Cochlear Mechanisms

Structure, Function, and Models

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TIP-LINK ORGANIZATION IN RELATION TO THE STRUCTURE AND ORIENTATION OF STEREOVILLAR BUNDLES

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Introduction

The linkages between stereovilli (stereocilia) of hair cells have been implicated both in the mechanical properties of the bundles and in the transmission of stimulus-induced movements to the individual mechanotransducer channels (e.g. Flock and Strelioff, 1984; Pickles et al., 1984; Hudspeth, 1985). Here, we describe new observations on the fine structure of the tip links between stereovilli. We also show that the spatial organization of the tip links in bundles of different conformations in a variety of species is appropriate for a role in transduction.

Methods

For transmission electron microscopy, temporal bones were extracted from anaesthetized guinea pigs. The bones were perfused with fixative as described previously (Pickles et al., 1987). Specimens were fixed for 0.5 h at 4° C in 2.5% glutaraldehyde containing 2% tannic acid and buffered with 0.05 M BES (NN-bis[2-Hydroxyethyl]-2-aminoethane sulfonic acid), adjusted to pH 7.4 with NaOH. After fixation tissues were postfixed in 1% OsO_4 in the same buffer for 5 min, followed by soaking in 2% tannic acid in distilled H_2O, with the pH adjusted to 7.0 with NaOH. Dehydration was accomplished with ethanol and the material stained *en bloc* with uranyl acetate and phosphotungstic acid before embedding in an Epon Araldite resin mixture (Mollenhauer, 1964). Sections were stained in methanolic uranyl acetate and lead citrate before examination in a JEOL 120 CXII transmission microscope operated at 80 kV.

For scanning electron microscopy in guinea pigs, the temporal bones were extracted as described above and fixed with 2.5% glutaraldehyde in 0.05 M BES buffer. For scanning electron microscopy in European lizards (*Podarcis muralis* and *P. sicula*), chicks (*Gallus gallus*), pigeons (*Columba livia*) and starling (*Sturnus vulgaris*), the animals were anaesthetized and perfused either transcardially or directly through the oval or round windows with fixative (pigeon: 2.5% glutaraldehyde in 0.1 M phosphate

buffer; chick, starling and lizard 1% or 2.5% glutaraldehyde and 15% saturated picric acid in 0.1 M phosphate buffer). Specimens were stored in 2% glutaraldehyde in 0.05 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 at 40 kV, and images were observed by a secondary-electron detector.

Results

THE FINE STRUCTURE OF TIP-LINKS IN GUINEA PIG HAIR CELLS

A fine, straight filament was visible in the centre of each tip link (Fig. 1). In many cases the fine central filament was surrounded by amorphous material, which had a variable conformation (Fig. 2). Similar material was also visible on the surface membranes of the stereovilli (Fig. 2). The fine central filament often appeared more lightly-staining than the surrounding material. Its mean diameter measured in sections taken at a range of original magnifications of 29,000 to 100,000 had a value of 5.5 nm (n=16), the same as the diameter of the actin filaments in the central core of the stereovilli measured in the same sections.

In sections cut perpendicular to the cuticular plate and perpendicular to the rows of stereovilli on an outer hair cell, the bundles had a triangular shape in lateral view, the stereovilli being closely apposed at the tips. The central filaments of the tip links running between the stereovilli of the three rows were nearly coaxial with each other, and also nearly coaxial with the shortest of the three stereovilli in the cross section (Fig. 3). In such sections, therefore, the two tip links and the shortest stereovillus lay along a nearly straight line. Moreover, in those sections where the rootlets were visible, each rootlet continued along the axis of the stereovillus (Fig. 4).

In addition to tip links, a broad band of links was visible running laterally between the rows of stereovilli, particularly visible between the tallest and second-tallest stereovilli in the bundle (arrowheads, Figs 3 and 4).

