were observed to adjoin inner phalangeal cells and one row of inner pillar cells. OHCs were arranged in a mosaic pattern comprised of alternating junctions with

one row of outer pillar cells (OPs) and three rows of Deiters 1 (D1), Deiters 2 (D2), and Deiters 3 (D3) cells (Fig. 2). IHCSs of stereocilia in *RFK* had regular, closely spaced stereocilia, whereas those of *MSF* had irregularly separated stereocilia bundles (Fig. 2). The two species had similar OHCs spacing patterns. In *RFK*, the innermost row (Fig. 2A, '①') and the middle row (Fig. 2A, '②') were distinctly separated by OPs. In contrast, in *RFK*, there was little space between the middle and outermost rows (Fig. 2A, '③') of OHCs.

Inner Hair Cells Stereocilia in the Two Species

In both species, IHCs stereocilia bundles were arranged linearly, which contrasted with the 'W' shaped arrangement of OHCs stereocilia bundles (Fig. 2). Stereocilia in *RFK* were usually independently freestanding (Fig. 3A), whereas the stereocilia of *MSF* were organized into bundles (Fig. 3B). IHCs stereocilia in the two species were tapered with club-like blunt tips (Fig. 3). In *RFK*, IHCs stereocilia decreased in length from a maximal value of 1.46 μm in the outermost row (Fig. 3A, '③') to 0.56 μm in the innermost row (Fig. 3A, '①'). Similarly, stereocilia lengths in *MSF* gradually decreased in length from a maximal of 1.56 μm in the outermost row (Fig. 3B, '⑤') to 0.28 μm in the innermost row (Fig. 3B, '①'). These results suggest that stereocilia lengths exhibit smaller gradients in *RFK* than in *MSF*. The numbers of IHCs stereocilia were greater in *MSF* than in *RFK* (*RFK*, 59 vs. *MSF*, 90; p<0.05), because IHCs in *RFK* possessed three rows of stereocilia, whereas *MSF* possessed five (Fig. 3). The lengths and numbers of IHCs stereocilia in *RFK* and *MSF* are summarized in Table 1.

Outer Hair Cells Stereocilia in the Two Species

In both species, stereocilia bundles of OHCs were arranged in a slight curve in microscopic view. There were three rows
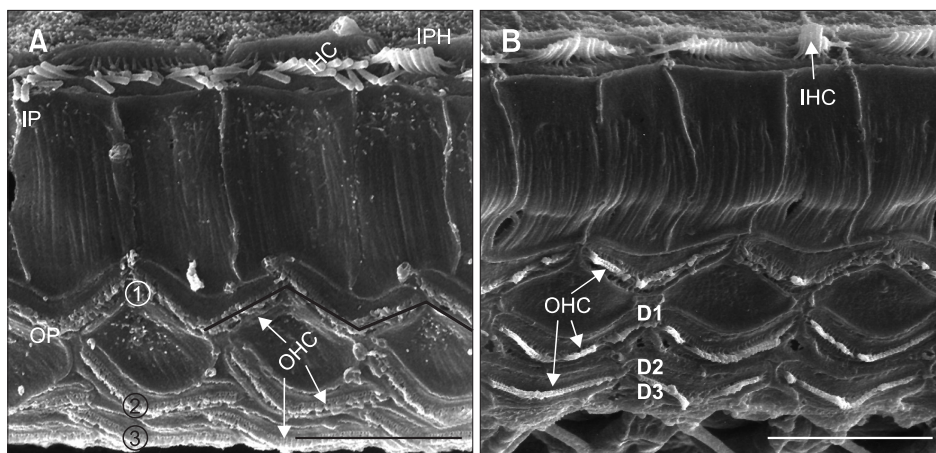

