Architecture of the Mouse Utricle: Macular Organization and Hair Bundle Heights

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Li A, Xue J, Peterson EH. Architecture of the mouse utricle: macular organization and hair bundle heights. J Neurophysiol 99: 718–733, 2008. First published November 28, 2007; doi:10.1152/jn.00831.2007. Hair bundles are critical to mechanotransduction by vestibular hair cells, but quantitative data are lacking on vestibular bundles in mice or other mammals. Here we quantify bundle heights and their variation with macular locus and hair cell type in adult mouse utricular macula. We also determined that macular organization differs from previous reports. The utricle has ~3,600 hair cells, half on each side of the line of polarity reversal (LPR). A band of low hair cell density corresponds to a band of calretinin-positive calyces, i.e., the striola. The relation between the LPR and the striola differs from previous reports in two ways. First, the LPR lies lateral to the striola instead of bisecting it. Second, the LPR follows the striolar trajectory anteriorly, but posteriorly it veers from the edge of the striola to reach the posterior margin of the macula. Consequently, more utricular bundles are oriented mediolaterally than previously supposed. Three hair cell classes are distinguished in calretinin-stained material: type II hair cells, type Ia hair cells contacting calretinin-negative (dimorphic) afferents, and type Ic hair cells contacting calretinin-positive (calyceal) afferents. They differ significantly on most bundle measures. Type II bundles have short stereocilia. Type Ic bundles have kinocilia and stereocilia of similar heights, i.e., KS ratios (ratio of kinocilium to stereocilia heights) ~1, unlike other receptor classes. In contrast to these class-specific differences, bundles show little regional variation except that KS ratios are lowest in the striola. These low KS ratios suggest that bundle stiffness is greater in the striola than in the extrastriola.

Introduction

Mice are important model systems for studies of vestibular mechanisms. A large and rapidly growing literature is characterizing the developmental (e.g., Davies et al. 2007; Denman-Johnson and Forge 1999; Fritsch et al. 2001; Géleoc et al. 2004; Goodyear et al. 2005; Kirkegaard and Nyengaard 2005; Lim and Anniko 1985; Mbiene et al. 1984, 1988; Sage et al. 2000; review: Quint and Steel 2003) and molecular (e.g., Bermingham et al. 1999; Deans et al. 2007; Delprat et al. 2005; Dumont et al. 2002; Gagnon et al. 2006; Lagziel et al. 2005; Phillips et al. 2006; Senften et al. 2006) biology of the murine vestibular labyrinth, its structure and immunochemistry (e.g., Cunningham et al. 2002; Desai et al. 2005; Fritsch et al. 2001; Morsli et al. 1998), hair cell (Géleoc et al. 1997; Holt et al. 1997; Rüscher and Eatock 1996; Rüscher et al. 1998), and afferent (Risner and Holt 2006) physiology, and central vestibular processing (Bagnall et al. 2007; Beraneck and Cullen 2007; Camp et al. 2006; Sekirnjak and DuLac 2002). It is therefore surprising that relatively little is known about the structure of vestibular hair bundles in mice, or in other mammals, in spite of the importance of hair bundles in signaling head movements and orientation in gravity space. Since the early work of Lim and colleagues (Lim 1971, 1973, 1976, 1979), only a few studies have described hair bundle structure in mice (Denman-Johnson and Forge 1999; Mbiene et al. 1984, 1988; Rzadzinska et al. 2004), and there have been no systematic attempts to quantify variation in bundle structure as a function of macular location and hair cell type. This information is necessary if we are to understand mechanisms of signal processing by the mouse vestibular periphery because hair bundle structure helps shape the mechanical response of hair cells to head movements and thus the signal that vestibular afferents send to the CNS.

To further our understanding of peripheral vestibular mechanisms in mice, we measured the heights of hair bundles across the utricular macula of adult (P30) mice using techniques designed to reflect in vivo dimensions as closely as possible. We focused on bundle heights because they have important consequences for hair cell signaling, including operating range, sensitivity, stiffness, and frequency selectivity (see Discussion, Hair bundle heights). To develop an appropriate sampling strategy for our studies of hair bundle heights, we first needed to characterize the gross architecture of the murine macula, including the location and trajectory of the striola, the line of polarity reversal (LPR), and the relation between them. The LPR is defined as the line at which hair bundles reverse orientation. In the utricle, the kinocilial (tall) ends of hair bundles face each other across this LPR. Thus they have opposite orientations. Bundle orientation, in turn, determines the direction of head movement that will excite utricular hair cells. In the course of examining the LPR and its relation to the striola, we made two findings that differ from previous reports on murine macular organization.

Next we asked whether hair cells differ significantly in hair bundle structure. Hair cells are classically divided into two types, type I and type II, based on the morphology of the afferent terminals they contact (Wersäll 1956). Afferents are divided into three groups based on their terminal structure: bouton, pure calyceal, and dimorphic (terminating in both calyceal and bouton endings) (review: Lysakowski and Goldberg 2004). Type II hair cells are found in all vertebrate vestibular epithelia; they contact bouton endings on bouton and dimorphic afferents. Type I hair cells are found in vestibular epithelia of amniotes (reptiles, birds, mammals); they contact cup-like calyceal endings on pure-calyceal and dimorphic af-

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The calcium-binding protein calretinin distinguishes these two classes of calyceal endings in mammals: calyces on pure-calyceal afferents are calretinin-positive, whereas calyces on dimorphic afferents are calretinin-negative (Desmadryl and Dechesne 1992; Desai et al. 2005). Accordingly, we labeled utricles with an antibody against calretinin and assigned hair cells to one of three classes: type II hair cells, type IC hair cells contacted by calretinin-positive calyces (i.e., pure-calyceal afferents), and type ID hair cells contacted by calretinin-negative calyces (i.e., dimorphic afferents). Then we asked whether the three hair cell classes differ significantly in hair bundle structure.

