Shaker-1 mutations reveal roles for myosin VIIA in both development and function of cochlear hair cells

Tim Self1, Mary Mahony1, Jane Fleming1, James Walsh2, Steve D. M. Brown2 and Karen P. Steel1,*

1MRC Institute of Hearing Research, University Park, Nottingham NG7 2RD, UK
2MRC Mammalian Genetics Unit and Mouse Genome Centre, Harwell, Didcot, Oxfordshire OX11 0RD, UK

*Author for correspondence (e-mail: karen@ihr.mrc.ac.uk)

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SUMMARY

The mouse shaker-1 locus, Myo7a, encodes myosin VIIA and mutations in the orthologous gene in humans cause Usher syndrome type 1B or non-syndromic deafness. Myo7a is expressed very early in sensory hair cell development in the inner ear. We describe the effects of three mutations on cochlear hair cell development and function. In the Myo7a816SB and Myo7a6D mutants, stereocilia grow and form rows of graded heights as normal, but the bundles become progressively more disorganised. Most of these mutants show no gross electrophysiological responses, but some did show evidence of hair cell depolarisation despite the disorganisation of their bundles. In contrast, the original shaker-1 mutants, Myo7a81, had normal early development of stereocilia bundles, but still showed abnormal cochlear responses. These findings suggest that myosin VIIA is required for normal stereocilia bundle organisation and has a role in the function of cochlear hair cells.

Key words: shaker-1, Mouse mutant, Myosin VIIA, Cochlea, Hair cell, Stereocilia, Hearing, Usher syndrome

INTRODUCTION

Very little is known about the molecular basis of sensory hair cell function or development, partly because the small number of hair cells in each inner ear makes a biochemical approach impractical. We have adopted a genetic approach to identifying molecules with key functions in hair cells, using positional cloning of mouse mutations known to lead to inner ear defects. The first gene to be identified using this approach and affecting the sensory hair cells directly was the myosin VIIA gene (Myo7a) in the shaker-1 mouse mutant (Gibson et al., 1995).

The shaker-1 mouse mutant was first described in the 1920s (Lord and Gates 1929), and shows the typical shaker-waltzer type behaviour often associated with inner ear defects: deafness, hyperactivity, head-tossing and circling. Defects are first seen in the inner ear neuroepithelia by light microscopy, with progressive degeneration of the sensory hair cells of the organ of Corti and saccule (Deol, 1956; Kikuchi and Hilding, 1965; Grüneberg et al., 1940; Mikaelian et al., 1965; Shnerson et al., 1983). The mutants show some behavioural and physiological responses to loud sounds for a short period following the onset of auditory function at around two weeks after birth (Mikaelian and Ruben, 1964; Grüneberg et al., 1940; Steel and Harvey, 1992; Harvey, 1989). Previous reports of the rate of hearing loss and the organ of Corti abnormalities in the original shaker-1 mutants vary, which might be explained by differences in genetic background between the various studies (eg Emmerling and Sobkovicz, 1990; Steel and Harvey, 1992).

The stria vascularis continues to function normally in generating a high resting endocochlear potential (Brown and Ruben, 1969).

We identified the gene affected by the shaker-1 mutations as encoding myosin VIIA, an unconventional myosin (Gibson et al., 1995). The same gene, MYO7A, is involved in Usher syndrome type 1B in humans, in which the congenital balance and hearing defects are accompanied by progressive retinitis pigmentosa (Weil et al., 1995; Weston et al., 1996; Lévy et al., 1997; Liu et al., 1997a), as well as in non-syndromic deafness (Liu et al., 1997b; Weil et al., 1997). The complete human (Chen et al., 1996; Weil et al., 1996; Kelley et al., 1997) and mouse (Mburu et al., 1997, accession number U81453) sequences for this large gene have been determined, and we have recently identified all seven available mutations at the shaker-1 locus (Mburu et al., 1997). Some of the mutations lead to premature stop codons early in the sequence, or are predicted to change highly conserved residues in functionally important parts of the motor head of the myosin; furthermore, at least two of the mutations in the mouse Myo7a gene are associated with very low levels (less than 1% of normal) of myosin VIIA protein, suggesting that these mutations are effectively null mutations (Hasson et al., 1997a).

