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Oncomodulin Identifies Different Hair Cell Types in the Mammalian Inner Ear

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Abstract

The tight regulation of Ca^{2+} is essential for inner ear function, and yet the role of Ca^{2+} binding proteins (CaBPs) remains elusive. Using immunofluorescence and RT-PCR, we investigated the expression of oncomodulin (Ocm), a member of the parvalbumin family, relative to other EF-hand CaBPs in cochlear and vestibular organs in the mouse. In the mouse cochlea, Ocm is found only in outer hair cells and is localized preferentially to the basolateral outer hair cell membrane and to the base of the hair bundle. Developmentally, Ocm immunoreactivity begins as early as postnatal day (P) 2 and shows preferential localization to the basolateral wall and hair bundle after P8. Unlike the cochlea, Ocm expression is substantially reduced in vestibular tissues at older adult ages. In vestibular organs, Ocm is found in type I striolar or central hair cells, and has a more diffuse subcellular localization throughout the hair cell body. Additionally, Ocm immunoreactivity in vestibular hair cells is present as early as E18 and is not obviously affected by mutations that cause a disruption of hair bundle polarity. We also find Ocm expression in striolar hair cells across mammalian species. These data suggest that Ocm may have distinct functional roles in cochlear and vestibular hair cells.

Keywords

Vestibular; Cochlea; ®-Parvalbumin; Ca^{2+} Binding Protein; Hair Cell

INTRODUCTION

A large number of Ca^{2+} binding proteins (CaBPs) of the EF-hand family play a role in buffering Ca^{2+} levels in hair cells and ganglion cells of the mechanosensitive end organs for hearing and balance (Hackney et al., 2005; Harter et al., 1999; Imamura and Adams, 1996; Pack and Slepecky, 1995; Thalmann et al., 1997). These proteins are all characterized by a common sequence of around 30 residues forming a helix-loop-helix motif (Lewit-Bentley and Rety, 2000). Calmodulin, calbindin, calretinin, and α -parvalbumin are present in the inner ear but at differing levels and, particularly within vestibular organs, have expression patterns that vary across species (Harter et al., 1999; Pack and Slepecky, 1995; Sage et al., 2000). In vestibular organs, CaBPs are better characterized within afferent terminals than they are within hair cells. Studies in mouse, rat, guinea pig, and gerbil find calretinin in ganglion cells, cochlear inner hair cells, and afferent calyces surrounding vestibular type I hair cells (Dechesne et al., 1994; Dechesne et al., 1993; Desai et al., 2005b; Kevetter and

Leonard, 2002b; Leonard and Kevetter, 2002; Zheng and Gao, 1997). Importantly, the presence of calretinin in calyx endings has been used to identify specific regions of vestibular sensory organs (Desai et al., 2005a; Desai et al., 2005b; Li et al., 2008). Calbindin is found in ganglion cells, cochlear hair cells, vestibular calyx endings, and vestibular hair cells (Dechesne and Thomasset, 1988; Dechesne et al., 1988; Pack and Slepecky, 1995). α -Parvalbumin is found in inner hair cells in the adult cochlea and some type I hair cells in vestibular organs (Pack and Slepecky, 1995; Soto-Prior et al., 1995; Yang et al., 2004; Zheng and Gao, 1997). Studies of CaBPs within the inner ear is further complicated by the fact that their expression changes during development and is not consistent across species. For example, α -parvalbumin is expressed in inner and outer hair cells at birth in the rat and is restricted to the inner hair cells after birth (Yang et al., 2004). In most species, calretinin is expressed in vestibular hair cells during development and is found in adult vestibular hair cells in some species but not others (Bermingham et al., 1999; Dechesne et al., 1994; Desai et al., 2005b; Zheng and Gao, 1997). Although oncomodulin (Ocm), a membrane of the parvalbumin family, has been studied for some time (MacManus, 1979) all we know is that it is found in outer hair cells (Celio, 1990; Pack and Slepecky, 1995; Sakaguchi et al., 1998; Soto-Prior et al., 1995; Yang et al., 2004). The role of Ocm in inner ear function remains elusive.

Given that Ocm is a CaBP its role in the regulation of Ca^{2+} concentration may be important to the function of cochlear outer hair cells (Hackney et al., 2005). While most CaBPs are found extensively throughout central and peripheral nervous systems, Ocm has a restricted expression pattern (Henzl et al., 1997; Sakaguchi et al., 1998; Thalmann et al., 1995; Thalmann et al., 1997; Yang et al., 2004; Yin et al., 2006). Recently, Ocm was found in activated macrophages in the eye and suggested to play a role in the regeneration of retinal ganglion axons (Yin et al., 2006) although this suggestion is controversial (Hauk et al., 2008). In the inner ear, macrophages or other blood tissue cells also have Ocm immunoreactivity (Yang et al., 2004). Although there have been reports that Ocm-like proteins are abundant within vestibular hair cells of nonmammalian species (Heller et al., 2002; Shin et al., 2007), there has been no comparable studies within mammalian vestibular organs. The discovery of Ocm in either hair cells or terminals elsewhere in the inner ear may contribute to uncovering whether Ocm function differs across cell types. Although little is known about the function of Ocm in the ear, it is reasonable to infer that Ocm plays a role in the function of Ca^{2+} -sensitive hair cell bundle processes such as transduction and adaptation as well as outer hair cell body motility because of its extensive expression (Sakaguchi et al., 1998; Shin et al., 2007; Yang et al., 2004). In this study, we investigated *Ocm* mRNA expression and Ocm immunoreactivity within the mouse inner ear. We compared Ocm expression with the expression of other CaBPs in vestibular and cochlear neuroepithelia. We found that Ocm may uniquely identify a subset of vestibular hair cells in the mouse as well as in other mammalian species. Further, Ocm may be compartmentalized within cochlear outer hair cells.

MATERIALS AND METHODS

Animal and tissue preparation

Mice (strains C57BL6, CBA-CaJ, CD1) were bred in-house or obtained from either Jackson Labs (Bar Harbor, ME) or Taconic Farms (Hudson, NY). Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Chinchilla ears were kindly provided by L. Hoffman (UCLA). Ears from the Looptail mutant mouse were kindly provided by Mark Warchol (Washington University in St. Louis). Animals were given near-lethal injections of sodium pentobarbital (Nembutal, 100 mg/kg, i.p.) and euthanized by decapitation. The day of birth (E19.5 – E20.5 for mice) represented postnatal day 0 (P0). All experimental procedures were approved by animal committees and conducted according to

the guidelines for Animal Research at Washington University school of Medicine and the University of California, Los Angeles.

