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A Balance of Form and Function: Planar Polarity and Development of the Vestibular Maculae

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Abstract

The mechanosensory hair cells of the inner ear have emerged as one of the primary models for studying the development of planar polarity in vertebrates. Planar polarity is the polarized organization of cells or cellular structures in the plane of an epithelium. For hair cells, planar polarity is manifest at the subcellular level in the polarized organization of the stereociliary bundle and at the cellular level in the coordinated orientation of stereociliary bundles between adjacent cells. This latter organization is commonly called Planar Cell Polarity and has been described in the greatest detail for auditory hair cells of the cochlea. A third level of planar polarity, referred to as tissue polarity, occurs in the utricular and saccular maculae; two inner ear sensory organs that use hair cells to detect linear acceleration and gravity. In the utricle and saccule hair cells are divided between two groups that have opposite stereociliary bundle polarities and, as a result, are able to detect movements in opposite directions. Thus vestibular hair cells are a unique model system for studying planar polarity because polarization develops at three different anatomical scales in the same sensory organ. Moreover the system has the potential to be used to dissect functional interactions between molecules regulating planar polarity at each of the three levels. Here the significance of planar polarity on vestibular system function will be discussed, and the molecular mechanisms associated with development of planar polarity at each anatomical level will be reviewed. Additional aspects of planar polarity that are unique to the vestibular maculae will also be introduced.

Keywords

Planar Polarity; PCP; Vestibular; Utricle; Saccule; Hair Cell; Stereocilia; Kinocilium

1. Introduction

The vestibular maculae are two sensory epithelia housed in the utricle and saccule of the inner ear that provide sensory input to the reflex circuits underlying balance and posture. Sensory receptor hair cells (HCs) in these epithelia are specially adapted to detect linear accelerations while also continuously monitoring the position of the head in reference to gravity. The maculae are part of the larger peripheral vestibular system which also contains the three semi-circular canal cristae that detect rotational movements (Fig. 1A). Each of these sensory end-organs contains HCs that directly respond to motion and relay that

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information to the CNS through afferent neurons of the VIIIth cranial nerve. Proper HC function depends upon the polarization and orientation of a specialized bundle of stereocilia located on the apical cell surface (Fig. 1B). This cellular polarization is a form of planar polarity because the axis of stereociliary bundle polarity is aligned in a two-dimensional field that is parallel to the plane of the epithelial surface. Apical structures of many epithelial cell types exhibit planar polarity including the multi-ciliated cells of the respiratory airway and ependymal cells lining the lateral ventricles of the brain. The diversity of planar polarity found in vertebrate and invertebrate systems has been reviewed [1, 2].

In many regards inner ear HCs of the mouse are an ideal model for studying vertebrate planar polarity because the stereociliary bundles of sensory HCs have a distinct morphological polarization that remains static once it has been established, is coordinated between adjacent cells, and can be easily visualized by fluorescence microscopy. These characteristics have been well demonstrated for the auditory HCs of the cochlea, and are also properties of vestibular HCs. However, unlike the cochlea the development of planar polarity in the vestibular system has received far less attention. One advantage of vestibular HCs as an experimental model is that they do not undergo the extensive convergent extension movements that pattern auditory HCs [3]. Since many developmental genes contribute to both planar polarity and convergent extension [4, 5] it can be difficult to separate their functional contributions to the two processes. Several anatomical characteristics also make the vestibular maculae an important complement to planar polarity studies in other systems. The most striking is that HCs in the utricle and saccule are divided between two groups that have opposite stereociliary bundle orientations (Fig. 1E). These groups contact each other at the Line of Polarity Reversal (LPR), a figurative line drawn by anatomists to demarcate the two populations. It is striking to note that the organization of vestibular HCs on either side of the LPR differs between the utricle and saccule. In the utricle, HCs are oriented with their polarized stereociliary bundles pointed towards the LPR while in the saccule the bundles point away [6]. These unique characteristics imply the existence of dedicated developmental mechanisms directing planar polarity and HC patterning about the LPR.

The purpose of this review is to discuss aspects of planar polarity relating to the development of HCs in the vestibular maculae. To accomplish this, planar polarity will be described at three anatomical scales; subcellular, cellular and tissue-wide, and a model will be presented for LPR formation. The primary experimental model that will be considered is the mouse because of the availability of powerful genetic approaches in the mouse and because anatomic, physiological and genetic similarities between the mouse and human inner ear make this an ideal system for developmental studies. Comparisons will also be made between mouse and analogous planar polarity systems in *Drosophila*. Overall the attributes of the vestibular maculae as an experimental model will be presented by emphasizing unique and important questions that can only be addressed using this system.