The gene is expressed in the cochlea, eye, kidney, testis and lung, although there is no obvious kidney, testis or lung phenotype associated with myosin VIIA mutations (Gibson et al., 1995; Weil et al., 1995; Smith et al., 1994). Using antibodies to the myosin VIIA protein, Hasson and colleagues
Heterozygous littermates. For the homozygote mutants, and 65 bp, 37 bp and 28 bp products in the heterozygote controls. The give 86 bp and 38 bp products in the homozygote mutants and 86 bp, 358

MATERIALS AND METHODS

Mice

The original spontaneous shaker-1 (Myo7a<sup>sh1</sup>) mutation was obtained from Harwell, UK, in the mid-1980s, backcrossed several times to the CBA/Ca inbred strain and is now maintained as a closed colony. Myo7a<sup>sh1</sup> was also a spontaneous mutation, occurring in the C57BL/6J strain and was kindly provided by Dr Wayne Frankel, The Jackson Laboratory, ME, on a mixed 25% BALBc, 75% C57BL/6J background (Letts et al., 1994). The Myo7a<sup>6J</sup> allele was derived from an ENU mutagenesis programme; the mutation was originally induced in a BALBc mouse, which was then repeatedly crossed to the BS inbred strain used at Oak Ridge National Laboratories, TN, and the stock was kindly provided by Dr Gene Rinchik (Rinchik et al., 1990). Mice were normally generated by heterozygote-to-homozygote matings to give segregating litters that included heterozygotes used as littermate controls. Timed pregnancies were used to generate prenatal stages, with the morning of the plug counted as 0.5 days post coitum (d.p.c.) for overnight matings, and the day of birth was 0 days. All mice were kept in full accordance with UK Home Office regulations.

Genotyping

Mice aged 12 days or older were classified as homozygous mutant or heterozygous control on the basis of the abnormal behaviour of the mutants. Mice younger than this were genotyped as follows. For the stock, 5 homozygotes and 8 heterozygote controls were studied at 3 and 12 days of age, and from the stock, 5 mutants and 8 littermate controls were studied at 3 and 15 days old. Inner ears were dissected under 2.5% glutaraldehyde, 0.25% tannic acid, pH 7.2, and fixed for 5 hours at 4°C. The osmium tetroxide-thiocarbohydrazide (OTOTO) procedure adapted from Hunter-Duvar (1978) was used to stain prior to dehydrating and critical-point drying. Specimens were sputter coated with gold and examined in a Jeol 6400 Winsem at 20 kV.

RESULTS

Myosin VIIA is expressed very early in hair cell development

We have looked at expression of the myosin VIIA gene during normal development by in situ hybridisation using a clone of the gene covering part of the head domain that has previously

In situ hybridisation

35S-labelled RNA probes were made by standard techniques, using T7 (sense) or SP6 (antisense) RNA polymerase to transcribe from the plasmid template. The template was a myosin VIIA clone in pCRII (Invitrogen) covering bases 1 to 1350 of the gene, corresponding to the motor head of the myosin molecule. A total of 26 CBA/Ca mice were used at daily intervals from 14.5 d.p.c. to 19.5 d.p.c. plus newborn, 3 days and 6 days after birth, together with 8 BALBc mice ranging from 17 dpc to 6 days after birth. Specimens were fixed in 4% paraformaldehyde and embedded in paraffin wax, and serial sections through the entire inner ear were cut at 8 µm. Every tenth section (or more) was used to hybridise with the labelled sense strand as a control for non-specific hybridisation, while the rest were hybridised with labelled antisense strand overnight at 55°C, essentially as described before (Wilkinson et al., 1987; Steel et al., 1992).
been shown by northern analysis to be specific for this gene (Gibson et al., 1995). Myosin VIIA was strongly expressed in sensory epithelia of the vestibular system (the saccular and utricle maculae and the three cristae) from the earliest stage we studied, 14.5 d.p.c. (data not shown). This is at about the time that sensory hair cells can be distinguished histologically (Mbiene et al., 1984). The labelling was located in the upper layer of the epithelia, suggesting it was restricted to developing sensory hair cells. In the cochlea, the first labelling was seen in the organ of Corti of the basal turn of the cochlear duct at 16.5 d.p.c. and, by 17.5 d.p.c., it was seen throughout the cochlea including the apex. This base-to-apex gradient in expression of myosin VIIA correlates with and pre-dates many other features of organ of Corti development which proceed in a base-to-apex direction (e.g. Rubel, 1978). Within the organ of Corti, the labelling appears to be localised to the region of the one row of inner hair cells and three rows of outer hair cells (Fig. 1), and no labelling above background levels was found in the developing supporting cells or the cochlear neurons. Myosin VIIA thus is expressed in cochlear hair cells a full day before we can detect the first signs of development of their characteristic ultrastructural features in mice of the same stock, suggesting that it may serve as a useful early marker for hair cell differentiation.