RT-PCR

Following anesthesia, temporal bones were removed and dissected free of extraneous tissues (nerve and muscle). Whole cochleas were separated from temporal bones while in RNALater (Ambion, Austin, Texas). Vestibular organs were microdissected separately in RNALater. Both cochlear and vestibular tissues were immediately frozen in liquid nitrogen and stored at -80°C . RNA was isolated using a Qiagen RNeasy mini RNA isolation kit according to manufacturer's instruction. cDNA was synthesized using either a Retroscript cDNA synthesis kit (Ambion) or a Superscript II cDNA synthesis kit (Invitrogen) with random hexamers as primers. To control for genomic DNA, parallel reactions were run without reverse transcriptase. Primers were designed specifically to amplify cDNA from mouse *Ocm*, α parvalbumin, calretinin, and calbindin (Table 1). TATA binding protein (Willems, 2006), β -actin and GAPDH primers were used as internal controls. PCR reactions were run at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 sec, 50°C (or the temperature specified in Table 1) for 1 minute, 72°C for 2 minutes, and followed by 72°C for 5 minutes and a 4°C hold. PCR products were separated on 1 or 2 % agarose gels.

In order to verify the presence of *Ocm* expression, we used a nested PCR strategy. Two sets of primers were designed, one pair inside of the other. In the first reaction, the outer pair was used. In the second reaction, the inner pair was used to amplify the *Ocm* amplicon generated in the first reaction.

Immunocytochemistry

For immunocytochemical experiments, we used a minimum of three (3) animals for each condition. Ears were typically prepared either as whole sensory organ preparations or sectioned on a Vibratome. In multiple labeling experiments, antibodies were applied to serial tissue section sets that included one section for multiple labeling and one section for single labeling control sections. Tissue was pre-treated with 0.3% Triton X 100 in PBS, then blocked in PBS containing 10% normal chick serum. Primary antibodies were made in the appropriate blocking solution and applied to tissues overnight at 4°C . Alexa fluor conjugated secondary antibodies (Invitrogen) made in chick were used at 1:500 dilution as appropriate and incubated for 2 hours at room temperature. Whole mount organs or sections were then mounted onto glass slides and coverslipped using Mount Quick Aqueous (Fisher). Immunostaining was visualized using confocal microscopy.

With all antibodies used, the pattern of cellular morphology and distribution in the inner ear were identical with previously published reports (Dechesne et al., 1994; Slepecky and Chamberlain, 1985; Slepecky and Ulfendahl, 1993; Thalmann et al., 1995; Usami et al., 1995; Zheng and Gao, 1997). Please see Table 2 for a list of all antibodies used and their immunogens, manufacturer, and dilutions.

Several control experiments were performed on primary and secondary antibodies used to visualize *Ocm*. As seen in Table 2, we used both a polyclonal rabbit anti-*Ocm* (OM3, Swant, Bellinzona, Switzerland) and a monoclonal mouse anti-*Ocm* (T. Henzl, University of Missouri, Columbia, MO USA) that were raised against recombinant full-length *Ocm*. For the OM3 antiserum, a recent study performed antibody control experiments that included incubation with normal rabbit serum instead of rabbit polyclonal anti-*Ocm*, and preadsorption of OM3 with an *Ocm* blocking peptide (Csillik et al., 2009). In both cases, there was an absence of staining. For the monoclonal anti-*Ocm*, specificity has been

demonstrated by immunoblot and immunohistochemistry in guinea pig and rat tissues (Henzl et al., 1997; Sakaguchi et al., 1998). Immunoblots of rat and guinea pig cochlear extracts displayed single bands around 12 kDa and isoelectric points comparable to recombinant Ocm. On cochlear sections, staining with both the monoclonal and polyclonal antibodies yielded nearly identical results. However, the monoclonal antibody did not label in vestibular whole mounts as well as the Swant polyclonal antibody. Both antibodies were also tested on inner ear tissues from mice with a targeted deletion of Ocm (Ocm^{tm1.1Ddsi}, MGI:97401). In all cases, there was no Ocm labeling in tissues from null mutants with either antibody (see supplementary Figure 1). Finally, omission controls were performed for the secondary antibodies used. In all such cases, there was no labeling by the secondary antibodies (data not shown).

The specificity of the rabbit anti-parvalbumin (PV-25, Swant, Bellinzona, Switzerland) was previously tested in the cerebral cortex of a parvalbumin knock-out mouse where there was an absence of labeling (Schwaller et al., 1999b). Parvalbumin labeling was found in the inner hair cells in adult mice and in inner and outer hair cells in newborn mice similar to previous studies (Pack and Slepecky, 1995; Yang et al., 2004).

The specificity of the mouse monoclonal anti-calbindin D-28k (300, Swant, Bellinzona, Switzerland) was previously demonstrated by immunohistochemical and immunofluorescent labeling in wild type mice where Purkinje cells were strongly labeled and an absence of labeling in knock-out mice, and by immunoblots and radioimmunoassay of rat brain tissues all showing a single protein band of roughly 28 kDa (Celio, 1990). Using this antibody, calbindin labeling is also found in vestibular and cochlear hair cells as described by previous reports (Dechesne and Thomasset, 1988; Pack and Slepecky, 1995).

We used two anti-calretinin antibodies. The specificity of the rabbit anti-calretinin (AB149/AB5054, Chemicon/Millipore, Temecula, CA) was tested by immunohistochemical staining of cerebellar neurons in human and rat tissues and by western blot of rat brain homogenates showing a protein band of appropriate molecular weight (according to manufacturer's product data sheet). The specificity of the goat anti-calretinin (CG1, Swant, Bellinzona, Switzerland) was tested by immunoblot and immunohistochemistry. The appropriate cellular targets in rat cerebellar tissue were labeled in all cases and the immunoblots of rat muscle homogenates demonstrated the common dimerized form at roughly 60 kDa (Schwaller et al., 1999a). Furthermore, we confirmed specificity by immunofluorescent staining of rat and mouse cerebellum as well as by staining of rat and mouse utricle. Both calretinin antibodies demonstrated identical patterns of labeling in the rat and mouse brain and inner ear tissues as previously described (Dechesne et al., 1994; Imamura and Adams, 1996; Pack and Slepecky, 1995).

The specificity of the mouse monoclonal anti-tubulin (T6793, Sigma-Aldrich, St. Louis, MO) was previously demonstrated by immunoblot of rat brain extracts and immunogold electron microscopy. Acetylated tubulin from the outer arm of *Strongylocentrotus purpuratus* (sea urchin) was used as the immunogen. In cultures of 3T3 and HeLa cells monoclonal anti-tubulin (6-11B-1) binds to primary cilia, centrioles, mitotic spindles, midbodies, and to subsets of cytoplasmic microtubules (LeDizet and Piperno, 1986; Morales and Fikova, 1991; Piperno and Fuller, 1985). Tubulin labeling of hair cell kinocilia and nerve fibers in the rat and mouse inner ear was identical to previous studies (Pack and Slepecky, 1995; Slepecky, 1995; Tannenbaum, 1995).