In addition to characterizing these class-specific differences, we also wished to quantify differences in hair bundle morphology as a function of macular locus. In particular, we wished to determine whether bundles in the striola differed from those in the extrastriola. The striola is a crescent-shaped band of specialized receptors and afferents near the LPR. It is a nearly universal feature of otoconial organs. Striolar afferents are physiologically distinctive; they are phasic (Baird and Lewis 1986) and phase advanced (Goldberg et al. 1990) relative to extrastriolar afferents. Striolar hair bundles are reported to differ from those in the extrastriola (reviews: Eatock and Lysakowski 2006; Lewis et al. 1985; Platt 1983) and understanding these structural differences may help clarify the role of striolar hair cells in detecting and encoding head movements and their contribution to the distinctive physiology of striolar afferents.

### Table 1. Number of hair cells in mouse utricle

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<tr>
<th>Total Hair Cells</th>
<th>Type I Hair Cells</th>
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<td>Medial, %</td>
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<tr>
<td>n</td>
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<tr>
<td>Median and range</td>
<td>1828–2025</td>
</tr>
<tr>
<td>Desai et al. (2005)</td>
<td>(average ± SE, n = 3)</td>
</tr>
<tr>
<td>Kirkegaard and Nyengaard (2005) (n = 2)</td>
<td>3615</td>
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</tbody>
</table>

Values in parentheses are percentages. Total utricular hair cells medial and lateral to the line of polarity reversal (determined by counting hair bundles) and total type I hair cells (determined by counting stained calyces in whole-mounts).

### Table 2. Striolar hair cells (n = 4)

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<th>Type IC</th>
<th>Type ID</th>
<th>Type II</th>
<th>Total</th>
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<tbody>
<tr>
<td>Medial to reversal line</td>
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<tr>
<td>Median</td>
<td>139</td>
<td>56</td>
<td>182.5</td>
<td>366.5</td>
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<tr>
<td>Percent total cells</td>
<td>37.9</td>
<td>15.3</td>
<td>49.8</td>
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<tr>
<td>Range</td>
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<tr>
<td>Lateral to reversal line</td>
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<tr>
<td>Median</td>
<td>6.5</td>
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<tr>
<td>Range</td>
<td>0–9</td>
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</table>

Proportions of three hair cell classes in the striola. Type I hair cells are classified according to the calretinin-immunoreactivity of their postsynaptic calyces. Medians are calculated separately for three hair cell classes and for total hair cells. Percentages are class median/total median (e.g., 139/366.5 = 37.9%).

### Table 3. Calyx complexity in the striola

<table>
<thead>
<tr>
<th>Calyx complexity</th>
<th>Calyceal Terminals by Complexity</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Calretinin-positive calyces</td>
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</tr>
<tr>
<td>Medial to reversal line</td>
<td>43</td>
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<tr>
<td>Lateral to reversal line</td>
<td>27–54</td>
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<tr>
<td>Total</td>
<td>45</td>
</tr>
<tr>
<td>Desai et al. (2005) (average ± SE, n = 3)</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>Calretinin-negative calyces</td>
<td>54</td>
</tr>
<tr>
<td>Total</td>
<td>33–81</td>
</tr>
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</table>

First number is median, second is range. Calyceal terminal clusters subdivided according to the number of calyces (cups) in the cluster. Medians for each cell calculated separately. More than one terminal cluster may arise from the same afferent. Numbers for Desai et al. 2005 refer to total calretinin-positive calyces per calyceal (calretinin-positive) afferent.

Desai et al. (2005) have argued that the presence of calretinin-positive calyces can be used to identify the striola in mammals because of its correlation with other distinguishing features of the striola including low receptor density, the presence of complex calyces, and the relation of the striola to the LPR. In this paper, we follow Desai et al. in equating the rodent striola with the band of calretinin-positive calyces. We first describe murine macular organization, including our two novel findings. Then we present data on hair bundle structure as a function of hair cell type and macular locus.

### Methods

We conducted all experiments on adult (P30) ICR mice of both genders (CD-1 out bred strain; Charles River Laboratories). We killed mice via intraperitoneal injection of Euthasol (390 mg pentobarbital sodium and 50 mg phenytoin sodium/ml; 0.1 ml) and transcardiac perfusion [brief rinse with 0.1 M phosphate buffer (PB), then 4% sodium and 50 mg phenytoin sodium/ml; 0.1 ml] and transcardiac perfusion [brief rinse with 0.1 M phosphate buffer (PB), then 4% paraformaldehyde in PB, both at pH 7.4]. We followed Ohio University Animal Care and Use Committee guidelines in all experiments. Fifteen mice (18 utricles) provided useful data.

### Table 4. Hair bundle dimensions (n = 328)

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<tr>
<th></th>
<th>Type IC</th>
<th>Type ID</th>
<th>type II</th>
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<tbody>
<tr>
<td>Kinocilium height, µm</td>
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<tr>
<td>Median</td>
<td>11.8</td>
<td>18.6</td>
<td>14.5</td>
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<tr>
<td>Range</td>
<td>9.6–16.0</td>
<td>8.4–26.6</td>
<td>6.8–22.6</td>
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<tr>
<td>Tall stereocilia height, µm</td>
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<tr>
<td>Median</td>
<td>12.5</td>
<td>12.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Range</td>
<td>9.4–17.5</td>
<td>7.4–20.7</td>
<td>2.7–15.4</td>
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<tr>
<td>KS ratio</td>
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<tr>
<td>Median</td>
<td>0.96</td>
<td>1.4</td>
<td>2.3</td>
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<td>0.8–1.5</td>
<td>1.0–2.3</td>
<td>1.1–4.5</td>
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<tr>
<td>Short stereocilia height, µm</td>
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<tr>
<td>Median</td>
<td>1.7</td>
<td>2.3</td>
<td>1.9</td>
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<tr>
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<td>Array length, µm</td>
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<td>2.6</td>
<td>2.2</td>
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<td>Range</td>
<td>1.6–3.8</td>
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<tr>
<td>Bundle slope</td>
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<tr>
<td>Median</td>
<td>4.2</td>
<td>4.7</td>
<td>2.8</td>
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<tr>
<td>Range</td>
<td>2.5–6.4</td>
<td>2.0–6.9</td>
<td>0.8–7.7</td>
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Dimensions of hair bundles on three classes of utricular hair cells; numbers are for all macular loci combined.
We removed the brain from the hemisected, undecalcified skull and the bone/cartilage medial and superior to the vestibule and then excised the utricles. Next we opened the membranous labyrinth overlying the macula and removed the otoconial membrane using a gentle stream of bathing medium (for wholemounts) or left the otoconial membrane intact (for utricular slices). Utricles to be visualized as slices (for measuring bundle heights) were embedded in 4% low melting point agarose (Bio-Rad, No. 161-3112) and sectioned at 60 μm using a Leica VT 100S vibrating blade microtome. Wholemounts or slices were immersed in a blocking solution at room temperature for 1 h (for slices; 24 h for wholemounts). The blocking solution consisted of 0.1 M phosphate-buffered saline with 5% fetal

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Differences in bundle dimensions for three classes of utricular hair cell as a function of macular locus. LES, lateral extrastriola; MES, medial extrastriola.