Mutants studied
We describe three shaker-1 mouse mutants here, with mutations affecting different parts of the motor head domain of the myosin VIIA molecule (Gibson et al., 1995, see Mburu et al., 1997 for detailed description of the mutations). The original shaker-1(Myo7a6J) mutation is an arginine to proline missense mutation, located in a surface loop that has a relatively unconserved amino acid sequence. The Myo7a6J mutation is also an arginine to proline missense mutation, but at a highly conserved position in the core of the motor domain, where it is expected to interact with a number of surrounding residues; this mutation is thus predicted to have a serious effect on protein stability and function. The third mutation examined here is the Myo7a816SB mutation that changes a splice acceptor site sequence, leading to skipping of a 30-base exon and predicted deletion of ten amino acids from the core of the myosin head, close to the proposed hinge domain. Such a deletion would be expected to have a severe effect on protein structure and function. The Myo7a6J and Myo7a816SB mutations appear to affect protein stability, because mRNA levels are normal but myosin VIIA levels are reduced to 21% and 6% of normal, respectively (Hasson et al., 1997a). The phenotypic effects of these two mutations may be partly due to protein instability in addition to abnormal function of the remaining myosin VIIA molecules.

Stereocilia bundles develop abnormally in two of the shaker-1 mutants
We examined the development of the upper surface of cochlear hair cells by scanning electron microscopy from 16.5 d.p.c. to 20 days after birth. At 16.5 d.p.c. in our stocks, hair cells cannot be distinguished from surrounding supporting cells except by their position in relation to the greater and lesser epithelial ridges, and there were no obvious differences between mutants and controls (not shown).

In normal littermate controls, there was a dramatic maturation of stereocilia bundles developing from a uniform covering of microvilli. By 18 d.p.c., hair cells could be clearly distinguished by the regular array of microvilli, some of which on the lateral side of each hair cell were elongating to form a V-shaped array with a kinocilium at the pole and a flexible appearance (Fig. 2A). 2 days later, in the newborn mouse, the tallest microvilli were arranged in more ordered rows of graded heights, and appeared straighter, taller and more rigid than previously (not shown). By 3 days after birth, the stereocilia bundle is located in the centre of the hair cell top, with a kinocilium and a patch of microvillus-free membrane on the lateral side and remaining microvilli on the inner (modiolar) side (Fig. 2C). In the basal turn, which is more advanced in development, there are fewer of these microvilli, and they appear to be resorbed progressively over the next few days until there are only stereocilia and no microvilli remaining by around 12 days after birth (Fig. 2E shows a mature stereocilia bundle). The kinocilium also regresses and disappears during this same period.

In the Myo7a6J mutants, the first signs of abnormalities can be detected as early as 18 d.p.c., when some of the growing microvilli show an irregular arrangement (Fig. 2B). Furthermore, the kinocilium is often eccentric in position, misplaced from its normal regular position at the lateral pole of each hair cell, and is often difficult to identify (Fig. 2B). By 3 days after birth, the formation of the stereocilia rows is very disordered with several small clumps of stereocilia appearing on the tops of some hair cells (Fig. 2D). As in normal mice, the basal turn develops ahead of the apical turn, and the disruption in development is thus more clearly seen in the base in the mutants. The disruption of the stereocilia rows and the erratic position of the kinocilium is more clearly seen in the outer hair cells, but is also present in the inner hair cells. The
stereocilia become more disorganised over the next few days, with many of them disappearing, possibly by falling off because stumps are often seen, until by 20 days after birth there are very few remaining (Fig. 2F).