Fig. 2. Scanning electron micrographs of IHCs and OHCs at the apical turns in the cochlea of *RFK* (A) and *MSF* (B). One row of IHCs and three rows of OHCs (①, innermost row; ②, middle row; ③, outermost row) were observed in both species. The distinguishing feature of IHCs stereocilia in *MSF* as compared with *RFK* was the wide spatial separation of stereocilia bundles. *RFK* had more regular, closely spaced freestanding stereocilia. OHCs stereocilia in both species were arranged in a 'W' manner (A). IP, inner pillar cell; IHC, inner hair cell; IPH, inner phalangeal cell; OP, outer pillar cell; OHC, outer hair cell; D1, D2, D3, three rows of deiter cells; *RFK, Rhinolophus ferrumequinum korai; MSF, Miniopterus schreibersii fuliginosus*. Scale bars=10 μm.
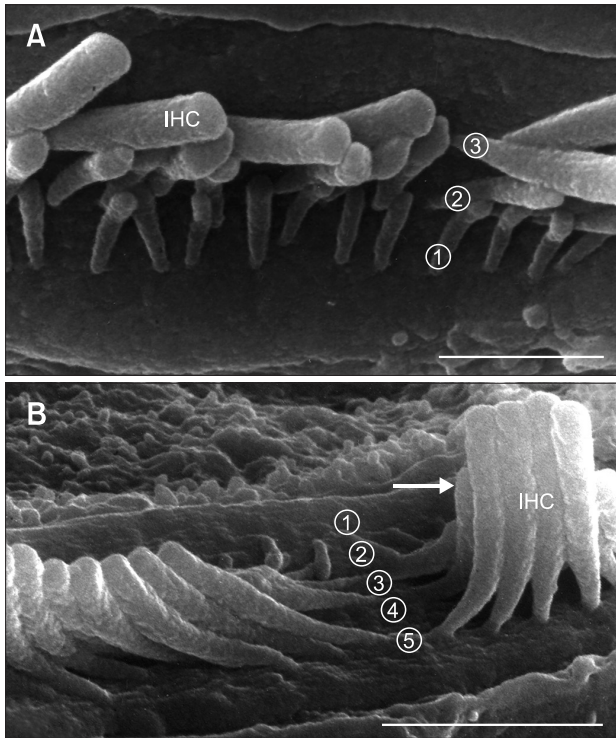
Fig. 3. Scanning electron micrographs of IHCs at the apical turn in the cochlea of *RFK* (A) and *MSF* (B). One row of IHCs possessed three rows of stereocilia in *RFK* (①, innermost row; ②, middle row; ③, outermost row). Note the one row of IHCs with five rows of stereocilia (①, innermost row; ②, inner row; ③, middle row; ④, outer row; ⑤, outermost row) and the fused form of stereociliary bundles (white arrow) (B) in *MSF*. IHC, inner hair cell; *RFK, Rhinolophus ferrumequinum korai; MSF, Miniopterus schreibersii fuliginosus*. Scale bars=1.5 μm.

Table 1. IHCs stereocilia lengths and numbers in the apical turn of the two bat species

Row	Rhinolophus ferrumequinum korai		Miniopterus schreibersii fuliginosus	
	Length (μm)	Number	Length (μm)	Number
IHC1 (innermost)	0.56±0.02	22±0.5	0.28±0.03	19±0.2
IHC2 (inner)	-	-	0.80±0.04	18±0.2
IHC3 (middle)	0.94±0.02	19±0.6	0.93±0.02	19±0.2
IHC4 (outer)	-	-	0.98±0.01	17±0.2
IHC5 (outermost)	1.46±0.03	18±0.3	1.56±0.03	17±0.4
Total	-	59±1.5	-	*90±0.7

Values are presented as mean±standard deviation. Number=3. IHC, inner hair cell. *p<0.05.