FIG. 1. Mouse utricular wholemount. This confocal micrograph shows spatial relations between a mouse utricle and cristae of the anterior and horizontal semicircular canals. The material is stained with α- and β-III tubulin (red), calretinin (green), and phalloidin (yellow). Afferents supplying the cristae (red) run under the macula and emerge from its lateral margin to reach the cristae. Calretinin-positive calyces form a band, the striola, near the center of the macula; they are also visible on the apices of the cristae. Calretinin and β-tubulin co-localize in striolar calyces; the calyces appear green at this magnification rather than yellow because the calretinin signal is stronger. Co-localization can be seen more clearly in higher magnification images (e.g., Fig. 4C). Stained hair bundles (yellow) outline the 3 neuroepithelia.
calf serum (FCS), 1% bovine serum albumin (BSA), and 2% Triton-X (for wholemounts) or 1% DMSO (for slices used to measure bundles because Triton-X can compromise details of bundle staining). Next they were labeled with a primary antibody “cocktail” (72 h at 4°C) consisting of blocking solution with one or more of the following. 1) We used a monoclonal mouse antibody against β-III tubulin (TUJ1; Covance MMS-435P, diluted 1:1,000) to visualize afferents because vestibular afferents strongly express this β-tubulin isotype (Perry et al. 2003). 2) We used a monoclonal mouse antibody against acetylated α-tubulin to label kinocilia (Sigma T6793, diluted 1:1,000) because acetylated tubulin is a marker for kinocilia in rodents (Ogata and Slepecky 1995). This method of labeling kinocilia yields kinocilia heights in fixed tissue that are within 5% of measurements in living utricular hair cells (Fontilla and Peterson 2000; Xue and Peterson 2006). 3) Finally, we used a polyclonal rabbit antibody against the calcium-binding protein calretinin (Chemicon AB149 or AB5054, diluted 1:1,000) to label pure-calyceal afferents (Desmadryl and Dechesne 1992; Desai et al. 2005).

We used AB149 in early experiments and AB5054 when the manufacturer discontinued AB149. Both antibodies produced the same pattern of labeling: a majority of type II somata and a 70- to 75-µm wide band of calretinin-positive calyces corresponding to the striola. AB149 labels a single 29-kD band in extracts from mouse labyrinth, suggesting that it does not cross-react with the closely related calcium-binding protein, calbindin d-28k (Desai et al. 2005). As an additional control, we double labeled two utricles (2 different mice) with AB5054 and a mouse monoclonal (Swant; clone 6B3) against recombinant human calretinin-22k, which does not cross-react with calbindin d-28k or other calcium-binding proteins (determined by immunoblots and its brain distribution; information from manufacturer). In these two utricles, all calretinin-positive calyces were labeled by both antibodies (data not shown); this provides further evidence that the calyceal label we observed was specific to calretinin.

After rinsing tissues in blocking solution (3 changes, 1 h each), we incubated tissues in a secondary antibody cocktail consisting of Alexa Fluor 633 goat anti-mouse IgG (H+L) and/or Alexa Fluor 488 goat anti-rabbit IgG (H+L), depending on the primary antibodies used, both diluted 1:200 in blocking solution, for 1 h at room temperature (for slices; ≤24 h for wholemounts), rinsed them in PBS (3 changes, 1 h each), and then stained stereocilia with phalloidin conjugated to Alexa-Fluor 546 (Invitrogen A-22283, diluted at 5 U/ml) for 30 min at room temperature. Phalloidin labels the filamentous actin that forms the core of stereocilia. Finally, we rinsed tissues in PBS (3 changes, 1 h each). To minimize shrinkage due to tissue processing, we did not dehydrate tissues after staining. Instead we mounted wholemounts and slices on glass slides in an aqueous medium (Slow Fade; Invitrogen), with or without (for immersible objectives) a cover slip, and scanned them using a Zeiss LSM 510 confocal microscope.

**FIG. 2.** Hair bundle distribution. This figure shows 1 utricle used to count hair cells in the murine macula (see Table 1). Each symbol represents a hair bundle (different utricle from those in Figs. 1, 3–5). The area of this macula is 0.172 mm². A: a band of low hair cell density is marked by red arrowheads. This low-density band corresponds to the band of calretinin-positive calyces (see Fig. 1). B: same plot as in A except that hair cells lateral (dark blue) and medial (light blue) to the line of polarity reversal are distinguished by different colors. The line of polarity reversal is shaped like a prone question mark with its stem oriented posteriorly (gray double arrowhead). Note that the line of polarity reversal lies at the lateral margin of the low-density area, i.e., the striola.
FIG. 3. Line of polarity reversal. This is a montage created from optical sections located as close as possible to the bases of utricular hair bundles. Stereocilia are labeled with phalloidin (yellow) and kinocilia with α-tubulin (red). 

A: low magnification view. A red line marks the line of polarity reversal. The dotted box delimits the area shown at higher magnification in B. The area of this macula is 0.209 mm².

B: high-magnification image showing that, posteriorly, the reversal line departs from its U-shaped trajectory to reach the posterior margin of the macula. On each side of the reversal line, bundles are rather loosely aligned. On some hair bundles, kinocilia were distorted by removal of the otoconial membrane. In such cases, we verified kinocilium position from the confocal stacks.