In the Myo7a^{816SB} mutants at 3 days old, a similar disorganisation was seen as in Myo7a^{6J}, but the abnormalities were more extreme, with more hair cells showing several small clumps of stereocilia and fewer hair cells having a single clump (Fig. 3). The clumps of stereocilia each contained rows of graded heights, but the orientation of these rows was erratic, and there were often clumps oriented in opposite directions on a single hair cell.

In both these mutants, the stereocilia appear to have grown to their normal lengths and proportions, and have formed rows of graded heights as they normally would. However, the stereocilia do not appear to be positioned correctly within the top of the hair cell.

In both Myo7a^{6J} and Myo7a^{816SB} mutants at later stages, hair cells have deteriorated with many being lost or showing signs of degeneration. However, a few hair cells do remain at the stages used for electrophysiology (12, 15 and 20 days), and some of the best-preserved hair cells are shown in Fig. 4 to allow correlation with the physiological recordings.

**Stereocilia bundles in the original shaker-1 mutant show only minor anomalies**

In contrast to the clear stereocilia bundle defects seen in the Myo7a^{6J} and Myo7a^{816SB} mutants, in the original Myo7a^{sh1} mutants the stereocilia bundles look reasonably normal at 3 d.p.c. (Fig. 5). No gross disorganisation of the hair cells can be seen by scanning electron microscopy at 3 or 15 days after birth, although many of the outer hair cells at 15 days had only two rows of stereocilia instead of the usual three rows (Fig. 4).

**Hair cell ultrastructure in shaker-1 mutants**

We examined the hair cells of the two most severely affected mutants, Myo7a^{6J} and Myo7a^{816SB}, by transmission electron microscopy, focusing particularly on the cuticular plate, into which the stereocilia become anchored as they mature, and the synaptic regions. The cuticular plate at 18 d.p.c. was loosely organised, being represented by a clearing of intracellular organelles in some less-developed cells (e.g. apical turn hair cells) while, in more-developed hair cells, the cytoskeletal matrix comprising the cuticular plate was beginning to condense (Fig. 6A). By birth, all hair cells examined showed a significant condensation of cuticular plate material, which looked essentially mature (Fig. 6B). There were regions of vesiculation forming a network around the periphery of the cuticular plate (the pericuticular necklace) and the area immediately around the insertion point of the kinocilium was also filled with many vesicles. Densely stained rootlets were seen extending from the core of the stereocilia into the cuticular plate by birth, and the cylindrical shape of each stereocilium narrowed towards the insertion point, forming the ankle (Fig. 6B).

Fig. 2. Scanning electron micrographs of the surface of the organ of Corti of Myo7a^{6J} homozygotes (B,D,F) and littermate controls (A,C,E), showing widespread disorganisation of stereocilia bundles in the mutants. (A,B) 18 d.p.c.; (C,D) 3 days after birth; (E,F) 20 days after birth. (A-D) Same magnification, scale bar at bottom of D represents 5 μm. (E,F) Same magnification, scale bar at bottom of E represents 5 μm.
of the cuticular plate and formation of rootlets between 18
d.p.c. and birth must presumably play a role in anchoring the
stereocilia.

In the mutants, there were no obvious abnormalities in the fine
structure of the cuticular plate material and it condensed over the
same period as in controls (Fig. 6A,B). Stereocilia structure,
including rootlets and ankles, also developed normally in the
mutants (Fig. 6C). However, there were more extensive areas of
vesicle-rich cytoplasm beneath the upper surface of the mutant
hair cells, including areas interspersed between patches of
cuticular plate, which was never seen in controls (Fig. 6D).
Furthermore, in some regions, stereocilia could be seen inserting
into these vesiculated regions (Fig. 6C) in contrast to controls,
in which all stereocilia inserted into cuticular plate. Irregular
bulges in the cuticular plate were often observed in mutants and,
as seen by scanning electron microscopy, the kinocilium could
be mislocated amongst the stereocilia.

