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Role of Myosin VI in the Differentiation of Cochlear Hair Cells

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The mouse mutant Snell's waltzer (*sv*) has an intragenic deletion of the *Myo6* gene, which encodes the unconventional myosin molecule myosin VI (K. B. Avraham *et al.*, 1995, *Nat. Genet.* 11, 369–375). Snell's waltzer mutants exhibit behavioural abnormalities suggestive of an inner ear defect, including lack of responsiveness to sound, hyperactivity, head tossing, and circling. We have investigated the effects of a lack of myosin VI on the development of the sensory hair cells of the cochlea in these mutants. In normal mice, the hair cells sprout microvilli on their upper surface, and some of these grow to form a crescent or V-shaped array of modified microvilli, the stereocilia. In the mutants, early stages of stereocilia development appear to proceed normally because at birth many stereocilia bundles have a normal appearance, but in places there are signs of disorganisation of the bundles. Over the next few days, the stereocilia become progressively more disorganised and fuse together. Practically all hair cells show fused stereocilia by 3 days after birth, and there is extensive stereocilia fusion by 7 days. By 20 days, giant stereocilia are observed on top of the hair cells. At 1 and 3 days after birth, hair cells of mutants and controls take up the membrane dye FM1-43, suggesting that endocytosis occurs in mutant hair cells. One possible model for the fusion is that myosin VI may be involved in anchoring the apical hair cell membrane to the underlying actin-rich cuticular plate, and in the absence of normal myosin VI this apical membrane will tend to pull up between stereocilia, leading to fusion.

Key Words: **myosin VI; genetic deafness; Snell's waltzer; mouse mutant; cochlear hair cells; microvilli; unconventional myosin.**

INTRODUCTION

Of the handful of genes involved in deafness identified so far, three have been found to encode unconventional myosin molecules. Mutations in *Myo7a* have been found in shaker1 mouse mutants, Usher type 1B in humans, and both dominant and recessive forms of nonsyndromic deafness in humans (Gibson *et al.*, 1995; Mburu *et al.*, 1997; Weil *et al.*, 1995, 1997; Liu *et al.*, 1997a,b). *Myo15* has been reported to underlie both the shaker2 mouse mutant and another form of human recessively inherited nonsyndromic deafness (Probst *et al.*, 1998; Wang *et al.*, 1998). Finally, *Myo6*, encoding the myosin VI molecule, is mutated in the Snell's waltzer (*sv*) mouse mutant (Avraham *et al.*, 1995).

The Snell's waltzer mouse mutant was first described by Deol and Green in 1966 and displays the typical shaker–waltzer behaviour often associated with inner ear defects: deafness, hyperactivity, head tossing, and circling. Using light microscopy, Deol and Green (1966) found hair cell degeneration and loss to progress rapidly from 12 days after birth. The responsible gene was identified by Avraham and colleagues in 1995, and the mutation in the Snell's waltzer mouse was found to be an intragenic deletion leading to an absence of detectable myosin VI protein in homozygotes. Immunofluorescence studies localised myosin VI to the inner and outer hair cells of the sensory epithelium in the normal adult mouse cochlea (Avraham *et al.*, 1995, 1997). Hasson *et al.* (1997) further reported that myosin VI was

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highly enriched in cuticular plates and the pericuticular necklace around the cuticular plate, present in the hair cell cytoplasm, but was not detected in the stereocilia in adult guinea pigs. Recently, myosin VI has been reported to be detected in presumptive hair cells from as early as 13.5 days of gestation, although the localisation within hair cells at early stages was not described (Xiang *et al.*, 1998).

It is estimated that sensory hair cells in the cochlea detect movements as small as 1 angstrom unit at the threshold of hearing, so it is not surprising that maintenance of their cytoskeletal structure to a high degree of precision is critical to their function. Unconventional myosins may participate in this maintenance, which might explain why hair cells are particularly susceptible to mutations in the corresponding genes. However, despite recent reports of stereocilia bundle defects in the shaker1 and shaker2 mutant mice (Self *et al.*, 1998; Probst *et al.*, 1998), we still do not understand the role of unconventional myosins in hair cell development or function. In this report, we describe the development of hair cells in Snell's waltzer mutants, along with some further observations of cochlear responses in these mutants.

MATERIALS AND METHODS.

Genotyping. Homozygous *sv* mutant mice can be distinguished from littermate controls from 12 days of age by their abnormal behaviour, but all mice were subjected to genotyping by PCR. Genomic DNA was amplified with primers that flank the deletion in the *sv* allele (Avraham *et al.*, 1995). Wild-type mice displayed a genomic fragment of 4.0 kb, *sv/sv* mice displayed a 2.9-kb fragment, and DNA from heterozygote mice generated both fragments. Primers used were as follows: primer M2404 is 5′-GAAGTTGACTACAAGTTTGGG-3′ (mouse *Myo6* cDNA nucleotides 2404–2424) and primer M2615R is 5′-GCTTCAGCTCGATATTTTATT-3′ (mouse *Myo6* cDNA nucleotides 2615–2595). The reaction mixture contained 200 ng genomic DNA, PCR buffer with 1.75 mM $MgCl_2$, 350 μM each dNTP, 300 nM each primer, and Expand Long-Template polymerase (*Taq* and *Pwo* DNA polymerases; Boehringer Mannheim). PCR was performed as follows: 2 min denaturing at 94°C; followed by 4 cycles of 10 s denaturation at 94°C, 30 s annealing at 56°C, 5 min extension at 68°C; followed by 24 cycles of 10 s denaturation at 94°C, 30 s annealing at 52°C, 5 min extension at 68°C; and finally an additional 5 min at 68°C.

