Research Article

Oncomodulin Expression Reveals New Insights into the Cellular Organization of the Murine Utricle Striola

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ABSTRACT

Oncomodulin (OCM, *aka* β -parvalbumin) is an EF-hand calcium binding protein that is expressed in a restricted set of hair cells in the peristriolar region of the mammalian utricle. In the present study, we determined the topologic distribution of OCM among hair cell phenotypes to advance our understanding of the cellular organization of the striola and the relationship of these phenotypes with characteristics of *tissue polarity*. The distributions of OCM-positive (OCM+) hair cells were quantified in utricles of mature C57Bl/6 mice. Immunohistochemistry was conducted using antibodies to OCM, calretinin, and β 3-tubulin. Fluorophore-conjugated phalloidin was used to label hair cell stereocilia, which provided the basis for determining hair cell counts and morphologic polarizations. We found OCM expression in striolar types I and II hair cells, though the distributions were dissimilar to the native striolar type I and II distributions, favoring type I hair cells. The distribution of OCM immunoreactivity among striolar type I hair cells also reflected nonrandom distribution among type I_c and I_d phenotypes (i.e., those receiving calretinin-positive and calretinin-negative calyces, respectively). However, many OCM+ hair cells were found lateral to the striola, and within the epithelial region encompassing OCM+ hair

cells, the distributions of OCM+ types I_c and I_d hair cells were similar to the native distributions of I_c and I_d in this region. Summarily, these data provide a quantitative perspective supporting the existence of different underlying factors driving the topologic expression of OCM in hair cells than those responsible for tissue polarity characteristics associated within the utricular striola, including calretinin expression in afferent calyces.

Keywords: β -parvalbumin, calretinin, calyx, hair cell, vestibular, bootstrap resampling

INTRODUCTION

There is an accumulating body of evidence indicating that the mammalian utricle exhibits a cellular organization far richer than depicted by the broad yet stereotyped distribution of hair cell morphologic polarization (Lindeman 1969). In particular, the region of epithelium immediately adjacent to the line of hair cell morphologic polarity reversal, known as the striola, exhibits several unique features that include afferent dendrites exhibiting primarily calyx-only morphologies (Fernandez et al. 1990) and calretinin expression (CALB2; Desai et al. 2005; Desmadryl and Dechesne 1992; Li et al. 2008). In fact, this region defined by the projection loci of calretinin-positive (CALB2+) calyces has come to represent the *de facto* striola (Desai et al. 2005). Furthermore, it has also been shown that hair cells

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receiving CALB2+ calyces (referred to as type I_{cc} hair cells) are primarily medial to the line of polarity reversal (LPR; Li et al. 2008)). Type I_{cc} hair cells are fundamentally similar with respect to the polarization of their stereocilia bundle and kinocilium location, summarily referred to as their *morphologic polarization vectors* (MPVs), in that they are primarily oriented lateralward (i.e., kinocilium at the lateral aspect of the stereocilia bundle; (Li et al. 2008)). Deans (2013) proposed that the systematic distribution of hair cell MPVs is an epithelia-level manifestation of planar cell polarity (PCP) that he categorized as *tissue polarity*. The close morphologic association between CALB2-positive, calyx-only afferents and their projection onto a restricted subset of striolar hair cells suggests a potential link between the factors responsible for these characteristics and the distinct elements of the sensory neuroepithelia. Furthermore, these relationships illustrate how planar polarity established within labyrinthine epithelia manifest into important characteristics of head movement coding (Deans 2013). For example, the relationship between striolar hair cells and the CALB2-expressing afferents forms the afferent limb of a lateralized head movement direction coding circuit originating within each labyrinth (Li et al. 2008).

As introduced above, Deans (2013) advanced a working framework organizing the relationship between morphologic features of the striola and hair cell polarity, the latter being a fundamental outcome of PCP molecular mechanisms. In this framework, the LPR represents a prominent feature of PCP at the level of *tissue polarity*, while the close association of morphologic polarizations between neighboring hair cells reflects a different level referred to as *cellular polarity* (Deans 2013). While the molecular mechanisms of each hierarchical level of PCP are not completely known, the superposition of specific afferent neuron characteristics onto this organization raises the possibility that they may be rooted in related mechanisms (Deans 2013). This also suggests that other striola-specific features may also be driven or constrained by molecular mechanisms of PCP.

However, not all cellular features closely associated with the utricular striola exhibit a strict dependence upon known PCP mechanisms. The expression of oncomodulin (OCM; aka β -parvalbumin) within hair cells in close proximity to the striola (i.e., the peristriolar region) may, at first glance, also reflect characteristics of tissue polarity. That is, OCM expression has been described as being confined to the striolae of the utricle and saccule, as well as the central zones of the cristae (Collado et al. 2011; Simmons et al. 2010) in patterns similar to the regions harboring CALB2+ afferent calyces. In fact, OCM expression has been referred to as a prominent hair

cell marker of the striola. Simmons et al. (2010) also reported that the qualitative pattern of OCM expression was preserved in the *looptail* mutant mouse, representing a model of PCP molecular pathway disruption resulting in disorganized arrangement of hair cell MPVs and the absence of a distinct LPR (Deans et al. 2007). Furthermore, it has recently been shown that OCM expression extends to the lateral extrastriola in hair cells that also express Emx2, a transcription factor that plays a crucial role in defining hair cell MPV and the LPR (Jiang et al. 2017). These findings indicate that OCM expression is not critically dependent upon molecular pathways resulting in phenotypes representing the hallmark of PCP. On the other hand, its close association with other features of tissue polarity may be indicative of upstream factors that are shared among these phenotypes. We implemented quantitative analyses and bootstrap resampling statistics to test hypotheses regarding the distributions of striolar hair cell phenotypes. The testing of these hypotheses is based upon the notion that these distributions represent the outcome of intrinsic factors (e.g., molecular and/or cellular PCP factors), and resampling analyses provides a quantitative metric to evaluate probabilities that the distributions depend upon similar underlying factors. Consequently, the present study was undertaken to determine the detailed relationship between characteristics of tissue polarity with the goal of providing a quantitative perspective of the cellular organization of the mammalian utricular striola.

METHODS

Animals and Specimen Preparation

The animals used to generate most of the data of the present study were young adults of the C57BL/6 background strain (aged 35–42 days). Though the mouse utricle exhibits maturity with respect to some phenotypes by 14 days of age (e.g., CALB2 expression in afferent calyces (Dechesne et al. 1994)), there is other evidence indicating that the murine utricle may still be undergoing maturation at 20–25 days (Schweizer et al. 2009). Therefore, we conducted additional analyses on older animals (aged 63–94 days) to verify that our principal findings regarding OCM distributions reflected that of the mature utricle. We also included our early experience with young animals aged 23–30 days. All procedures involving them were approved by the Chancellor's Animal Research Committee, and conformed to standards established in the *NIH Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication, revised 2011), and the principles presented in the *Guidelines for the Use of Animals in Neuroscience Research*

by the Society for Neuroscience (available from the Society for Neuroscience). Mice were deeply anesthetized with isoflurane and rapidly decapitated, after which fixative (4 % paraformaldehyde in 0.1 M phosphate buffer) was immediately infused into the vestibule. Vestibular sensory epithelia were further exposed to fixative by opening the membranous labyrinth and removing the utricular otolithic membrane with a gentle stream of fixative. Temporal bone specimens were then removed from the skull and immersion-fixed and agitated for 2 h. Specimens were then thoroughly rinsed (including copious flushing of the vestibule) with 0.1 M phosphate-buffered saline (PBS), and microdissected to remove the utricular epithelia.

We also used utricle specimens from animals with a targeted deletion of the OCM coding sequence ($OM^{tm1.1Ddsi}$; MGI:97401) to verify the specificity and the topographic region of expression of the OCM antibodies used in this investigation (Simmons et al. 2010; Tong et al. 2016). These animals are designated $Actb^{cre}OCM^{lox/lox}$, and were a mixed strain with C57Bl/6 and 129Sv/J DNA and were approximately 60 days of age. Utricle specimens representing this genotype were dissected and processed using procedures identical to those described above.