Ribbon-type synaptic vesicle arrays were seen in both

**Fig. 3.** Scanning electron micrographs of the organ of Corti of a *Myo7a^816SB* homozygous mutant at 3 days after birth, showing outer hair cells with extensive disruption of the stereocilia bundles. Scale bar represents 5 μm.

**Fig. 4.** Scanning electron micrographs of inner (left column) and outer (right column) hair cells from controls at 15 days (A,B), *Myo7a^sh1* mutants at 15 days (C,D), *Myo7a^6J* mutants at 15 days (E,F), and *Myo7a^816SB* mutants at 12 days (G,H). These cells are chosen to illustrate the best-preserved hair cells found at the ages used for electrophysiology, as these are the cells that are most likely to be giving any response seen, and many hair cells elsewhere in the cochlear duct are in later stages of degeneration. Only minor anomalies are seen in the *Myo7a^sh1* hair cells, such as the predominance of outer hair cells with only two rows of stereocilia instead of the more usual three (compare D with B). Both outer and inner hair cells are abnormal in the *Myo7a^6J* and *Myo7a^816SB* mutants (E-H). The kinocilium (arrow) is misplaced in both *Myo7a^816SB* inner hair cells shown, being on the side of the left hair cell and on the modiolar rather than lateral pole in the right hair cell (G). Note some tip links present on the left side of A. In all micrographs, lateral pole is at the top, and scale bar shown in H represents 5 μm.
mutants and controls, inner and outer hair cells, with no obvious differences between the genotypes (Fig. 7).

**Cochlear dysfunction in shaker-1 mutants**

Cochlear function was assessed using a recording electrode on the round window, a well-established method for assessing cochlear activity giving response thresholds close to those of single units (Johnstone et al., 1979). We looked at three responses, representing activity of different elements of the cochlea. Compound action potentials (CAP) consist of one or two sharp negative deflections at the start of the toneburst, representing synchronous firing of many cochlear neurons. Summating potentials (SP) are d.c. shifts in the waveform sustained for the duration of the toneburst stimulus and represent the gross counterpart of intracellular d.c. responses of sensory hair cells in the cochlea (e.g. Dallos et al., 1972; Dallos, 1986; Harvey and Steel, 1992). Cochlear microphonics (CM) are an a.c. response with a frequency identical to that of the stimulus and CM measured from the round window is thought to be primarily generated by the basal turn outer hair cells (e.g. Patuzzi et al., 1989, Dallos and Cheatham, 1976). In normal mice, CM and SP responses can be recorded from around 8-9 days using this approach but CAP responses are only detected from 11-12 days and responses gradually mature over the following few days (e.g. Harvey, 1989). We looked at mice during this period of maturation at 12, 15 and 20 days, to ensure that we did not miss any time window of activity.

The three different mutants showed different responses to sounds. Homozygotes for the Myo7a<sup>816SB</sup> allele gave no CM, CAP or SP responses at all at any of the ages studied (12, 15 and 20 days after birth) up to the maximum sound intensities used (100 dB SPL for CM; up to 130 dB SPL depending on frequency for CAP and SP).

Of the eleven Myo7a<sup>6J</sup> mutants examined, most showed no response to sound stimuli, but two of the mutants aged 20 days did show some response. These two mice gave an SP response, but only at very high intensities and only to a restricted range of frequencies (Fig. 8B). The responses are genuine SP responses despite the high thresholds because the waveforms are very similar in shape, size and latency to waveforms close to threshold in control mice and, furthermore, they could be measured repeatedly. These were the only responses that could be detected in these mutants.

The original shaker-1 mutant, Myo7a<sup>sh1</sup>, shows more extensive responses than the other two alleles. The mutants showed CM responses with thresholds only slightly higher than in controls, indicating that outer hair cells can function at these ages (Fig. 8A). Myo7a<sup>sh1</sup> homozygotes also show SP responses, albeit at raised thresholds compared with littermate controls (Fig. 8B), suggesting some sensory hair cells depolarise in response to sound stimulation. Finally, in this series, no CAP responses could be detected, although we have previously recorded severely abnormal CAPs in some but not all mutants (Harvey, 1989; Steel and Harvey, 1992).