of OHCs and each possessed three rows of stereocilia of a different length, resembling steps (Fig. 4). In *RFK* (a CF/FM bat), OHCs stereocilia decreased in length from a maximal value of 0.38 μm in the outermost row (Fig. 4A, '③') to 0.19 μm in the innermost row (Fig. 4A, '①'), whereas OHCs stereocilia in *MSF* (a FM bat) decreased in length from a
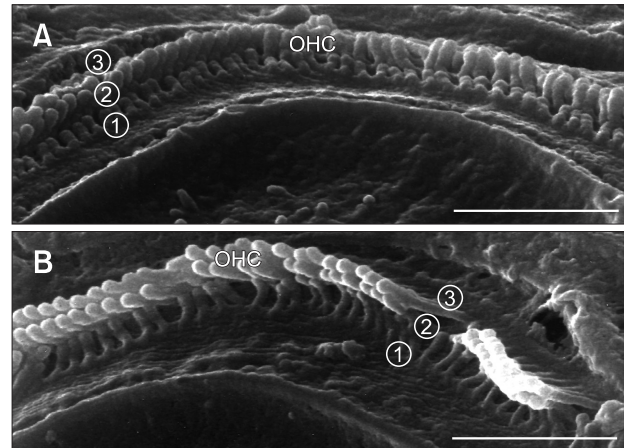

Fig. 4. Scanning electron micrographs of OHCs at the apical turn in the cochlea of *RFK* (A) and *MSF* (B). Each row of OHCs in both species possessed three rows of stereocilia (①, innermost row; ②, middle row; ③, outermost row). OHC, outer hair cell; *RFK, Rhinolophus ferrumequinum korai; MSF, Miniopterus schreibersii fuliginosus*. Scale bars=1.5 μm.

Table 2. OHCs Stereocilia lengths and numbers in the apical turn of the two bat species

Row	Rhinolophus ferrumequinum korai		Miniopterus schreibersii fuliginosus	
	Length (μm)	Number	Length (μm)	Number
OHC1 (innermost)	0.19±0.01	*50±0.5	*0.26±0.01	38±0.7
OHC2 (middle)	0.28±0.01	*58±0.4	*0.47±0.01	43±0.5
OHC3 (outermost)	0.38±0.03	*64±0.3	*0.56±0.02	45±0.3

Values are presented as mean±standard deviation. Number=3. OHC, outer hair cells. *p<0.05.

maximal value of 0.56 μm in the outermost row (Fig. 4B, '③') to 0.26 μm in the innermost row (Fig. 4B, '①'). OHCs stereocilia lengths in *RFK* were shorter than in *MSF* for all three rows (p<0.05). Opening angles and OHCs stereocilia numbers were found to show irregularities. The opening angle of stereocilia bundles in *RFK* increased from the innermost row (124°) to the outermost row (148°) (Fig. 4A, '①~③'), and numbers of stereocilia from the innermost to the outermost row increased from 50 to 64 depending on opening angle (Fig. 4A, '①~③'). In *MSF*, the opening angles of stereocilia bundles were smaller; opening angles and numbers of stereocilia from the innermost row to the outermost row were 117° to 121° and 38 to 45, respectively (Fig. 4B, '①~③'). The number of OHCs stereocilia in *RFK* were greater than in *MSF* for all three rows (p<0.05). These results suggest that the lengths and numbers of OHCs stereocilia in *RFK* are shorter and more numerous than those of *MSF*. The lengths and numbers of OHCs stereocilia of *RFK* and *MSF* are summarized in Table 2.