Imaging

Using a laser scanning confocal microscope (Zeiss LSM 5 or BioRad Radiance 2000, Thornton, NY, USA), inner ear sensory organs were sequentially scanned at high (500–

1000 $\times$) magnification, exciting the green (488 nm), red (543 nm) and far-red (637 nm) channels. Fluorescent emissions were separated with appropriate blocking and emission filters, scanned at slow (50 lines/s) scan speeds for high resolution, and independently detected with 8-bit accuracy by photomultiplier tubes using, if necessary, accumulation to increase signal and reduce noise. Three-dimensional images of serially reconstructed image stacks from the confocal microscope were rendered using Volocity (v4.xx; Improvision, PerkinElmer, Shelton, CT). Whole mount images were captured in the X–Y plane and digitally rotated and viewed in the X–Z plane. Z-projections of image stacks were routinely performed. Single images were exported to Canvas (vX, ACD Systems, Canada) or Photoshop (Adobe Systems, USA) and image quality (brightness/contrast or histogram levels) adjusted to maximize signal and minimize background.

RESULTS

Endogenous expression of Ocm in the inner ear

In the mammalian ear, Ocm has only been shown in cochlear outer hair cells. We wanted to address two questions. First, is Ocm restricted to cochlear hair cells and not found in vestibular hair cells within mammals? Second, are there differences between the developmental expression of Ocm and other EF hand CaBPs in the inner ear? We used RT-PCR to perform a qualitative investigation of Ocm expression in cochlear and vestibular tissues. The endogenous expression of Ocm was investigated in older (10 month) adult (Figure 1a) and young postnatal (Figure 1b) mice. Since Ocm is closely related to α PV, we designed primer sets that separately amplified Ocm and α -parvalbumin sequences. In Figure 1a, results from an RT-PCR are shown in groups of five with and without reverse transcriptase (RT) reaction: cochlea +RT, cochlea –RT, vestibular +RT, vestibular –RT, and no DNA control. In cochlear tissues from older adult mice, both α -parvalbumin and Ocm demonstrated high levels of mRNA expression as expected from previous studies (Yang et al., 2004). In contrast, vestibular tissues from 10-month-old adult mice showed high levels of α -parvalbumin mRNA expression but little, if any, detection of Ocm mRNA expression. Additionally, we detected only minimal levels of calbindin or calretinin mRNA (data not shown). The relative level of α -parvalbumin mRNA expression in vestibular tissue was higher than in the cochlea. We performed several controls; shown are negative RT, negative cDNA, and TATA box mRNA expression controls. Additional β -actin and G3PDH gene controls were also used (not shown).

Young postnatal mice contrasted with older adult mice in our ability to detect CaBP expression. Inner ear tissue was collected from P3, P5 and P8 mice. Using RT-PCR analysis, we found mRNA expression for α PV, Ocm, calretinin, and calbindin in both cochlear and vestibular tissues (Figure 1b). Additionally for Ocm mRNA expression, we used a nested RT-PCR strategy to increase the specificity of DNA amplification and reduce potential background due to non-specific amplification of DNA. TATA binding protein, G3PDH and β -actin were used as additional gene controls (not shown). Vestibular and cochlea tissues demonstrate differences with respect to Ocm expression as a function of age. The presence of Ocm mRNA in vestibular tissues from younger animals and our inability to detect Ocm in vestibular tissues from older animals is consistent with reports suggesting a generalized decrease in CaBPs within the vestibular periphery as animals age (Kevetter and Leonard, 2002a).

Localization of Ocm immunoreactivity in the mouse cochlea

Although Ocm immunoreactivity has been previously described in the cochlea (Hackney et al., 2003; Sakaguchi et al., 1998; Yang et al., 2004), there is little information on its subcellular localization and colocalization with other CaBPs during development. To assess

Ocm subcellular localization at the light-microscopic level, we used both monoclonal and polyclonal antibodies directed against rat recombinant Ocm. As previously described in the rat (Yang et al., 2004), we found Ocm immunoreactivity in cochlear outer hair cells (Figure 2). In the basal regions of the P10 mouse cochlea, Ocm labeling was confined to outer hair cells. However, the mouse also demonstrates weak Ocm immunoreactivity in inner hair cells located in the apical turn (data not shown). Ocm localizes to outer hair cells with no obvious distinction among outer hair cell rows. Within basal turn outer hair cells, Ocm immunoreactivity was preferentially localized to the basolateral membrane and separately to the lower portion of the hair bundle and upper portion of the cuticular plate region (Figure 2a). However, Ocm did not colocalize with phalloidin labeling. The lateral wall is a distinctive structure of outer hair cells consisting of the plasma membrane highly-enriched with the motor protein prestin, an actin-spectrin cortical lattice, and one or more layers of subsurface cisternae (Holley et al., 1992; Jensen-Smith and Hallworth, 2007). In longitudinal views of an adult mouse organ of Corti, we could see intense Ocm labeling along the apical portion of the cuticular plate (Figure 2b). Again, this view demonstrated very little, if any, overlap between phalloidin staining and Ocm immunoreactivity. Longitudinal views of basal turn organ of Corti also show the most intense Ocm localization (Figure 2b,d) associated with the lateral wall. Some punctuated labeling was also observed at the synaptic pole of the outer hair cell. Using high threshold, narrow band tuning (Figure 2b **inset**), confocal scans show Ocm immunoreactivity localized to the basal wall and to the base of the hair bundle suggesting that the highest Ocm concentrations may be in these regions.

In guinea pigs, components of the protein phosphorylation cascade—IP₃ receptors and calmodulin-dependent protein kinase type IV (CaMK-IV)—immunolocalize to the lateral wall of outer hair cells (Frolenkov et al., 2000). To determine whether Ocm colocalizes with proteins associated with the lateral wall, we immunolabeled for Ocm and CaMK-IV. In basal turn outer hair cells, labeling for both Ocm and CaMK-IV was concentrated at the cell cortex, along the lateral wall between the nucleus and the cuticular plate (Figure 2c,d). In the P10 mouse, CaMK-IV, but not Ocm, also showed extensive labeling in the cytoplasmic region between the nucleus and cuticular plate (Figure 2c). Some punctuated colabeling was also observed at the synaptic pole of the outer hair cell, for both Ocm and the CaMK-IV. In P10 rat outer hair cells, Ocm labeling demonstrated a continuous association with the lateral wall while the CaMK-IV labeling was more punctate (Figure 2d). The pattern of labeling observed for Ocm and CaMK-IV suggests an association with the cortical cytoskeleton or the plasma membrane.