2. Planar Polarity and Vestibular Hair Cell Structure and Function

Linear and gravitational accelerations are detected by HCs located in the vestibular maculae of the utricle and saccule. The maculae develop within the bony labyrinth of the inner ear the utricle oriented in the horizontal plane and the saccule oriented in the vertical plane. Although they are only connected to each other by the narrow utriculo-saccular foramen they are likely derived from a common developmental precursor [7–10]. Similar hypothesis argue that the cochlea is also a developmental extension of the saccule [11] and some evidence suggests that the mammalian saccule is also sensitive to low frequency sound [12, 13].

HCs extend a polarized bundle of stereocilia from their apical surface adjacent to a single kinocilium. The kinocilium is always displaced to one side of the cell surface, and the stereocilia are of graded heights and arranged in an ascending cluster with the tallest located adjacent to the kinocilium (Fig. 1C). Kinocilia are true cilia assembled from microtubules, whereas stereocilia are actually specialized microvilli composed of filamentous actin. As a result the planar polarity of the HC bundle, from the shortest stereocilium to the kinocilium, is easily visualized using cytoskeletal markers. Within a bundle, shorter stereocilia are connected to their taller neighbors by protein fibers called tip-links that are an important component of the mechanotransduction apparatus [14]. As a result deflections of a stereociliary bundle in the direction of the kinocilium leads to ion channel opening and HC depolarization. The stereociliary bundle and kinocilium also contact an overlying otoconial membrane that is composed of extracellular matrix proteins such as Otolin and embedded with dense otoconial crystals (Fig. 1B) [15, 16]. Inertial forces exerted on the otoconial membrane during head tilt or linear head movements are sufficient to deflect the stereociliary bundles. Due to the polarized organization of the stereociliary bundle and the mechanotransduction apparatus, only deflections of the stereociliary bundle towards the kinocilium generate the tension required to open mechanotransduction channels [17, 18].

The range of motion that is detected by an individual vestibular HC is determined by the planar polarity of the stereociliary bundle [19]. In order to detect the broadest range of motions, HCs of the vestibular maculae are arranged so that their stereociliary bundles are oriented across ~360 degrees. This organization is highly stereotyped and displays three significant features. First, with one important exception, the orientation of stereociliary bundles on neighboring HCs is highly similar (Fig. 1D). Second the orientation of bundles gradually changes between HCs that are separated by increasing distance so that the difference in bundle orientation between the most distantly placed cells approaches 180 degrees. Finally in both the utricle and saccule the HCs are divided into two groups of opposite stereociliary bundle polarity that are separated by the LPR (Fig. 1E). The LPR is the exception where neighboring cells may have stereociliary bundles of opposite orientation. As previously described, in the utricle HCs at the LPR are oriented with their excitatory axes pointed towards each other while in the saccule they are pointed away. As a result movement of the overlying otoconial membrane produces a combination of excitatory and inhibitory responses as it simultaneously deflects the bundles in these two groups of cells.

This organization is relatively simple in comparison to patterns of planar polarity that have evolved in species of fish in which inner ear sensory organs like the saccule have dual vestibular and auditory functions [20, 21]. In these species saccular HCs are arranged into at least four quadrants separated by one LPR where HCs point away from each other and a second LPR where opposing cells are arranged in an anti-parallel configuration (Fig. 1E). In mammals the LPR is closely associated with the striola, a specialized region of the vestibular maculae with unique cellular, sensory and synaptic characteristics that are specially adapted for detecting higher frequency movements [22, 23]. Although ‘striola’ and ‘LPR’ are often used synonymously in the literature, for the purpose of this review ‘striola’ will be reserved to describe the regional specialization and ‘LPR’ to describe the cell boundary between HCs with opposite bundle polarity.

3. Vestibular Planar Polarity Occurs at Three Anatomical Scales

The morphological characteristics of vestibular HCs and their organization within the vestibular maculae suggest that planar polarity is represented at three distinct anatomical scales (Fig. 1C–E). This distinction is not restricted to the inner ear and similar distinctions in planar polarity organization are found in tissues from other systems and species [1, 24].