Antibodies and Immunohistochemical Processing

The antibodies used to characterize the distribution of OCM-immunopositive hair cells among other hair cell phenotypes (i.e., including type I_c) within the murine utricle are listed in Table 1. In this table are the specifications for immunogen, host in which it was raised, and the controls that verify antibody efficacy. Initial experiments were conducted using the rabbit-host anti-OCM antibody, providing a general understanding of the distribution of OCM expression among other striolar hair cell phenotypes. However, to investigate more specific associations between OCM expression and striolar hair cells, we used the goat-host anti-OCM antibody. This enabled us to conduct multi-labeling experiments using a rabbit-host anti- β -3-tubulin (anti-TUBB3) antibody, which our laboratory routinely uses as a very reliable marker of afferent calyces and, consequently, type I hair cells. These latter experiments provided the predominant portion of the data for this report.

The immunohistochemical procedures were applied to intact utricles. Specimens were first immersed in blocking solution for 2 h at room temperature containing 0.1 % Triton X-100 and 1.0 % BSA solution in PBS, and were then incubated for 24 h at 4 °C in cocktails of the primary antibodies diluted in blocking solution. Fluorophore-conjugated, host-specific secondary antibodies were then applied for

Table 1

Antibody inventory used to investigate OCM expression in mouse utricle

Target	Manufacturer (product #)	Immunogen	Host	Working concentration	Positive control	Negative control
Oncomodulin (OCM)	Swant (OM3)	Recombinant full length rat Oncomodulin	rabbit	1:500	Simmons et al. 2010	Conditional knockout
Oncomodulin (OCM)	Santa Cruz (sc-7446)	Peptide mapping N-terminus of h-OCM	goat	1:500	Present study using Swant Ab	Primary Ab omission
Calretinin (Calb2)	Chemicon (MAB1568)	Recombinant full length rat Calretinin	mouse	1:500	Cerebellum (immunopositive granule cells); Li et al. 2008	Primary Ab omission
β 3-Tubulin (TUBB3)	Covance (PRB-435P)	Class 3 β -Tubulin from rat brain	rabbit	1:500	—	Primary Ab omission

h-OCM human-OCM, Ab antibody

2 h at room temperature. Utricles labeled with anti-OCM, anti-TUBB3, and anti-Calb2 were used to investigate the relationship between OCM-positive (OCM+) hair cells, TUBB3-positive (TUBB3+) calyces, and CALB2-positive (CALB2+) calyces. Fluorophore-conjugated phalloidin (Biotium CF405M) was added to the secondary antibody solutions for stereocilia labeling, which provided the basis for determining hair cell morphologic polarization and navigating the topography of the utricle during confocal imaging. Secondary antibodies were used at 1:250 concentration and included Alexa 488 donkey anti-mouse (Life Technologies A21202), Alexa 555 donkey anti-goat (Life Technologies A21432), and Alexa 647 donkey anti-rabbit (Life Technologies A31573). After the final washes in PBS, intact utricles were whole-mounted on glass slides with aqueous mounting media. Silicone spacers (S-24735; Invitrogen) were used to create a small chamber on the glass slide within which the utricle specimens were mounted, thereby minimizing distortion of in situ structure by the placement of the coverslip.

Imaging and Analysis

The majority of the critical data of the present investigation were obtained using a Zeiss LSM 710 confocal microscope and a Plan Apochromat 63X oil-immersion objective (1.4 N.A.). Image stacks covering the entire striola of each utricle were obtained, along with representative stacks of the medial extrastriola representing epithelia containing hair cells previously reported to be OCM-negative (Collado et al. 2011; Simmons et al. 2010). Optical sections were collected at a spatial resolution (in pixels) of 1000×1000 . The entire striola was captured within 4–5 image stacks, where adjacent stacks included sufficient overlap to insure all OCM+ hair cells and CALB2+ calyces were imaged. This process was documented by photobleaching the phalloidin-fluorophore. Low objective power (i.e., using $\times 10$, 0.4 N.A. objective) image stacks were also obtained that included the entire utricle.

We established quantitative criteria with which hair cells positive for anti-OCM immunolabeling were objectively identified. Because the majority of utricular hair cells express CALB2 (Desai et al. 2005; Li et al. 2008), we were able to clearly identify hair cells well outside the striola (defined by the epithelial region harboring CALB2+ calyces), and therefore outside the region within which OCM+ hair cells were located (Collado et al. 2011; Simmons et al. 2010). Background fluorescence intensity was measured for > 150 extrastricular hair cells (approximately 50 in each of three utricles) utilizing imaging parameters identical to those used to visualize and capture anti-OCM

labeling. These measures represented the average pixel intensity within $12.6 \mu\text{m}$ (Collado et al., 2011) (circular region with radius of $2 \mu\text{m}$) of selected hair cells in a single optical section. These data provided the basis for determining the distribution of background intensities resulting from the processing and imaging parameters used in all specimens (i.e., illumination intensity, detector gain, and detector offset values were set to predetermined values). The distribution of background intensities was found to be statistically similar to a normal distribution (Kolmogorov-Smirnov test, $p > 0.05$; data not shown), from which the mean (I_{bf}) and standard deviation (∂_{bf}) of background fluorescence intensity were determined. From these parameters, we established the maximum fluorescence intensity corresponding to 99.99 % of hair cells exhibiting only background fluorescence as $I_{bf} + 3.63 \cdot \partial_{bf}$. This value also represented the minimum fluorescence intensity representing OCM+ immunolabeling. That is, the minimum intensity criterion enabled the interpretation that there was an extremely low probability ($p < 0.0001$) that a hair cell exhibiting only background fluorescence would be associated with intensities greater than this emission intensity value.

Utilizing the criteria described above, we identified all OCM+ hair cells. For the most critical data of this investigation, utricles immunolabeled with anti-OCM were also labeled with anti-TUBB3 and anti-Calb2, providing the basis for identifying all type I hair cells, and the subset of type I_c hair cells. Confocal stacks containing all three antibody emission channels, as well as the emission channel representing phalloidin labeling, were separated for analyses for which *NeuroLucida*[®] (MBF Bioscience Inc.) was utilized to identify OCM+, type I, and type I_c hair cells from the same stacks. Hair cells determined to be OCM+ as well as type I_c were categorized as *colabeled*. Additionally, the location of each hair cell relative to the LPR (i.e., medial or lateral) was determined from its morphologic polarization elucidated through phalloidin labeling of the stereocilia bundle and the position of the kinocilium that is clearly identified as unlabeled (Schweizer et al. 2009). Stereocilia labeling also provided the basis for quantifying total striolar hair cell counts.

Analysis of Topologic Distributions

Confocal stacks obtained using the $\times 63$ objective and representing the entire striolae from three utricles were imported into *NeuroLucida* (MBF Bioscience Inc.) and were tiled to form complete reconstructions containing all striolar hair cells and calyces. A closed contour (e.g., shown as the solid black line in Figs. 2 and 4) for each utricle representing each striola was

constructed by closely tracing the phalloidin-labeled tight junctions around CALB2+ calyces. Open contours (e.g., dashed lines in Figs. 2 and 4) representing the respective LPRs were traced in similar fashion by following the tight junction between hair cells with opposite MPVs. Hair cell phenotypes were determined as defined above, and markers representing each phenotype were placed within the “overlay,” from which final counts for each utricle were established. This analysis provided the basis for the topologic reconstructions in Figs. 4 and 5. Additionally, distances of each hair cell to the LPR were provided by the *NeuroLucida* software, and these data provided the basis for Fig. 8 and subsequent analyses.

Bootstrap Resampling Statistics

Quantitative perspectives of the distributions of OCM+ hair cells among other striolar hair cell phenotypes was established by testing hypotheses that the counts of specific OCM+ hair cell subtypes (OCM+ type I and II, and OCM+ type I_c and I_d) were derived from random association with the underlying hair cell phenotypes (e.g., total striolar type I and II, and total OCM+ type I hair cells). These hypotheses were tested through bootstrap resampling strategies implemented through custom scripts developed in the *Igor Pro* software environment (version 6.37, Wavemetrics Inc.). In general, 100,000 resamples were obtained, and if the computed native statistic was outside the distribution of the 100,000 resampled computed statistics, the probability was reported as less than 0.00001.

We also utilized resampling strategies in conducting Kruskal-Wallis (nonparametric analyses of variance) and Dunn-Holland-Wolfe (nonparametric post-hoc multiple comparison tests) analyses of distance-to-LPR measures for striolar hair cells. In short, analyses were conducted on native distributions to determine the test statistic (i.e., the *H* or *Q* statistics for Kruskal-Wallis and Dunn-Holland-Wolfe tests, respectively), which was compared to distributions of these statistics found by resampling 100,000 times. As noted above, if the computed native statistic was outside the distribution of the 100,000 resampled computed statistics, the probability was reported as less than 0.00001.

