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THE SEARCH FOR THE MORPHOLOGICAL BASIS OF MECHANO-TRANSDUCTION IN COCHLEAR HAIR CELLS

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INTRODUCTION

Hensen (1863) described the stereocilia on the hair cells of the cochlea, and suggested that stereociliary movement might initiate mechanosensory transduction. Over the next hundred and more years, much indirect evidence accumulated to support this contention. However, it was not until 1977, as a result of the micromanipulation experiments of Hudspeth and Corey, that a direct confirmation of the hypothesis appeared.

It has not so far been possible to obtain direct evidence on the next stage, that is, to identify unambiguously the site of the transducer channels on the stereocilia, or to define unambiguously the way that the stimulus is coupled to them. Nevertheless, new anatomical and electrophysiological observations in recent years have led to further hypotheses in this area, with the real possibility that the actual site of the mechanotransducer channels has now been identified.

EVIDENCE ON THE SITE OF TRANSDUCTION

A key observation which needs to be explained by any theory of transduction, is the relation between the functional and morphological polarization of hair cells. As originally shown by Lowenstein and Wersäll (1959), deflection of the hair bundle in the direction of the kinocilium is always excitatory, and deflection in the opposite direction always

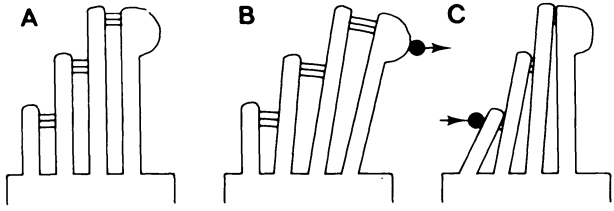

Figure 1. A: The stereocilia on a hair cell are graded in height, and in some cases there is a kinocilium (shown here with a bulb at the top) situated next to the tallest stereocilia. B and C: Both these manipulations lead to channel opening, but have opposite effects on the separation of the stereocilia. Circle: probe.

inhibitory. As a related observation, anatomical descriptions show the stereocilia to be universally graded in height, being always tallest on the side of the bundle next to the kinocilium, and shortest on the side furthest away from the kinocilium (Fig. 1A).

Kinocilium Theories

The association of the kinocilium with the axis of functional polarization led to hypotheses that the kinocilium might be directly involved in transduction. It was for instance suggested that transduction might involve distortion of the cell membrane at the site of insertion of the kinocilium (Fig. 1A) into the cuticular plate, or in the cuticle-free region or *opening* in the cuticular plate at this point (e.g. Hillman, 1969; Dallos, 1973). Such hypotheses have three disadvantages: (i) No kinocilium exists in the mature mammalian cochlea. It cannot therefore be involved in transduction in this organ. (ii) Even in cells which have a kinocilium, transduction depends on the stereocilia rather than the kinocilium. This was shown by Hudspeth and Jacobs (1979), who separately manipulated the kinocilium and stereocilia in frog saccular hair cells. (iii) In anomalous saccular hair cells with two well separated sub-bundles, only one of which had a kinocilium, manipulation of the sub-bundle which lacked a kinocilium nevertheless produced receptor potentials (Hudspeth and Jacobs, 1979). Therefore, transduction is not likely to depend on a normal relation between the stereocilia and the opening in the cuticular plate, or other structures normally found at the point of insertion of the kinocilium.

Since the kinocilium or associated structures do not seem directly involved in transduction, there remains the possibility that the gradation in heights of the stereocilia in some way determines the functional polarization.