Moreover these anatomical distinctions suggest that different sets of genes and molecular mechanisms may act at each level, and the final pattern of planar polarity organization likely requires the coordinated overlap of mechanisms from each level. With these considerations in mind, development of planar polarity within the vestibular maculae will be discussed at the following three levels:

1. *Subcellular Planar Polarity* – Within individual cells planar polarity is manifest in the asymmetric localization of molecules or cellular structures where polarization is parallel to the plane of the surrounding epithelium. In a HC, subcellular planar polarity is evident in the polarized structure of the stereociliary bundle and the position of the kinocilium on one side of the apical cell surface (Fig. 1C).
2. *Cellular Polarity or Planar Cell Polarity (PCP)* – At this intermediate scale, planar polarity is manifest in the orientation of polarized cells within the two-dimensional surface of the epithelium. In the vestibular maculae this is evident in the coordinated orientation of stereociliary bundle polarity between neighboring cells (Fig. 1D). For HCs of the inner ear, and in particular in the cochlea, PCP is the level of planar polarity that has been studied in the greatest detail.
3. *Tissue Polarity* – This is the largest spatial scale of planar polarity organization within organs or the entire organism. In the maculae a striking example of tissue polarity is seen in the division of vestibular HCs into two groups divided by, and patterned around the LPR (Fig. 1E). The developmental mechanisms regulating this level of planar polarity are the least understood.

3.1. Subcellular Planar Polarity and Polarization of the Stereociliary bundle

The subcellular planar polarity of a HC is reflected by the polarized organization of the stereociliary bundle. In the mouse vestibule, bundle polarization initiates at the earliest stages of HC differentiation. When viewed by scanning electron microscopy (SEM) at embryonic day 12.5 (E12.5) the first HCs can be distinguished from adjacent precursors by specialization of their apical surface. At this stage a single cilium emerges from the center of the cell surface and is surrounded by elongated microvilli [25, 26]. This cilium lengthens to form the kinocilium, and subcellular planar polarity is established as the cilium migrates to one side of the apical cell surface. The microvilli subsequently elongate to form the staircase array of stereocilia [25]. The early emergence and movement of the kinocilium argues that this structure directs subsequent stereociliary bundle polarization [27], and linkages between the kinocilium and adjacent stereocilia have been proposed to guide polarized bundle formation [28].

Polarization of the stereociliary bundle is cell intrinsic and occurs independently from mechanisms directing planar polarity at the level of PCP or tissue polarity. This distinction is demonstrated in the majority, if not all, mutant mice that have been used to study PCP. For example as described in greater detail below, auditory and vestibular HCs in *vangl2* knockout [29] and *frizzled 3/6* double knockout mice [30] are incorrectly oriented relative to neighboring cells. Nonetheless mutant HCs still form polarized stereociliary bundles indicating that their subcellular planar polarity is intact. In contrast fewer mutations have been described that only affect subcellular planar polarity. One example is a conditional knockout mouse disrupting the function of IFT88/Polaris; an essential factor for intraflagellar transport and cilia formation [31]. In auditory HCs in these mice, the position of the basal body is disrupted, occasionally remaining at the center of the apical cell surface. Although a kinocilium is not formed, mutant HCs still form stereocilia but the polarized organization is lost, sometimes resulting in a symmetrical, circular patterns. A similar pattern of symmetrical stereocilia bundling occurs in Protocadherin 15 mutants that have disrupted links between stereocilia and between the kinocilium and stereocilia [28]. These

genetic analyses provide additional evidence that the kinocilium directs polarized bundle morphogenesis. Together these data are consistent with the idea that the translocation of the kinocilium to one side of the HC surface is a critical early step in establishing subcellular planar polarity.

Some of the molecular signals that direct formation of polarized bundles are beginning to be uncovered. These require the activity of the P21-activated kinase PAK, which is asymmetrically localized in the vicinity of the kinocilium [32]. PAK function is further regulated by the small GTPase Rac1, which is itself a well characterized regulator of actin dynamics [33]. Following Rac1 gene deletion or pharmacological inhibition of PAK activity, stereociliary bundles are misformed and the kinocilium is frequently positioned incorrectly within the bundle [32]. The Rac1-PAK signaling pathway is also dependent upon the function of Kif3a [34], a component of the Kinesin II motor complex that is necessary for plus-end microtubule trafficking and anterograde intraflagellar transport (reviewed by [35]). One function for Kif3a in HCs is the coupling of basal body position and stereocilia assembly, thereby directing the formation of cohesive, polarized bundles. Kif3a likely acts by localizing PAK activity to one side of the developing HC [34]. Although these studies show an important role for the Kinesin II motor complex in subcellular planar polarity, they do not demonstrate that Kinesin II is the motor that translocates the kinocilium. An outstanding question is the identity of this motor protein because its identification may show how subcellular planar polarity is coupled to PCP and tissue polarity.