DISCUSSION

Studies on the audition systems of echolocating bats have demonstrated that they are intricately adapted to the requirements of species-specific acoustic foraging strategies (Neuweiler, 2003). For instance, *R. rouxi*, which pursue flying insects in or around dense vegetation, have evolved a sophisticated cochlear fovea and neuronal specializations that allow the selective detection of wing-beating targets among echo-clutter. In this bat, the morphological organizations of cochlear hair cells are probably related to the tuning capacities and sensitivities of receptor organs (Vater & Lenoir, 1992). The Korean greater horseshoe bat in the Republic of Korea uses CF and FM components during echolocation. In the present study, we analyzed and compared the basic and characteristic stereocilia structures of *RFK* and *MSF* which utilize CF/FM and FM echolocation patterns, respectively. In particular, we examined the fifth half-turn (8.1~8.5 mm from the basal end) of the cochlea which corresponds with distances of between 69% and 66% (*RFK*) and 69% and 71% (*MSF*) from the basal end, respectively. Based on cochlear innervation features, such as, densities of spiral ganglion and of inner and outer receptors, the cochlea of greater horseshoe bats can be subdivided into three regions: (1) the basal region 1.3~5.4 mm (8%~33%) from the basal end where CF segments of echolocation signals are detected (around 83 kHz); (2) the middle region 5.4~8 mm (33%~50%) from the basal end where FM segments of echolocation signals are detected (40~80 kHz); and (3) the apical region from 8 mm to the apex (16 mm) where frequencies lower than 40 kHz are detected (Bruns & Schmieszek, 1980). In another study, *Tadardia brasiliensis* (a FM bat), the most sensitive and sharpest tuning region between 35% and 80% distant from the base was found to detect frequencies between 30 and 12 kHz (Vater & Siefer, 1995). These results suggest that apical turn of *RFK* and *MSF* processes lower frequencies than that of basal turn.

Inner Hair Cells Stereocilia in the Apical Turns of *Rhinolophus ferrumequinum korai* and *Miniopterus schreibersii fuliginosus*

In the present study, IHCs stereocilia length ranges in *RFK* (a CF/FM bat) and *MSF* (a FM bat) were 1.46~0.56 μm and 1.56~0.28 μm, respectively. The small range of IHCs stereocilia length found in the apical turn of *RFK* than in *MSF* is believed to be due to adaption to narrow frequency bands. In contrast, the broad range of stereocilia length in the IHCs of *MSF* may be compensated for an extended hearing range than in *RFK*. IHCs stereocilia in the basal turn of the cat are 2.3 μm long in the 38 kHz region and 7.1 μm long in the apical turn (the 0.175 kHz region) (Frishkopf & DeRosier, 1983). Moreover, IHCs stereocilia length in the basal turn

of the mouse is 3.5 μm in the 20 kHz region (Lim, 1986). Shorter stereocilia lengths of IHCs in the apical turns of *RFK* and *MSF* than in non-echolocating mammals may provide an explanation for their higher hearing frequency ranges. Furthermore, these results indicate that the hearing range of echolocating bats does not reach as far into the low frequency ranges as in the non-echolocating mammals.

In the present study, the numbers of IHCs stereocilia in *MSF* were larger than in *RFK* (*MSF*, 90 vs. *RFK*, 59). It is well known that IHCs are the main sensory cells in the auditory system and their activities are relayed to hearing centers in the brain. On the other hand, OHCs contribute importantly to the sensitivity and frequency resolution provided by cochlear mechanics, but only indirectly to auditory perception (Dallos et al., 2008). These ideas are consistent with known differences between organizations of the two receptor cell populations (IHCs and OHCs) at the ultrastructural (Lim, 1986) and in terms of innervation density (Pujol & Lenoir, 1986), the coupling of stereocilia to the tectorial membrane (Lim, 1986), and motile properties (Brownell et al., 1985; Zenner, 1986; Ashmore, 1987). If transduction channels depend on the number of stereocilia (Osborne et al., 1984; Hudspeth, 1985), then the IHCs of *MSF* have more transduction sites than those of *RFK*, and hence, probably improve the sensitivity echolocation perception. This suggests that the cochleas of *MSF*, a FM bat, are sensitive to a lower range of frequencies than *RFK*, CF/FM bat. The expanded frequency range of *T. brasiliensis* (a FM bat) has a frequency best hearing band at 21~27 kHz (Pollak et al., 1978), which according to Simmons & Stein (1980) corresponds to the lower frequency end of FM echolocation signals.