C: hair bundle orientation for the same macula shown in Figs. 3A and 5. Each bundle is represented by an arrowhead; arrowhead orientation indicates the orientation of the hair bundle. Arrowheads were drawn by eye from the center of the bundle to the kinocilium, using a magnified image of the montage on a computer monitor. Inset: magnified image of one bundle from this montage showing how we used an arrowhead to indicate bundle orientation. Gray line: line of polarity reversal. Dotted box: area shown by high magnification confocal micrograph in Fig. 3B.
We classified hair cells as follows. Hair cell somata surrounded by a calyx were classified as type I (Wersäll 1956). We identified calyces and distinguished them from other afferent terminals and from supporting cells by following them through confocal stacks to establish their smooth, flask-like shape. Theoretically there could be some ambiguity in distinguishing a striolar calretinin-positive type I hair cell soma contacted by a calretinin-negative calyx from a calretinin-positive type II soma. But these profiles differ in size and three-dimensional shape, and any ambiguity is not likely to be a serious concern because only 5% of type I somata are calretinin-positive in the striola (Desai et al. 2005).

We adopted the following terminology to distinguish between the two classes of type I hair cell. Numerous reports suggest that, at least in mammals, calretinin is selective for pure-calyceal afferents (reviewed in Desai et al. 2005). Therefore we refer to type I hair cells contacted by calretinin-positive calyces as type IC (for calyx) and type I hair cells contacted by calretinin-negative calyces as type IN (for dimorph). This terminology has the advantage of simplicity, but it is important to realize that there are exceptions (Desai et al. 2005 report one calretinin-positive dimorph) and that this terminology is inappropriate for some nonmammals (some bouton endings in turtle posterior canal are calretinin-positive; Monk and Peterson 1995; Lysakowski, Holt, and Goldberg, unpublished observations, reported in Desai et al. 2005).

**Organization of the macula**

We used four utricular wholemounts (4 different mice) to count hair bundles. We stained one utricle with phalloidin (to visualize stereocilia), created a montage of the utricle by scanning it at ×40 magnification, and counted the hair bundles. We stained the remaining three utricles with phalloidin and α-tubulin (to visualize kinocilia) and created montages at ×40 (2 utricles) or ×63 (1 utricle) magnification. We used these three cases to identify the line of polarity reversal (LPR) and to count the number of hair cells medial and lateral to the LPR (Table 1).

For one of the double-labeled utricles, we also approximated the orientation of all hair bundles. To do this, we created a Photoshop montage of the epithelial surface using optical sections as close as possible to the bases of utricular bundles. Then we viewed a magnified image of the montage on a computer monitor and drew an arrowhead, by eye, from the center of each bundle to its kinocilium (Fig. 3C, inset). Because removal of the otoconial membrane can result in bent kinocilia, occasional bundle profiles were difficult to interpret unequivocally from the on-screen montage. In such cases, we checked bundle orientation in the original confocal stacks.

We used three utricular whole mounts (2 different mice) to determine the number of type I hair cells. We stained these utricles with β-III tubulin and phalloidin with or without calretinin. We counted the number of calyces to determine the number of type I hair cells (Table 1).

We used four additional utricles (4 different mice) stained with phalloidin, α-tubulin, β-tubulin, and calretinin to delimit the striola and determine its relation to the region of low hair cell density and the LPR (1 of these was also used for the hair cell counts summarized in Table 1). We determined the relation of calyces to the LPR by following each type I hair cell up through the confocal stack to its hair bundle. The stacks were created at ×40 magnification. We also used these utricles to quantify the number of hair cells of each class in the

![Image](http://jn.physiology.org/)

**FIG. 4.** The band of calretinin-positive calyces corresponds to the region of low hair cell density. Two different channels from the same confocal projection are shown separately (A and B) and superimposed (C). The line of polarity reversal (solid line) was identified by following hair cells up to their bundles. Different utricle than those in Figs. 1–3 and 5. A: calretinin staining. The band of calretinin-positive calyces is visible just medial to the reversal line. Only 3 calyces (arrowheads) are lateral to the reversal line. Green profiles above and below the band of calyces are hair cell somata; most of these are type II (Desai et al. 2005). Three examples are marked by asterisks. B: β-III tubulin staining. All calyces are labeled. A low-density band runs from left to right near the mid-point of the image. In adjacent lateral (top) and medial (bottom) extrastriolae, numerous calretinin-negative calyces (from presumed dimorphic afferents) are labeled. C: calretinin and tubulin staining. The channels shown in A and B are superimposed. Co-localization of the 2 labels (yellow) occurs in the striola. Calretinin-positive somata (solid green profiles) appear between calyces. They are larger on the right than on the left because the 2 regions of this image are at slightly different depths.
striola (Table 2). For these experiments, we identified type I hair cells by their postsynaptic calyces, and we estimated the number of type II hair cells by counting the number of hair bundles in the striola and subtracting the total number of calyces from the total number of bundles. We delimited the striola as follows. Examination of calyx position relative to the LPR revealed that very few calretinin-positive calyces are lateral to the LPR (see Results). Therefore we identified the striola as the band of type IC hair cells medial to the LPR with any intercalated type ID and type II hair cells, plus the few type IC hair cells lateral to the LPR. We did not include any type II or type ID hair cells lateral to the LPR in our striolar counts because there were so few lateral type IC hair cells that it was impossible to define a striolar border lateral to the LPR.

Finally, we used these four utricles to count the number of calyces per terminal cluster for calretinin-positive and -negative calyceal terminals in the striola (Table 3). An afferent that branches will give rise to more than one terminal cluster, but we were not always able to trace clusters back to their parent axon with confidence. So numbers in Table 3 refer to the number of calyces per terminal cluster not per afferent. Fernandez et al. (1990) report that in chinchilla utricle, such branching occurs in fewer than 10% (3/31) of calyceal afferents.