Onset of Ocm immunoreactivity in cochlear hair cells

Although the onset of Ocm immunoreactivity in the mouse cochlea occurs about 1–2 days later than we found in rats, it follows the onset of synapse formation from brainstem cholinergic axon terminals similar to rats (Osman et al., 2008; Simmons, 2002). Immunoreactivity to Ocm in the mouse is found first in basal regions of the postnatal cochlea and then in apical regions. The earliest immunoreactivity to Ocm was found in only 1 out of 3 mice examined at P2. Immunoreactivity was found in 2 out of 4 mice at P3 (Figure 3a). Immunoreactivity was always present in basal regions of the cochlea by P4 (n=5). In the cochlear apex, Ocm immunoreactivity was present by P6.

In adult animals, we could not find any overlapping immunoreactivity between Ocm and α -parvalbumin, calbindin or calretinin in basal regions of the cochlea. However when Ocm immunoreactivity first appears between P2 and P4, it overlaps extensively with calretinin and calbindin-D28k. In the P3 cochlea, calretinin and calbindin label both inner and outer hair cells with slightly more intense labeling at the cuticular plate (Figure 3a). In contrast, Ocm shows only diffuse labeling in outer hair cells with little or no immunoreactivity in the

cuticular plate. At P6, calretinin immunoreactivity is reduced in the outer hair cells and Ocm immunoreactivity is more intensely uniform in the outer hair cells (Figure 3b). However, Ocm immunoreactivity is not readily detected near the cuticular plate or in the stereocilia. Additionally, apical inner hair cells show very weak Ocm immunoreactivity as well as calretinin labeling. Our previous studies with α -parvalbumin in the rat suggest that α -parvalbumin is also present in inner and outer hair cells during early postnatal development suggesting that outer hair cells express α -parvalbumin, calbindin and calretinin at the onset of Ocm expression (Yang et al., 2004). By P8, outer hair cells in basal cochlear regions uniquely express Ocm whereas inner hair cells, for example, express α -parvalbumin (Figure 3c). Also around P8, Ocm immunoreactivity begins to label preferentially the lateral wall of the outer hair cell. As previously mentioned, the lateral wall is a distinctive structure of outer hair cells and previous studies have associated lateral wall F-actin and prestin as being the primary determinants of outer hair cell mechanical properties before and after hearing onset, respectively (Jensen-Smith and Hallworth, 2007). Preferential labeling of the lateral wall at P8 would be consistent with a role for Ocm in outer cell motility. In the very apex of the adult mouse cochlea, we found weak Ocm labeling in the inner hair cells as well as weak α -parvalbumin labeling in the outer hair cells (data not shown). Similar to α -parvalbumin, calbindin labels only inner hair cells and not outer hair cells in basal regions of the adult cochlea (data not shown).

Ocm is associated with striolar and central regions of vestibular sensory organs

Our RT-PCR analysis suggests that Ocm is present in the vestibular system of young animals but not older (10 month or greater) adult animals (see Figure 1). Utricles and cristae from young (6 week) adult mice were immunolabeled for Ocm. Immunoreactivity was absent from most regions of the sensory epithelia. However, as shown in Figure 4a, a limited, centralized band of hair cells consistently labeled for Ocm. We also found significant differences in Ocm immunoreactivity between young and old adult mice. At six weeks, Ocm-positive cells formed a relatively dense band of hair cells with uniform immunostaining (Figure 4a). However at six-to-eight months, Ocm immunoreactivity became limited to a much more sparse band of hair cells with irregular shapes and immunostaining (Figure 4b). We also investigated whether Ocm immunoreactivity labels a similar band of vestibular hair cells in other species. In both the rat (see Figure 4f) and chinchilla (see supplementary Figures), we found a centralized band of Ocm-positive hair cells in utricle, saccule, and cristae.

The subcellular pattern of immunolocalization in vestibular hair cells differed considerably from cochlear outer hair cells (Figure 4c,d). Cross sections taken from the adult utricle showed intense Ocm labeling in a subset of utricular hair cells. Typical of vestibular Ocm labeling, these hair cells labeled diffusely throughout their cytoplasm with reduced or diminished labeling near the cell nucleus. Unlike cochlear outer hair cells, intense Ocm labeling extended into the cuticular plate and throughout most of the hair bundle (Figure 4c,d). In vestibular hair cells, labeling for both Ocm and CaMK-IV differed between the mouse and rat. The label for Ocm and CaMK-IV colocalized mostly at the narrowed neck region of utricular hair cells from the P10 mouse (Figure 4e). Both Ocm and CaMK-IV diffusely labeled in the hair cell cytoplasm. In the P10 rat utricle, Ocm and CaMK-IV were found near the plasma membrane (Figure 4f). The CaMK-IV labeling was concentrated near the cell surface whereas the Ocm was also found diffusely throughout the cytoplasm.

To investigate the relation between Ocm immunoreactive hair cells and the striolar region, we determined hair cell morphological polarization patterns and the presence of calretinin-labeled calyceal endings. Similar to previous reports regarding the distribution of calretinin-labeled calyceal endings in striolar and central zones of the mouse (Desai et al., 2005a; Desai et al., 2005b), Ocm-positive hair cells in the adult mouse form a distinctive crescent-

or J-shaped band of cells in the utricle (Figure 5a), an S-shaped band of cells in the saccule, and an oval-shaped band of cells in the cristae. The region that is medial to or encompasses the reversal line where hair bundle morphological polarization reverses is referred to as the striola in macular organs (Desai et al., 2005a; Desai et al., 2005b; Li et al., 2008; Schweizer et al., 2009). Typically, the morphological criteria used to delineate the striola include: a lower density of hair cells, the presence of complex calyx type endings, and the line of polarity reversal. Consistent with recent reports on the polarization patterns of striolar hair cells (Li et al., 2008; Schweizer et al., 2009), most Ocm-labeled hair cells had similar hair bundle orientation and were medial to the line of polarity reversal (Figure 5b,c). The morphological polarization vectors of the hair bundle can be determined with the actin-filament marker phalloidin (Li et al., 2008; Schweizer et al., 2009). The actin-containing stereocilia and cuticular plate are stained by phalloidin but not the kinocilium, which is made up of tubulin. There is an absence of phalloidin-staining where the kinocilium is located. The morphological polarization vector of hair cells was determined by locating the central point of its apical surface and drawing a vector to the kinocilium. The line of polarity reversal was determined as the location where hair bundle orientation vectors (Figure 5b, **arrows**) reversed. Recent studies also suggest that the presence of calretinin-positive calyces coincides with the striola and central zones in mammalian vestibular organs (Desai et al.; Desai et al.; Kevetter and Leonard, 2002b; Leonard and Kevetter, 2002; Li et al., 2008). In our Ocm and calretinin double-stained organs from adult mice (Figure 5d,e), vestibular hair cells in the utricle and cristae were distinguished by presence or absence of Ocm labeling and calretinin-positive calyceal endings. Most, if not all, Ocm-positive hair cells had calretinin-positive calyceal endings. In a very few cases, weak calretinin-positive calyceal labeling was also found on Ocm-negative hair cells. During development, there were calretinin positive hair cells, but none of these calretinin-positive hair cells had calretinin-positive calyceal endings (data not shown). In the adult utricle, calyceal endings were also labeled by α -parvalbumin and were found extensively on Ocm-positive hair cells (Figure 5f). Such calyceal labeling indicates that the majority, if not all, Ocm-labeled hair cells are also type-I hair cells.