3.2. Planar Cell Polarity (PCP) and the Coordinated Orientation of Adjacent Hair Cells

The molecular basis of planar polarity is best understood at the level of PCP; the coordinated orientation of stereociliary bundle polarity between neighboring HCs. Many essential molecules regulating this level of planar polarity have been identified through genetic screens in *Drosophila*. These include the core PCP proteins Frizzled, Disheveled, Van Gogh, Prickle, Diego and Flamingo. Their identification and function during planar polarity development have been reviewed extensively [1, 36, 37]. Conservation between *Drosophila* and mouse PCP gene function was first demonstrated in the cochlea for *vangl2* [38] and *CELSR1* [39] the vertebrate orthologs of *van gogh* and *flamingo*. In these mutants the coordinated orientation of stereociliary bundle polarities between neighboring HCs is disrupted while subcellular planar polarity remains intact. Subsequent studies demonstrated similar phenotypes for *frizzled* and *disheveled* mutants [3, 30].

With the exception of *vangl2*, the function of core PCP genes during maculae development has not been reported, although PCP protein function is likely to be conserved between auditory and vestibular HCs. In *vangl2* knockout mice the PCP phenotype is restricted to HCs located in the striola region [29]. This limited effect may be due to compensation from the closely related *vangl1* gene in extrastriolar regions [40]. Consistent with this idea, the maculae phenotype is greater in *Looptail* mice, in which a semi-dominant mutation in *vangl2* inhibits trafficking of both Vangl1 and Vangl2 to the cell surface [29]. While these phenotypes do not exclude an additional function for Vangl2 in guiding kinocilia movements, the absence of symmetric stereociliary bundles strongly suggests that the core PCP proteins like Vangl2 are not required for subcellular planar polarity. Moreover, in mouse mutants where subcellular planar polarity is disrupted there is no change in core PCP protein expression, further arguing that these two levels of planar polarity are distinct [28, 31, 34].

Core PCP proteins are asymmetrically localized at the apical boundaries of epithelial cells in both vertebrates and invertebrates (Fig. 2). In *Drosophila*, the transmembrane receptor Frizzled, and the cytosolic factors Disheveled and Diego are present at the distal side of wing epidermal cells opposite to the transmembrane protein Van Gogh and its cytosolic

binding partner Prickle on the proximal side. Flamingo, an unusual cadherin with seven membrane spanning segments, is present at both sides of the cell [36]. Thus at a cell:cell boundary the extracellular domain of Frizzled from one cell is opposed to the extracellular domain of Van Gogh from the adjacent cell (Fig. 2A). Although resolving protein distributions between neighboring cells at points of cell-cell contact is difficult using standard fluorescence-based techniques this problem was elegantly solved in the fly by assaying protein localization at the boundaries of mutant clones (for an example see [41]).

For vertebrates genetic approaches to resolve PCP protein distribution are more difficult than in *Drosophila*. Despite this, the majority of experiments from the mouse inner ear are consistent with the hypothesis that PCP protein distribution is conserved between *Drosophila* and mouse. For example, electroporation of GFP-tagged constructs show asymmetric localization of Fz6 to the lateral side of vestibular supporting cells where it opposes Prickle-like 2 (Pk2); a Vangl2 associated protein that is enriched at the medial side of vestibular HCs (Fig. 2B–C) [42]. Similarly in the cochlea genetic mosaic analyses show Fz6 localization at the proximal side of auditory HCs [30]. In this location the extracellular domain of Fz6 opposes Vangl2 present at the distal side of the adjacent supporting cell as recently shown by GFP-tagged Vangl2 distribution, and super resolution STED microscopy of endogenous protein [43]. Therefore for both auditory and vestibular HCs, and similar to *Drosophila*, Frizzled6 in one cell is opposed to Vangl2/Pk2 from the adjacent cell (Fig. 2B). Curiously Pk2 (and likely Vangl2) is enriched at the HC side of the HC:SC boundary in the utricle [42] whereas Vangl2 is enriched at the supporting cell side of the HC:SC boundary in the cochlea [43]. Although Vangl2 and Dishevelled proteins are asymmetrically localized in vestibular HCs and supporting cells [44, 45], which side of the HC:SC boundary carries these proteins has not been unequivocally established.