RESULTS

Specificity of OCM Immunolabeling in Mouse Utricle

The general pattern of OCM expression in the mouse utricle (C57BL/6, CBA-CaJ, and CD1 strains) was described by Simmons et al. (2010) and confirmed by Collado et al. (2011). OCM expression was restricted to hair cells located in a discrete swath of utricular

epithelium similar to the area that harbored the CALB2+ subset of afferent calyces (Fig. 1a–c). As expected, we found a very similar general pattern of expression in wild-type C57BL/6 mice with both antibodies used in the present investigation (Fig. 1d, e). The specificity of this immunolabeling was verified by its absence in utricles from mice exhibiting targeted OCM deletion (*Actb^{cre}OCM^{flox/flox}*; (Tong et al. 2016)). In these specimens, CALB2 expression was similar to that described previously for the mouse utricle (Fig. 1a–c; (Desai et al. 2005; Li et al. 2008)), while OCM expression was completely absent (Fig. 1f). These data confirm the specificity of OCM immunolabeling that resulted from use of the antibodies employed in the present investigation (Table 1).

OCM expression in vestibular epithelia was shown to be attenuated in aged animals (i.e., 10 months; Simmons et al. 2010). The summary data in Table 2 present cursory results of OCM+ hair cell counts in specimens obtained from animals aged younger (i.e., 23–30 days) and older (i.e., 63–97 days) than those upon which the detailed analyses of the present study were conducted. This illustrates that the distributions of OCM+ hair cells are stable in specimens from C57BL/6 mice aged through approximately 100 days. As noted above, this includes those representing the source of the data in the balance of this report (i.e., 35–42 days).

OCM Expression Is Not Restricted to Type I Hair Cells

In their evaluation of OCM expression in the mouse utricle, Simmons et al. (2010) reported that it was found primarily in type I hair cells. The observed association with CALB2+ calyces further supported the notion that it was restricted to the striolar region. A more detailed analysis of OCM expression among vestibular hair cell phenotypes would elucidate whether, in fact, it was confined to type I hair cells. If it was not restricted to type I, then quantitative analyses would reveal whether or not OCM expression resulted from uniform distribution among all striolar hair cells. Such a finding would provide insight into factors contributing to OCM expression and the cellular organization of the utricle, i.e., it would imply the existence of a general factor restricting OCM expression within the striola but not to general hair cell phenotype. In the present study, we objectively defined the utricular striola as that region harboring CALB2+ calyces, and then quantified OCM expression among striolar type I and II hair cells.

Detailed analyses of OCM+ hair cells revealed important characteristics that refined the previously reported qualitative pattern of expression (Collado et al. 2011; Simmons et al. 2010). We found that OCM

Fig. 1. OCM immunolabeling is absent in utricular hair cells in a conditional OCM knockout, verifying the specificity of antibodies used in the present investigation. **a–c** Low power maximum intensity projection micrographs of mouse utricles labeled with antibodies to Calb2. Many hair cells and afferent calyces (white arrowheads) are labeled by anti-Calb2 immunostaining in both WT (**a, b**) and knockout utricles (**c**). The knockout specimen was harvested from a conditional knockout *Actb^{cre}OCM^{fllox/fllox}* mouse (Simmons et al. 2010), processed and imaged in parallel to WT specimens. Anti-Calb2 calyces can be seen in this specimen (**c**), demonstrating that other immunohistochemical phenotypes of the knockout utricle are unaffected in this conditional knockout. The utricle perimeter is outlined by the dotted white line in all micrographs. The approximate in situ specimen orientations are indicated by the compass (top right in **d**). **d, e** OCM-positive hair cells (**d, e**) are confined to a region of the utricle similar to that occupied by CALB2+ calyces (white arrowheads in **a, b**). OCM-immunolabeled hair cells are completely absent in the conditional knockout specimen (**f**), verifying the specificity of the immunolabeling in utricular hair cells. Scale shown in **a** (100 μm, bottom left) is identical for all micrographs

expression was not confined to type I hair cells, nor was every striolar type I hair cell found to be OCM+. These findings are illustrated in Fig. 2. Figure 2a represents a single optical section from a striolar confocal stack showing OCM-immunolabeled hair cells (red) and TUBB3-immunolabeled calyces (green). Most type I hair cells in this micrograph (i.e., those encapsulated by TUBB3+ calyces) are

Table 2
Distribution of OCM+ and type I_c hair cells in utricles from young adult and adult C57BL/6N mice. Values represent means (± standard deviation)

Age (days)	n	Phenotype					
		Striolar HCs		Type I _c		Colabeled	
		Count	Count	Count	Count	Count	Count
23–30	3	430	315	206	181	181	181
		(10.2)	(15.0)	(15.5)	(8.1)	(8.1)	(5.1)
63–97	5	432	337	234	181	181	181
		(39.4)	(70.0)	(46.9)	(32.4)	(32.4)	(9.5)

%* proportion relative to total striolar hair cell counts, %** proportion relative to OCM+

OCM+, though a few are OCM-negative (OCM−; white arrowheads). OCM+ type II hair cells are clearly represented by the immunopositive hair cells without calyces (white asterisks). The volume reconstructions of OCM-immunolabeled hair cells (Fig. 2b, c) further illustrate OCM expression in type II hair cells (Fig. 2c), identified by the absence of a TUBB3+ calyx and its stereotyped morphology represented by a broad neck (cyan arrowheads) and narrow base (yellow arrowheads). This contrasts the morphology of a type I hair cell illuminated by the volume reconstruction shown in Fig. 2b, which was encapsulated by a TUBB3+ calyx.

A broader perspective of OCM expression among type I and II hair cells is shown in the striolar hair cell map from a representative mouse utricle in Fig. 2d. In this schematic representation, the striola is outlined by the solid black line, determined by tracing the phalloidin-labeled tight junctions around the exterior-most type I_c hair cells (Desai et al. 2005; Li et al. 2008). The LPR (dashed purple line) is also shown, determined by tracing the phalloidin-labeled tight junctions between hair cells exhibiting diametrically opposed MPVs (Li et al. 2008). Hair cells encapsulated by a TUBB3-labeled calyx were identified as type I; those without a calyx were identified as type II. Positive (OCM+) or negative (OCM−) OCM immunolabeling is indicated by filled and unfilled symbols, respectively. This map illustrates the widespread distribution of type I OCM+ hair cells (I•OCM(+); filled red circles) throughout the striola. These appear to vastly outnumber both type I OCM− (I•OCM(−); open red circles) and type II OCM+ hair cells (II•OCM(+), filled blue squares). In contrast, II•OCM(+) hair cells are outnumbered by type II OCM-negative hair cells (II•OCM(−); open blue squares).

In addition to a global perspective of OCM expression among striolar hair cells, the map in Fig. 2d also illustrates their topographic distribution, and a few observations will be highlighted here ahead of more thorough analyses to follow. The most obvious finding is that while CALB2+ calyces are primarily medial to the LPR (Li et al. 2008), OCM expression was not similarly confined and many OCM+ hair cells were located lateral to the LPR. Most, but not all, OCM+ hair cells located lateral to the LPR were type I. Two more subtle findings can also be noted in this specimen. First, most I•OCM(−) hair cells (open red circles) were located close to the medial striolar border, and only a few were found in the central striola. And second, while OCM expression among type I hair cells appeared to be generally uniform across the striola, II•OCM(+) hair cells were found in the lateralmost striolar area (i.e., few filled blue squares close to the medial striolar border). As noted, the spatial distribution of OCM expression will be

analyzed in a separate subsection to follow (see subheading “Topology of OCM Expression”).

A quantitative summary of OCM expression among striolar type I and II hair cells is plotted in Fig. 2e. It is important to note that these data depict only those hair cells within the striola (e.g., within the solid line border in Fig. 2d). The striolar hair cell subpopulations in our specimens were comprised (mean ± standard deviation) of 57.2 % (± 2.7 %) type I and 42.8 % (± 2.7 %) type II hair cells (Fig. 2e, leftmost two bars in red), and proportions that were comparable to those previously reported (Desai et al. 2005; Li et al. 2008). The fraction of OCM+ type I hair cells among all striolar hair cells was found to be 47.3 % (± 2.6 %), while II•OCM(+) (Fig. 2d, rightmost two bars in green) comprised only 9.8 % (± 1.7 %). These data indicate that a greater fraction of striolar hair cells are I•OCM(+) compared to those that are II•OCM(+). Since striolar hair cells exhibit a greater fraction of type I (Fig. 2d, left bars), even random (e.g., nonselective) OCM expression would yield a greater fraction of I•OCM(+) than II•OCM(+). Therefore, we utilized bootstrap resampling methods to test the hypothesis that the distribution of OCM expression among striolar hair cells was based upon the underlying distribution of type I and II striolar hair cells. If this hypothesis was not supported by the analysis, we would conclude that other factors must be driving the empirical distribution of OCM expression among the underlying hair cell phenotypes.