Onset of Ocm immunoreactivity in cochlear and vestibular hair cells

Vestibular hair cells showed an earlier onset of Ocm immunoreactivity than cochlear hair cells. At P3 when cochlear Ocm immunoreactivity was absent in half of the mice investigated, it was present in macular organs (Figure 6a,b) and cristae (Figure 6c,d) in all mice examined (n=4). Similar to young adult ears, Ocm immunoreactivity at these early postnatal ages was limited to a distinct band of centralized hair cells. Vestibular hair cells at birth (P0) as well as in the embryo (E18) also expressed Ocm immunoreactivity. At birth, Ocm immunoreactivity is extensive and labels nearly one-quarter to one-third of hair cells in, for example, the cristae (Figure 6e,f). The Ocm-positive hair cells form a very dense band with a high percentage (~80–90%) of hair cells that had phalloidin-labeled hair bundles also labeling for Ocm. However unlike seen in young adult ears, the Ocm labeling intensity in these embryonic and P0 ears was variable: the most intensely labeled hair cells are towards the center of the band and the least intensely labeled hair cells are towards the peripheral edge of the band. At E18, Ocm immunoreactivity is also found in all vestibular organs as a distinct centralized region of labeling (Figure 6g,h).

We also investigated whether the topography of Ocm labeling was preserved in mutants with defects in their polar cell polarity (PCP) organization. For example, the murine ortholog of *vang*, called *vangl2*, is mutated in *Looptail* (Lp) mice, resulting in individual hair cells that remain polarized but are misoriented relative to each other (Deans et al., 2007). The Lp mutant has vestibular sensory epithelia that lack a line of reverse polarity and raises the question of whether they have an organized striola. Since these animals typically die at

birth, we were limited to studies of embryonic Ocm immunoreactivity. Similar to wild-type mice, Ocm immunoreactivity shows a distinct band of centralized hair cells in all vestibular sensory epithelia of the Lp mutant (Figure 7a,b). Neurofilament-labeled afferent endings formed calyces on Ocm immunoreactive hair cells (Figure 7c). We could find no qualitative differences between wild-type and Lp mutant Ocm immunoreactivity. Defects in PCP apparently do not change the position or pattern of Ocm-positive hair cells in vestibular organs.

DISCUSSION

The major findings of this study are illustrated in the schematic shown in Figure 8. In the cochlea, our new finding is that β -parvalbumin CaBP, Ocm, may be localized to the basolateral wall of outer hair cells as well as in the hair bundle. In the peripheral vestibular system, our findings lead us to hypothesize that Ocm identifies striolar hair cells of the utricle and saccule as well as central zone hair cells of the cristae. Additionally, our results suggest Ocm may be found preferentially in the vestibular type I hair cells found in the striola and central zone. Compared to other CaBPs investigated in the inner ear, our results show that Ocm has a unique expression pattern that differs across sensory neuroepithelia. In young adult ages, we can find no evidence of any significant overlapping expression of Ocm with other CaBPs in hair cells, but during embryonic and early postnatal ages Ocm expression overlaps with the expression of other CaBPs. Our studies further suggest that Ocm onset and localization differs between cochlear outer hair cells and vestibular striolar hair cells, which may have functional consequences for the role(s) Ocm has in these sensory hair cell types.

Ocm in Vestibular Hair Cells

That Ocm expression defines a subpopulation of hair cells restricted to striolar and central zone regions of maculae and cristae, respectively, is a hypothesis put forward by this study. Our data show that Ocm expression in vestibular hair cells not only has an earlier embryonic onset than cochlear hair cells, but also is down-regulated in adult vestibular hair cells before cochlear hair cells. Such down-regulation of Ocm expression is similar to reports suggesting a generalized decrease in CaBPs in vestibular organs as animals age (Kevetter and Leonard, 2002a). Further, Ocm regulation appears independent of genes that control for hair bundle polarization patterns (Deans et al., 2007). Striolar and central hair cell regions give rise to vestibular afferents that have more phasic, irregular responses compared to afferent responses originating outside of these regions (Goldberg et al., 1992). Striolar regions can be distinguished by several morphological characteristics such as smaller overlying otoconia, larger, less densely packed hair cells, presence of complex calyceal endings, and shorter hair-cell bundles (Denman-Johnson and Forge, 1999; Fernandez et al., 1990; Lindeman, 1969). More recent studies propose that striolar regions are distinguished by the presence of simple and complex calretinin-positive calyceal endings (Desai et al., 2005b; Li et al., 2008). In the present study, Ocm-positive hair cells have calyceal endings that label for both calretinin and α -parvalbumin. This calretinin labeling of calyceal endings suggests that Ocm-positive hair cells may define the striola. The striolar region has long been associated with the line of polarity reversal (Desai et al., 2005b). In a meticulous study of the polarization patterns of hair bundles in the mouse utricle, the striola as defined by the central band of calretinin-positive calyces is almost entirely medial to the line of polarity reversal (Li et al., 2008). Our study is in agreement with this finding as we observe the majority of Ocm-positive cells lie mostly medial to the line of polarity reversal. Given that striolar hair cells uniquely express Ocm, and that the striola gives rise to unique physiological responses from vestibular afferents, striolar hair cells might be expected to exhibit differences in Ca^{2+} -dependent physiological processes such as mechanotransduction and adaptation responses.

To date however, there have been few, if any, reports of differences between striolar and extrastriolar hair cell adaptation and transduction mechanisms (Vollrath and Eatock, 2003), but in light of more recent morphological studies, such differences may have been obscured. It is quite possible that differences between striolar and extrastriolar hair cells were obscured by the mistaken assumption about the location of the reversal line within the striola.