Hair bundle heights

We used two adjacent, 60 μm-thick utricular slices from eight utricles (6 different mice) to measure bundle heights; the arrowheads in Fig. 6A (inset) show slice orientation. These two slices sample ~26% of total striola length and over 22% of total anterior-posterior macular length (Desai et al. 2005). We stained the slices with phalloidin, α-tubulin, β-tubulin, and calretinin, mounted them on glass slides, and scanned them using an alpha plan-fluar ×100 oil-immersion objective (NA = 1.45) to create montages of complete medial-to-lateral transects through each utricle. We imported the resulting stacks into a computerized morphometry program (Neurolucida, Microbrightfield) and used this program to measure bundle heights as described previously (Xue and Peterson 2006). Bundles were considered measurable if they met the following criteria: they could be assigned unambiguously to hair cell class (type II, type IC, type ID) and they were sufficiently intact that we could trace kinocilia and stereocilia with confidence. Accordingly, we omitted bundles that were badly distorted or those deep in the slice where staining is less bright than at the surface. Using these criteria, we were able to classify and measure 25–30% of all bundles in each slice with confidence.

We measured kinocilia and stereocilia heights by following them in three dimensions to minimize underestimates of heights due to foreshortening, and we quantified heights of the kinocilium, the tallest and shortest stereocilia, and array length (3-dimensional distance from the tallest to the shortest stereocilia, measured parallel to the hair cell’s apical surface). We used these measurements to calculate a KS ratio (height of kinocilium/height of tallest stereocilia) and bundle slope ([height of tallest stereocilia - height of shortest stereocilia]/array length). The schematic hair bundles in Fig. 7 show measured/calculated variables in red.

Exploratory and inferential statistical analyses were implemented in Statistica (ver. 6.1; StatSoft) or S+ (ver. 6.2; Insightful). We used robust statistics for our analyses (Wilcox 2005) because
some variable distributions were markedly nonnormal (see DISCUSSION in Xue and Peterson 2006). We implemented all robust functions in S+. To compare bundle classes or bundles at different locations on the macula we used t1way (robust analog of 1-way ANOVA) and lincon (for multiple comparisons; Wilcox 2005; chapt. 7). To summarize spatial trends across the macula, we used Loess fits, a smoothing method that captures patterns in bivariate data that cannot be fit with simple linear or quadratic equations (Cleveland 1993). We used a local quadratic fit, a Gaussian weighting function, and a span of 0.3–0.5 (see DISCUSSION in Xue and Peterson 2006). We implemented Loess fits in S+. Quantitative data for 328 hair bundles are illustrated in Figs. 7–9 and summarized in Tables 4 and 5.

RESULTS

Organization of the macula

The macula is kidney-shaped, with its long axis oriented approximately antero-posteriorly (Fig. 1). In maculae viewed as unflattened wholemounts, median macular area is 0.185 mm² (range: 0.152–0.209 mm²; n = 4). This is probably an underestimate due to foreshortening of the curved macular perimeter. The median number of utricular hair cells is 3,613; they are almost equally divided between medial and lateral sides of the reversal line (Table 1). The median number of type I hair cells, estimated by counting calyces labeled with β-III tubulin in three utricles, is 2,520. Table 1 compares these counts of total hair cells and total type I hair cells with results from two previous reports on mouse utricle.

Figure 2 illustrates two features of macular organization. First, there is a central region of low hair cell density (Fig. 2A, red arrowheads), and this low-density region is medial to the LPR (Fig. 2B; the position of the LPR is indicated by symbol color; hair cells medial and lateral to the LPR are represented by light blue and dark blue symbols, respectively). Second, the LPR follows the trajectory of the low-density area in the anterior macula, continuing to the medial macular perimeter as expected from previous reports (Desai et al. 2005). But posteriorly it departs from this trajectory and heads toward the posterior (rather than medial) margin of the macula (Fig. 2B, gray double arrowhead). Thus the LPR resembles a prone question mark with its stem directed posteriorly.

The trajectory of the LPR is shown for a different utricle in Fig. 3A. Details of the posterior LPR are shown in Fig. 3B, which is a high-magnification view showing hair bundles of type I and type II hair cells. Medial extrastriola. Some type II bundles are marked by arrows. Their somata are often calretinin-positive, and their stereocilia are much shorter than those on type I bundles. Thus their KS ratios (height of kinocilium/height of tallest stereocilia) are greater.

The region of low hair cell density just medial to the LPR (Fig. 2B; the position of the LPR is indicated by symbol color; hair cells medial and lateral to the LPR are represented by light blue and dark blue symbols, respectively). Second, the LPR follows the trajectory of the low-density area in the anterior macula, continuing to the medial macular perimeter as expected from previous reports (Desai et al. 2005). But posteriorly it departs from this trajectory and heads toward the posterior (rather than medial) margin of the macula (Fig. 2B, gray double arrowhead). Thus the LPR resembles a prone question mark with its stem directed posteriorly.

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et al. 2005). This is illustrated in Fig. 4 (different utricle from those in Figs. 1–3), which shows two channels from the same confocal stack individually (A and B) and superimposed (C). One channel (Fig. 4A) shows calretinin staining of pure-calyceal afferents. Calretinin also stains some hair cell somata; examples are marked by asterisks. The second channel (Fig. 4B) shows staining with $\beta$-III tubulin, which stains all afferents. Figure 4C shows the relation between calretinin and $\beta$-III tubulin immunoreactivity. A band of double-labeled (yellow) calyces runs across the middle of the image and, throughout the image, calretinin-positive hair cell somata are seen between circular, calyceal profiles.

The LPR in Fig. 4 was identified by following hair cell somata up to their apical hair bundles. Note that the region of low hair cell density (clearest in Fig. 4B) corresponds to the band of calretinin-positive calyces (Fig. 4, A and C) and that, with three exceptions (Fig. 4A, arrowheads), calretinin-positive calyces are medial to the LPR. This is summarized in Fig. 5 (same utricle as in Fig. 3), which shows the complete band of calretinin-positive calyces (arrowheads) and its relation to the LPR and to the macula as a whole. In this utricle, we found only four calretinin-positive calyces lateral to the LPR. Thus the striola, defined as the band of calretinin-positive calyces, lies almost entirely medial to the LPR.

Table 2 shows the median number of type II, type I$_C$, and type I$_D$ hair cells in the striola of four utricles. In these maculae, the number of type I$_C$ hair cells lateral to the LPR ranged from 0 to 9 (Table 2). As reported by Desai et al. (2005), calretinin-positive calyceal afferents form terminal clusters that are either simple (1 calyx) or complex (2–3 calyces). Calretinin-negative calyces in the striola and calretinin-positive calyces lateral to the LPR were almost entirely simple calyces (Table 3).