The bootstrap resampling strategy enabled a test of the hypothesis that the distribution of I•OCM(+) and II•OCM(+) hair cells resulted from the nonselective expression of OCM among the underlying distribution of type I and II hair cells. This was implemented by randomly resampling 765 hair cells (the sum of all OCM+ hair cells in three utricles) from the total population of 1327 striolar hair cells (sum of all striolar hair cells in three utricles) distinguished by primary hair cell phenotype (i.e., type I or II). In our sample of striolar hair cells among three mouse utricles, 758 were type I and 569 were type II. The resampling was repeated 100,000 times, and the distribution of resampled I•OCM(+) hair cells (i.e., based upon random resampling 765 OCM+ hair cells) is shown in the histogram in Fig. 2e. These data illustrate that the expected three-utricle distribution of I•OCM(+) hair cells, based upon random expression of OCM among the underlying distribution of type I and II hair cells, would exhibit a mean count of 437.1 ± 13.76 hair cells (histogram mean; range 391–494), vastly lower than the total empirical count of 628 (Fig. 2e, red arrowhead). We conclude that the probability of obtaining 628 I•OCM(+) hair cells through random OCM expression among the underlying striolar subpopulations of type I and II hair cells

was less than 0.00001, supporting rejection of the hypothesis. This conclusion strongly infers that the factors driving OCM expression among striolar hair cells are different than those responsible for the underlying distribution of principal hair cell types in this region, resulting in a much more extensive expression of OCM among type I hair cells. The

corollary conclusion can also be made; that is, while OCM expression is not restricted to type I hair cells, the count of II•OCM(+) is much lower than expected from random expression among striolar hair cells. Simply stated, peristriolar (the epithelia region within or near the striola) OCM expression is not exclusive to type I hair cells, but is preferential to this phenotype.

Fig. 2 OCM is expressed in both type I and II hair cells of the utricular striola. **a** Optical section from a confocal series illustrating the anti-OCM (red) and anti-TUBB3 (green) immunolabeling. Most TUBB3+ calyces encapsulate an OCM+ hair cell, though notable exceptions are seen in which a I•OCM(–) hair cell is paired with a I•OCM(+) hair cell within double TUBB3+ calyces (white arrowheads). II•OCM(+) hair cells are identified by the absence of an associated calyx, and are highlighted by the white asterisks. **b, c** These micrographs illustrate volume-rendered type I (**b**) and II (**c**) OCM+ hair cells. The arrowheads in each micrograph highlight the stereotypical differences in neck (white arrowheads) and base (yellow arrowheads) width for the type I and II hair cells. **d** Each hair cell within one utricular striola specimen is identified and plotted to illustrate the spatial distribution of OCM expression among type I and II hair cells. The striola boundary was drawn close to the outermost CALB2+ calyces, following the phalloidin-labeled tight junctions at the epithelial surface. Very few II•OCM(+) hair cells were found outside the striola, while many more I•OCM(+) hair cells were located outside the striola boundary. The arrowheads highlight cases where the striola was extended to closely surround type I_c hair cells; these were lateral to the LPR (cyan dashed contour). **e** Distributions of striolar hair cell phenotypes. The leftmost two bars (red) represent the mean ($\pm$ standard deviation) count of type I and II hair cells as a percentage of total striolar hair cell count. The two bars at right (green) represent the mean count ($\pm$ standard deviation) of OCM+ type I (stripes) and II (solid) hair cells, computed as a percentage of the total striolar hair cell count. These data show that while the ratio of type I/II hair cells among all striolar hair cells was approx. 3:2, the I/II ratio of OCM+ hair cells was approx. 4:1. The mean count for each phenotype is shown at the base of each bar, along with the total count among the three specimens analyzed. **e** Histogram of expected OCM+ type I hair cells based upon random resampling ($n = 100,000$) of the native counts of type I and II hair cells among the three striola specimens examined in the present study. This graph illustrates that the probability of counting a total of 628 I•OCM(+) hair cells from random resampling of 758 total hair cells (type I and II) of native distribution is well under 0.00001

OCM Expression Among All Striolar Hair Cell Phenotypes

Striolar type I hair cells can be parsed into two general phenotypes based upon characteristics of the afferent calyx with which they are associated. Type I_c hair cells represent those that receive a CALB2+ calyx, and reportedly are associated with afferents exhibiting a calyx-only dendritic morphology (Desmadryl and Dechesne 1992; Li et al. 2008). Type I_d represent hair cells whose encapsulating calyx (TUBB3+) is calretinin-negative (CALB2–) and are associated with dimorphic dendritic morphologies (Li et al. 2008). Including type II hair cells and in the context of OCM expression, therefore, six striolar hair cell phenotypes were distinguished, namely OCM+ and OCM– categories of type I_c, type I_d, and type II (i.e., I_c•OCM(+), I_c•OCM(–), I_d•OCM(+), I_d•OCM(–), II•OCM(+), and II•OCM(–), respectively). The micrographs in Fig. 3a–d provide examples of each phenotype, representing maximum

intensity projections from a three-channel confocal stack demonstrating immunolabeling for anti-CALB2 (Fig. 3a), anti-TUBB3 (Fig. 3b), and anti-OCM (Fig. 3c). The channels are merged in Fig. 3d, in which each OCM-distinguished phenotype is identified as a–f. Corresponding orthogonal views of six striolar representative hair cell phenotypes are shown in the row of orthogonal plane micrographs labeled Fig. 3a–f at the bottom of the figure.

The six hair cell phenotypes were identified in the utricular whole mounts, which provided a convenient method for analyzing their spatial distributions with respect to the topography of the utricular neuroepithelium. These distributions are shown for one utricle in Fig. 4, representing the same specimen depicted in Fig. 2c with the additional distinctions of striolar type I phenotypes (i.e., type I_c and I_d). Figure 4a illustrates the spatial distributions of all striolar hair cell phenotypes based upon OCM expression, including all OCM+ hair cells outside the striola. Fig. 4b–d represent hair cell types I_c, I_d, and II, where OCM+ and OCM– cells are depicted as filled and unfilled symbols, respectively. Also shown on each schematic is the LPR (cyan dashed line), which provided an additional functional landmark in the epithelia. Numerical summaries of the distributions are shown in Table 3, while principal distributions of striolar and OCM-expressing hair cell phenotypes are shown in Fig. 5.

The defining hair cell phenotype of the mammalian striola is the type I_c, the distribution of which is shown in Fig. 4b. They represent 44.0 ± 2.0 % of all striolar hair cells, and most (91.7 ± 0.4 %) were located medial to the LPR as previously described (Li et al. 2008). Figure 4b illustrates that most type I_c hair cells were positive for OCM expression (89.9 ± 1.9 %), and most I_c•OCM(–) hair cells were found close to the medial striolar border (11 of 15 I_c•OCM(–) in the specimen shown in Fig. 4b). In addition, most I_c hair cells lateral to the LPR were OCM+. These data corroborate the previous observation of a close association of type I_c hair cells with those that were OCM+ (Simmons et al. 2010).

The distribution of type I_d hair cells is shown in Fig. 4c, illustrating the relatively small fraction of striolar type I hair cells that are represented by this phenotype (i.e., within the striolar boundary). Many I_d•OCM(+) are located adjacent to but lateral to the LPR. As found for I_c•OCM(–), striolar I_d•OCM(–) hair cells were close to the medial striolar border. Notable is the paucity of I_d hair cells in the central and lateral striola (i.e., those within the striola border).

The spatial distribution of striolar type II hair cells is shown in Fig. 4d. A small number of II•OCM(+) hair cells were found immediately lateral to the LPR.