Differences between Ocm expression in Cochlear and Vestibular Hair Cells

The most notable difference between Ocm expression in vestibular and cochlear hair cells was the subcellular localization of Ocm immunoreactivity as schematized in Figure 8. In cochlear outer hair cells, Ocm immunoreactivity was mostly restricted to the basolateral wall and the lower portion of the hair bundle (at or near the rootlet region of cuticular plate) whereas in vestibular hair cells, Ocm immunoreactivity was diffuse throughout the cytoplasm and hair bundle. It has been hypothesized that hair cells have fast-acting and highly mobile Ca^{2+} buffers in order to isolate Ca^{2+} signals (Parsons et al., 1994). If true, then one expects hair cells to have a high concentration of cytoplasmic Ca^{2+} buffer with a diffuse localization. Indeed, such a diffuse localization has been found for parvalbumin 3, a homologue of Ocm in nonmammalian vertebrates (Heller et al., 2002). Similar to Ocm-positive vestibular hair cells, parvalbumin 3 labeling is observed throughout the cytoplasm and extends into the hair bundle and is estimated at 3 mM, which is similar to estimates of Ocm in mammalian hair cells (Hackney et al., 2005; Heller et al., 2002). Such an extensive, cytoplasmic distribution, in addition to its Ca^{2+} binding properties makes Ocm a likely candidate to contribute to the Ca^{2+} buffering capacity of non-mammalian hair cells as well as of striolar and central zone mammalian hair cells (Heller et al., 2002). There is also a high level of Ocm in mammalian outer hair cells and this observation has led some investigators to suggest Ocm provides the bulk of the Ca^{2+} buffer capacity found in the outer hair cell, approximately 4–6 mM (Hackney et al., 2005). However, this suggestion assumes that Ocm is freely diffusible. In contrast to vestibular hair cells, cochlear outer hair cells demonstrate a clear subcellular compartmentalization of Ocm except for the first postnatal week. In the present study, our localization of Ocm differs from previous reports of diffuse, cytoplasmic labeling of Ocm (Hackney et al., 2005; Sakaguchi et al., 1998). In an immunoelectron microscopy study performed in rats, Hackney et al. (2005) found Ocm immunogold labeling throughout the cytoplasm and nucleus, but not in mitochondria. Since our study used the same antibodies used by Hackney et al. (2005), it is most probable that the labeling differences are due to either differences in techniques used or the use of apical hair cells, which in our study demonstrate a more diffuse pattern of Ocm labeling. A recent report showing upregulation of Ocm in a-parvalbumin deficient mice suggests that Ocm may be compartmentalized and distributed non-homogeneously in calyciform presynaptic terminals of the reticular thalamic nucleus (Csillik et al., 2009). The addition of glutaraldehyde may also have sufficiently altered labeling sites so as to give a different labeling pattern and/or greater nonspecificity (Birrell et al., 1987). For example, the high level of nuclear labeling in their study raises questions about the degree of antibody specificity. A further explanation for the labeling pattern observed in our study is that the Ocm antibody may be capable of recognizing different conformational forms of Ocm such as Ca^{2+} -bound versus Ca^{2+} -free conformations under the conditions used. Indeed unlike α -parvalbumin, recent reports suggest that Ca^{2+} -free Ocm adopts a conformation distinct from that of the Ca^{2+} -bound state, which is much more similar to the changes that occur in calmodulin (Henzl et al., 2008; Henzl and Tanner, 2007). The global rigidity and relatively high intracellular mobility of α -parvalbumin make it a prime candidate as a Ca^{2+} buffer, while the Ca^{2+} -induced conformational changes in Ocm make it more likely to be a Ca^{2+} sensor similar to calmodulin (Schwaller, 2009). Thus, binding Ca^{2+} leads to changes in the conformation of Ocm, which may distinguish membrane associated and cytosolic forms. The membrane association of Ocm labeling in basal-turn outer hair cells suggests a more specialized role

for Ocm in localized Ca^{2+} signaling. It is possible that Ocm may be localized in distinct microdomains to influence local fluxes of Ca^{2+} and/or to serve as a Ca^{2+} sensor similar to calmodulin (Blum and Berchtold, 1994; Klug et al., 1994; MacManus et al., 1984; Mutus et al., 1988).

Unlike the diffuse hair bundle labeling found in striolar and central hair cells of vestibular organs, Ocm labeling of the hair bundle in cochlear outer hair cells was localized preferentially to regions near the base of the hair bundle. We could find no evidence of hair bundle labeling in outer hair cells prior to the second postnatal week. The localization of Ocm immunoreactivity to the hair bundle suggests a type of functional compartmentalization consistent with Ca^{2+} signaling (Beurg et al., 2008; Dallos, 1992; Fettiplace, 2006; Mellado Lagarde et al., 2008). Both the plasma membrane CaATPases (PMCA) and calmodulin are also highly concentrated in the hair bundle of outer hair cells (Dumont et al., 2001; Furness et al., 2002; Yamoah et al., 1998). Ocm labeling closely resembles labeling for PMCA2, which is highly concentrated in the hair bundle stereocilia and to a lesser extent at the basal poles of mammalian outer hair cells (Dumont et al., 2001; Spiden et al., 2008). Further, the upregulation of Ocm in cochlear outer hair cells parallels the expression of PMCA (Furuta et al., 1998; Schulte, 1993). The lack of Ocm in the hair bundles of Ocm-negative hair cells indicates that either other CaBPs substitute for Ocm function or that Ocm has a particular function that is missing or lacking in Ocm-negative hair cells.

The Ocm labeling pattern we find in this study also shows a high degree of similarity with other proteins involved in Ca^{2+} dependent processes that affect the motor function of outer hair cells. Our results document colocalization of Ocm with CaMK-IV. Previous studies find CaMK-IV localized to the region underlying the lateral cell membrane of outer hair cells in both guinea pig (Frolenkov et al., 2000) and gerbil (Koyama et al., 1999), a distribution consistent with the subsurface cisternae. The presence of CaM kinases is thought to be a key element in the activation of Ca^{2+} -dependent phosphorylation cascades that relate to outer hair cell motility (Koyama et al., 1999; Puschner and Schacht, 1997). The association of CaATPases with the subsurface cisternae is also consistent with the idea that the subsurface cisternae functions as a Ca^{2+} reservoir (Schulte, 1993). The preferential distribution to the lateral wall supports Ocm involvement in Ca^{2+} signaling cascades associated with motility. The Ocm labeling pattern in basal-turn outer hair cells is reminiscent of prestin, which is found densely packed along the basolateral wall of outer hair cells with weaker or intermittent density below the nucleus (Adler et al., 2003; Belyantseva et al., 2000; Yu et al., 2006; Zheng et al., 2002; Zheng et al., 2000). Additionally, prestin is absent at the cuticular plate, again similar to Ocm. Phosphorylation of both cytoskeletal and prestin proteins is modulated by intracellular Ca^{2+} . As a potential buffer, Ocm may help to titrate or isolate release of Ca^{2+} from the subsurface cisternae. Increases in intracellular Ca^{2+} lead to a decrease in axial stiffness and trigger Ca^{2+} dependent slow motility (Mammano et al., 2007). Alternatively as a potential Ca^{2+} sensor, Ocm may mediate the activation of Ca^{2+} -calmodulin-dependent phosphorylation of cytoskeletal proteins and/or possibly even prestin that leads to changes in outer hair cell stiffness and subsequently motor function (Frolenkov et al., 2003; Sziklai et al., 2001; Szonyi et al., 2001).