At its point of attachment on the taller of two stereovilli, the central filament of the tip link ran to the centre of an electron-dense area, which formed a bridge between the external plasma membrane and the central actin filaments of the stereovillus (arrowheads, Figs 1 and 2). At its lower end, the central filament met the plasma membrane at the tip of the shorter stereovillus, which was often pulled out into a point. This point overlay a clear area, with a denser cap over the ends of the actin filaments immediately underneath, visible in sections cut in a suitable plane. In some sections, fine filaments could be seen connecting the plasma membrane to the underlying density. The dense cap was only visible at the ends of the actin filaments immediately underlying the tip link: the other filaments ended without any obvious association with an electron-dense area. This was particularly apparent in the tips of stereovilli of the second tallest row on inner hair cells, which ended with a wider tip.

TIP-LINK ORGANIZATION IN RELATION TO THE HAIR-CELL AXIS

We have previously suggested that tip links on guinea pig inner and outer hair cells are organized such that the horizontal component in their orientation runs parallel to the hair cell axis of bilateral symmetry (Pickles et al., 1984; Comis et al., 1985). This axis defines the axis for excitatory and inhibitory deflections of the stereovilli (Flock, 1965). We have further investigated the point in bird and lizard basilar papillae, where the hair bundles show a quite different conformation and, sometimes, a systematically-varying orientation with respect to the papillar edge (Miller, 1980; Gleich and Manley, 1988).

In micrographs of bundles viewed perpendicular to the cuticular plate, the stereovilli were hexagonally packed (Fig. 5). The tip links were oriented along a 1,0 axis of the hexagon, parallel to the axis of bilateral symmetry, and at right angles to the long axis of the bundle as a whole (arrows: Fig. 5). The stereovilli were therefore organized into columns, running parallel to the hair-cell axis of bilateral symmetry. These observations were made consistently in bundles from lizards and all birds.

In micrographs of bundles viewed at an acute angle to the cuticular plate, the organization of stereovilli into columns, connected by tip links, was also obvious (Fig. 6). In some bundles, within the general gradation in height of the stereovilli from shortest on one side of the bundle to tallest on the opposite side, all the stereovilli in one column were a little taller, or a little shorter, than the corresponding stereovilli in adjacent columns (e.g. arrows, Fig. 6). Examples of this behaviour were found in all ears examined and in all regions of the papillae.

Where bundles had separated, perhaps due to mechanical trauma during preparation of the specimen, the stereovilli separated most commonly into columns running parallel to the axis of bilateral symmetry of the hair cell (Fig. 7). These are the columns defined in the previous paragraphs on the basis of the tip-link connections. When this occurred, there was no obvious debris on the upper ends of the separated, lateral, surfaces of the stereovilli in the gap (Fig. 7).

Discussion

The present observations on the fine structure of tip links suggests that the links have a fine central filamentous core, surrounded by an amorphous coat. We do not have information on the nature of the central filament, although it may well be protein. Like actin, the central filament reacts poorly with positive stains. Moreover, its diameter is similar to that of a single actin filament. On the other hand, the amorphous surrounding coat may well be an extension of the coat on the surface of the individual stereovilli, and may therefore consist of glycocongugates (Santi and Anderson, 1987).

One or both ends of the tip links are possible sites for the transducer channels, on the hypothesis that stretch of the tip links opens the transducer channels (Pickles et al., 1984). The densities associated with the ends of the filament probably represent anchorage points at which the forces are coupled to the internal cytoskeleton. At its lower end, the tip link joins to a conical extension of the membrane at the tip of the shorter

