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The fine structure and organization of tip links on hair cell stereovilli

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Introduction

We have recently described a set of links between the stereovilli of hair cells in the mammalian cochlea. The links emerge from the tips of the shorter stereovilli in the bundle, and extend upwards to join the side-wall of the adjacent taller stereovillus (e.g. Pickles *et al.*, 1984; Comis *et al.*, 1985). The tip links may be particularly interesting, because the evidence suggests that they are likely to be involved in sensory transduction, coupling the stimulus-induced movement to the transducer channels, perhaps situated at one or both of their points of insertion into the stereovilli (e.g. Hudspeth 1985; Pickles, 1985). In the present experiments, we have further investigated the fine structure of the tip links, in order to see how the structure might be related to a possible role in transduction. We have also investigated the organization of the links in stereovilli from a variety of species, since if they are involved in transduction, we might expect them to be universally present on mechano-receptor hair cells of acousticolateral origin and to be oriented parallel to the excitatory-inhibitory axis of the cell.

Methods

For transmission electron microscopy, guinea pigs were anaesthetised, the bullae extracted, and perfused with 2.5% glutaraldehyde and 2% tannic acid in 0.05 M BES buffer, adjusted to pH 7.4 with NaOH (method modified from Little and Neugebauer, 1985). After extraction of the modioli, the specimens were fixed for 0.5 h in the same buffer but including in addition 2% tannic acid, followed by postfixation in 1% OsO_4 in the same buffer for 5 min, and soaking in 2% tannic acid in distilled H_2O, at pH 7.0. Dehydration was accomplished with ethanol and the material stained *en bloc* with uranyl acetate and phosphotungstic acid, before embedding in an Epon Araldite epoxy resin mixture. Sections were stained in methanolic uranyl acetate and lead citrate before examination.

For scanning electron microscopy in lizards, pigeons and starlings, the

subjects were anaesthetised and were perfused either intravenously or directly through the oval or round windows with fixative (pigeon: 2.5% glutaraldehyde in 0.1 M phosphate buffer; starling and lizard: 1% glutaraldehyde and 15% saturated picric acid in 0.1 M phosphate buffer). Specimens were stored in 2% glutaraldehyde in 0.05 M or 0.1 M phosphate buffer until further treatment. They were then dehydrated in acetone, dried by the critical point technique with liquid CO_2, and sputter-coated with platinum to a nominal depth of 25 nm. Specimens were examined in a JEOL 120 CXII microscope with a scanning attachment, and images were observed by a secondary electron detector.

In order to measure the electrophysiological responses of the chick basilar papillae, chicks (1 - 14 days old) were anaesthetised, and the basilar papillae removed via an intracranial approach. The papillae were mounted in bird ringer or buffered saline (pH 7.4, 285 mOsm) in a recording chamber, with electrodes on either side of the sensory epithelium. The tectorial membrane was moved directly by means of a piezoelectric pusher (Burleigh), usually at 200 Hz and a peak to peak amplitude of 70 nm, the microphonic being detected with the help of a spectrum analyzer (Hewlett-Packard 3580A). Responses were tested every 5 min; after responses had been shown to be stable for 20 min, enzyme solution was introduced into the chamber, and responses were monitored at 10 min intervals for a further 40 - 60 min. The papillae were then fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy as above.

For enzyme experiments in the guinea pig, temporal bones were extracted as above, the cochleae perfused with 2.5 % glutaraldehyde, and the cochleae opened under fixative, giving a total time in fixative of 5 min. The opened modioli were immersed in enzyme solution at 4°C for 10 or 20 min, and then processed for scanning electron microscopy as above.

Results

The fine structure of tip links

In sections stained with uranyl acetate and lead citrate, a fine relatively straight filament was visible in the centre of the tip link (Fig. 1). The central filament was surrounded by darkly-staining material with a variable appearance. In some sections, the central filament appeared to be negatively stained. The central filament, including the heavily darkly-staining material immediately surrounding it, was measured as having a diameter of 5.5 nm ± 0.4 nm (sem, n = 16).