Theories Related to Gradation in Height of the Stereocilia

Malcolm (1974) suggested that if the stimulus was preferentially coupled to the tallest stereocilia, the stereocilia of the different rows would tend to separate when the tallest stereocilia were pulled away from the shorter ones, i.e. moved in the excitatory direction (Fig. 1B). Conversely, the stereocilia would tend to close up if the tallest stereocilia were pushed in the direction of the shorter, i.e. in the inhibitory direction. He suggested that the opening of the spaces between the stereocilia would reduce the total access resistance for currents flowing through the apical surface of the hair cell. As a related hypothesis, it can be suggested that transduction might depend on stretch of links connecting the stereocilia together laterally, so that separation of the rows of stereocilia would stretch the links and open channels at the points of insertion. Both these hypotheses can be countered by the manipulation summarized in Fig. 1C. If the stereocilia are moved by a probe attached to the shortest stereocilia, rather than one attached to the tallest stereocilia as in Fig. 1B, movements in the direction of the tallest stereocilia still produce excitation, although the effects on the separation of the stereocilia, and the lateral linkages between them, can be expected to be the opposite to those shown in Fig. 1B (e.g. Hudspeth, 1982).

Tip Link Theories

A theory which successfully explains the relation between the functional and morphological polarization of hair cells, as well as the more recent data obtained from micromanipulation of hair cells, was put forward as a result of the discovery of tip links by Pickles et al. (1984) and Osborne et al. (1984). A fine link emerges from the tip of each shorter stereocilium in the hair bundle, and runs upwards to join the next tallest stereocilium of the adjacent row (Fig. 2). In addition, it was observed that the stereociliar bundle was tapered, with the stereocilia of the different rows tilting in towards each other so they nearly touched at their upper ends (Fig. 3). In this region, the stereocilia were joined by laterally-running links as well as by tip links. It was suggested that deflection of the whole bundle would result in a sliding or shearing motion between the stereocilia of the different rows. A deflection in the excitatory direction, i.e. in the direction of the tallest stereocilia, would tend to stretch the tip links (Fig. 3). The geometry is such that we would expect this to occur

irrespective of whether the stereocilia were pulled or pushed (cf Figs. 1B and C). Stretch of links would open transducer channels situated at one or both of the points of attachment of the links (Pickles, 1985).

The tip link theory has the following advantages:

(i) It explains the relation between the morphological and functional polarization of hair cells, since the excitatory-inhibitory axis runs parallel to the gradation in heights of the stereocilia.

(ii) It explains why the gradation in height of stereocilia is found in mechanotransducing acousticolateral hair cells.

(iii) As required by the hypothesis, tip links are found apparently universally in hair cells. In the mammals, they have been described in human and guinea pig cochlea and vestibular systems (Pickles et al., 1984; Rhys-Evans et al., 1985), and in chinchilla and rat cochleae (Lim, 1986; Lenoir et al., 1987). In birds, they have been described in chick, pigeon and starling basilar papillae (Pickles et al., 1988, 1989), and in reptiles in the lizard basilar papilla (Pickles et al., 1988, 1989). They have been described for the fish vestibular system by Little and Neugebauer (1985). We have also seen them in the saccular macula of the frog *Rana pipiens*, an example of the vertebrate class remaining.

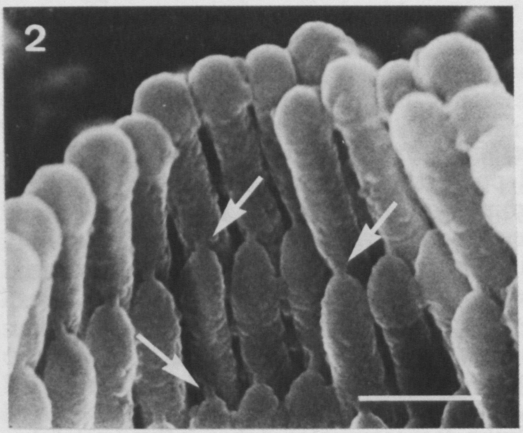

Figure 2. Tip links (e.g. arrows) seen in the apex of the 'V' of an outer hair cell in the guinea pig cochlea. Scale bar: 500 nm.

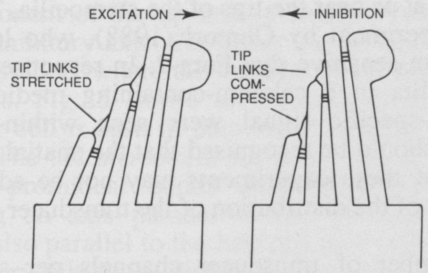

Figure 3. If preparation damage is avoided, stereocilia are seen to be closely apposed at their tips. Deflection of the bundle produces a sliding motion between the stereocilia, detected by the tip links.