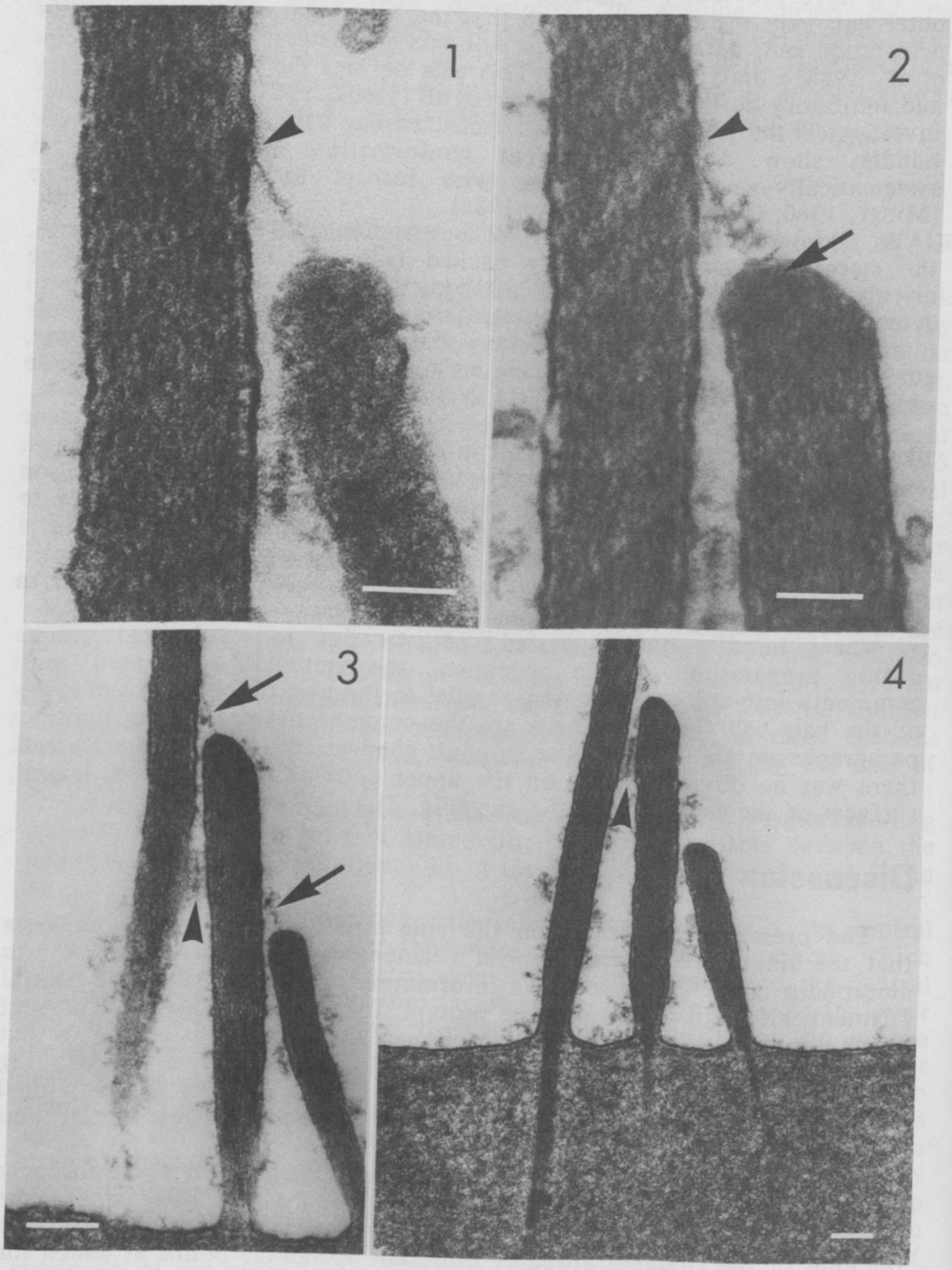
1
2
3
4

Fig. 1. Tip link on guinea pig outer hair cell, showing central filament. Arrowhead: upper dense point of attachment. Scale bar: 100 nm.