Mosaic analyses of mutant clones in *Drosophila* show that an essential function of core PCP proteins is to relay polarity information between adjacent cells, thereby coordinating their intrinsic subcellular planar polarity [1]. The similarity of PCP protein distribution in HCs and supporting cells of the mouse maculae and the *Drosophila* wing suggest that a similar relay occurs in the vestibular maculae. Several molecular mechanisms have been proposed for this relay including receptor:ligand interactions between the two transmembrane proteins, Frizzled and Van Gogh across the extracellular space [46]. Alternatively, homodimeric interactions between Flamingo proteins expressed by adjacent cells could relay PCP signals between adjacent cells [47]. However in order to function in the vestibular maculae these signals must also propagate through the intervening network of supporting cells that surround HCs (Figs. 1D, 2B). This is likely to occur as judged by the results of an experiment in which alterations in PCP signaling in a subset of cells in the auditory organ of chick led to non-autonomous effects on the orientation of adjacent, unmanipulated HCs [48]. Furthermore, PCP proteins are expressed in supporting cells of the chick utricle and are able to direct polarized stereociliary bundle development of regenerating HCs after ototoxic trauma [49]. Despite these observations, the cue that initiates the PCP cascade coordinating stereociliary bundle polarity between vestibular HCs has not been identified. One candidate is the Wnt class of extracellular ligands which bind Frizzled receptors and initiate intracellular signaling via the canonical β -catenin dependent pathway or the alternative PCP pathway [50]. However, as described in greater detail below, Wnt-signaling may function primarily at the level of Tissue Polarity.

In *Drosophila* the Fat/Dachsous signaling pathway is also an important regulator of wing hair and photoreceptor PCP, and the orthologous gene Fat4 contributes to planar polarity in the mouse [51]. Fat and Dachsous are atypically large cadherins that form a receptor:ligand pair. Polarity information is encoded by the relative activity of Fat signaling on opposite sides of the cell and this is dependent on a gradient of Dachsous expression and is

modulated by the cadherin kinase four-jointed [52]. While it has been suggested that the Fat signaling pathway functions upstream of core PCP signaling the emerging consensus is that these two pathways act in parallel [53]. Depending upon the tissue, the contribution from one parallel pathway may be more significant than the other. In the mouse cochlea, Fat/Dachsous signaling appears to be less significant because only subtle changes in polarity are present in *fat4* knockouts, *dachsous1* knockouts or *fat4; dachsous1* double knockouts [54]. Similarly the related *fat3* knockouts lack HC planar polarity defects despite expression of Fat3 throughout the inner ear sensory epithelia [55]. Although this does not rule out a function for Fat/Dachsous signaling in HC development, it strongly suggests that the core PCP proteins have a more significant role in HCs.

3.3. Tissue Polarity and Formation of the LPR

Tissue polarity in the vestibular maculae is most evident in the patterning of HCs about the LPR. As noted earlier, utricular HCs are oriented with their bundles pointed towards the LPR while saccular HC stereociliary bundles point away from the LPR (Fig. 1E). Based upon morphological criteria, formation of the LPR is completed in the mouse by E15.5 [25, 26]. Between E13.5 and E15.5 the maculae contain HCs that can be divided between groups of opposite stereociliary bundle polarity, however planar polarity is not tightly coordinated between adjacent cells [25]. This raises the possibility that rotational movements might refine the final orientation of stereociliary bundles or contribute to formation of the LPR. Indeed, during auditory HC maturation in the mouse, a period of refinement tightly couples stereociliary bundle orientation with the neural:abneural axis of the cochlea [56], and chick HCs undergo rotational movements during maturation of the basilar papillae [57]. In the mouse cochlea, these refinement events are dependent upon Wnt-signaling and can be disrupted *in vitro* by Wnt antagonists [56, 58]. Although together these observations indicate that stereociliary bundle orientation can change over time, the significance of bundle rotation has not been established for development of tissue polarity in the maculae.