Possible physiologic roles of Ocm

As noted in our previous study (Yang et al., 2004) as well as above, it is possible that Ocm is associated with efferent function and outer hair cell electromotility. The effects of acetylcholine on the electromotility of outer hair cells are Ca^{2+} and calmodulin dependent and may be mediated by Ca^{2+} -dependent phosphorylation of components of the outer hair cell cortical cytoskeleton (Dallos et al., 1997; Frolenkov, 2006; Frolenkov et al., 2000; Sziklai et al., 2001). Since CaMK-IV is also believed to play a role in efferent induced

electromotile responses, Ocm could presumably work as a specialized Ca^{2+} buffering system suited to the action of acetylcholine on the outer hair cells. In addition to the basolateral wall labeling, we observed punctuated regions of intense immunoreactivity at the basal poles. These regions are typically where efferent terminals contact the outer hair cells. There is substantial evidence that SK-type, Ca^{2+} -activated K^+ channels participate in acetylcholine-induced hyperpolarization of outer hair cells at efferent synapses. The biochemical pathway targeting outer hair cell stiffness either after acetylcholine application or following the increase of internal Ca^{2+} may share a common mechanism that is related to Ocm function (Mammano et al., 2007).

The postnatal onset of Ocm expression in rats and in mice not only coincides with the arrival of the medial olivocochlear efferent terminals, but also the down regulation of αPV , the appearance of the subsurface cisternae lining the basolateral wall, and the increased expression of the cytoplasmic CaATPase in the cisternae (Ito et al., 1995; Jensen-Smith and Hallworth, 2007; Schulte, 1993; Vater et al., 1997; Weaver et al., 1994; Weaver and Schweitzer, 1994; Yang et al., 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Adult (10 month) and postnatal expression profiles of Ca^{2+} binding proteins in the inner ear. **a.** Adult RT-PCR with parvalbumin (α PV), oncomodulin (Ocm), TATA binding protein gene control, and a 100 bp ladder. The PCR was done with and without reverse transcriptase (RT) reaction as an additional control. **b.** Postnatal RT-PCR for calretinin (top), Ocm-230, nested Ocm-180, α PV, and calbindin (bottom). Lanes 1–4 are P3 cochlea and vestibular with + / – RT, respectively. Lanes 5–8 are P5 cochlea and vestibular with + / – RT, respectively. Lanes 9–14 are P8 cochlea1, cochlea2, and vestibular with + / – RT, respectively. Lane 15 is the blank.

Figure 2.

Ocm immunoreactivity in the cochlea of the mouse. The scale bars in each panel represent 10 μm . **a.** A cross section through the organ of Corti of a P10 cochlear basal turn shows outer hair cells (OHCs) immunoreactive for Ocm. The inner hair cells (IHCs) did not show any Ocm immunoreactivity. Ocm labels the lateral wall of outer hair cells and the hair bundle (arrow). Phalloidin staining labels the cuticular plate (arrow) and stereocilia. The Ocm labeling in the hair bundle is more intense than the phalloidin labeling. **b.** A confocal image projection of the organ of Corti in an adult mouse shows Ocm labeled outer hair cells. The Ocm labeling in the hair bundle is predominately toward the outer portion of the apical surface of the outer hair cell (arrow). Ocm also labeled the basolateral wall of the outer hair cell. Punctuated Ocm labeling (asterisk) is found at the base of outer hair cells. Phalloidin staining is shown in the cuticular plate and distal (apical) portion of the hair bundles. Phalloidin staining is also shown on the hair bundles of inner hair cells. The inset shows a single section where Ocm labeling is restricted to the hair bundle and the basal pole of outer hair cells. **c.** Colocalization of Ocm and CaMK-IV in cochlear outer hair cells in the P10 mouse. Ocm and CaMK-IV colocalized (yellow) to the lateral wall of outer hair cells (OHCs). A projection image shows punctuated regions of colocalization (arrows) as well as non-overlapping regions of labeling (asterisks). **d.** Colocalization of Ocm and CaMK-IV in cochlear hair cells in the P10 rat organ of Corti. A 3d reconstruction of a longitudinal view of three rows of outer hair cells (OHCs) shows Ocm and CaMK-IV concentrated at the peripheral edges of the outer hair cell lateral walls. The arrows identify regions of colocalization (yellow) between Ocm and CaMK-IV. See Supplemental Figure 2 for the same green/magenta image.

Figure 3.

Comparisons of Ca^{2+} binding protein immunoreactivity in the early postnatal cochlea. **a.** A confocal image shows a P3 mouse cochlear section from the basal turn. Calretinin (CR, green) and calbindin (CB, grey) label inner and outer hair cells. Ocm labels outer hair cells. Afferent fibers below the inner hair cell are labeled by calretinin. Phalloidin staining is also present. The insets show labeling in the separate confocal channels. The scale bar represents 10 μm . **b.** A confocal image of a section taken from the basal turn of a P6 mouse cochlea labeled with phalloidin, Ocm, and calretinin. The scale bar represents 10 μm . **c.** A confocal image shows a section from the midbasal turn of a P8 mouse cochlea. Parvalbumin labels only the inner hair cells whereas Ocm labels only the outer hair cells. The arrow identifies

lateral wall labeling. The scale bar represents 15 μm . See Supplemental Figure 3 for the same green/magenta image.

Figure 4.