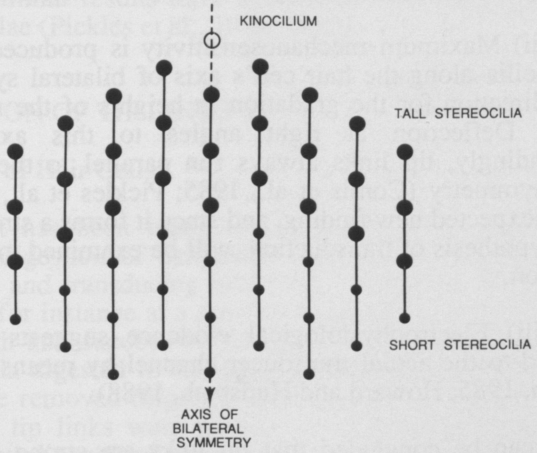

Figure 4. Stereocilia are hexagonally packed (large spots mark the positions occupied in V-shaped bundles, e.g. mammalian outer hair cells). Tip links run in the direction shown by the solid lines, parallel to the excitatory-inhibitory axis and parallel to the axis of bilateral symmetry.

(iv) The hypothesis explains why transduction is associated with the stereocilia rather than the kinocilium, even in cells which possess a kinocilium, since the tip links are associated with stereocilia.

(v) We would expect the transducer current to flow into the cell at or near the insertions of the tip links, and we would expect the transducer current to flow down the stereocilia themselves. The former point agrees with the observations of Hudspeth (1982), made in hair cells of the bullfrog sacculus. By exploring with an extracellular electrode during manipulation of the stereocilia, he showed that the transducer current

flowed into the cells at or near the tips of the stereocilia. The second point was tested in an experiment by Ohmori (1988), who loaded chick hair cells with the calcium-sensitive dye Fura-2. In response to small deflections of the stereocilia in a calcium-containing medium, the greatest changes in calcium-specific signal were seen within the stereociliar bundle. However, it should be recognised that the spatial resolution of the techniques in both of these experiments may not be adequate for more than a rough analysis of the distribution of the transducer currents.

(vi) The number of transducer channels per stereocilium was estimated by Holton and Hudspeth (1986) to be about four, and by Russell (1983) to be about one. The small number of transducer channels per stereocilium would agree with the idea that the stimulus is connected to each stereocilium at one, or only a few, sites on its surface.

(vii) Maximum mechanosensitivity is produced by deflection of the stereocilia along the hair cell's axis of bilateral symmetry, which is also the direction for the gradation in heights of the rows of stereocilia (Fig. 4). Deflection at right angles to this axis is ineffective. Correspondingly, tip links always run parallel to the hair cell axis of bilateral symmetry (Comis et al., 1985; Pickles et al., 1988, 1989). This was an unexpected new finding, and since it forms a strong support for the tip-link hypothesis of transduction, will be examined in greater detail in a later section.

(viii) Electrophysiological evidence suggests that the force is transmitted to the actual transducer channel by means of an elastic link (Hudspeth, 1985; Howard and Hudspeth, 1988).

It can be concluded that tip links are strong candidates for the structures which couple the mechanical stimulus to the transducer channels. While so far it has not been possible to perform an absolutely critical test of the hypothesis (e.g. by selectively removing only the tip links and seeing whether transduction disappears), some of the issues examined above, such as the spatial organization of the tip links, certainly would have had the potential for disproving the hypothesis.