Remarkably the abrupt change in stereociliary bundle orientation that occurs at the LPR does not require corresponding changes in the subcellular distribution of core PCP proteins. For example the polarity protein Pk2 is adjacent to the kinocilium in cells located on one side of the LPR and opposite to the kinocilium in cells located on the other side of the LPR (Fig. 2C)[42]. Thus while the core PCP proteins are essential at the level of Planar Cell Polarity for coordinating the orientation of adjacent cells, they are not sufficient at the level of Tissue Polarity to direct LPR formation. An interesting possibility is that the PCP proteins establish an underlying polarity (referred to hereafter as “ground polarity”) that is shared by all cells, while a second planar polarity signal directs the way that the ground polarity is interpreted based upon cellular location. This ground polarity hypothesis was proposed by Green and colleagues to describe an analogous cellular patterning event in *C. elegans* that also uses cellular polarity established by the core PCP proteins and a non-canonical Wnt signal [59]. In this system a second Wnt, now acting through canonical pathways, reverses the interpretation of ground polarity in half of the cells thereby generating a line of symmetry analogous to the LPR. If a similar mechanism acted to direct tissue polarity in the vertebrate ear then the source and diffusion characteristics of the secreted morphogen, perhaps also a Wnt, would determine the position of the LPR.

An alternative possibility is that HCs located on opposite sides of the LPR have unique transcriptional profiles and are thus genetically encoded to respond to the PCP-based ground polarity information in opposite manners. One advantage of this mechanism is that transcriptional domains could be established by restricted patterns of transcription factor expression that are self-sustained for the remainder of development. A candidate regulator for transcriptional patterning is *Emx2* because in *emx2* mutants the LPR is not formed and all vestibular HCs point in a common direction [60]. In the developing cortex where it has

been studied in greater detail, *Emx2* functions in concert with other transcription factors to pattern cortical areas [61]. Thus *Emx2* could be an upstream regulator that patterns the maculae while planar polarity is enacted by downstream effectors with regional patterns of expression. *Emx2* mutant analysis is consistent with the idea that the position of the LPR is determined by transcriptional patterning of the maculae rather than a morphogen. Despite these possibilities, mechanisms regulating tissue polarity and patterning the LPR in conjunction with the core PCP proteins have not been identified.

4. Planar Polarity and the Developmental Origins of the Sensory Precursors

Several lines of evidence, including the distribution of molecular markers and phenotypic analysis of mutant mice, support a hypothesis that the utricular and saccular maculae are derived from a common prosensory domain [8]. First the expression of *lunatic fringe*, a molecular marker common to the utricular and saccular maculae initiates in the anteroventral portion of otocyst between E9-E10.5 and is maintained as these sensory organs separate into distinct structures [62]. Second, genetic fate mapping of the neurogenic region of the otocyst labels cells that contribute to both the mature utricle and saccule, in addition to vestibular ganglion neurons [63, 64]. Furthermore, the common origin of the vestibular maculae is demonstrated by the phenotype of *Imx1a* mutants and *N-myc* conditional knockout mice because in these animals the utricle and saccule fail to separate resulting in a single gravistatic organ [7, 9, 10]. However not all vestibular sensory epithelia are derived from a common origin, and molecular marker studies also show that the semi-circular canal cristae have distinct lineages [62, 64].

Inner ear morphogenesis and formation of the utricle and saccule occurs during a rapid embryonic period that coincides with the initiation of HC planar polarity. Analyses using the paint fill technique demonstrate that the utricle is formed at E12 and that the utricle and saccule are distinct structures in the mouse at E13 [62]. This period of utricle and saccule separation overlaps with the earliest stages of stereociliary bundle formation in differentiated HCs at E12.5 [25, 26]. In addition, the core PCP protein Pk2 is asymmetrically localized in nascent HCs prior to movement of the kinocilium to one side of the apical cell surface [42]. Together, these data suggest that prior to E12.5 a utricular/saccular precursor may already contain the molecular cues necessary to initiate planar polarity and maintain HC orientation through later stages of development.

In a scenario in which the utricular and saccular maculae are derived from a common origin, how could a single developmental precursor give rise to two sensory epithelia with complimentary patterns of stereociliary bundle organization at the LPR? A simple model that accommodates this anatomy can be proposed based upon the coordinated development of planar polarity at the PCP and tissue polarity levels (Fig. 3). In this model the coordinated orientation of stereociliary bundles is determined by the PCP-based ground polarity established in the utricular/saccular precursor and maintained through the morphogenic separation of the utricle and saccule (Fig. 3A). Since the subcellular distribution of PCP proteins does not determine the absolute orientation of the stereociliary bundle (Fig. 2C), formation of the LPR requires a second mechanism. Potential alternatives include a diffusible morphogen or a restricted domain of transcription factor expression that only effect HCs destined for one side of the future LPR. Regardless of the precise molecular mechanism, in this model the purpose of the second signal is to divide the macular precursor into two domains, shown here as an annulus (Fig. 3B), in which HCs in the center respond to the PCP-based ground polarity with stereociliary bundle polarities opposite to that of HCs in the surround. As a result, division of the annulus would create a single LPR in each half (Fig. 3C). Moreover the change in stereociliary bundle orientation along one LPR would be the reverse of the change in bundle orientation along the other, thereby forming