Fig. 3. Six striolar hair cell phenotypes can be distinguished by OCM expression and calyx phenotype. The micrographs in **A–C** represent projection images from a single confocal stack illustrating striolar immunolabeling for Calb2 (**A**), TUBB3 (**B**), and OCM (**C**). The merged image is shown in **D**. The scale bar in **D** corresponds to the micrographs in **A–C**, as well. Each of the six phenotypes are highlighted by a white

arrowhead and italicized lowercase letter; these are then isolated as orthogonal plane micrographs at the bottom and correspond to (**a**) $I_c \bullet OCM(+)$, (**b**) $I_c \bullet OCM(-)$, (**c**) $I_d \bullet OCM(+)$, (**d**) $I_d \bullet OCM(-)$, (**e**) $II \bullet OCM(+)$, and (**f**) $II \bullet OCM(-)$. Scale bar (**f**) = 10 μm (applies to **a–f**). The white arrowheads in **e** and **f** highlight the structures of interest in the micrographs

While type II hair cells represented 42.8 % of all striolar hair cells, only a quarter of those (i.e., 24 % of striolar type II) were OCM+. Type II hair cells were found throughout the striola, though $II \bullet OCM(+)$ appeared to be located primarily in the lateralmost area in this specimen. Interestingly, these appear to be located in the area of the striola sparsely populated by type I_d hair cells.

Quantitative summaries of OCM expression among hair cell phenotypes are shown in Fig. 5. Figure 5a depicts the subpopulation of OCM+ hair cells within types I_c , I_d , and II, illustrating that the largest fraction was among type I_c at 0.57 ± 0.0008 , while the fraction

of I_d and II were $0.25 (\pm 0.006)$ and $0.18 (\pm 0.007)$, respectively. This large representation of type I_c among all OCM+ hair cells was rooted in the finding that 89.9 % of type I_c hair cells were OCM+ (± 1.9 %; Fig. 5b), while OCM expression was found in 76.5 % of type I_d hair cells (± 3.7 %; Fig. 5b). In contrast, only approximately 27.4 % of type II hair cells expressed OCM+ (± 2.7 %; Fig. 5b).

The data of Fig. 5 illustrate that OCM expression among type I hair cells (Fig. 2) is further broken down into type I_c and I_d hair cells that were depicted in the spatial maps of Fig. 4. We tested whether this distribution reflects random expression among type I

Fig. 4. Spatial distribution of OCM expression among striolar type I_c, I_d, and II hair cells. **a** Map of all hair cells in the striola of a representative utricle, identified by OCM expression, principal phenotype (type I or II), and, if type I, its subtype. The striola boundary is indicated by the solid contour, while the LPR is indicated by the dashed line (cyan). All OCM+ hair cells are represented to include those outside the striola boundary; these were only found lateral to the LPR. The general orientation of the striola within the

mouse temporal bone is indicated by the “compass” at right. **b–d** Maps of type I_c (**b**), I_d (**c**), and II (**d**) hair cells, where positive and negative OCM expression is indicated by filled and open symbols, respectively. Type I_c and II hair cells appear to be uniformly distributed within the striola, while type I_d hair cells tend to be concentrated around the striola perimeter. The exception to this is the rostral striola, where type I_d hair cells populate the striola interior

hair cell subtypes. Hence, we tested the hypothesis that the relative distributions of I_c•OCM(+) and I_d•OCM(+) were similar to the native distributions of striolar I_c and I_d hair cells. The alternative, of course, is that OCM expression is not randomly distributed among type I_c and I_d hair cells, but is more closely associated with one type. This test was achieved

through bootstrap resampling of the 628 total number of striolar OCM+ type I hair cells (i.e., only those within the striola across the three mouse utricles; Table 3) among the native subpopulations of type I_c and I_d hair cells. The restriction to intrastriolar hair cells was based upon the apparent lateral boundary of I_c hair cells represented by the LPR, and therefore

Table 3

Hair cell counts by oncomodulin expression and phenotype in the utricular striola and extrastriola. Extrastriola counts include only OCM+ hair cells outside the striola boundary, determined by the projection locus of CALB2+ hair cells. Hence, all I_c hair cells were contained within the striola. All counts reflect the mean of three specimens; standard deviations (i.e., *st. dev.*) are included within parentheses

Hair cell phenotype	Striola			Extrastriola
	Total (<i>st. dev.</i>)	OCM+ (<i>st. dev.</i>)	OCM- (<i>st. dev.</i>)	OCM+ (<i>st. dev.</i>)
All	442.3 (25.93)	255.0 (4.58)	187.3 (21.94)	50.7 (3.51)
I	252.7 (8.39)	209.3 (0.58)	43.3 (8.14)	42.3 (2.08)
I _c	193.3 (3.79)	173.7 (0.58)	19.7 (4.04)	—
I _d	59.3 (4.73)	35.7 (0.58)	23.7 (4.16)	42.3 (2.08)
II	189.7 (22.3)	45.7 (4.04)	144.0 (18.36)	8.3 (5.13)

Fig. 5. Summary of OCM expression among striolar hair cell phenotypes. **a** Distribution of total OCM+ hair cell comprised of mean fractions ($\pm$ standard deviation) represented by each primary phenotype. While most OCM+ hair cells were found to be type I_c , OCM was expressed by type I_d and II hair cells, as well. **b** This bar graph depicts the mean fraction ($\pm$ standard deviation) of each striolar hair cell phenotype that also exhibited OCM expression, illustrating that OCM was expressed 90 and 78 % of type I_c and I_d , respectively, while only approximately 25 % of type II hair cells exhibited were OCM+

this first analysis of OCM+ type I phenotypes also respected that spatial border. The expected distributions of $I_c \bullet \text{OCM}(+)$ and $I_d \bullet \text{OCM}(+)$ derived from the native I_c and I_d counts within the striolae of three specimens are represented by the histograms in Fig. 6a, the mean counts ($\pm$ standard deviation) for which are 480.6 ($\pm$ 10.7) and 147.4 ($\pm$ 10.7), respectively. The total empirical counts of $I_c \bullet \text{OCM}(+)$ and $I_d \bullet \text{OCM}(+)$ from the three utricles were 521 and 103 hair cells, respectively. The joint probability that these

counts resulted from random OCM expression among striolar type I hair cells was 0.00002. Therefore, when considering only OCM+ expression within the striola, this result indicates that OCM expression is strongly associated with type I_c hair cells that received CALB2+ calyces.

Although type I_c hair cells were largely confined to the striola bordered by the LPR at its lateral aspect, OCM+ hair cells were not. Consequently, the analysis resulting in Fig. 6a excluded $I_d \bullet \text{OCM}(+)$ type hair cells located lateral to the LPR. To elucidate a more comprehensive view of OCM expression among type I hair cells, we analyzed the associations within the utricular region harboring OCM+ hair cells, the results for which are shown in Fig. 6b. The histograms illustrate the distributions of $I_c \bullet \text{OCM}(+)$ and $I_d \bullet \text{OCM}(+)$ resulting from random resampling of 755 OCM+ hair cells based upon the native distributions found in this region. Note that the horizontal axes of Fig. 6a, b are identical, and the expected increase of $I_d \bullet \text{OCM}(+)$ (i.e., due to OCM+ type I hair cells lateral to the LPR) is readily apparent. The empirical counts for $I_c \bullet \text{OCM}(+)$ and $I_d \bullet \text{OCM}(+)$ in the three utricle specimens were 521 and 243, respectively, and the joint probability that these could have resulted from random expression of OCM among type I_c and I_d hair cells was 0.12. This supports the conclusion that, within the region of utricular epithelium in which OCM+ hair cells are located, OCM expression is associated with the native distribution of type I_c and I_d hair cells, and therefore can be considered to reflect the random distribution among type I hair cells found in that region.