Outer Hair Cells Stereocilia in the Apical Turns of *Rhinolophus ferrumequinum korai* and *Miniopterus schreibersii fuliginosus*

Supernumerous OHCs and missing OHCs are typical features of the cochlear apex in non-echolocating mammals (Lenoir et al., 1987), but were not observed in the apical cochlea of *RFK* or *MSF* in the present study. The three rows of OHCs stereocilia bundles play an important role in determining cochlear frequency response characteristics (Strelioff et al., 1985). In the present study, stereocilia lengths at the apical turn in both species decreased gradually from the outermost to innermost row. In non-echolocating mammals, such as, the guinea pig and chinchilla, clear gradations were also observed in middle and apical cochleas (Wright, 1984; Lim, 1986). Furthermore, this gradation has also been reported in the middle and apical turns of the echolocating bats, *R. rouxi* and *Hipposidero bicolor* (Vater & Lenoir, 1992). Therefore, gradation of OHCs stereocilia lengths may be a common anatomic feature at the apical and middle turns of echolocating and non-echolocating mammals.

The stereocilia lengths in the innermost to outermost rows in the apical turn of *RFK* (with FM values of 49 kHz) were 0.19 to 0.38 μm, which are shorter than the reported values of *Pteronotus parnellii* another CF/FM echolocating bat (CF value of 61 kHz and stereocilia lengths of 0.7~0.8 μm) (Suga et al., 2003). In the present study, stereocilia lengths of the innermost to outermost rows in *MSF* (with FM values of 80~20 kHz) were 0.26 to 0.56 μm. *T. brasiliensis* employs a broadband system (Vater & Siefer, 1995), and has a frequency range extending from 40 to 75 kHz and a stereocilia length range of 0.8~1.0 μm. These results suggest that the lengths of stereocilia in echolocating bats depend on hearing frequency and species. In particular, longer OHCs stereocilia in the apical turn of the cochlea in *MSF* implies that this feature is used for lower resonance frequencies than in *RFK*. In contrast, the shorter stereocilia in *RFK* probably represent an adaptation to high frequency hearing. Hudspeth & Corey (1977) also demonstrated that shorter OHCs stereocilia in vertebrate hair cells represent a more delicate response to resonant motion when sound waves enter the ear. Therefore, differences between OHCs stereocilia lengths in *RFK* and *MSF* appear to be closely related to specialized hearing frequency ranges and susceptibilities to motions.

In the chinchilla cochlea, a widening of the opening angle of stereocilia bundles is accompanied by an increase in the number of OHCs stereocilia (Lim, 1986). In the cochleas of *RFK*, the opening angles of stereocilia bundles from the innermost to the outermost row and numbers of stereocilia per row were found to be greater than at corresponding positions in *MSF* (*RFK*, 124°~148° and 50~64 vs. *MSF*, 117°~121° and 38~45). Wide opening angles of stereocilia bundles appear to be correlated with the processing of high frequencies (Vater & Lenoir, 1992). In *R. rouxi*, opening angles and numbers of OHCs stereocilia varied from 90° and

40 to 130° and 63, respectively, from the apical to the basal coil. A similar observation was made in the chinchilla, in which the variation ranged from 60° and 22 to 120° and 35, respectively, from the low- to the high-frequency region (Vater & Lenoir, 1992). Thus, we would expect that the apical turn of *RFK* represents a higher frequency range than that of other horseshoe bats and non-echolocating mammals.

CONCLUSIONS

The length gradient and the number of IHCs stereocilia at the apical cochlea of *Rhinolophus ferrumequinum korai* (*RFK*) were found to be smaller than in *Miniopterus schreibersii fuliginosus* (*MSF*). Additionally, the lengths and numbers of OHCs stereocilia in *RFK* were shorter and greater in number than those observed in *MSF*. These features suggest that the apical cochleas of *RFK* (a CF/FM echolocater) are adapted for the processing of higher frequency echolocation calls whereas the apical cochleas of *MSF* (a FM echolocater) more sensitively process low frequency components of echolocation calls.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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