**Hair bundle heights**

We used utricular slices to measure hair bundle heights (Fig. 6), and we subdivided these hair cells into three classes: type II hair cells, type I$_C$, and type I$_D$. The three hair cell classes differ in bundle structure. Each occupies a distinct niche in our variable space. This is illustrated in Fig. 7, which is a matrix of two-dimensional scatter plots that show the relations between five major structural variables. The tallest stereocilia on type II bundles (green symbols) are shorter than in both classes of type I hair cells (blue symbols); thus they have higher KS ratios and shallower bundle slopes. They also have shorter array lengths than any type I hair cell. Thus in lateral view, the stereocilia of type II hair cells form a much smaller bundle than those of type I hair cells (Fig. 6B, arrows).

The first row of the matrix plot shows that bundles of type I$_C$ hair cells (Fig. 7; dark blue symbols) have shorter kinocilia than those of type I$_D$ hair cells (light blue symbols); as a result their KS ratios are shorter. In addition, their array lengths are longer than those of all other utricular bundles. Differences between bundles of the three hair cell classes are quantified in box plots (Fig. 8) and in Table 4. All differences are statistically significant except for the following: tall stereocilia on the two subgroups of type I hair bundles do not differ nor do short
sawstereocilia on type IC and those on type II bundles. Significance levels were $P < 0.0001$ for all comparisons but one (bundle slope on type $I_C$ vs. type $I_D$ hair cells; $P < 0.01$).

In contrast to the marked differences between bundles of different hair cell classes, type $I_D$ and type II hair cells (the only classes to span the striola and the extrastriola) show little regional variation in bundle structure (Fig. 9). For most variables, data points for type $I_D$ bundles and type II bundles form relatively flat distributions: they vary little from LES, to striola, to MES. But values for the two hair cell classes generally occupy different values on the ordinate. The major exceptions are kinocilium height (significantly shorter in the striola) for both hair cell classes (Fig. 9, $A$ and $B$) and, thus KS ratio (lower in striola; Fig. 9, $E$ and $F$). There are no regional differences in the height of the shortest stereocilia (Fig. 9, $G$ and $H$) or in bundle slope (Fig. 9, $K$ and $L$). Regional differences in the remaining two variables, height of tallest stereocilia (Fig. 9, $C$ and $D$) and array length (Fig. 9, $I$ and $J$) are small and often occur in only one of the two hair bundle classes. These differences between bundles in different macular zones are summarized in Table 5. The regional differences in type $I_D$ and type II hair cells indicated by nonoverlapping confidence intervals of the median in box plots (Fig. 9) and Table 5 were confirmed by statistical analysis ($P$ values from 0.012 to $<0.0001$). Figure 10 summarizes class- and region-specific differences in bundle heights.

**DISCUSSION**

This study is part of a long-term effort to understand how hair cell properties contribute to signal processing in the mouse utricle (Rüsch and Eatock 1996; Rüsch et al. 1998; Vollrath and Eatock 2003). One goal was to create a detailed picture of murine macular organization that can serve as a framework for characterizing regional differences in the properties of hair cells and afferents. A second goal was to quantify hair bundle structure in mouse utricle. We focused on bundle heights because models and experiments suggest that the heights of kinocilia and stereocilia are important determinants of hair bundle mechanics and, thus of hair cell signaling (see Hair bundle heights). In addition, we compare the present data on bundle heights with previously published results from turtle (Xue and Peterson 2006) and bullfrog (Baird 1994a) because common features may shed light on general organizational principles of vertebrate utricles. Our most important results are first, that the LPR and the relation of the LPR to the striola differ from published reports and, second, that there are significant differences in the structure of hair bundles on hair cells of different type and macular loci that are, in many respects, similar to the differences observed in other vertebrate utricles.

**Organization of the macula**

Several reports of macular areas are available (Denman-Johnson and Forge 1999; Desai et al. 2005; Fritzsch et al. 2001;...
Kirkegaard and Nyengaard 2005; Mbiene et al. 1984), but differences in methods and developmental stage make comparisons problematic. For adult mice, our results are in general agreement with previous descriptions of macular area and the total number of utricular hair cells (Desai et al. 2005; Kirkegaard and Nyengaard 2005; see Table 1). Our estimates of type I hair cell numbers, which are based on counts of stained calyces in wholemounts, are similar to those of Kirkegaard and Nyengaard (2005) and higher than those of Desai et al. (2005; see Table 1). The reason for this difference is unclear; one possibility is difference in mouse strains. A second possibility is differences in method (we counted calyces in wholemounts, whereas the previous studies used stereological methods); but this is unlikely because counts of type I hair cells in the two previous studies differed, but both used the same stereological (dissector) method.

The murine macula exhibits two types of irregularities in bundle alignment. First the alignment of neighboring hair cells is somewhat irregular (Fig. 3, B and C). This has also been reported in several qualitative studies (review: Lewis et al. 1985) and at least one quantitative analysis (turtle utricle: Platt 1977; turtle: Rowe and Peterson 2006; pigeon: Si et al. 2003; Zakir et al. 2001). Our results on the trajectory of the reversal line differ from those of previous reports. By distinguishing kinocilia and stereocilia, we were able to map the reversal line in six utricular wholemounts. The result differs from Lindeman’s iconic utricle, which was based on data from guinea pigs (Lindeman 1969). Our results also differ from a recent report on the striola of mice and other rodents (Desai et al. 2005). These authors made the reasonable assumption that the LPR continues the trajectory of the striola to end at the medial margin of the macula. This proved to be incorrect. The LPR follows the trajectory of the striola anteriorly, but it departs from this trajectory posteriorly, running toward the posterior margin of the macula. Thus instead of forming an inverted “U”, the reversal line resembles a prone question mark, with its stem directed posteriorly (Figs. 2B and 3). To our knowledge, this is the first such mapping of the reversal line in mammals.