Topology of OCM Expression

The data in Fig. 4 illustrate that striolar hair cell phenotypes may exhibit unique spatial distributions. For example, while the LPR represents the predominant feature of tissue polarity (Deans 2013), Fig. 4 shows that the distribution of type I_c hair cells did not strictly adhere to this boundary with a few hair cells exhibiting MPVs consistent with the lateral extrastriola. At the same time, OCM expression was also found in hair cells immediately lateral to the LPR with MPVs opposite the OCM+ hair cells found medial to the LPR. We examined the distribution of OCM+ hair cells with respect of the LPR to evaluate the extent to which these deviations were simply an extension of the distributions found within the striola. As argued for analyses of hair cell phenotype distributions throughout this investigation, such analyses provide insight into common factors that are responsible for the spatial distributions. At the same time, the absence of OCM expression was conspicuous among hair cells along the medial striolar border, and suggested a spatial attribute of OCM expression not specifically

Fig. 6. Resampled distributions of OCM+ type I_c and I_d drawn from native distributions in the striolae of three utricles. These histograms reflect the distributions of OCM+ type I_c (red) and I_d (blue) that resulted from 100,000 random resamples from all type I hair cells in the three specimens. The empirical counts of OCM+ I_c and I_d in these specimens are shown in red and blue arrowheads, respectively. **a** Resampled distributions of OCM+ hair cells within the striola (e.g., within the area delineated by the regions harboring CALB2+ calyces). The lateral border for this region is principally the LPR. Given these native distributions, the joint probability that the

empirical counts ($I_d \bullet \text{OCM}(+) = 103$, blue arrowhead; $I_c \bullet \text{OCM}(+) = 521$; red arrowhead) were derived from nonspecific OCM expression was 0.00002. **b** Resampled distributions of OCM+ hair cells from the region defined by all OCM expression, extending lateral to the LPR to include all OCM+ hair cells. The joint probability that the empirical counts were derived from the underlying type I_c and I_d distributions was 0.12, supporting this null hypothesis (e.g., OCM expression is randomly distributed among type I hair cell phenotypes)

associated with other striolar characteristics (e.g., medial to the LPR, and encompassing the region harboring CALB2+ calyces). Therefore, we further explored the spatial relationships of hair cell phenotypes relative to the LPR (Figs. 7 and 8), beginning with simple proportions based upon their MPVs indicating their position either medial or lateral to the LPR.

Throughout this report, we adhered to a definition of the striola as the region harboring CALB2+ calyces, which is bordered laterally by the LPR (Li et al. 2008). This is illustrated by Fig. 4, which also demonstrates that a small number of type I_c hair cells were located lateral to the LPR and exhibited MPVs consistent with these loci. Figure 4 also illustrates that while most OCM+ hair cells were medial to the LPR, several were lateral to the LPR and were represented by type I_c , I_d , and II phenotypes. The spatial distributions of OCM+ hair cells relative to the LPR are shown in Fig. 7. Figure 7a exemplifies how hair cell MPVs are determined through phalloidin labeling (Fig. 7a, inset), which establishes the basis for identifying hair cells with diametrically opposed orientations and, subsequently, the LPR. This confocal stack was obtained from the caudolateral portion of the striola. Figure 7b represents an immunostained optical section from the stack represented in A, to exemplify OCM+ hair cells (shown in red) found on either side of the LPR (the LPR from A was superimposed on this micrograph as the dashed white open contour). Type I

hair cells were identified by the TUBB3+ calyces (appearing as green “rings” in this single optical section). Type I_c hair cells associated with CALB2+ calyces are also identified (yellow asterisks), though the CALB2 immunolabeling was omitted from this micrograph in the interest of clarity. The summary distributions of OCM+ hair cells in relation to the LPR are shown in Fig. 7c, which depict the fractions of OCM+ hair cells within each phenotype that exhibited MPVs consistent with medial LPR hair cells. We termed these the *medial fractions*, and Fig. 7c illustrates that the medial fractions of $I_c \bullet \text{OCM}(+)$ and $\text{II} \bullet \text{OCM}(+)$ were $0.93 (\pm 0.007)$ and $0.85 (\pm 0.082)$, respectively. However, the medial fraction of $I_d \bullet \text{OCM}(+)$ hair cells was considerably less (0.51 ± 0.031), indicating that the medial fraction of $I_d \bullet \text{OCM}(+)$ hair cells was approximately equal to the fraction located lateral to the LPR. These data indicate that the factor(s) responsible for the spatial distribution of OCM expression in type I_d hair cells was (were) less tightly coupled to those governing the location of the LPR and other morphologic features of the striola, such as CALB2+ calyx projection loci.

The analysis shown in Fig. 6 demonstrated that the distribution of OCM+ type I hair cells *within the striola* (i.e., medial to the LPR) could not be accounted for by random expression among the native distribution of type I_c and I_d hair cells. However, expanding the region harboring OCM+ type I hair cells to include those lateral

Fig. 7. OCM+ hair cell distributions relative to the LPR. **a** Maximum intensity projection micrograph of a confocal stack from a phalloidin-labeled utricle. Phalloidin labeling of hair cell stereocilia enabled identification of the eccentric kinocilia loci, represented by the “voids” in labeling as highlighted by the white arrowheads in the inset micrograph. The LPR (blue dashed line) was delineated by tracing the tight junctions between hair cells with opposing MPVs. The lower and left region of the micrograph represents the striola, while the upper and right represents the lateral extrastriola. **b** Single optical section micrograph of region of utricular epithelium shown in **a**, albeit from deeper in the confocal stack and illustrating OCM and TUBB3 immunolabeling. CALB2 immunolabeling is not shown in the interest of sparing confusion with three labels, although type I_c hair cells (encapsulated by a CALB2+ calyx) were positively identified and indicated by the yellow asterisks. The LPR determined in **a** is superimposed on this micrograph to illustrate hair cell phenotypes on either side (white dashed line). The cyan dashed line represents the lateral border of the striola. I_c •OCM+ and I_d •OCM+ (hair cells encapsulated by TUBB3+ calyces with and without yellow asterisks, respectively) hair cells in the lateral extrastriola are

indicated by the red arrowheads. Hair cell loci on either side of the LPR were unequivocally identified by tracking to the surface of the confocal stack; this 3D feature is not possible in the 2D micrograph shown and therefore gives the appearance of a few hair cells that are “bisected” by the LPR. The scale bar (bottom left) represents 10 μ m, and corresponds to the large micrographs in both **a** (not the inset) and **b**. **c** Mean fractions of OCM+ type I_c , I_d , and II hair cells found medial to the LPR. **d** This graph summarizes the results of a resampling analysis testing the hypothesis that the distribution of OCM+ hair cells lateral to the LPR (total of 144 hair cells in three utricles) was based upon the native striolar distributions of type I_c and I_d hair cells (shown as the histograms). The empirical counts of I_c •OCM(+) and I_d •OCM(+) are shown as red and blue arrowheads, respectively, reflecting a joint probability of supporting the above-stated hypothesis was <0.00001 . This result supports rejection of this hypothesis, indicating that the lateral striola distribution of OCM+ type I hair cells is derived from factors different than those leading to the native intrastricola distribution of I_c •OCM(+) and I_d •OCM(+) hair cells

to the LPR led to a different interpretation, such that within this region OCM expression *did* reflect a random distribution among type I hair cells. We further explored this difference in topology through an analysis for OCM+ type I hair cells lateral to the LPR, which numbered 144 among the three utricles analyzed, to determine whether

their distribution was similar to the native distribution of striolar type I_c and I_d hair cells. The results of this analysis are shown in Fig. 7d, illustrating the predicted distributions of I_c and I_d OCM+ hair cells with lateral MPVs if they were governed by factors similar to those responsible for the native intrastricola distribution. As expected, these

Fig. 8. Spatial correlates of OCM+ hair cell loci. **a** Representative utricular striola illustrating the locations of all type I (triangles) and II (squares) hair cells. Each striolar hair cell is symbolized according to OCM expression (OCM+, filled symbols; OCM-, open symbols). The LPR is depicted by the dashed contour (cyan). This map demonstrates that I•OCM(-) hair cells (open triangles) are found predominantly along the medial striolar border. **b** Box-and-whisker plots showing the distributions

of hair cell locations (as distance from the LPR) by principal phenotype and OCM expression. I•OCM(+), II•OCM(+), and II•OCM(-) hair cells exhibited similar distances to the LPR (resampled Kruskal-Wallis test, $P < 0.00001$), while those of I•OCM(-) exhibited the greatest distance (resampled Kruskal-Wallis test, and Dunn-Holland-Wolf multiple comparison tests, $p = 0.00003$)

findings demonstrated that the probability the empirical distribution could have resulted from factors driving the native intrastriolar distributions was less than 0.00001. These findings further indicate that the factors responsible for the topologic distribution of OCM+ hair cells relative to the LPR are quite different from those affecting type I_c hair cells.