Scanning electron microscopy. A total of 25 *sv* homozygotes and 32 littermate controls were examined at birth and 1, 3, 7, 12, and 20 days after birth (DAB); all controls were +/*sv* except one +/+ at 7 DAB. Inner ears were rapidly removed under a fixative of 2.5% glutaraldehyde in 0.1 M phosphate buffer with 0.25% tannic acid and 3 mM $CaCl_2$, pH 7.2. Openings into the coils were made by piercing the apex and rupturing both the oval and the round windows. The specimens were fixed for 5 h, buffer washed, and then dissected further to reveal the surface of the organ of Corti. The osmium tetroxide–thiocarbohydrazide procedure adapted from Hunter-Duvar (1978), Furness and Hackney (1986), and Davies and Forge (1987) was used to stain prior to dehydrating and critical point drying. Specimens were finally sputter coated with gold and examined in a Jeol 6400 Winsem electron microscope.

Transmission electron microscopy. A total of 47 (13 inner hair cell, IHC, and 34 outer hair cell, OHC) homozygote *sv/sv* hair cells and 30 (8 IHC and 22 OHC) control hair cells were examined at birth and 20 days after birth from two mutants and two control mice (one +/+ and one +/*sv*). Cochleas were fixed in 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer with 3 mM $CaCl_2$, 0.25% tannic acid, and 1% sucrose, pH 7.2 for 5 h at 4°C. After buffer washing the cochleas were postfixed in 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol, and stained *en bloc* with 1% phosphotungstic acid and 1% uranyl acetate, before being embedded in Araldite epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate before examination on a Jeol 1010 electron microscope at 80 kV. For counts of raised membranes between stereocilia, sections with good profiles of the cuticular plate and stereocilia bases were chosen (i.e., stereocilia were shown in longitudinal section). Counts were made from 8 control and 9 mutant IHCs plus 22 control and 21 mutant OHCs in newborn specimens.

Endocytosis. We assessed the ability of mutant hair cells to undergo endocytosis by using the fluorescent dye FM1-43, in 6 mutants and 15 control mice at 1 and 3 days of age. FM1-43 is a styryl dye used extensively to investigate activity-dependent vesicle recycling, endocytosis, and membrane recycling (e.g., Cochilla *et al.*, 1999). The dye fluoresces brightly when bound to a membrane, but shows little fluorescence in aqueous solution. Cochlear coils were dissected in Hanks' balanced salt solution at room temperature, transferred to Dulbecco's modified Eagle's medium containing 10 mM Hepes (GIBCO BRL), and cultured for 2 h at 37°C. They were then stained with the dye FM1-43 (Molecular Probes) at a concentration of 3 μM for 15 s at 37°C. This exposure time was chosen because it gave optimal labelling of focal spots of fluorescence presumed to represent newly endocytosed vesicles, and longer periods of labelling led to an intense, diffuse labelling within hair cells without the focal pattern that gives confidence that the labelling was indeed of vesicles. Samples were subsequently washed with PBS, fixed with 4% paraformaldehyde, and mounted before viewing on a Leica confocal microscope. Confocal images were examined and assessed blind by two independent observers in addition to the authors.

Electrophysiology. Eight *sv/sv* mutants and eight of their littermate controls (+/*sv* or +/+) at 20 or 30 days after birth (four pairs at each age) were used for measuring cochlear responses. The mice were kept in full accordance with UK Home Office regulations. The mice were anaesthetised with urethane, a recording electrode was positioned on the round window of the cochlea, and responses recorded as described previously (Steel and Smith, 1992). Round window responses are a well-established method for assessing cochlear activity and give response thresholds close to those of single units (Johnstone *et al.*, 1979). Three types of response were measured. Compound action potentials (CAPs) consist of one or two sharp negative deflections at the start of the tone burst, representing synchronous firing of many cochlear neurones. Summating potentials (SPs) are DC shifts in the waveform sustained for the duration of the tone burst and are thought to represent the gross counterpart of intracellular dc responses of sensory hair cells in the cochlea (e.g., Dallos *et al.*, 1972; Dallos, 1986), and they may be positive or negative in polarity depending on stimulus frequency and intensity. Cochlear microphonics (CMs) are an AC response with a frequency identical to that of the stimulus, and the CM measured from the round window is considered to be primarily generated by the basal turn outer hair cells (e.g., Patuzzi *et al.*, 1989; Dallos and Cheatham, 1976).

RESULTS

Stereocilia Start to Develop Normally, but Then Fuse Together to Form Giant Stereocilia

In the littermate controls, stereocilia bundles show a normal pattern of development, with basal turn hair cells more advanced than apical turn hair cells. At birth, the cluster of microvilli at the top of each hair cell is already polarised, with a crescent-shaped array of microvilli on the lateral (outer) edge of each cell growing taller. Later, these microvilli become the stereocilia, while microvilli in the centre and modiolar (inner) edge of each hair cell are shorter and in the process of being resorbed. A kinocilium is located at the lateral pole of each hair cell. Over the next few days, the stereocilia grow to form rows of graded heights, with the tallest on the lateral edge; the short microvilli are cleared from the surface; and the kinocilium is lost (Fig. 1). During these few days after birth, outer hair cell stereocilia form a distinct V-shaped array of thin, apparently more rigid stereocilia, generally with three rows per hair cell, while inner hair cell stereocilia retain their crescent-shaped organisation and grow thicker than those of outer hair cells (Fig. 1).