**DISCUSSION**

We found a good correlation between the ultrastructure, physiological responses and the nature of the mutation in the three alleles of myosin VIIA studied here. The Myo7a<sup>816SB</sup> mutation results in a 10-amino-acid deletion in the core of the motor head of the myosin molecule (Gibson et al., 1995; Mburu et al., 1997) and leads to the most severe disruption of development of stereocilia bundles seen among the mutants and a complete absence of stimulus-related cochlear potentials. The Myo7a<sup>6J</sup> mutation is a missense mutation also within the core of the myosin VIIA head associated with abnormal
Myosin VIIA in hair cell development

stereocilia bundle development and little or no cochlear responses. In contrast, the Myo7a sh1 mutation is a missense mutation located in a poorly conserved surface loop of the myosin head and this change is associated with the mildest of the pathological effects seen among the three mutants studied here: stereocilia appear to develop nearly normally and hair cells can generate CM and SP responses (albeit at raised thresholds).

Myosin VIIA clearly has a role in the development of stereocilia bundles. Stereocilia develop from the microvilli that cover the upper surface of the hair cell at early stages (16.5 d.p.c.), with lateral microvilli in a crescent-shaped array growing taller while microvilli on the modiolar (inner) side of the hair cell regress as the cell matures (Tilney et al., 1986, 1988, 1992; Kaltenbach et al., 1994). Myosin VIIA does not seem to be required for the initial establishment of this lateral-modiolar polarity of hair cells because this polarisation is clearly visible in the Myo7a 6J mutants at 18 d.p.c. despite early signs of disorganisation (Fig. 2B). Myosin VIIA also does not seem to be required for the ultrastructural maturation of stereocilia from the microvilli, which are their precursors, and the stereocilia in the mutants show apparently normal elongation to form rows of graded heights. However, myosin VIIA is required, either directly or indirectly, for maintaining the normal arrangement of the stereocilia in an ordered V-shaped array at the top of the hair cell, because in the Myo7a 6J and Myo7a 816SB mutants, the stereocilia form small clusters arranged in diverse patterns and orientations within the top of the hair cell. (See later for discussion of Myo7a sh1.)

There are several possible explanations for the progressive disorganisation of the bundle. Firstly, myosin VIIA may be acting as an anchor molecule and immature hair cells in the frog saccule show particularly strong labelling for myosin VIIA. There is abundant actin within the mature cuticular plate (eg De Rosier and Tilney, 1989), which could interact with myosin in anchoring the rootlets of stereocilia. If myosin VIIA does act as an anchor, it seems unlikely to be the only molecule involved because a greater amount of stereocilia disorganisation might be expected if there was no effective anchor.

An alternative explanation for the disrupted stereocilia array is that myosin VIIA is required for cohesion of the cuticular plate, such that in the two most severely affected mutants, the cuticular plate does not form a single coherent mass but is interrupted by areas of vesicle-rich cytoplasm. This in turn might allow movement of the pieces of cuticular plate with role as an anchor molecule and immature hair cells in the frog saccule show particularly strong labelling for myosin VIIA. There is abundant actin within the mature cuticular plate (eg De Rosier and Tilney, 1989), which could interact with myosin in anchoring the rootlets of stereocilia. If myosin VIIA does act as an anchor, it seems unlikely to be the only molecule involved because a greater amount of stereocilia disorganisation might be expected if there was no effective anchor.