The maps of OCM expression among striolar hair cell phenotypes shown in Fig. 4 indicated that their spatial distribution may not be uniform, whereby

I•OCM(-) hair cells were found primarily along the medial striolar border. A similar topologic expression pattern was found for type II hair cells, but is manifested somewhat differently in that II•OCM(-) hair cells were distributed throughout the striola and II•OCM(+) hair cells appeared to exhibit locations closer to the LPR. These data are illustrated in Fig. 8a, in which all type I and II hair cells are shown for the same specimen mapped in Fig. 4, and hair cells positive or negative for OCM expression are shown as filled and unfilled

symbols, respectively. For this analysis, type I hair cells were considered in aggregate and not distinguished as I_c or I_d . The distance of each hair cell to the LPR was determined, and numerical distributions of these measures by phenotype (i.e., $I \bullet OCM(+)$, $I \bullet OCM(-)$, $II \bullet OCM(+)$, $II \bullet OCM(-)$) for all striolae ($n = 3$) are illustrated by the box-and-whisker plots shown in Fig. 8b. We first rejected the hypothesis stipulating similarity in the distance-to-LPR distributions across the four phenotypes (single factor resampling Kruskal-Wallis analysis of variance; $H = 180.6$; $p < 0.00001$).

Nonparametric multiple comparison tests were then conducted to identify differences in phenotype pairs, whereupon two principal findings were elucidated. First, comparison of the distance-to-LPR for $I \bullet OCM(-)$ and $I \bullet OCM(+)$ hair cells confirmed the observation apparent in Figs. 4 and 8a. This analysis revealed that the probability of the $I \bullet OCM(-)$ distances arising from a random sample of distances exhibited by all type I hair cells was considerably less than 0.00001 (Dunn-Holland-Wolf nonparametric multiple comparison; $Q = 5.56$, $Q_{c,100,000} = 4.13$). Second, the comparison between $II \bullet OCM(+)$ and $II \bullet OCM(-)$ phenotypes also revealed that the distance-to-LPR measures could not have derived from the same underlying distribution ($p = 0.00003$; Dunn-Holland-Wolf nonparametric multiple comparison; $Q = 4.25$, $Q_{c,99,997} = 4.19$, $Q_{c,99,998} = 4.26$). Therefore, these data support the conclusion that, among striolar type I and II hair cells, those that were OCM+ were located closer to the LPR than those that were OCM-.

DISCUSSION

Comprehensive Investigation of OCM Expression Refines Its Characterization as a Striolar Marker

Previous investigations have proposed OCM to be a hair cell marker for the mammalian utricular striola, largely by virtue of its expression in the similar topographical region harboring CALB2+ calyces (Collado et al. 2011; Simmons et al. 2010). Furthermore, these qualitative observations provided the impression that OCM was principally confined to type I hair cells (Collado et al. 2011). The present investigation provides more comprehensive analyses of OCM expression that revealed important deviations from the original observations that are critical for and expand the interpretation of these findings.

The current definition of the mammalian utricular striola represents a refinement of the criterion that includes the epithelial region populated by CALB2+ calyces (Desai et al. 2005), and it is now established that it is bordered laterally by the LPR (Li et al. 2008). While most OCM+ hair cells are contained within the striola, approximately 20 % were found lateral to the LPR and, therefore, outside of what is currently considered the

lateral striolar boundary. A similar observation can be gleaned from the data presented by Collado et al. (2011), who also showed OCM+ hair cells lateral to the LPR. Furthermore, Jiang et al. (2017) have recently reported that expression of the transcription factor *Emx2* in hair cells of the lateral extrastriola is responsible for the LPR, as it is required for MPVs opposite those of the medial extrastriola. Careful inspection of these most recent data (i.e., Fig. 1 in Jiang et al. (2017)) revealed that the lateral border of OCM-expressing hair cells extended beyond the medial border of *Emx2* expression. On the other hand, datasets from the present investigation as well as that of Li et al. (2008) would strongly indicate that the medial border of *Emx2* expression and the lateral border of CALB2-expressing calyces form a boundary with strong functional implications (e.g., physiologic polarization of hair cells and afferents). Furthermore, OCM expression does not extend to those hair cells closest to the medial striolar border, defined by the medialmost loci of CALB2+ calyces. Since the LPR is the most prominent morphologic landmark of the macular epithelia, closely associated with two other cellular phenotypes in hair cell *Emx2* and calyx CALB2 expression, it seems most prudent to use these as the principal defining characteristics of the striola. Hence, OCM cannot be considered an unequivocal marker for the striola, but exhibits a stereotyped lateral displacement from the striola proper.

Another key finding of the present investigation, promoting amendment of previous suggestions, is the expression of OCM within type II hair cells (Fig. 2). Earlier observations of what appeared to be close associations between OCM+ hair cells and type I-defining calyces suggested that OCM may be specific to the type I phenotype (Collado et al. 2011; Simmons et al. 2010). Through the use of quantitative criteria for defining OCM expression, we found that approximately 25 % of type II hair cells were OCM+. This enabled us to refute the hypothesis that OCM expression was random among striolar hair cells (Fig. 2e), and though not under the strict control of type I-specific factors (i.e., OCM is not expressed in all type I hair cells), it is strongly biased toward type I hair cells.

Tissue Polarity Correlates in Striolar Organization

A framework for the organization of the vestibular epithelia in the context of planar cell polarity was offered by Deans (2013), who advocated that the epithelia exhibit characteristics reflecting attributes of planar cell polarity that are best appreciated from the perspective of the entire epithelium, and therefore reflect a level of PCP termed *tissue polarity*. This included two iconic characteristics of utricular epithelia: the patterning of hair cells with opposing MPVs forming the LPR, and the restricted projection loci of afferent CALB2+ calyces on the medial side of the

LPR (Deans 2013; Li et al. 2008). As discussed above, these characteristics collectively form the striola (Li et al. 2008). Hair cell OCM expression also appeared to meet the criteria of being a tissue polarity characteristic (Collado et al. 2011; Jiang et al. 2017; Simmons et al. 2010), being constrained to peristriolar regions of epithelia. While the present study does not shed light on the cellular or molecular mechanisms through which putative tissue polarity may result, analyses of hair cell and afferent dendrite phenotypes that may reflect PCP could provide insight into the likelihood that they result from similar or shared underlying mechanisms.

Jiang and colleagues very elegantly showed that the patterning of utricular hair cell MPVs to form the LPR was dependent upon positive expression of *Emx2* in lateral extrastriolar hair cells, forming a stark contrast to medial LPR hair cells that were negative for *Emx2* expression (Jiang et al. 2017). Since most CALB2+ calyces project medial to the LPR (Fig. 7), there appears to be a strong negative topologic correlation between *Emx2* expression and CALB2+ calyx projection locus. This is highly suggestive of a potential cellular correlate linking these tissue polarity phenotypes. However, because *Emx2* is a hair cell transcription factor, the correlate must be indirect to affect CALB2 expression in afferent neurons. Any putative relationship between *Emx2* expression and CALB2 projection loci cannot be entirely responsible for these characteristics of tissue polarity, as the CALB2+ calyx projection loci extend only partially along the stark line of differential *Emx2* expression on either side of the LPR. Other factors must be acting to corral CALB2 expression in afferent neurons to the utricular striola.

Through the present analyses, we found features of hair cell OCM expression that infer a close association with underlying factors responsible for the distribution of CALB2+ calyces. Although OCM expression was not restricted to type I hair cells, its distribution was not the result of a general “striola factor” that would result in nonspecific expression in type I and II hair cells. Rather, it was overwhelmingly associated with type I hair cells. Analysis of OCM expression among type I hair cells indicated a nonspecific pattern in a region that did not conform to the LPR (and, therefore, boundaries established by *Emx2* expression) or CALB2+ calyx loci. This region, therefore, appears topologically distinct, and therefore appears to be derived from factors not specifically related to factors driving patterning of other striolar characteristics.

While the discussion above elucidates features of OCM expression that are different from other PCP-related phenotypes, there are also features that are similar to tissue polarity characteristics. These include the rostro-caudal extent of OCM expression, which is similar to that exhibited by the projection region of

CALB2+ calyces (Fig. 4). Therefore, OCM expression appears to be somewhat of a hybrid phenotype, exhibiting similarities and differences from phenotypes that are more closely related to PCP phenotypes. It may suggest that PCP molecular pathways render phenotypes broader than those related strictly to stereocilia polarization. On the other hand, if OCM expression is not manifested by PCP pathways the present data suggest that other factors contribute to phenotypes that resemble tissue polarity characteristics (i.e., these features are not dependent upon PCP pathways). Either way, the present evaluation of OCM expression reveals new avenues of investigation to more fully understand the factors responsible for the utricle's exquisite cellular organization.