DIRECTION OF TIP LINKS AND SPATIAL ORGANIZATION OF STEREOCILIA

The hair cell shown in Fig. 2 was photographed in a direction looking nearly parallel to the hair cell axis of bilateral symmetry, and so nearly parallel to the excitatory-inhibitory axis of the cell (as defined in Fig. 4). The tip links run nearly along the line of view (i.e. away from the observer) rather than across it. This forms an example of the way that the

horizontal component of the tip link orientation runs parallel to the hair cell excitatory-inhibitory axis. The point can be made clearly in hair cells of bird basilar papillae, where the bundles of stereocilia are compact and have many rows. Fig. 5 shows an example from the starling basilar papilla. The hair cell is seen in plan view, looking at right angles to the cuticular plate. The tall stereocilia are at the top of the micrograph. The tip links form prominent bands connecting the hexagonally-packed stereocilia along an axis parallel to the gradation in heights of the stereocilia, and also parallel to the hair cell axis of bilateral symmetry, the expected excitatory-inhibitory direction. In many micrographs the groups of stereocilia joined by tip links are seen to form columns running parallel the hair cell axis of symmetry (Fig. 6), and in bundles distorted during preparation the groups of stereocilia tend to separate into columns joined by the tip links. Similar results have been shown for chick, pigeon and lizard basilar papillae (Pickles et al., 1988, 1989).

THE COMPOSITION OF TIP LINKS

Enzyme Digestion of Hair Cells

An attempt has been made to test the tip link hypothesis by selective enzyme digestion of hair cells. A favourite technique for the isolation of viable and transducing hair cells is digestion in collagenase (Sigma Type IV), for instance at a concentration of 1 mg/ml for about 10 min (e.g. Hudspeth and Jacobs, 1979). Fig. 7 shows a hair cell from a chick basilar papilla digested in 460 μg/ml collagenase for 1 hr, with the tectorial membrane removed (Sigma Type IV, at 20°C, pH 7.7). Over all the cells counted, tip links were present on 82% of possible sites, as against 78% in control cochleae incubated in buffer for the same length of of time. Digestion in 2.22 mg/ml collagenase for 1 hr produced 72% survival (not significantly different by 2-tailed t-test). It can be concluded therefore that collagenase preserves the anatomical integrity of the linkages on the stereocilia, as well as the functional integrity of transduction. It can also be concluded that tip links do not consist of collagen.

Further enzyme digestion experiments in the guinea pig showed that while crude protease (Sigma Type I, P-4630, 200 μg/ml for 10 min) or trypsin (Sigma Type III-S, T-2395, 72 μg/ml for 20 min) removed the tip links as soon as they produced other observable changes, chondroitinase (Sigma C-3509, 1 unit/ml for 10 min), where it left tip links on the hair cells, left the links looking thinner (Pickles et al., 1988). These results suggest that tip links have an essential protein component, but gain thickness from a chondroitinase-sensitive component.

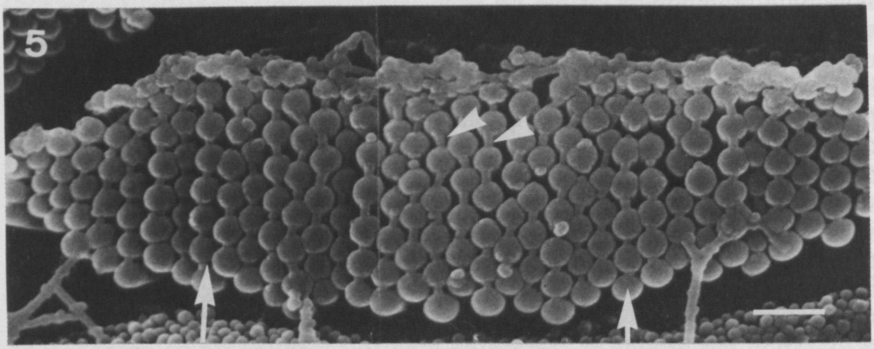

Figure 5. Hair cell from starling basilar papilla in plan view shows hexagonal packing of stereocilia, and tip links (arrowheads). Arrows: columns of stereocilia joined by tip links. A few tip links are not preserved. Scale bar: 500 nm.