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Fig. 6. Transmission electron micrographs of hair cells at (A) 18 d.p.c., (B-D) newborn. (A) The developing cuticular plate from an inner hair cell from a Myo7a 6J mutant, in which cuticular plate condensation is indistinguishable from that in controls. (B) Control inner hair cell cuticular plate 2 days later, at birth, shows extensive condensation of the matrix, and the vesicle-rich area close to the insertion of the kinocilium is shown (to the right in this view, arrow and inset). (C) Myo7a 816SB mutant outer hair cell at birth, showing insertion of stereocilia rootlets into a vesicle-rich area. Rootlet and ankle formation seen here are essentially the same as in controls, but rootlets always insert into cuticular plate in controls. (D) Myo7a 816SB mutant outer hair cell at birth, showing an interspersed vesicle-rich area (arrow and inset). The orientation of the section is mid-modiolar, and the position of the kinocilium inserting into a vesicle-rich area would normally be to the left of the view. The location of a vesicle-rich area between two patches of stereocilia in this view is abnormal. Scale bar A,B,D (marked on A) represents 1 μm. Scale bar on C represents 500 nm.
attached stereocilia within the top of the hair cell. However, it seems just as likely that the abnormal presence of vesiculated regions within the cuticular plate is secondary to abnormal distribution of stereocilia, rather than being the cause of stereocilia disorganisation.

We were able to rule out a third possible explanation for the disorganisation: a delay in condensation of the cuticular plate in mutants, allowing a prolonged period of stereocilia mobility within the top of the hair cell. Our sections of hair cells during the period of condensation showed no such delay in mutants.

A fourth possible explanation for the abnormal organisation of stereocilia in the two mutants is that the kinocilium is primarily affected by the mutations and that this interferes with a putative role of the kinocilium in organising the stereocilia array (eg Sobkowicz et al., 1995; Kelley et al., 1992). A primary cilium defect has been proposed as an explanation for the link between retinal defects, vestibular dysfunction and hearing impairment in Usher syndrome, and ultrastructural defects have been observed in the photoreceptor cilia of three individuals with Usher syndrome type 2 (Barrong et al., 1992; Hunter et al., 1986; Berson and Adamian, 1992). In the Myo7a6J and Myo7a816SB mutants, in cells where the kinocilium could be clearly identified it was often some distance from its normal location (eg Fig. 4G), and there was no obvious relationship between the position of the kinocilium and the arrangement of the stereocilia. Thus, the observations in these two severely affected mutants suggest that, either the kinocilium is not involved in organising the stereocilia bundle in normal mice, or the kinocilium requires functional myosin VIIA to control stereocilia bundle organisation.

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**Fig. 7.** (A) Synaptic vesicles arranged in a typical ribbon formation (arrow) at the base of a control outer hair cell at 3 days after birth. (B) Synaptic vesicles of a Myo7a6J mutant inner hair cell look normal (arrow). Scale bar represents 500 nm.

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**Fig. 8.** Physiological responses of the cochlea. (A) Cochlear microphonic thresholds for a 2 μV response ± s.e.m. at a range of frequencies in controls (dashed lines) and Myo7a6J homozygotes at 15 and 20 days. Solid line at top represents maximum output of sound used, and Myo7a6J and Myo7a816SB homozygotes gave no CM responses up to this level. Thresholds for the Myo7a6J mutants are slightly higher than for the controls, and control thresholds improve between 15 and 20 days. CM amplitude was also analysed (not shown). Linear input/output functions are obtained from both genotypes, and the mean CM amplitude for the controls was slightly higher than for the mutants. CM responses at 3, 6, 12 and 18 kHz were analysed by analysis of variance for repeated measures: the test of between-subject effects showed no effect of age at any frequency (eg 6 kHz: f=0.39, df=15.1, P=0.544) and no significant effect of genotype at 12 and 18 kHz (12 kHz f=1.30, df=15.1, P=0.272; 18 kHz f=2.96, df=15.1, P=0.106), but a significant effect at the 5% level of genotype for 3 and 6 kHz stimuli (3 kHz: f=6.77, df=15.1, P=0.020; 6 kHz: f=5.69, df=1, P=0.031). Thus, there were significantly smaller CM amplitudes in the mutants at low frequencies but no significant differences at higher frequencies. (B) Thresholds (±s.e.m.) for visual detection of a summating potential response in the Myo7a6J mutants and littermate controls. Control thresholds improve from 15 to 20 days after birth, showing continuing maturation of the responses, but mutant thresholds deteriorate further during this time. Solid line at top represents the maximum sound intensity used, and all Myo7a816SB and most Myo7a6J homozygotes gave no SP response up to this level. Two Myo7a6J mutants did give a clear SP response, but only at 18 or 24 kHz and with very raised thresholds; these are shown by the black triangles. (C) Compound action potential response thresholds ±s.e.m. in the control group. Thresholds improve between 15 and 20 days. No CAP responses were seen in any of the homozygote mutants studied here at any age, up to the maximum intensities used shown in B.
Apical membrane turnover in the hair cell may play a role in normal positioning of stereocilia, so a fifth possible explanation for the observed stereocilia disorganisation is that this process requires myosin VIIA. However, in a separate study (Richardson et al., 1997), we show that cationic ferritin uptake by hair cells of Myo7a<sup>-/-</sup> mutants is normal, suggesting that non-specific apical hair cell endocytosis is probably normal, arguing against this explanation for the stereocilia disorganisation.