complimentary patterns as seen in the mature utricle and saccule. An additional morphological event would be required to invert the saccule intermediate into its mature form to account for Pk2 localization opposite of the kinocilium in HCs in the inferior extrastrilar region [42] (Fig. 3D). This model for vestibular maculae development incorporates in a simple way, the observed distribution of core PCP proteins and the unique anatomy of HC patterning at the LPR.

5. Planar Polarity and Innervation of the Vestibular Maculae

The vector component of sensory information relayed to the CNS by vestibular ganglion neurons is determined by the polarized orientation of the HCs contacted by each neuron. Therefore an important component of vestibular system wiring is matching the response characteristics of a vestibular ganglion neuron and the central targets of that neuron. The majority of afferent neurons contact two or more HCs (Fig. 4). Invariably these HCs are located on the same side of the LPR and have similarly oriented stereociliary bundles. For example in the striola, Type I HCs are engulfed by large calyceal synapses formed by the Calyx-only class of afferent neurons. These afferents frequently contact multiple HCs in a synaptic cluster. Immunolabeling [22] or tracer labeling [65] of Calyx-only afferent endings demonstrates that the HCs within a cluster are restricted to one side of the LPR. Thus the Calyx-only class of afferent neuron only responds to motion in a single direction despite receiving input from multiple HCs. Similarly while the Bouton-only class of afferent neurons form conventional synapses with multiple HCs and Dimorphic neurons form calyx and conventional synapses with a pair of Type I and Type II HCs, each class of afferent neuron still only contacts cells located on one side of the LPR [65]. Together, these anatomical observations argue that bundle orientation and afferent innervation are actively coordinated during development. However the cellular events enacting this coordination and whether these events are genetically encoded or activity dependent has not been determined.

Stereociliary bundle orientation is also coordinated with the central targets of the afferent neurons. Neural tracing experiments using lipophilic dyes show that neurons contacting HCs located on opposite sides of the LPR project to distinct central targets [66]. In these experiments, dye placement in the vestibular nucleus labeled neurons that contact HCs in the striola and medial extrastrilar regions of the utricle and saccule. In contrast dyes placed in the cerebellum only labeled afferent neurons from lateral extrastrilar regions. Thus, afferent innervation patterns are coordinated with the position of the LPR in the vestibular maculae so that second order neurons contact HCs with similar bundle polarity and send projections to distinct central targets.

6. Conclusions

Comparative studies between mouse and *Drosophila* have led to rapid advances in understanding planar polarity as conserved mechanisms of PCP protein function have been identified. There are also unique examples of planar polarity that have evolved in vertebrates and, as this review illustrates, a number of scientific questions that can be addressed using the vestibular system of the mouse. For example, the vestibular maculae is a model where alternative molecular mechanisms directing subcellular planar polarity and tissue polarity can be identified, and the ways they interact with core PCP protein signaling can be interrogated. It is also important to understand how planar polarity is coordinated with other tissue-specific aspects of organ development. For example within the maculae, what prevents afferent neurons from contacting multiple HCs located on opposite sides of the LPR? And how is planar polarity influenced by the rapid and dynamic processes of inner ear morphogenesis? While work in the mouse has been limited to a certain degree by the neural tube defects associated with mutations in the core PCP genes, this problem should be

resolved in the near future through the study of conditional mutants and the application of Cre/LoxP technologies. When this limitation is removed, many of these outstanding questions of planar polarity will be addressed, and studying the vestibular maculae is likely to advance our understanding of planar polarity mechanisms in the auditory system and other developmental processes.