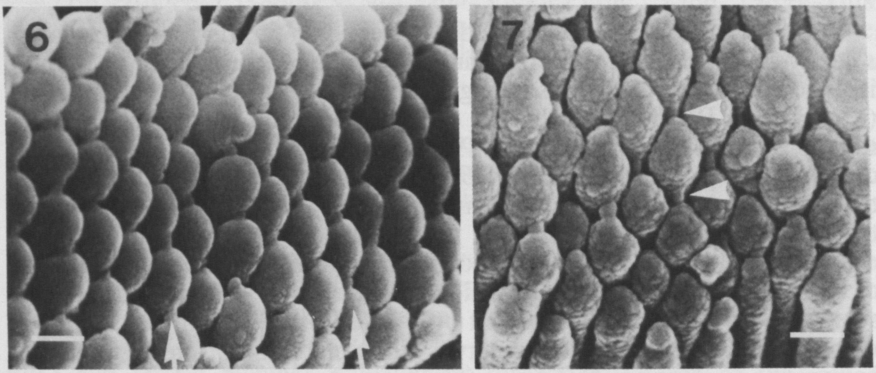

Figure 6. Columns of stereocilia (arrows) joined by tip links. The columns run from shortest stereocilia in the bundle (bottom of picture) to tallest (at top). Starling. Scale bar: 200 nm.

Figure 7. After digestion in collagenase, a high proportion of tip links remain. Chick basilar papilla, incubated in 460 μg/ml collagenase for 1 hr. Scale bar: 200 nm.

The Fine Structure of Tip Links

These results are in agreement with the observations of the fine structure of tip links, which show tip links to possess a fine central filament surrounded by an amorphous coat (Osborne et al., 1988). The fine central filament, approximately 6 nm in diameter, reacts poorly with positive stains. It almost certainly consists of protein, and as suggested by the protease and trypsin experiments, the protein is likely to be important for the structural integrity of the tip link. A fine central filament within the tip link would be ideal for transferring the stimulus-induced forces from the stereociliar bundle as a whole to a minute area of membrane. This would be necessary if the stimulus energy were to be coupled efficiently to the small number of membrane-bound transducer channels. Apart from the lack of reaction to collagenase, we do not have further information on the composition of the protein component. Since transduction can survive deflections of the stereocilia which stretch the tip links by more than 100% (Howard and Hudspeth, 1988), the central filament of the tip link must on the tip link hypothesis be able to stretch by at least this amount. This suggests that it is composed of a resilient, elastic protein such as elastin. Elastin itself reacts poorly with positive stains, as does the central filament of the tip link, and is relatively resistant to degradation by proteolytic enzymes except for the elastases. Preliminary experiments in our laboratory suggest that the tip links are indeed digested by elastase. On the other hand, it must be remarked that elastin itself does not normally form thin filaments. By contrast, a filamentous protein such as actin is a poor candidate for the central filament of the tip link, since actin breaks with stretches of more than a few percent (Kishino and Yanagida, 1988).

The amorphous coat around the tip link is likely to be glycoconjugate, and appears to be continuous with the glycocongugate cell coat around the stereocilia (Santi and Anderson, 1986; Osborne et al., 1988). Like the coat around the stereocilia, it reacts positively with cationized ferritin (Neugebauer and Thurm, 1987). The variability in the thickness of tip links from micrograph to micrograph is likely to represent differences in the preservation of this coat.

THE DEVELOPMENT OF TIP LINKS, IN RELATION TO THE DEVELOPMENT OF THE HAIR CELL AXIS OF SYMMETRY

One intriguing question is, how do the tip links form, and how do they always come to run parallel to the hair cell axis of bilateral symmetry? Are the tip links present as soon as the stereocilia emerge from the cell surface, or do they jump across between adjacent stereocilia once the stereocilia have formed? Scanning electron microscopy in immature