Fig. 2. Tip link on guinea pig outer hair cell, showing variable thickness of amorphous material attached to central filament of tip link. Arrowhead: upper dense point of attachment. Arrow: site of lower density under the surface membrane of the stereovillus. Scale bar: 100 nm.

Fig. 3. Tip links on guinea pig outer hair cell, showing that the tip links between the different rows are nearly coaxial, and coaxial with the shortest stereovillus on the bundle. Arrows: tip links. Arrowhead: lateral (inter-row) links. Scale bar: 200 nm.

Fig. 4. Guinea pig outer hair cell, showing that the rootlets are coaxial with the individual stereovilli, and that the stereovilli slope towards each other at their tips. Arrowhead: sideways (inter-row) links. Scale bar: 200 nm.

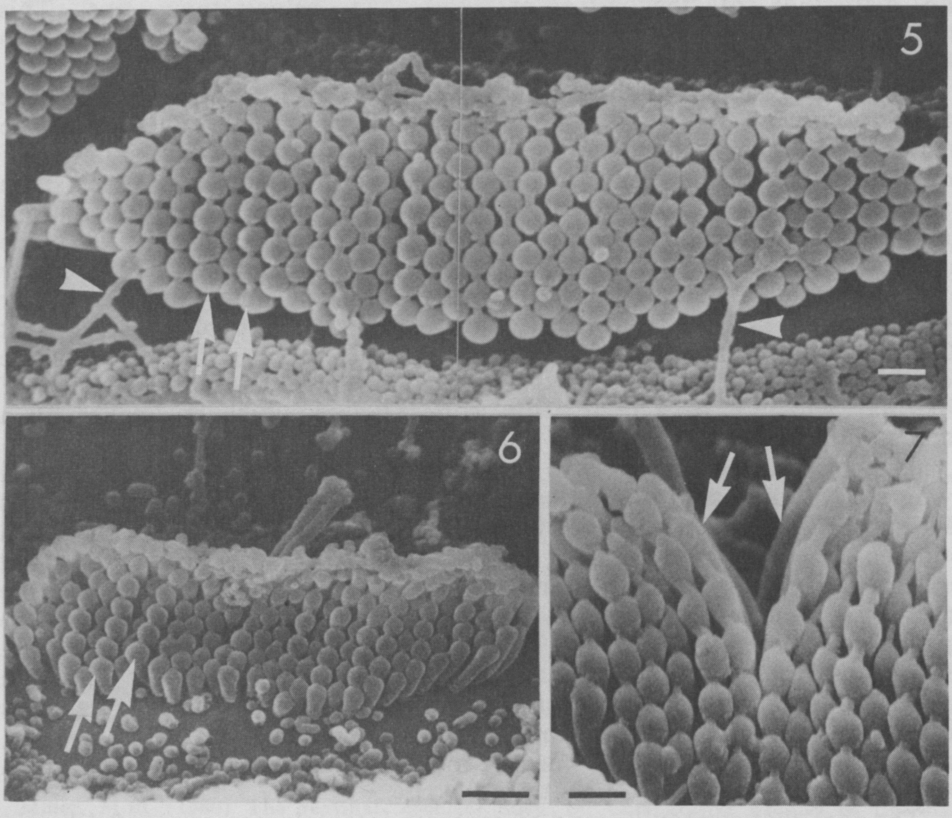

Fig. 5. Hair cell from neural edge of starling basilar papilla, viewed nearly perpendicular to the cuticular plate. Arrows indicate the axes of columns formed by the tip links. The tallest stereovilli in the bundle are at the top of the bundle. Arrowheads: extraneous material, perhaps from tectorial membrane, lying over stereovilli. Scale bar: 500 nm.

Fig. 6. Hair cell of starling basilar papilla. Arrows indicate two columns of stereovilli, joined by tip links. the stereovilli in these columns are a little taller than those in the adjacent columns. Neural edge of papilla. Scale bar: 1 μm.