Patterns of Ocm labeling across vestibular organs. **a.** A confocal image taken from a 6-week-old mouse utricle labeled for Ocm and phalloidin. The scale bar represents 100 μm . **b.** A confocal image taken from an 8-month-old mouse utricle labeled for Ocm and phalloidin. The scale bar represents 50 μm . **c.** A 3d-reconstruction Ocm labeling in an adult mouse utricular striola shows Ocm labeling throughout the cytoplasm and hair bundle (arrows). The cells are predominately flask shaped. In contrast to the tight cluster at P3, the Ocm labeled hair cells are interspersed with non-Ocm labeled cells represented by their phalloidin-labeled hair bundles. **d.** A 3d reconstruction of the central zone of the cristae from an adult mouse shows Ocm labeling and phalloidin staining. Ocm labels throughout the cytoplasm and hair

bundle (arrow). The scale bar represents 40 μm . **e.** Ocm and CaMK-IV immunoreactivity in hair cells of the utricular striola. In the P10 mouse utricle the CaMK-IV labeling was mostly concentrated to the narrowed neck region of the hair cell and Ocm labeling was diffuse throughout the cytoplasm. **f.** In the striola of the P10 rat utricle CaMK-IV labeling was punctate along the basolateral surface whereas Ocm was diffuse. Scale bar, 10 μm . See Supplemental Figure 4 for the same green/magenta image.

Figure 5.

a. A 3d reconstruction of an adult mouse utricle (Utr) and two cristae (Crst) shows labeling for phalloidin, Ocm, and calretinin. The scale bars represent 100 μm . **b, c, d.** 3d reconstructions of the inset taken from the adult mouse utricle (**a**) identify the line of polarity reversal (solid white line). The scale bar represents 40 μm in **b, c**, and **d**. Using only the phalloidin channel (**b**), the line of polarity reversal is shown. The arrows indicate hair bundle polarity vectors (i.e., direction of the kinocilium). The line divides the utricle into medial (M) and lateral (L) regions. The addition of the Ocm channel (**c**) reveals the relation between Ocm-positive hair cells and the line of polarity reversal. The addition of the calretinin channel (**d**) reveals the calretinin positive calyces. **e.** Confocal image of an adult

mouse cristae section. Calretinin labels afferent calyces. Ocm labels a central band of hair cells. Calbindin intensely labels calyx endings of hair cells in the adult mouse cristae. The hair bundles and cuticular plates are labeled with phalloidin (cyan). The scale bar represents 10 μm . **f.** A confocal image shows an adult mouse reconstruction of the utricular striola. Parvalbumin labels calyx type endings and Ocm labels a center band of hair cells. The inset identifies two Ocm-positive cells that are contacted by a parvalbumin-labeled complex ending. The scale bar represents 20 μm . See Supplemental Figure 5 for the same green/magenta image.

Figure 6.

Neonatal and embryonic Ocm labeling in vestibular organs. **a, b.** Confocal images of a section taken from a P3 mouse saccule labeled with phalloidin (**a**) and Ocm (**b**). **c, d.** A confocal image of a section taken from a P3 mouse cristae labeled with phalloidin (**c**) and Ocm (**d**). **e, f.** A P0 mouse cristae with phalloidin staining (**e**) and Ocm labeling (**f**). Scale bar represents 50 μm . **g, h.** An E18 utricle with phalloidin staining (**g**) and Ocm labeling (**h**). Scale bar represents 50 μm .

Figure 7.

Embryonic Ocm labeling in mutant mice lacking a line of polarity reversal. **a.** A flat mount of a normal E18 inner ear with utricule, saccule and cristae labeled for tubulin, Ocm, and phalloidin staining. Scale bar represents 100 μm. **b.** A flat mount of an E18 Looptail mutant inner ear labeled for tubulin, Ocm, and phalloidin staining (blue) as above. Scale bar represents 40 μm. Ocm immunoreactivity is found in a subset of tightly clustered hair cells in an E18 utricule of the Looptail mutant. **c.** A 3d reconstruction of Ocm labeling in the utricular striola is diffuse and extends throughout the cell body. Neurofilament-positive endings surround the Ocm-labeled hair cells. The scale bar represents 30 μm. See Supplemental Figure 7 for the same green/magenta image.

Figure 8.

A schematic summarizing the different patterns of Ocm labeling (magenta) found in cochlear and vestibular hair cells. In the cochlea, Ocm labels intensely three regions within outer hair cells: the basal portion of the hair bundle (1), the lateral wall (2), and basal pole (3). This is in contrast to Ocm labeling of vestibular striolar and central zone hair cells, which are diffusely labeled throughout the cytosol and hair bundle. Afferent (blue) and efferent endings are shown in contact with hair cells.

Table 1

Description	Genbank Accession #	Forward Primer	Reverse Primer	Primer Targets	Amplicon Size
Mouse alpha parvalbumin	NM_013645	AAAAAGAAACCCGGATGAGGT	GCCAGAAAGCGTCTTTGTTTC	134, 266	152
Mouse calbindin 1-28K	NM_009788	ATCCACACCTGCAGTCAATCTC	TTCCGGTGATAGCTCCAAATC	147, 290	163
Mouse oncomodulin	NM_033039	GAGCATCACGGACATTCTGA	CGCTCTGGAAACCTCTGTAGG	133, 331	218
mouse oncomodulin, nested		GCGCTGATGACATTGCAG	TGAGCTCATCTTTCATCCAGGT	153, 303	178
Mouse calretinin	NM_007586	CTCCTGAAGAAAGGCCAACAG	TATAGCCGGTTCCATCCTTG	485, 694	229
Mouse calmodulin	NM_009790	ACTGGGTCAGAACCCCAACAG	GTTCGCCGCACCTGATGTAA	291, 471	200
Mouse beta-Actin	NM_007393	ATGGAGGGGAATACAGCCC	TTCTTTGCAGCTCCTTCGTT	33, 163	149
TATA Binding Protein	U_63933	GGCCTCTCAGAAGCATCACTA	GCCAAAGCCCTGAGCATAA	128, 277	167

Table 2

Table of Primary Antibodies Used

Antigen	Immunogen	Manufacturer	Dilution used
α -Parvalbumin	rat muscle parvalbumin	Swant (Bellinzona, Switzerland) Cat# PVG-214 polyclonal goat (ascites, no preservative, lyophilized)	1:2000
Calbindin D-28k	calbindin D-28k, purified from chicken gut	Swant (Bellinzona, Switzerland) mouse monoclonal #300	1:250
Calretinin	recombinant full length human calretinin	Swant (Bellinzona, Switzerland) goat polyclonal #CG1	1:500
Calretinin	recombinant full length rat calretinin	Chemicon/Millipore (Temecula, CA) rabbit polyclonal #AB149/AB5054	1:500
Oncomodulin	Recombinant full-length rat oncomodulin	Swant (Bellinzona, Switzerland) rabbit polyclonal #OM3	1:500 – 1:2000
Oncomodulin	Recombinant full-length rat oncomodulin	M.T. Henzl (University of Missouri, Columbia)	1:100
α -tubulin, acetylated (6-11B-1) IgG2b	acetylated tubulin from the outer arm of sea urchin	Sigma Aldrich #T6793, mouse monoclonal	1:500