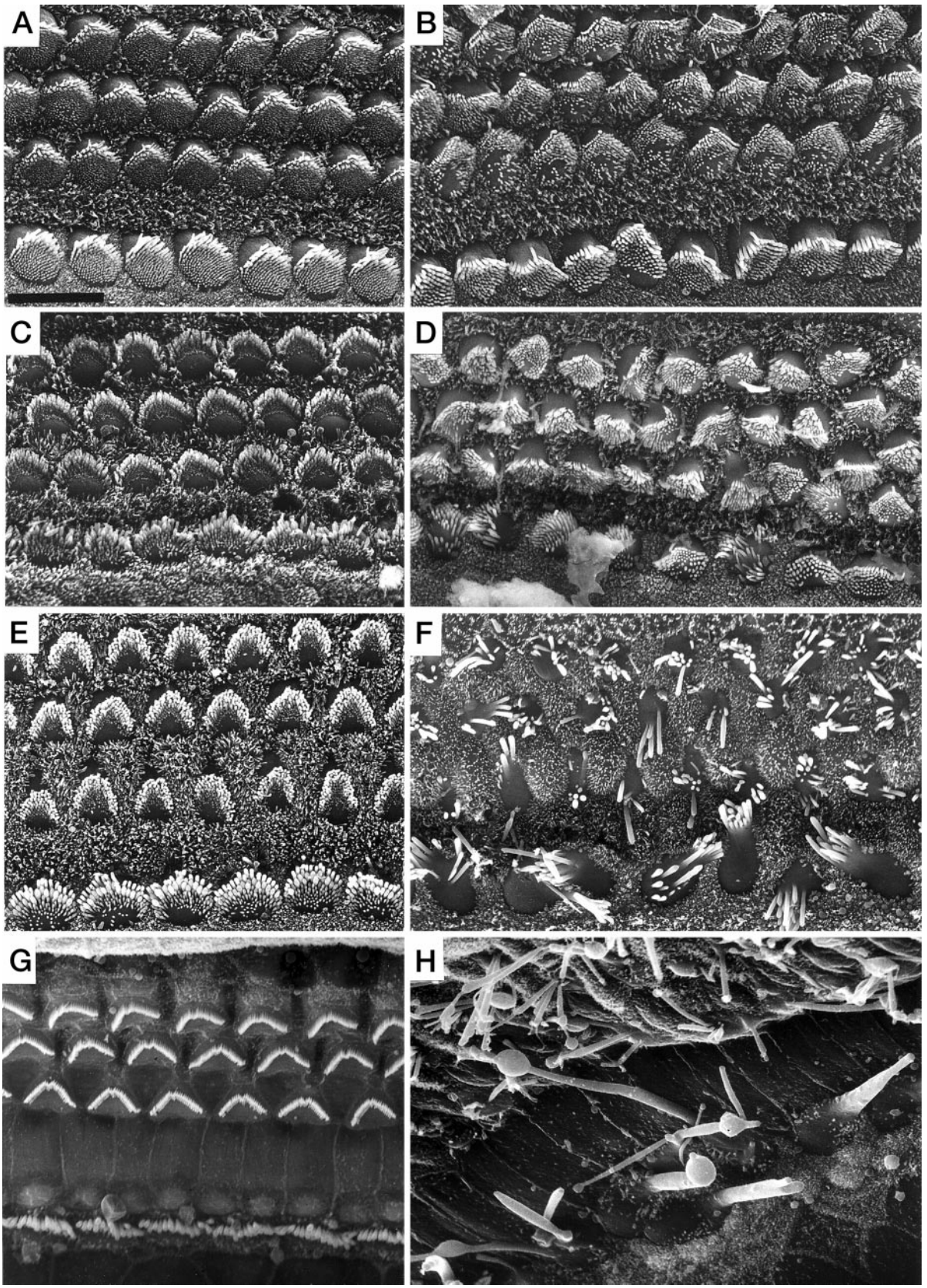
In the Snell's waltzer mutants, the general organisation of the stereocilia bundles appears broadly normal at birth, with the crescent-shaped arrays of growing stereocilia located at the lateral poles of hair cells, but there are some minor signs of disorganisation (Fig. 1B shows 1 DAB). In places, the stereocilia bundles show a disorganised, swirled appearance, and close inspection by scanning electron microscopy reveals early signs of fusion of stereocilia, starting at their bases, in a few hair cells (Figs. 1B and 2A). There were no obvious abnormalities in the cuticular plates of the newborn mutant hair cells when examined by transmission electron microscopy, but occasionally the apical plasma membrane between adjacent stereocilia was raised slightly, and this appeared to occur more often in the mutant than in the control (Fig. 3). For IHCs, no interstereocilia gaps showed raised membranes in controls (0/49), while 22% of these gaps (10/46) showed visibly raised membranes in mutants. For OHCs, 8% of gaps showed raised membranes in controls (15/194), compared with 20% in mutants (37/185). Material similar in appearance to the cuticular plate filled the area below the raised membranes. A day later, further signs of disorganisation and fusion are seen, and by 3 DAB most hair cells are affected (Figs. 1D and 2B). At 7 DAB, there is extensive fusion and excess growth of stereocilia, resulting in just one or a few large protrusions on the top of each hair cell (Figs. 1F and 2C). The process of fusion and extension of these protrusions, or giant stereocilia, continues. By 20 DAB, there are no hair cells anywhere in the cochlear duct showing normal stereocilia, and some gaps are seen in the regular array of hair cells, suggesting that they are beginning to degenerate, almost certainly as a secondary effect (Avraham *et al.*, 1995) (Fig. 1H). The protrusions often lie across the surface of the organ of Corti, including across neighbouring support cells, rather than remaining perpendicular to the upper surface, and sometimes appear to be fused to the underlying apical surface membrane (Fig. 2D). The protrusions can show bifurcations and often have bulbous regions at or near their tips (Fig. 1H). These giant stereocilia appeared to contain diffuse actin-like material, membrane whorls, and other cell contents not normally found inside stereocilia (Fig. 4). Many hair cells had disappeared by 20 DAB, and degenerating hair cells were commonly seen in sections of a 20-DAB mutant.

Mutant Hair Cells Show Evidence of Endocytosis

As unconventional myosins have been implicated in membrane trafficking, and apical membrane turnover might influence stereocilia organisation, we used a membrane dye, FM1-43, to assess whether mutant hair cells showed evidence of endocytosis. Serial confocal images through the organ of Corti showed that there were focal spots of intense fluorescence located within the hair cell cytoplasm, both above and below the nuclei, and they were particularly numerous just below the apical surface of the hair cells (Fig. 5). The intensity of these focal spots varied between samples, so we do not suggest that these observations are quantitative or can be taken to indicate that membrane trafficking is completely normal. Nonetheless, these spots were seen in both mutant and control preparations at 1 and 3 days of age and can be taken to indicate that endocytosis has occurred. In transmission electron micrographs of newborn specimens, pits looking like coated pits were seen at the apical surface of both mutants and control hair cells (Fig. 5).