At its upper end, the central filament ran to the centre of a density on the sidewall of the taller stereovillus, the density forming a bridge between the external membrane of the stereovillus and the internal actin paracrystal. At its lower end, the filament ended on what usually appeared as a conical extension of the membrane of the stereovillus (Fig. 2). Below this, there was a clear area, and below that, there was a dense cap over the ends of the filaments of the actin paracrystal (Fig. 2). In some cases it was just possible to see filaments

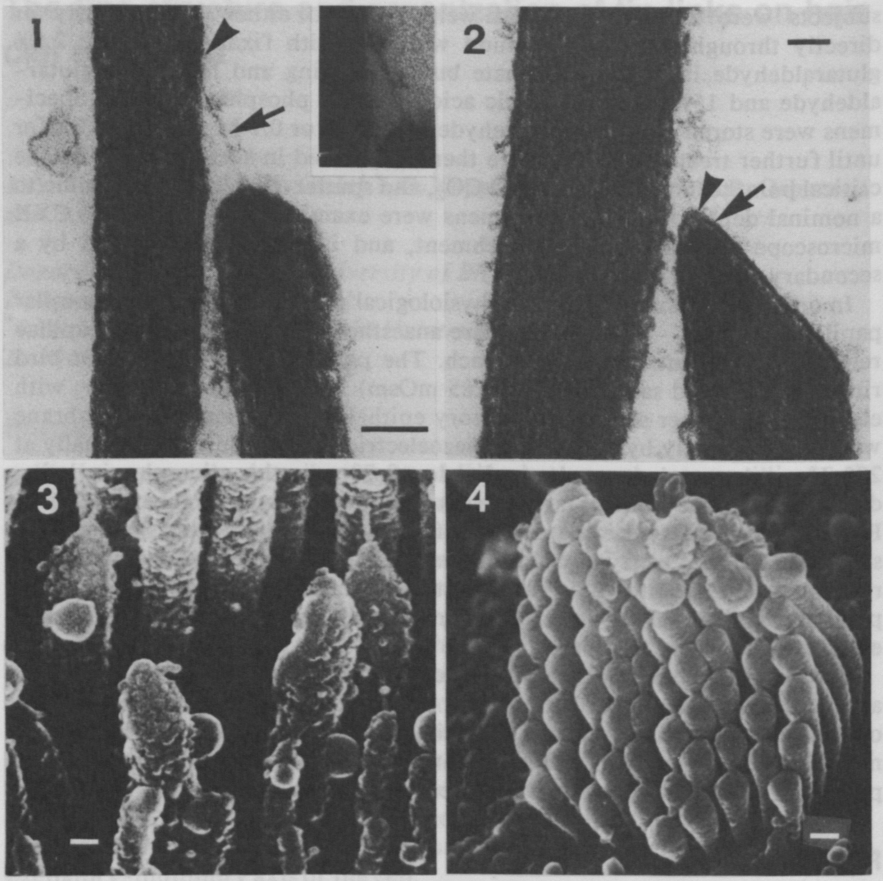
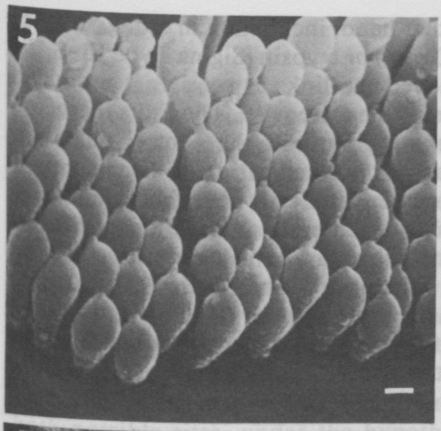
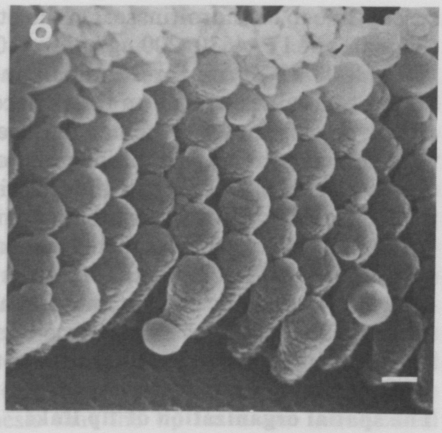


Figure 1. Tip link with central filament (arrow), and upper dense point of attachment (arrowhead). Insert: same, more densely printed. Guinea pig outer hair cell. Scale bar: 100 nm.
Figure 2. Dense cap (arrow) over actin filaments just under tip link. Filaments (arrowhead) are just visible, connecting density to surface membrane just under tip link. Guinea pig inner hair cell. Scale bar: 100 nm.
Figure 3. Chondroitinase-digested inner hair cell, showing some tip links remaining, but thinner than in control cochleae. Insert: detail. Guinea pig. Scale bars: 200 nm and 50 nm.
Figure 4. Hair bundle from the lizard Podarcis sicula, showing columns of stereocilia joined by tip links (arrows). Scale bar: 500 nm.

running between the dense cap and the overlying membrane near the point of insertion of the central filament of the tip link.