The trajectory of the LPR has consequences for hair bundle orientation. Hair bundle orientation is roughly perpendicular to the LPR. Because of the LPR trajectory, many hair bundles in posterior murine macula (especially those in postero-medial macula) have a significant mediolateral component to their
orientation. This is similar to the orientation of bundles in central macula (Fig. 3C). Thus more utricular hair cells are mediolaterally oriented than would be expected if the LPR were shaped like an inverted “U” (Desai et al. 2005).

Like the reversal line, the mouse utricular striola does not form an inverted “U”, with anterior and posterior limbs directed medially. As first noted by Lorente de No (1926), the striola is better described as hook-shaped or “J”-shaped with its posterior limb directed more posteriorly than medially (Fig. 1). Lindeman (1969; p. 53) noted that in this respect it differs from the striolae he examined (guinea pig, rabbit, cat, squirrel monkey, human).

The striola corresponds to the central region of low hair cell density (Figs. 2A and 4) and to the band of calretinin-positive calyces (Fig. 5). Desai et al. (2005) have suggested that calretnin in these calyceal afferents may underlie their distinctive discharge irregularity (Goldberg et al. 1990). Throughout its trajectory, the striola is almost entirely medial to the line of polarity reversal (Fig. 5). In four utricles for which we labeled calretinin-positive calyces and mapped the reversal line (by staining kinocilia and stereocilia to determine bundle orientation), there were 0–9 type Ic hair cells lateral to the reversal line (<5% of type Ic hair cells). These results differ significantly from previous reports which depict the striola in mammals, including mice, as straddling the reversal line (e.g., Desai et al. 2005; Lim 1977; Lindeman 1969; Rosenhall 1972). Our data indicate that striolar hair cells will be excited by a limited subset of head movement directions: linear displacements in the plane of the macula toward the contralateral side or ipsilateral head tilt (e.g., for a left utricle, head displacement to the right or left ear down).

It is unclear why the striola is (largely) restricted to one side of the LPR, but this finding has two implications for central processing of signals from left and right labyrinths. First, striolar signals in rodents are distinctive (e.g., phase advanced relative to extrastriolar signals) (Goldberg et al. 1990). Thus at least in mice, these distinctive signals are lateralized: short-latency (monosynaptic) striolar drive to vestibular secondary neurons will only be triggered by a subset of head movement directions. Second, our results may be relevant to recent theories about the genesis of the translational vestibuloocular reflex (TVOR). Angelaki (2004) has reviewed data suggesting that the TVOR is an adaptation to maintain high acuity foveae on a visual target, it is robust in frontal-eyed, foveate primates but poorly developed or absent in lateral-eyed vertebrates, including rats, and the TVOR is triggered from the lateral striola. One hypothesis suggested by these data is that the lateral striola will be poorly developed or absent in afoveate,
lateral-eyed species. Our results are consistent with this hypothesis. Mice have little or no lateral striola (present results), and they are lateral-eyed, with low visual acuity (Artal et al. 1998) and detectable but poorly developed retinal specializations for frontal vision (Drager and Olsen 1981). One difficulty for this hypothesis is that pigeons possess a lateral striola (Si et al. 2003), although they appear to lack a TVOR, at least under certain experimental conditions (Dickman and Angelaki 1999). But as these authors point out, it is possible that pigeons exhibit a TVOR in the presence of a frontal target.

A third implication of our finding that the striola in mice lies medial to the LPR is that reported similarities in the transduction properties of striolar and extrastriolar hair cells of mice (Vollrath and Eato 2003) may need to be reassessed. If hair cells immediately lateral to the reversal line were erroneously assigned to the striola, this may have obscured real differences between the transduction properties of striolar and extrastriolar hair cells in mouse utricle.

Hair bundle heights

Several structural features of hair bundles are thought to be important for mechanotransduction, including stereocilia number and spacing, the complement of actin filaments within each stereocilium and their cross-linking, dimensions of the stereociliary shaft and pivot, and interstereociliary links (reviews: Peterson 2006). But from the earliest descriptions of hair bundles, particular attention has been paid to bundle heights (see discussions in Rowe and Peterson 2006; Xue and Peterson 2006). Briefly, bundle heights have important effects on the hair cell’s operating range (tip Peterson 2006; Xue and Peterson 2006). We applied these methods to hair bundles of mouse utricle.

Functional significance of bundle heights

Absolute bundle heights influence experimental values for hair cell operating range, sensitivity, and lineal stiffness because all three depend on measuring the displacement of bundle tips (either kinocilium or the tallest stereocilium). For example, geometry dictates that to produce a given angular rotation, the tips of taller bundles must undergo a greater linear displacement than the tips of shorter bundles. Thus they will have a greater operating range and, because operating range and sensitivity are inversely related, a lower sensitivity. This relation between bundle height, operating range, and sensitivity as been demonstrated experimentally in bullfrog utricle (Baird 1994b), chick otoconial organs (Ohmori 1987), and mouse (by comparing vestibular and auditory hair cells) (Géléeolé et al. 1997). Similarly, the tip of a tall bundle will deflect further than the tip of a short bundle when subjected to the same force, resulting in a smaller linear stiffness (displacement vs. force) even though the rotational stiffness, which factors out differences in bundle height, is the same for the two bundles (Géléeolé et al. 1997).

Given the mechanical importance of kinocilium and stereocilia heights, it is surprising that no previous studies of vestibular hair bundles have analyzed systematic variation in kina- and stereocilia heights as a function of hair cell type and epithelial locus in any mammal. Differences might provide an important source of hypotheses about origins of physiological diversity in utricular afferents of different types and macular loci (Goldberg et al. 1990). Furthermore, the few existing descriptions of bundle heights are likely to be somewhat inaccurate given the problems inherent in measuring kinocilium and stereocilia heights by conventional light and electron microscopic methods (discussion in Xue and Peterson 2006). To address this, we attempted to develop measurement methods that are accurate, closely reflect in vivo conditions, allow identification of hair cell type and location, and provide samples that are large enough for statistical analysis (Fontilla and Peterson 2000; Xue and Peterson 2006). We applied these methods to hair bundles of turtle (Xue and Peterson 2006) and mouse (Xue et al. 2005a; present results) utricle.