Snell's waltzer Mutants Show Minimal Responses to Sound Stimulation

The control mice showed compound action potentials, summating potentials, and cochlear microphonics that were essentially identical to previously described responses in mice (e.g., Steel and Smith, 1992; Harvey and Steel, 1992) (data not shown). For example, the controls showed best (lowest) CAP thresholds at 18 kHz (mean 25.3 dB SPL $\pm$ 1.6 SEM), which is around the usual best frequency for mice. As reported previously (Avraham *et al.*, 1995), Snell's waltzer homozygotes showed no evidence of a CAP or CM response at any frequency or intensity of stimulus used. Six of the eight mutants also showed no sign of an SP response. However, in 2 of the 30 DAB mutants, SP responses were recorded (Fig. 6). In both cases, these SP responses were recorded only in response to 18-kHz stimuli and not to any other frequency used in the range 3 to 30 kHz, and they were recorded in response to very high intensity stimuli, above 125 dB SPL. These responses were considered true SP responses despite the high thresholds because (a) the waveforms were very similar in shape, size, and latency to waveforms close to threshold in control mice (e.g., compare control waveforms at 25 and 30 dB with mutant waveforms



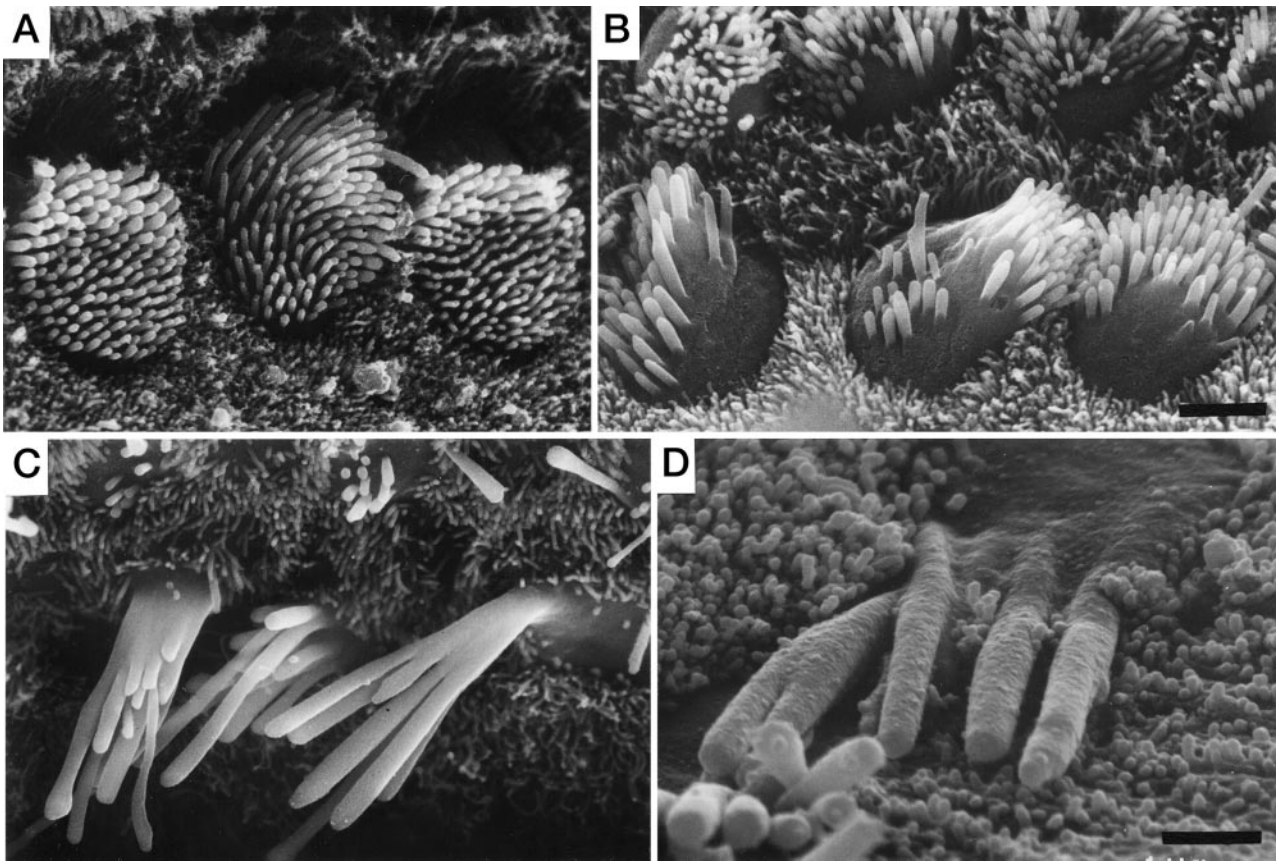


FIG. 2. Detail of stereocilia fusion in inner hair cells of Snell's waltzer mutants. (A) Newborn mutant, basal turn. The middle bundle appears to have an abnormal orientation, with its tallest microvilli on the right rather than at the lateral pole towards the top of the image, while the bundles on the left and right have a normal appearance for this stage. (B) 3 DAB mutant, basal turn. There are signs of fusion starting from the bases of the stereocilia in these three inner hair cells. Some disorganised outer hair cell bundles are just visible at the top of the image. (C) 7 DAB mutant, basal turn. Fusion is much more extensive than at earlier stages, and the number of stereocilia that can be distinguished is reduced. Some irregular bulges in the fused stereocilia can be found (e.g., extreme left). (D) 12 DAB mutant, apical turn. Fusion of these inner hair cell stereocilia is extensive, and they lie parallel with the upper surface of the epithelium, apparently fused to the underlying cell membrane. Scale bars represent 2 μm (A same as B, C same as D).

at 128 and 130 dB in Fig. 6), (b) the SP responses could be repeatedly measured in the two mutant mice, and so were not spurious noise-related artefact, and (c) the amplitude of the response increased with increasing stimulus intensity, indicating that it was a genuine biological response.