Finally, Hasson et al. (1997b) suggested that myosin VIIA may be involved in crosslinks between the shafts of adjacent stereocilia. Abnormal crosslinks resulting from defective myosin VIIA might lead to a lack of cohesion of stereocilia within the bundle and consequent disruption of the bundle (Myo7a<sup>+/+</sup> and Myo7a<sup>-/-</sup>) or excessive resorption of stereocilia that are not firmly linked to the rest of the stereocilia bundle (Myo7a<sup>sh1</sup>). It would be interesting to know the ultrastructural localisation of myosin VIIA during development of stereocilia to assess this and the other possible explanations for the derangements observed.

The observations on the original shaker-1 mutation, Myo7a<sup>sh1</sup>, were particularly interesting because they suggest that myosin VIIA may have a role in hair cell function in addition to its role in stereocilia bundle development. The mutant myosin VIIA molecule in these mutants appears to have sufficient activity to support normal early development of the stereocilia bundles, although there are some minor anomalies seen at 15 days. Outer hair cells seem to function moderately well judging by the nearly normal CM recorded in these mutants, despite many outer hair cells having only two rows of stereocilia instead of the more usual three. However, the raised thresholds for SP responses and grossly abnormal CAP responses suggest that hair cells require normal myosin VIIA activity for normal function, and that the missense mutation of the Myo7a<sup>sh1</sup> allele interferes with this role. The nature of this requirement is not known. The simplest explanation is that myosin VIIA may be required for normal transduction, and the same process affected in the mild Myo7a<sup>sh1</sup> mutant, be it stereocilia anchoring, stereocilia crosslinking, cuticular plate cohesion or some other process, is severely affected in the two more extreme alleles giving rise to much earlier signs of abnormality in the form of stereocilia disarray.

The progressive hearing loss observed in the Myo7a<sup>sh1</sup> mutants suggests that some MYO7A mutations in humans may be involved with hearing impairments less severe than that seen in typical Usher syndrome type 1B and may lead to progressive hearing loss observed in some cases (Liu et al., 1997c).

The finding of an SP response in two of the Myo7a<sup>sh1</sup> mutants is particularly interesting, given the gross disorder of their stereocilia bundles. It suggests that some hair cells at 20 days after birth are able to depolarise in response to sound stimuli. We have recently shown that individual Myo7a<sup>sh1</sup> mutant hair cells in organ of Corti culture at the equivalent of 3 days after birth do produce transduction currents when their stereocilia bundles are stimulated (Richardson et al., 1997).

Finally, a disrupted pattern of stereocilia bundles has been described in one other mutant to date, the Jackson shaker (js) mutant (Kitamura et al., 1991a,b, 1992). The js gene product may be involved in the same process of stereocilia bundle organisation and may interact with myosin VIIA. Disorganised stereocilia bundles or multiple clumps of stereocilia have also been observed in presumed regenerating hair cells in the chick inner ear following damage (e.g. Cousillas and Rebillard, 1988; Hashino et al., 1991; Duckert and Rubel, 1993), indicating that the molecular or mechanical processes that lead to ordered development of the bundle in normal development are not always properly co-ordinated in regeneration and myosin VIIA may be involved in this process.

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