The material of variable appearance surrounding the central filament of





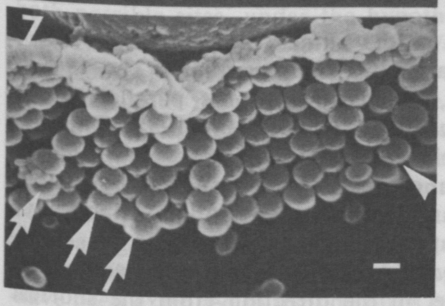


Figure 5. Hair bundle in starling, showing tip links, with stereocilia organized into columns running at right angles to the rows (i.e. along the axis of bilateral symmetry). Basal end of papilla, near abneural edge. Scale bar: 500 nm.
Figure 6. Tip link organization in pigeon. Abneural edge, middle of papilla. Scale bar: 500 nm.
Figure 7. Anomalous bundle in pigeon, with tip links over left and some of right of bundle oriented towards the right (arrows), and with those on the extreme right running towards the left (double-headed arrows). This hair cell was oriented 30 degrees differently from its neighbours. The latter were oriented such that their rows of stereovilli ran at right angles to the arrows on the left of the figure, with their tip links running parallel to the arrows. The large structure at the top is a bleb, probably associated with the hole in the cuticular plate. Middle of papilla, neural edge. Scale bar: 200 nm.

the tip link appeared to be continuous with the variable material surrounding the external membranes of the stereovillus. It may therefore consist of glycoconjugates. This hypothesis was tested by enzyme digestion in the guinea pig. Opened cochleae were incubated in chondroitinase (Sigma C-3509, 1 unit/ml) for 10 min at 4°C. While some of the tip links were missing, those that did survive were usually finer than in control cochleae (Fig. 3), suggesting that a surrounding coat had been removed, and that it was

susceptible to chondroitinase. On the other hand, incubation in crude protease (Sigma Type I P-4630, 200 μg/ml for 10 min) or trypsin (Sigma Type III-S, T-2395, 1000 72 μg/ml for 20 min) removed the links as soon as it produced other observable changes on the surface of the stereovilli. The links therefore may well contain a protein component, perhaps in the central filament, although we have no further information on the composition of the filament.

A common procedure for the isolation of viable hair cells from the mammalian cochlea involves incubation in collagenase (e.g. Zajic and Schacht, 1987). Digestion of the isolated chick basilar papilla in collagenase (Sigma type IV C-5138, 1 mg/ml) for up to 1 hr at 20°C had little effect on the evoked microphonic current, and was associated with survival of the tip links, although changes in the membrane texture sometimes made them difficult to see. This suggests that the links may not be composed of collagen.

The spatial organization of tip links

We have previously reported that in the guinea pig, tip links have a horizontal component in their orientation which is parallel to the axis of bilateral symmetry of the hair cell, or in other words parallel to the excitatory-inhibitory axis (Pickles *et al.*, 1984; Comis *et al.*, 1985). This was true in both inner and outer hair cells, i.e. in bundles of very different conformation. The presence and spatial organization of tip links was further investigated in hair cells of the bird and lizard basilar papillae, which have conformations quite different from that of the mammalian cochlea.

In the lizards *Podarcis sicula* and *Podarcis muralis* the tip links ran parallel to the axis of bilateral symmetry, i.e. in the direction from the shortest stereovilli to the tallest (Fig. 4). The stereovilli and their associated tip links in fact formed columns, running parallel to the axis of symmetry, sometimes clearly separating out into groups (Fig. 4). The same columnar organization was found in the starling (Fig. 5), pigeon (Fig. 6) and chick basilar papillae.

Where anomalous tip link orientations were found, they were generally associated with the following: (i) the stereovilli had been obviously disorganized in preparation, so that the links had been distorted, and (ii) there were other abnormalities in the bundle. In some cases moreover extraneous material lay over the surface of the bundle, making it difficult to identify the tip links. In only a few hair cells (an average well under 1%) was it possible to find anomalously-oriented tip links in an otherwise normal bundle. Mostly there were other abnormalities as well. In the case illustrated in Fig. 7, the tip links on the whole of the left half and on part of the right half ran towards the right; those on the extreme right of the bundle ran towards the left. This bundle was also anomalous in that the bundle as a whole was tilted with respect to its neighbours, with the orientation of the rows as a whole being some 30° different from those on either side. The orientation was such that the tip links over most of the bundle (i.e. over the left half and most of the right half) ran in a direction nearly parallel to the tip links on the adjacent hair cells.