Fig. 7. Hair cell of starling basilar papilla, showing separation of bundle along the columns defined by the tip links. No debris is visible on the lateral, separated, walls of the stereovilli (arrows). Scale bar: 500 nm.

stereovillus. Fine strands connect the membrane to the underlying dense cap over the ends of the actin filaments. The conical extension of the membrane is one possible site for the transducer channel(s). It is interesting that the conical extension of the membrane at the tips of shorter stereovilli, with the underlying density, was first noted by Flock (1965). The association of densities with attachment sites at both ends of the tip links was first noted by ourselves (Pickles, Osborne and Comis, Society for Experimental Biology, 5 January, 1984).

The geometrical arrangement shown in Figs 1 and 2 would be expected to produce a stretch of the tip links, in response to an angular deflection of the stereovilli. Indeed, the arrangement shown would tend to couple the deflections into the tip links with minimal stretch or compression of the other links, for instance of the lateral links which in guinea pigs join the stereovilli of the different rows just below their tips. This arrangement would therefore be efficient at coupling stimulus energy to the tip links, as opposed to other structures of the hair bundle.

The fine filament within each tip link is ideal for transmitting the stimulus-induced forces to a minute area of membrane. This would be mandatory if energy is to be coupled efficiently to the small number of transducer channels, perhaps between one and four, thought to exist on each stereovillus (Russell, 1983; Holton and Hudspeth, 1986). Calculations from the effective collecting area of the ear (Rosowski et al., 1986), the number of hair cells per mm of cochlear duct (Ulehlova et al., 1987), our own counts of the number of tip links on human inner and outer hair cells, and the critical bandwidth expressed as a length of cochlear duct (Greenwood, 1961), lead us to conclude that the forces collected from the acoustic wavefront would be concentrated onto the central filaments of tip links which have a total cross-sectional area which is smaller by a factor of 10^8 times.

The orientation of the tip links parallel to the axis of bilateral symmetry of the hair cell, and so parallel to the excitatory-inhibitory axis (Flock, 1965), is particularly obvious in the compact, hexagonally-packed bundles of birds and lizards. In addition, the observation of strict orientation of the tip links along the axis of bilateral symmetry of the individual bundle, irrespective of the bundle's orientation within the epithelium, is consistent with the hypothesis that the tip links are associated with the transduction process. This association of the direction of the tip links with the axis of bilateral symmetry of the hair cell was found even though in birds all orientations of hair cell axis could be seen, ranging from 0^0 to 90^0 with respect to the neural edge, depending upon position in the papilla.

The fact that stereovilli tend to separate into columns connected by tip links suggests that any sideways connections between different columns are weaker than the connections running within each column. Moreover, when the stereovilli had separated into columns, no debris was visible on the lateral surfaces of stereovilli near their upper ends. This suggests that there are no, or only sparse, lateral links connecting the stereovilli of the different columns in this region. It is likely therefore that the upper end of the bundle is relatively weakly braced against deflections which are oblique to the axis of bilateral symmetry. Again, this would be appropriate if the main mechanical input was parallel to the axis of

bilateral symmetry, i.e. parallel to the excitatory-inhibitory axis. At the moment, we do not know if the apparently stronger intra-column connections result only from the tip links, or whether there is a contribution from lateral links running below the tips of the stereovilli. On the other hand, where it was possible to look deep into the gaps where stereovilli had separated, it was possible to see remains of lateral links at the lower ends of the stereovilli, consistent with the rich lateral network of links in this region, as previously reported by Csukas et al. (1987) for the lizard.

Sometimes the stereovilli within one column were, within the general gradation in height of the stereovilli, all a little taller, or a little shorter, than the stereovilli in adjacent columns. This suggests that a factor which governs the heights of the stereovilli during development has a component which is expressed along each column, the column being as defined above on the basis of the tip links connections. It appears therefore that the heights of the stereovilli and tip link orientation are closely related by common factors in development.

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