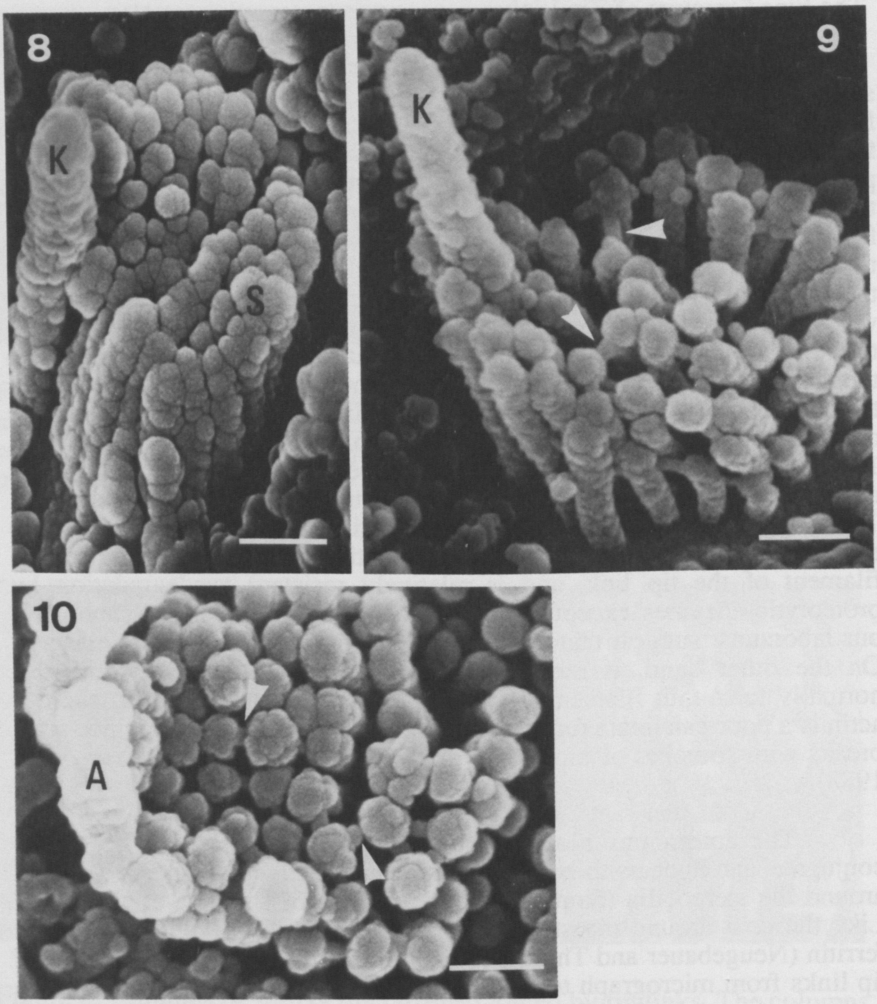

Figure 8. Tightly-packed stereocilia (S) in the developing hair bundle of a 10-day embryonic chick. K: kinocilium. Scale Bar: 200 nm.

Figure 9. When the immature stereocilia separate, material (arrowheads) can be seen connecting their upper ends. Extreme apex of papilla of 13-day chick embryo. K: kinocilium. Scale bar: 200 nm.

Figure 10. Material (arrowheads) connecting immature stereocilia, seen in plan view. No columnar organization of the immature stereocilia is visible (compare with Fig. 5). To the left of the bundle (A), some stereocilia and the kinocilium have lengthened. Scale bar: 200 nm.

hair bundles from the chick basilar papilla shows that at 10 - 11 days embryonic age, the stereocilia form a tightly-packed clump without any gradation in height (Fig. 8; confirmed by stereo views). A kinocilium is situated to one side of the bundle. In some bundles the immature stereocilia tend to separate slightly (Fig. 9). In these cases, material can often be seen connecting the upper ends of the separated stereocilia, in a layer just below their tips (Fig. 9). However, links do not appear to run preferentially parallel to the axis of bilateral symmetry, nor is there any indication that the stereocilia tend to group into columns running parallel to that axis (Fig. 10).

As described previously by Tilney et al. (1988), the stereocilia on the side of the hair cell nearest the kinocilium begin to grow in height first, and adjacent rows then progressively lengthen. As this wave of development spreads across the bundle, links can be seen which have a dual appearance, looking both like the material connecting the immature stereocilia just below their tips, and the tip links seen in mature bundles. It is suggested therefore that the tip links are differentiated from an initial uniform population of links, by the differential growth of the stereocilia. As the gradation in height of the bundle appears, it is suggested that those lateral connections running in a direction corresponding to the steepest gradient in height are pulled to a more vertically-pointing position, to differentiate into the tip links (Fig. 11). The links are then carried upwards as the stereocilia grow in height. In this way, the tip links would tend to run in the direction of gradation in heights of the stereocilia, and so parallel to the hair cell axis of bilateral symmetry.