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Highlights

- Planar polarity of vestibular hair cells enables the detection of linear movements
- Vestibular planar polarity is manifest at three distinct anatomical scales
- Planar polarity is tightly coordinated with afferent neuron innervation

Figure 1. Planar Polarity and the organization of vestibular HCs in the utricle and saccule

(A) The vertebrate inner ear is composed of six sensory epithelia (blue shading) that contain the HCs mediating hearing and balance. Vestibular function is divided between the utricular and saccular maculae that detect linear acceleration, and three semi-circular canal cristae that detect head rotation. Hearing is mediated by HCs located in the organ of Corti of the cochlea. (B) Vestibular HCs are surrounded by supporting cells, and extend stereociliary bundles into the otoconial membrane; an extracellular matrix embedded with otoconia. Inertial movements of the membrane lead to bundle deflections. (C–E) The planar polarity of vestibular HCs is seen at three anatomical scales. (C) Subcellular Planar Polarity is evident in the polarized organization of the stereociliary bundles. Individual stereocilia are grouped in a staircase array of increasing heights with the tallest adjacent to a kinocilium. The kinocilium is always displaced to one side of the bundle. (D) Planar Cell Polarity (PCP) is the coordinated orientation of stereociliary bundles between adjacent HCs. (E) Tissue Polarity is the organization of PCP with respect to the anatomy of the maculae. This is best illustrated by HC patterning about the Line of Polarity Reversal (LPR, red line) in the utricle and saccule. In the utricle, groups of HCs (illustrated by black arrows) are oriented with

their stereociliary bundles pointed towards the LPR while in the saccule stereociliary bundles point away from the LPR. In mammals HCs in the utricle and saccule are divided by a single LPR. However in some species of fish, as illustrated for the deep sea Elopomorph *Synaphobranchus bathybius* [20] the saccular maculae contains multiple lines of reversal and more elaborate patterns of Tissue Polarity. Lateral (L), Medial (M), Superior (S), Inferior (Inf.).

Figure 2. The subcellular distributions of core PCP proteins are likely conserved between mouse and *Drosophila* and are not altered at the LPR

(A) Core PCP proteins are asymmetrically localized in epidermal cells of the developing *Drosophila* wing with Frizzled and Dishevelled enriched at the distal cell boundary near the site of hair formation, and Van Gogh and Prickle enriched at the proximal side. Flamingo is present at both cell boundaries. (B) In the developing mouse utricle the core PCP proteins Fz6 and Pk2 are located on opposite sides of HCs and supporting cells, similar to the relative distribution of Frizzled and Prickle in *Drosophila*, suggesting that other PCP proteins are similarly distributed. The remaining core PCP proteins in mouse are asymmetrically localized at cell boundaries however proximal versus distal distributions have not been established. (C) Pk2 (green) is enriched at the same side of vestibular HCs regardless of stereociliary bundle orientation or cellular position relative to the LPR. Bundle orientation is illustrated using antibodies against Spectrin (red) which labels the cuticular plate beneath the stereocilia but not the basal body of the kinocilium. (C') Annotated stereociliary bundle

orientation (arrowheads) for HCs illustrated in (C) relative to Pk2 protein distribution. The dashed line indicates the position of the LPR.

Figure 3. Theoretical Model for LPR formation in a common utricular/saccular precursor

(A) In this model, the subcellular distribution of PCP proteins like Pk2 (green) establishes a ground polarity throughout the utricular/saccular precursor prior to HC development. (B) The precursor is then patterned into two domains containing HCs that produce opposite stereociliary bundle orientations (arrows and arrowheads) in response to the ground polarity. In this simplified model the utricular/saccular precursor is drawn as an annulus though other configurations are possible. The two domains are indicated by blue and white shading. (C) Separation of the utricular/saccular precursor during inner ear morphogenesis produces the utricle and a saccule intermediate. Both sensory organs contain an LPR however HC patterning at the LPR is complimentary. (D) When this model is based upon an annular patterning event, additional morphogenic movements (illustrated by red arrows) are required in order produce the mature saccular conformation.

Figure 4. Afferent Innervation is Coordinated with Bundle Polarity and the LPR

(A) The maculae contain two types of HCs that are distinguished from each other by the synaptic structure formed with afferent neurons. Type I HCs receive large calyx endings that surround the cell body while Type II HCs make Bouton-like contacts. The two HC classes are innervated by three types of afferent neurons. The Calyx-only class of neurons (orange) contacts clusters of Type I HCs in the striola region. Dipmorphic neurons (blue) contact HCs throughout the sensory epithelia, forming calyces with Type I HCs and boutons with Type II HCs. Bouton-only afferents (green) only contact Type II HCs located outside of the striola. (B) Each class of afferent neuron may contact multiple HCs, and the Dimorphic and Bouton-only neurons contact groups of HCs located on either side of the LPR. Despite the range of bundle orientations present in the utricle, afferent neurons only contact HCs with similar stereociliary bundle orientations. Developmental mechanisms coordinating neuronal innervation and stereociliary bundle orientation are not known. Bold arrows indicate the stereociliary bundle orientation.