DISCUSSION

We found that in Snell's waltzer mutants with no detectable myosin VI, cochlear hair cells appear to start to develop normally, forming correctly oriented arrays of growing stereocilia on their upper surface, but from around birth these arrays become progressively more disorganised and the stereocilia fuse. This process occurs rapidly, with very few abnormal cells seen at birth, but by 3 DAB, practically all hair cells are affected, and stereocilia fusion is extensive by 7 DAB. By 20 DAB, giant stereocilia are seen and hair cells are starting to degenerate. Our observation of hair cell degeneration in the mutants is consistent with the earlier reports of Deol and Green (1966) and Avraham *et al.* (1995),

FIG. 1. Scanning electron microscopy to show maturation of stereocilia bundles in control littermates (left column) and Snell's waltzer mutants (right column), at ages 1 DAB (A, B, basal turn), 3 DAB (C, D, midbasal turn), 7 DAB (E, F, apical turn), and 20 DAB (G, H, midapical turn). The basal turn is more advanced in its development than the apex, so mutant and control images are matched for location. Scale bar represents 10 μm (shown on A).

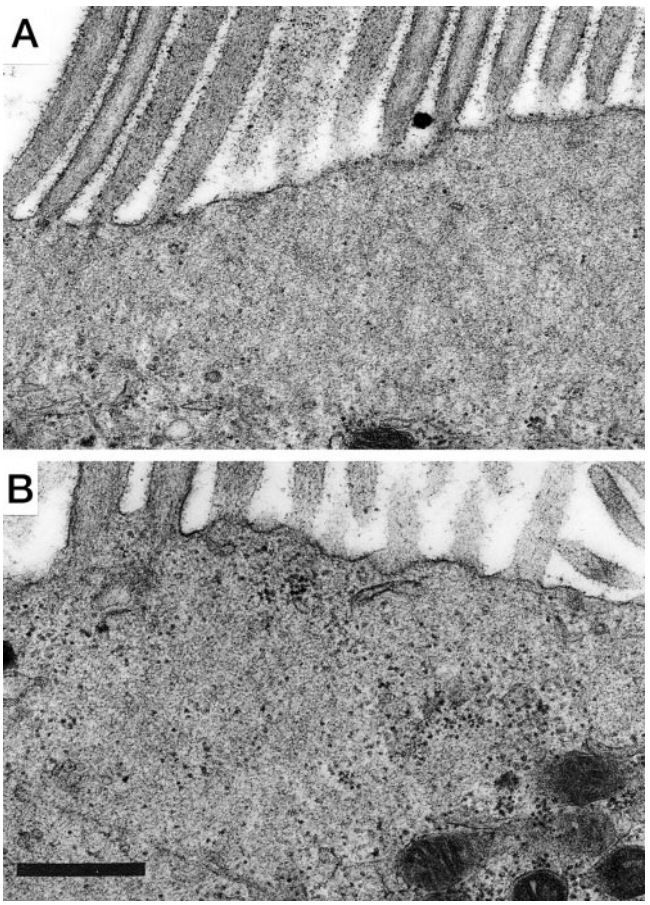


FIG. 3. Transmission electron micrographs of upper surface of hair cells of a newborn control (A, basal turn) and a newborn mutant (B, midapical turn). The bases of the stereocilia are shown inserting into the organelle-free cuticular plate. A region of membrane between adjacent stereocilia is shown in the mutant (B, left); these regions of raised membrane are seen in mutants and littermate controls, but seem to occur more frequently in the mutants. Scale bar represents 500 nm.

although neither work described the ultrastructure of the hair cells or looked at their development. Our ultrastructural findings suggest that myosin VI is essential for organising and maintaining the structural integrity of the stereocilia bundle upon the apical surface of the hair cell.

The fusion and growth of giant stereocilia appear to be a specific response to the lack of myosin VI, rather than a general feature of hair cell degeneration. In other deaf mouse mutants such as *Myo7a* mutants (shaker1) and *Myo15* mutants (shaker2), no stereocilia fusion is seen (Self *et al.*, 1998; Probst *et al.*, 1998). However, extensive stereocilia fusion early in development has been reported in the waltzing guinea pig (Sobin and Anniko, 1983); the responsible gene has not yet been identified, but *Myo6* is an obvious candidate. Fusion of stereocilia and formation of giant stereocilia have been reported previously as a rare event in damaged cochleas (e.g., Engström, 1983; Wright, 1982) and from experimental manipulations of surface charges (Neugebauer and Thurm, 1987), but these do not occur during development as we find in Snell's waltzer mutants. Nonetheless, the gross similarity between the giant stereocilia seen in Snell's waltzer and in damaged ears suggests that disturbance of myosin VI might be implicated in the process of damage observed previously in mature mammals.