Functional properties of OCM expression

The contribution of OCM to the physiology of the 57 % hair cells in which it is expressed has yet to be investigated. It is tempting to consider its molecular physiology to be similar to that of α -parvalbumin (α -PV), about which considerably more is known. α -PV has been associated with biexponential kinetics of Ca^{2+} buffering, which functions largely to steepen Ca^{2+} removal following a rapid influx and thereby truncate the action of free Ca^{2+} (Schwaller 2010). Buffering Ca^{2+} in this way would render functional characteristics similar to a high-pass filter. At the presynaptic active zone, this action could transform depolarization-mediated increase in local Ca^{2+} (i.e., via voltage-gated Ca^{2+} channels) to a transient increase, resulting in concomitant transient neurotransmitter release. Though it is not known whether oncomodulin exerts similar function in hair cells, these characteristics are consistent with the response dynamics of afferents projecting from the utricular striola (Goldberg et al. 1990). This characteristic of afferent dynamics is not likely to be due solely to OCM, though it is likely to be a contributing factor.

If OCM alters hair cell physiology, the present study indicates that the resulting coding alterations is not restricted to type I hair cells, and therefore reveals a physiologic subset of type II hair cells. Of course, the contribution of this hair cell phenotype to afferent response dynamics is unknown, but this finding reveals a new factor that should be considered in the factors that contribute to vestibular hair cell biology. In fact, the role of OCM may be easier to untangle in studies of type II hair cells, since the lateral striola harbors plenty of type II hair cells exhibiting both phenotypes, expediting the problem of electrophysiologic sampling.

In the cochlea, OCM is found exclusively in outer hair cells where it has been recently shown to play a crucial role in cochlear function (Tong et al. 2016). OCM-null animals exhibit large threshold increases in auditory

brainstem response and distortion product otoacoustic emission audiometry. These animals also exhibited partial outer hair cell loss in the more basal cochlear region. However, the function of OCM in the cochlea may have a unique role in outer hair cells that may not be generalized to vestibular hair cells. Its subcellular location is restricted to the base of the stereocilia bundle and along the basolateral membrane, quite different than the more widespread cytosolic distribution in vestibular hair cells (Simmons et al. 2010). In addition, OCM may be linked to hair cell electromotility through the function and expression of prestin (Tong et al. 2016), and while prestin is expressed in vestibular hair cells it does not convey electromotile function (Adler et al. 2003).

The findings of the present study support those of previous studies demonstrating a different pattern of expression for OCM from that of α -PV in adult mouse vestibular epithelia (Dememes et al. 1993; Simmons et al. 2010). Previous investigations showed that α -PV was also expressed in type I hair cells of the peristria region (epithelial region in the vicinity of the striola; the striola was not specifically delineated) of the mouse utricle and some Scarpa's ganglion neurons (Dememes et al. 1993). We found no evidence for OCM expression in the dendrites of Scarpa's ganglion neurons, while clearly identifying OCM in a subpopulation of type II hair cells. Meanwhile, Simmons and colleagues showed that α -PV expression persisted well into late adulthood, whereby OCM expression wanes with age and was absent in mice that were 10 months of age (Simmons et al. 2010). This age-dependent differential expression pattern was demonstrated with RT-PCR methods, providing further evidence supporting differential expression of the two parvalbumin isoforms. Collectively with our findings, these studies attest to the veracity of the immunohistochemical methods for elucidating OCM-specific expression.

Summary

Through the present analysis, we have used statistical methodologies to obtain insight into associations of hair cell and afferent phenotypes in investigating critical characteristics of the mammalian utricular striola. These analyses have demonstrated that the expression of OCM is sufficiently different from other striolar characteristics to conclude that the underlying factors responsible for its expression are distinct from other characteristics. These findings provide a foundation upon which further investigations can be launched to ameliorate our understanding of the mechanisms for cellular correlates of tissue polarity in vestibular epithelia.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

REFERENCES

- ADLER HJ, BELYANTSEVA IA, MERRITT RC JR, FROLENKOV GI, DOUGHERTY GW, KACHAR B (2003) Expression of prestin, a membrane motor protein, in the mammalian auditory and vestibular periphery. *Hear Res* 184(1-2):27–40. [https://doi.org/10.1016/S0378-5955\(03\)00192-8](https://doi.org/10.1016/S0378-5955(03)00192-8)
- COLLADO MS, THIEDE BR, BAKER W, ASKEW C, IGBANI LM, CORWIN JT (2011) The postnatal accumulation of junctional E-cadherin is inversely correlated with the capacity for supporting cells to convert directly into sensory hair cells in mammalian balance organs. *J Neurosci* 31(33):11855–11866. <https://doi.org/10.1523/JNEUROSCI.2525-11.2011>
- DEANS MR (2013) A balance of form and function: planar polarity and development of the vestibular maculae. *Semin Cell Dev Biol* 24(5):490–498. <https://doi.org/10.1016/j.semcdb.2013.03.001>
- DEANS MR, ANTIC D, SUYAMA K, SCOTT MP, AXELROD JD, GOODRICH LV (2007) Asymmetric distribution of prickle-like 2 reveals an early underlying polarization of vestibular sensory epithelia in the inner ear. *J Neurosci* 27(12):3139–3147. <https://doi.org/10.1523/JNEUROSCI.5151-06.2007>
- DECHESNE CJ, RABEJAC D, DESMADRYL G (1994) Development of calretinin immunoreactivity in the mouse inner ear. *J Comp Neurol* 346(4):517–529. <https://doi.org/10.1002/cne.903460405>
- DEMEMES D, EYBALIN M, RENARD N (1993) Cellular distribution of parvalbumin immunoreactivity in the peripheral vestibular system of three rodents. *Cell Tissue Res* 274(3):487–492. <https://doi.org/10.1007/BF00314545>
- DESAI SS, ZEH C, LYSAKOWSKI A (2005) Comparative morphology of rodent vestibular periphery. I. Saccular and utricular maculae. *J Neurophysiol* 93(1):251–266. <https://doi.org/10.1152/jn.00746.200300746.2003> [pii]
- DESMADRYL G, DECHESNE CJ (1992) Calretinin immunoreactivity in chinchilla and guinea pig vestibular end organs characterizes the calyx unit subpopulation. *Exp Brain Res* 89(1):105–108
- FERNANDEZ C, GOLDBERG JM, BAIRD RA (1990) The vestibular nerve of the chinchilla. III. Peripheral innervation patterns in the utricular macula. *J Neurophysiol* 63(4):767–780. <https://doi.org/10.1152/jn.1990.63.4.767>
- GOLDBERG JM, DESMADRYL G, BAIRD RA, FERNANDEZ C (1990) The vestibular nerve of the chinchilla. IV. Discharge properties of utricular afferents. *J Neurophysiol* 63(4):781–790. <https://doi.org/10.1152/jn.1990.63.4.781>
- JIANG T, KINDT K, WU DK (2017) Transcription factor Emx2 controls stereociliary bundle orientation of sensory hair cells. *elife* 6. <https://doi.org/10.7554/eLife.23661>
- LI A, XUE J, PETERSON EH (2008) Architecture of the mouse utricle: macular organization and hair bundle heights. *J Neurophysiol* 99:718–733. <https://doi.org/10.1152/jn.00831.2007>
- LINDEMAN HH (1969) Studies on the morphology of the sensory regions of the vestibular apparatus with 45 figures. *Ergeb Anat Entwicklsgesch* 42(1):1–113
- SCHWALLER B (2010) Cytosolic Ca²⁺ buffers. *Cold Spring Harb Perspect Biol* 2:a004051. <https://doi.org/10.1101/cshperspect.a004051>
- SCHWEIZER FE, SAVIN D, LUU C, SULTEMEIER DR, HOFFMAN LF (2009) Distribution of high-conductance calcium-activated potassium

- channels in rat vestibular epithelia. *J Comp Neurol* 517(2):134–145. <https://doi.org/10.1002/cne.22148>
- SIMMONS DD, TONG B, SCHRADER AD, HORNAK AJ (2010) Oncomodulin identifies different hair cell types in the mammalian inner ear. *J Comp Neurol* 518(18):3785–3802. <https://doi.org/10.1002/cne.22424>
- TONG B, HORNAK AJ, MAISON SF, OHLEMILLER KK, LIBERMAN MC, SIMMONS DD (2016) Oncomodulin, an EF-hand Ca^{2+} buffer, is critical for maintaining cochlear function in mice. *J Neurosci* 36(5):1631–1635. <https://doi.org/10.1523/JNEUROSCI.3311-15.2016>