At the moment the question remains open as to whether this mechanism is sufficient to explain the very great regularity seen in the organization of the tip links, and whether further organizing principles are required. There is also the intriguing possibility that tip links are involved in development in a more intimate way. Tilney et al. (1988) have speculated that pull on the tip links, by activating the transducer channels, induces growth of the shorter stereocilia. By progressive effects on the different rows, this could be responsible for the spread of growth across the stereociliar bundle. Tilney's speculation receives support from our own micrographs, since it was commonly found that, within the normal gradation in height of the stereocilia, all the stereocilia in one tip-link column could be a little taller, or a little shorter, than the corresponding stereocilia in adjacent columns. This suggests that some factor, which governs the heights of the stereocilia, has been transmitted along a column during development.

If Tilney's hypothesis is indeed valid, we would also expect transducer channels to be operative as soon as the gradation in height of the stereocilia begins to appear. In this context, it can be noted that the

earliest microphonic potentials in response to sound are found in chicks at 10-11 days embryonic age (Saunders et al., 1973). This is the age at which the gradation in heights of the stereocilia is just beginning to appear (Tilney et al., 1988).

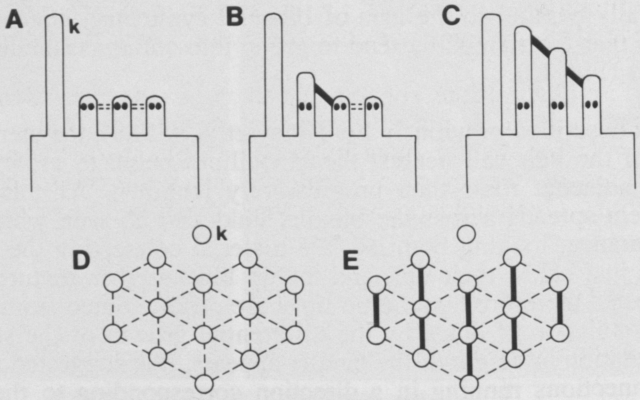

Figure 11. Hypothesis for the formation of tip links running parallel to the hair-cell axis of bilateral symmetry. In A, the immature stereocilia are connected in all directions by lateral links (dotted lines- in plane of figure; spots- coming out of plane of figure). As the stereocilia nearest to the kinocilium (k) progressively lengthen, links running parallel to the gradient in height are pulled to a more vertically-pointing position to form the tip links (thick lines; B and C). Thus the orientation of tip links seen in the mature bundle (plan view: E) is produced from the population of lateral links seen in the immature bundle (D). No connections between the kinocilium and the stereocilia are drawn in this figure, because in the scanning electron micrographs of this paper they were obscured by the close apposition of the cilia.

SUMMARY AND CONCLUSIONS

Recent years have seen a remarkable convergence between the anatomical and physiological information available on transduction in hair cells. While completely conclusive evidence that the tip links are involved in mechanotransduction is still lacking, the hypothesis is supported by a large amount of indirect evidence. Moreover, it explains details which are difficult to account for under any other hypothesis.

If tip links are indeed involved in transduction, we would expect the transducer channels to be situated at one, or less likely both, of the links' points of attachment to the stereocilia. At the upper end, the central filament within the link runs directly to the centre of an osmophilic spot in the sidewall of the taller of the two stereocilia to which it is connected. At the lower end, the central filament attaches to the membrane at the tip of the shorter of the two stereocilia. The membrane in this region is attached to the underlying actin paracrystal by fine strands, and so a pull on the tip link would tend to develop a stretch in the membrane at this point.

Further observations suggest that there is a close relation between the tip links and the spatial organization of the stereocilia, and even possibly a role for the tip links in the development of the stereocilia. Future studies of the morphological correlates of transduction may therefore ultimately illuminate a wider range of hair-cell processes.

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