Little is known about the function of unconventional myosins such as myosin VI, although a number have been suggested to have a role in vesicle transport in diverse cell types (e.g., Durrbach *et al.*, 1996; Hill *et al.*, 1996; Govindan *et al.*, 1995; Geli and Riezman, 1996; Mochida *et al.*, 1994; Hasson and Mooseker, 1994; Mermall *et al.*, 1998). A *Drosophila* myosin VI, 95F, has been shown to transport

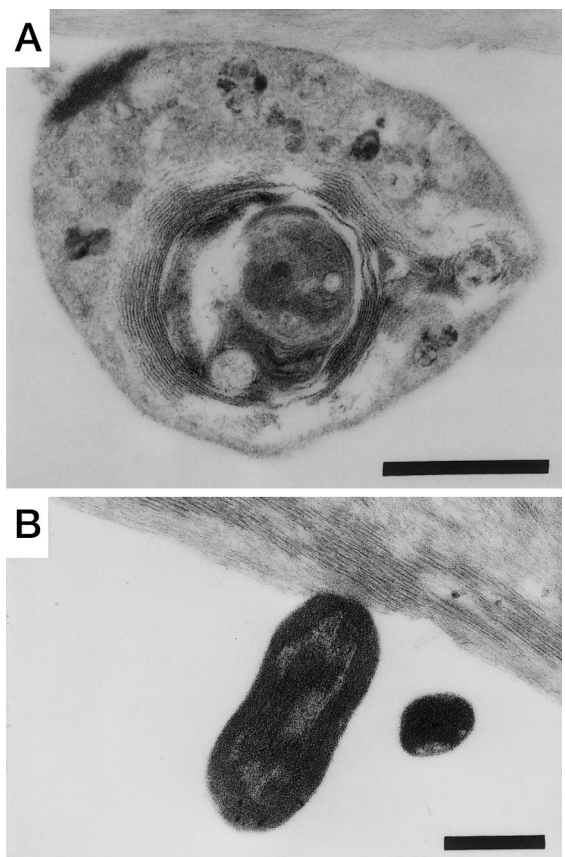


FIG. 4. Transmission electron micrographs showing sections through the fused stereocilia of a 20-DAB mutant. Much extraneous material, such as membrane whorls, is present in places (A), while other sections appear to show dense arrays of actin-like material (dark-stained material) (B). One stereocilium is shown in (A) and sections through two in (B); all are membrane bound. The extracellular material at the top of each image is the tectorial membrane. Scale bars represent 500 nm.

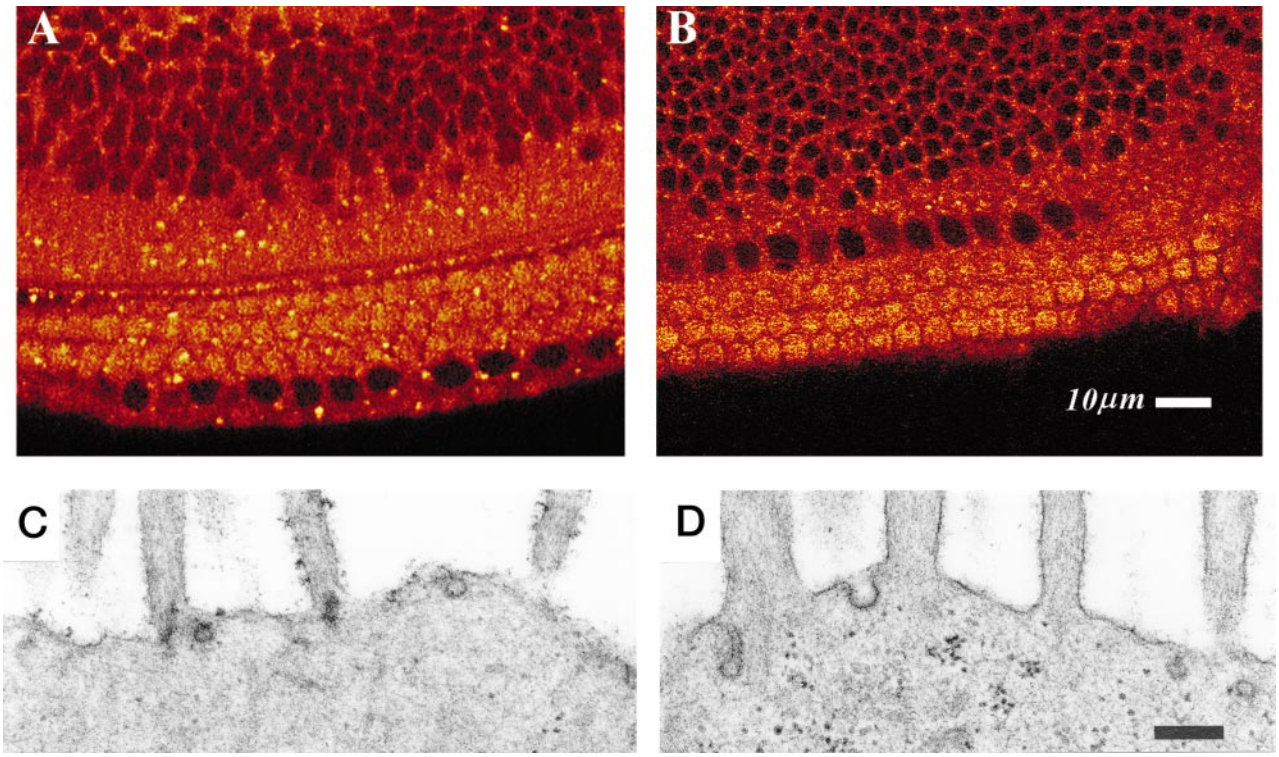


FIG. 5. (A and B) Confocal images from the region of inner and outer hair cells. Images from each series have been chosen to show the region just below the cuticular plate of outer hair cells. (A) Control (+/*sv*) at 3 DAB. (B) Mutant at 3 DAB. The mutant image is in a plane that includes the inner hair cell nuclei (regular line of dark patches), while the control image is in a plane that includes Henson's cell nuclei just lateral to the outer hair cells (at the bottom of the image). The bright yellow labelling represents vesicles labelled with the dye FM1-43, and the red is low-level background labelling. (C and D) Transmission electron micrographs showing pits and vesicles with densely stained coats at or close to the apical surface of hair cells. (C) Control (+/*sv*) at 1 DAB. (D) Mutant at 1 DAB. Many such pits and vesicles were observed near the surface of hair cells at this stage. Scale bar represents 200 nm.