Discussion

The results show that tip links have two components, namely a fine central filament, and a variable surrounding coat. We do not have information on the nature of the filament, although it is presumably protein. It has the same diameter as actin filaments within the paracrystal of the stereovilli in our material. Like actin, the filament is negatively-staining, and is susceptible to osmium tetroxide (Comis *et al.*, 1985). A fine filament would be ideal for concentrating the stimulus-induced movements onto a small area of membrane, suitable for opening the 1 - 4 transducer channels associated with each stereovillus (Russell, 1983; Holton and Hudspeth, 1986).

We do not know at which end of the link the transducer channels might be situated. The density at the upper point of attachment, or the conical extension of the stereovillar membrane at the lower point of attachment, are both possible sites, under the hypothesis that the tip links are involved in transduction (Pickles *et al.*, 1984). We note that the membrane around the lower point of insertion, at the tip of the shorter stereovillus, is anchored by filaments onto the dense cap on the underlying actin paracrystal, so that stimulus-induced shear can be concentrated on the membrane at that point.

The finding that tip links are present in a wide range of species supports the notion that they have an important functional role. They have so far been reported for the mammalian cochlea and vestibular system, including the guinea pig, rat and man (Pickles *et al.*, 1984; Rhys-Evans *et al.*, 1985) and the fish vestibular system (Little and Neugebauer, 1985). Here, the observations have been extended to include birds (chick, starling and pigeon) and reptiles (lizards). Investigation of these species had advantages, in that the hair bundles have a compact form, with the stereovilli being tightly packed into a hexagonal array. In such bundles, the orientation of the tip links parallel to the hair cell axis of symmetry is particularly obvious. The fact that the stereovilli tend to split into columns which run parallel to the axis of bilateral symmetry (i.e. along the excitatory-inhibitory axis) suggests that the mechanical connections along this axis are stronger than those in the other directions. This again emphasises the possible importance of the axis in hair cell function.

The laying down of the axis of the hair cell during ontogeny, and the development of the tip links and their organization (Neugebauer, 1986), are particularly interesting issues. Anomalously-oriented tip links may give suggestive evidence here. We have as yet only preliminary evidence on this point. We have previously reported in a group of guinea pigs where hair cells were found with anomalous axes, that tip links nevertheless tended to run at right angles to the long axis of the individual rows of stereovilli (Pickles *et al.*, 1986). This suggests that tip link organization is more closely related to the hair cell itself rather than to its surroundings. Here, however, we report a case where anomalous organization of the tip links was associated with an anomalous orientation of the bundle as a whole. This suggests that the orientation of the tip links can be affected by the surroundings of the cell, as

well as by factors internal to the cell.

Summary

Tip links on hair cells of the guinea pig cochlea have a fine (6-nm) central core, surrounded by a variable outer coat. The fine central filament is related to densities at its points of attachment on the stereovilli (stereocilia). We report here tip links for a wider range of systems, including birds (starling, pigeon and chick) and lizards. With very few exceptions, the tip links run parallel to the axis of bilateral symmetry of the hair cell, and the stereovilli are organized into columns parallel to this axis. When the organization of the tip links does not fit into this scheme, there are generally other anomalies in the hair bundle.

Acknowledgements

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Comments

Slepecky:

In your diagram you show the tip links parallel to the direction of bilateral symmetry, which is also parallel to the direction of hair cell excitation in the radial direction. This arrangement of stereocilia may be true for the basal region of the cochlea, but at the apical region the axes of bilateral symmetry of the stereocilia bundle is not exactly radial, and the fibers of the tectorial membrane curve. Does the orientation of the tip links vary with the shape of the stereocilia bundle, along the length of the cochlea?

Reply by Pickles:

There is almost certainly a longitudinal component to the travelling wave in the cochlear duct, so it should not be surpising that the axis of bilateral symmetry of the hair cells does not always run in a direction which is radial across the duct.

There are problems in analyzing the three-dimensional organization of stereovillar bundles in the apex of the mammalian cochlea, since the stereovilli are rather long and are easily disturbed during the preparation. However, it should be noted that in species such as birds, the angle of the hair cell axis changes in a regular and systematic manner across the width of the papilla, undergoing a change in orientation of up to 90°. In these cases, the columns formed by the tip links, and the tip links themselves, nevertheless continue to run parallel to the axis of bilateral symmetry of the bundle, in spite of the change in orientation.