In the present study, we subdivided mouse utricular hair cells into three classes, and we asked two questions. First, do the three hair cell classes differ in bundle structure? Second, does the bundle structure of a single class vary as a function of macular locus? Our first finding is that the three classes of hair cells differ significantly in nearly all measured variables (Fig. 8; Table 4). Compared with type II hair cells, type I receptors have taller stereocilia, lower KS ratios, longer arrays, and steeper bundle slopes. Within the type I group, hair cells contacted by calretinin-positive calyces have shorter kinocilia, lower KS ratios, longer array lengths, and shallower bundle slopes than type I receptors contacted by calretinin-negative calyces. Our second finding is that, in contrast to this class-specific variation, there is little spatial variation in the bundles of each mouse hair cell class except that kinocilia are shorter, and thus KS ratios are lower, in the striola than in the extrastriola.
The ratio of kinocilium height to the height of the tallest stereocilia (KS ratio) will also affect bundle stiffness because, when force is applied to a kinocilium, the mechanical advantage of taller stereocilia allows them to more effectively resist kinocilium deflection than shorter stereocilia. This is suggested by models of turtle utricular hair cells (Silber et al. 2004) and by experiments. There is an inverse relation between measured stiffness and KS ratio in turtle utricular striola, such that KS ratio increases and stiffness declines with distance from the line of hair cell polarity reversal toward the medial extrastriola (Spoon et al. 2005). Statistical analyses suggest that KS ratio is the major structural determinant of hair bundle stiffness in turtle utricle (Moravec et al. 2005). This provides further support for the suggestion made in the preceding text that type Ic hair cells are stiffer than other utricular hair cells (because they have the lowest KS ratios; Figs. 7–9, E and F) and that, as a result, they may be especially suited for signaling high head accelerations or high-frequency components of head movements.

Finally, type I hair bundles are more steeply sloped than those of type II hair cells (Fig. 9L), and they are also wider (perpendicular to the bundle’s excitatory axis) (Xue and Peterson, unpublished data). This may translate into bundle responses that are phase advanced relative to those of type II hair cells because the steeper, wider type I bundles may be more readily stimulated by endolymph flow (Nam et al. 2005; see DISCUSSION in Rowe and Peterson 2006; Xue and Peterson 2006).

### TABLE 6. Height of kinocilia, tallest stereocilia, and KS ratio

<table>
<thead>
<tr>
<th></th>
<th>Striola</th>
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<tr>
<td><strong>Kinocilium Height, μm</strong></td>
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<tr>
<td>Mouse utricle</td>
<td>12.9 ± 2.4</td>
<td>12.8 ± 2.3</td>
<td>18.7 ± 2.8</td>
<td>15.5 ± 3.5</td>
<td>12.3 ± 1.6</td>
<td>7.1 ± 2.6</td>
<td>12.8 ± 2.3</td>
<td>6.6 ± 2.5</td>
<td>1.1 ± 0.2</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>Turtle utricle*</td>
<td>10.9 ± 1.6</td>
<td>10.4 ± 1.8</td>
<td>—</td>
<td>14.1 ± 4.3</td>
<td>7.6 ± 1.7</td>
<td>4.4 ± 1.1</td>
<td>—</td>
<td>4.6 ± 2.0</td>
<td>1.5 ± 0.5</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Guinea pig saccule† (Lapeyre et al. 1992)</td>
<td>7.9</td>
<td>4.2</td>
<td>11.0</td>
<td>4.3</td>
<td>9.8 ± 1.1</td>
<td>12.2 ± 1.9</td>
<td>8.9 ± 1.1</td>
<td>3.8 ± 1.0</td>
<td>1.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Bundle heights as a function of hair cell type and macular locus: comparison of four otoconial organs. *Recalculated from Xue and Peterson 2006. Striolar values are from striolar zone 3 only to factor out effects of macular locus (striolar zone 3 is the only utricular zone in turtles in which type I and type II hair cells exist side by side). †Values for extrastriola are from measurements of the “periphery”. SDs not available. ‡Values for the extrastriola are averages for type B bundles; only a small fraction of these (6/55) come from the striola. Striolar values are for type E bundles, which occupy the innermost striolar rows. Values for other striolar bundle types are generally similar. KS ratios were calculated from measurements of kinocilium and tallest stereocilia heights given in Baird (1994a), Table 1.
Relation to previous work on hair bundle heights

Comparisons with other species are difficult because there have been few systematic attempts to quantify heights of hair bundles in vestibular organs as a function of hair cell type and macular locus. Table 6 summarizes the available data. There are two consistent findings. First, kinocilia of both type I and II bundles in the striola are shorter than those in the extrastriola, and KS ratios are lower. This may be true for some fish species as well (review: Platt 1983). Because low KS ratios tend to increase bundle stiffness (Moravec et al. 2005; Silber et al. 2004; Spoon et al. 2005), these height data are consistent with the suggestion that striolar bundles in vertebrate otocural organs are stiffer than those in the extrastriola and, therefore, striolar hair cells may be specialized for signaling high head accelerations or high-frequency head movements (Baird 1994a; Eatock and Lysakowski 2006).

Second, at any given macular locus in amniotes, the tallest stereocilia on type I hair cells are taller than those on type II hair cells. If force is applied to the kinocilia of these hair cell types at the same height (either because they have similar kinocilia heights or because they contact the otoconial membrane at the same height above the epithelial surface), these differences in the heights of the tallest stereocilia raise the possibility that neighboring hair bundles will differ significantly in stiffness (because they differ in KS ratio). If so, such stiffness differences might be overwhelmed if the otoconial membrane moves as a rigid plate during head movement. Alternatively, differences in coupling between bundles and the otoconial membrane such as those seen in turtle utricle (Xue et al. 2007) may allow these stiffness differences to be expressed.

Only one previous study, on turtle utricle, has compared hair bundles of type I hair cells contacted by calretinin-positive and -negative calyces (Xue et al. 2005b). In both mouse and turtle, KS ratio is lower for type Ic hair cells, compared with type Id hair cells at the same macular locus. Here again, neighboring hair cells (the 2 classes of type I hair cell in the striola) may differ in stiffness (because they differ in KS ratio), raising questions about how or whether these stiffness differences might be expressed in intact utricles.

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References