cytoplasmic particles *in vivo* (Mermall *et al.*, 1994; Bohrman, 1997). In keeping with the proposed transport role of unconventional myosins, myosin VI might have an indirect effect on stereocilia organisation by transporting an essential cytoplasmic element to the apical surface of hair cells, the abnormal distribution of which results in destabilisation of the apical membranes. Alternatively, myosin VI might be directly involved in stabilising the stereocilia array, which would not be surprising given that myosins by definition interact with actin, and the apical surface of hair cells is very rich in actin. In adult mammals, myosin VI is observed in the cuticular plate of hair cells but not in the actin-filled stereocilia (Hasson *et al.*, 1997; Avraham *et al.*, 1997), so any explanation for the fusion seen in Snell's waltzer hair cells must account for this distribution. However, it should be noted that the distribution of myosin VI in developing hair cells has not yet been described. We consider four possible explanations for the role of myosin VI in hair cells, and we focus upon stereocilia fusion because this is the earliest defect we have seen and other defects are quite likely to be secondary to the fusion.

First, there is evidence of extensive membrane turnover at the apical surface of immature hair cells, as shown for example by the presence of endocytotic pits (Souter *et al.*, 1995), and this turnover may be involved in controlling the growth and organisation of stereocilia bundles. As many other unconventional myosins, including myosin VI, have been proposed to have a role in membrane trafficking (e.g., Mermall *et al.*, 1994; Hasson and Mooseker, 1994; Buss *et al.*, 1998), perhaps myosin VI has a similar role in the transport of materials during membrane recycling in hair cells. This membrane recycling seems particularly active at the vesicle-rich band around the cuticular plate, the pericuticular necklace, which is known to be rich in myosin VI (Kachar *et al.*, 1997, Hasson *et al.*, 1997). If myosin VI is involved in membrane recycling at the hair cell apical surface, its absence could lead to abnormal turnover and secondary disorganisation and fusion of stereocilia bundles. However, we observed uptake of the dye FM1-43 by both mutant and control preparations, indicating that endocytosis was occurring in the mutant hair cells, even though these observations are not quantitative so cannot be taken

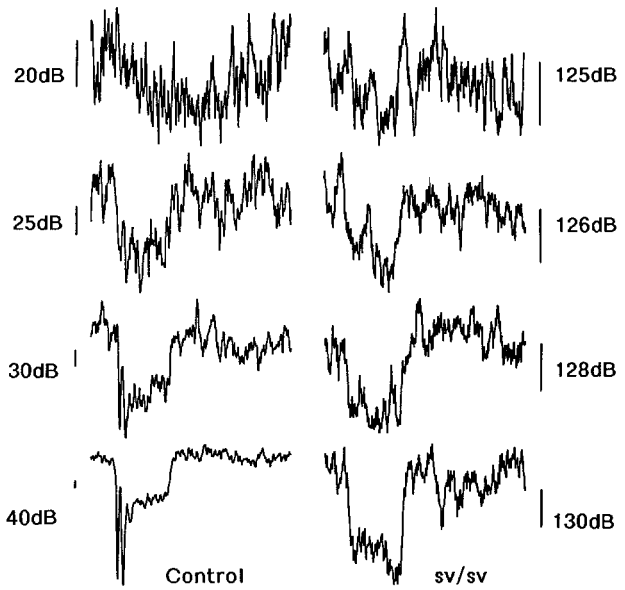


FIG. 6. Waveforms from an *sv*/*sv* mutant (right) and a littermate control (left), both at 30 DAB, in response to an 18-kHz tone burst at varying stimulus intensity levels. 40 ms of the averaged waveform is shown, and the summating potential is the DC offset sustained for the 15-ms duration of the tone burst. For these stimulus conditions, the summating potential is a downward, negative deflection. Vertical axes are scaled to the same height, and the scale bars represent 1 mV. Top waveform in each column shows no response, and increasing sound levels (dB SPL) give progressively larger amplitude summating potentials. The control waveforms for 30 and 40 dB SPL stimuli also show a compound action potential response, shown by the two sharp negative deflections at the start of the response.

to indicate that membrane trafficking is completely normal in mutants. Thus, we were unable to obtain experimental support for this explanation for stereocilia fusion.

Second, myosin VI is present around the rootlets of the stereocilia, where they insert into the cuticular plate. This localisation led to the suggestion that myosin VI may serve to pull down on the rootlets, using actin as a substrate, thus helping to anchor the stereocilia and provide resistance to deflection (Hasson *et al.*, 1997; Mermall *et al.*, 1998). If stereocilia are not well anchored, they could presumably more easily dislocate and pull out from their attachment in the cuticular plate, which might account for the extension of stereocilia observed in the mutants. However, this explanation does not account for the fusion of stereocilia seen.

Third, the localisation of myosin VI in the cuticular plate and around the rootlets of stereocilia may result from a role for this myosin in transporting essential components to the stereocilia. Although the preferred end for addition of new actin monomers is at the tip of the growing stereocilia, much of the growth (at least in chicken stereocilia) results from addition to the actin filaments at the root of the stereocilia (Pickles *et al.*, 1996). This suggests that the delivery of stereocilia components to the site of construction is controlled, and myosin VI may be involved in this process. If delivery of components is not in correct balance, the result may be the uncontrolled growth observed in mutant stereocilia and inclusion of cell components that would not normally be found in stereocilia. Again, this hypothesis fails to explain the initial fusion of stereocilia observed.

Fourth, myosin VI might be acting to pin down the apical membranes between stereocilia, via the tail, to the actin in the cuticular plate, via the motor domain. The natural state of a lipid bilayer in an aqueous environment is to form a sphere to minimise surface tension, and energy must be expended to maintain any other shape. Thus, the array of closely packed stereocilial membranes must be actively held in place to avoid their tendency to fuse, particularly near the bases where the ankle-shaped insertions of the stereocilia into the cuticular plate (Figs. 3 and 7) already provide a curved area of membrane close to a sphere. Another unconventional myosin, myosin I, has been proposed to perform the function of anchoring the actin filaments in the core of brush border microvilli to the plasma membrane (Heintzelman and Mooseker, 1992). Myosin VI may act in a similar way, attaching the apical plasma membrane to the actin of the cuticular plate of hair cells (Fig. 7). In the absence of myosin VI, as in the Snell's waltzer mutant, inadequate anchoring of the apical membrane between stereocilia could result in the membrane between adjacent stereocilia rising up by surface tension and effectively zipping up the stereocilia (Fig. 7). This explanation would account for the progressive fusion, starting from the bases of stereocilia, seen in Snell's waltzer mutants and for the early disorganisation of stereocilia bundles, but not necessarily the extension to form giant stereocilia. However, if the stereocilia bases are fused, there may be fewer limitations on access of cell contents to stereocilia, leading to the gross stereocilia extensions and abnormal cytoplas-

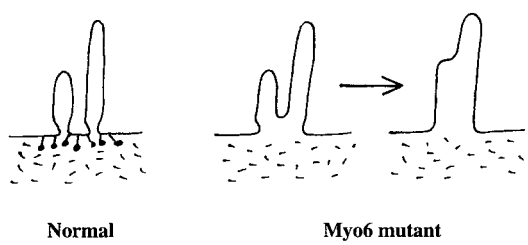


FIG. 7. One model for the role of myosin VI in maintaining stereocilia structure. Left: In the normal hair cell, myosin VI may help to hold down the surface membrane by anchoring the membrane with its tail domain and using the abundant actin in the cuticular plate (dashes) to attach the motor domain. Right: In the absence of myosin VI, there may be insufficient force to hold down the surface membranes, which may then proceed to fuse starting at the base and continuing to zip up to the stereocilia tips.

mic inclusions observed within them. The zipping up model has some support from observations on *Drosophila* embryos treated with antibody to 95F, the *Drosophila* myosin VI (Mermall and Miller, 1995). In these developing syncytia, deep furrows in the plasma membrane normally form to separate adjacent dividing nuclei, but in the antibody-treated embryos, the furrows are not so deep, resulting in inappropriate spindle interactions and disorganisation of the blastoderm. There is an obvious parallel in the formation or maintenance of deep furrows, in which the myosin VI may help to hold down the membrane against surface tension forces, and the maintenance of the closely apposed stereocilia membranes of hair cells. Further support for this suggestion comes from the recent observation by Buss *et al.* (1998) that chicken myosin VI is enriched at the plasma membrane of membrane ruffles, filopodia, and microvilli in cultured fibroblasts, regions of extensive invaginations of cell membrane; myosin VI may be involved in helping to anchor membranes here as well.

If myosin VI is required to anchor apical hair cell membrane to the actin cytoskeleton of the cuticular plate and thus permit the stereocilia to remain as separate entities, how do the microvilli, which later develop into stereocilia, form in the first place? We presume there must be another anchoring mechanism which is sufficient for early development of microvilli, but which is not adequate to prevent fusion of membranes at the time that the microvilli acquire the characteristic features of stereocilia around the time of birth. Any mutation that prevented the formation of microvilli might be expected to be lethal, as these structures are so important in diverse organs such as kidney and gut. Myosin VI has a distribution in the gut brush border similar to that in hair cells, being localised mainly to the terminal web, an actin-rich region just below the microvilli forming the brush border (Hasson and Mooseker, 1994). In mammalian brush border, no myosin VI is found in the microvilli, just as none is found in mammalian stereocilia (Hasson and Mooseker, 1994). In brush border, myosin VI has a generalised distribution within the enterocyte cell body during early development, but it becomes concentrated to the terminal web during maturation of microvilli, at the same time as they are growing longer (Heintzelman *et al.*, 1994; Hasson and Mooseker, 1994; Smith *et al.*, 1984). If myosin VI does have a critical role in the maturation of microvilli, then we might expect Snell's waltzer brush border to remain in an immature state, and our preliminary evidence suggests that this may be the case. The brush border microvilli of the mature mutant gut are indeed shorter than normal (at 18 weeks, one *sv/sv* gut sample had a mean length of 1.41 μm, compared with 2.10 μm in the littermate control, half-way along the villus), and microvillus fusions near their roots occur more frequently in young mutants than in controls (at 3 DAB, there were 28.7 fusions per 100 μm in one *sv/sv* mutant compared with 5.4 fusions per 100 μm in the littermate control). The rapid turnover time of enterocytes (2.7 days; Smith *et al.*, 1984) may ensure that any tendency for brush border microvilli to fuse is not lethal to the mouse, but hair cells differ from enterocytes in that they are never replaced, and continued fusion of stereocilia is not life threatening.

The primary function of sensory hair cells is reflected in the electrical potentials they generate when stimulated with sound, and the ultrastructural abnormalities we saw in the *sv/sv* mutant hair cells suggested that there may be very little physiological response from them. Six of the eight *sv/sv* mutant mice examined showed no sign of any stimulus-related cochlear responses even at very high sound intensities, indicating a profound cochlear impairment. However, the finding of a summating potential response in two of the mutant mice is surprising in that it indicates that the cochlear hair cells can, for certain stimulus conditions, still respond to sound by depolarising. Furthermore, the fact that an AC stimulus is converted into a DC response in the mutants indicates that the nonlinear behaviour of the hair cells, an important aspect of their function (e.g., Cheatham and Dallos, 1993), is to some extent preserved. As a careful examination of the cochlear duct by scanning electron microscopy revealed no hair cells with a normal stereocilia array (e.g., Fig. 1H), it seems unlikely that this summating potential in the mutants might be generated by the normal transduction mechanism, but might instead be mediated by putative stretch receptors in the basolateral cell membranes.

Finally, as myosin VI is widely expressed throughout the body (Hasson and Mooseker, 1994), it may have different roles in different places, so we should not rule out the possibility that it may have more than one role in hair cells.

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