Shaker Mice and a Peek into the House of Usher

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“To be deaf is a greater affliction than to be blind...”

Helen Keller

Hear, Hear!

The curious phenomenon of hearing yields only to the curiosity that is the inner ear—that wonder of miniaturisation which has been described laconically by Hudspeth as “a three-dimensional inertial-guidance system and an acoustical amplifier and frequency analyser compacted into the volume of a child’s marble” [23]. Because the ear is such a complex organ in terms of organisation, its development and maintenance must be the charge of many interacting genes and proteins. The drawback of such an arrangement is that there is a great potential for mis-development or breakdown in function. Add that to the fact that the sound-transducing cells of the inner-ear are exposed to violent shearing forces of fluid movement, plus the ear’s sensitivity to pharmaceuticals and infection, and we can appreciate the scale of the problem of deafness that epidemiologists have painted.

It is estimated that in the U.K., 16 to 25% of adults suffer from hearing difficulty. Many of these people suffer from an impairment of more than 25 dB HL in their better ear [11]. Across the Atlantic, it is thought that more than 36% of Americans will suffer from significant hearing impairment by the time they are 75. At any one time, 2 million Americans are estimated to

be profoundly deaf [36]. Also, a recent large scale study by a colleague on profound deafness in the Sichuan province of China [29]—the region is thought to be a good demographic representation of the whole country—unearthed a prevalence of 2 in 1,000 Chinese with hearing impairment exceeding 90dB HL.

The possibility of a genetic component in the aetiology of hearing impairment was first discussed only in the latter half of the nineteenth century, by the otologist William Wilde of Dublin [40]. After more than a century, despite the current golden era of molecular genetics, the true effect of nature over nurture (or rather the lack thereof!) on hearing loss is still not known. Currently, the estimated proportion of cases of hearing loss caused by genetic factors range from 3% to a disturbing 78% [29, 49]. Out of the cases of profound deafness, 30% [36] to 50% [16, 27] are thought to be caused by some genetic defect. If we regard the hypothesis that presbycusis in humans may have a strong genetic component [36], as is thought to be the case for mice [28], then, taken as a whole, hereditary deafness is the single most important cause of hearing loss in humans.

Whither Deafness Genes?

Perhaps it is fortunate that most cases of hearing loss are hereditary in nature. For we are in an age when, although still a formidable task, pathfinding into a genome and looking for a disease gene is not only

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(Received 8 October 1996 / Accepted 20 October 1996)

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possible, but getting easier with the continual development of more sophisticated tools. Finding a deafness gene would not only mean the possibility of diagnostic tests but perhaps therapy in the future. For the moment, studying these genes gives us the opportunity to dissect and better understand the development and the physiology of the hearing apparatus.

The main problem with finding deafness genes via the conventional route of linkage analysis in affected families is several fold. Firstly, as already alluded to above, the genetic causes of deafness are heterogeneous. Secondly, an estimated 60 to 75% of these genes are thought to be autosomal recessive [16, 36]. Couple these facts together and the number of families needed to demonstrate linkage for the majority of deafness causing genes becomes uncompromisingly large [5, 7]. The alternative is to use large families or inbred, isolated populations. But these are rare.

As a result our attention has been directed elsewhere—to finding these genes using the spring-board of mouse models. Several reasons make the mouse amenable to such a strategy:

- the mouse cochlea is anatomically similar to that of humans [44];
- pathological features in hereditary inner-ear abnormalities have been shown to be similar in both species [6, 42, 44];
- syntenic linkage groups are often conserved in terms of gene-content and order between the two species [6];
- large genetic crosses can be performed to aid linkage analysis;

- transgenics can be created to examine and validate the candidate genes;
- there are many mouse deafness mutants already described and studied [32, 43].

Recently, as a result of this strategy, the first gene involved in a neuroepithelial hearing defect was cloned from the *shaker-1* (*sh1*) mouse [18]. The human homologue was quickly implicated as the aetiological agent of Usher type 1B syndrome [46]. However, an earlier prediction that the non-syndromic autosomal recessive deafness disease, DFNB2 (previously known as NSRD2) [19], as being the human equivalent of the *shaker-1* model still cannot be discarded.

Shaker Mice and the Hunt for a Deafness Gene

Physiology and morphology

The *shaker-1* mouse was first described by Lord & Gates in 1929 [31]. The original mutant displayed a progressive hearing loss, was hyperactive, had a tendency to circle and toss its head and slowly lost its ability to swim [31, 32]. Since then, the mutant has been studied extensively both physiologically and anatomically.

The genetic strain within which the gene of interest nestles is thought to have an influence on its phenotypic expression—a caveat to bear in mind when interpreting results from different laboratories. This appears to be especially important for *shaker-1* [45]. The hearing apparatus of *shaker-1* appears to develop normally but hearing starts to deteriorate rapidly from 12 days post-

Test	heterozygous litter-mate (+/ <i>sh1</i>)	<i>shaker-1</i> (<i>sh1/sh1</i>)	Inference
Cochlear Microphonics (CM) (Threshold defined as 2 μ V)	CM thresholds (at several frequencies) stabilise and/or continue to improve 15 days post-natal.	CM improves at same rate as +/ <i>sh1</i> after birth but begins deteriorating after day 14.	Outer hair cells (OHC's) develop and function normally until day 14.
Positive Summating Potentials (+SP)	+SP thresholds (at several frequencies) improve and stabilise after around 16 days post-natal.	+SP improves at similar rate as +/ <i>sh1</i> after birth but does not reach the same low thresholds.	Inner hair cells (IHC's) develop and function normally until day 12.
Compound Action Potential (CAP)	First CAP detected at day 11 and improves until around day 16 post-natal for several frequencies.	First CAP detected at day 13 and levels out at day 15. About 50% showed no CAP even up to day 30.	The IHC to afferent innervation pathway is dysfunctional.

natal [20, 45]. This indicates that the nature of the defect is neuroepithelial [44]. Extensive physiological examination [20, 45] showed the following:

These studies seem to implicate the cochlea, and more specifically, the hair cells, as the site of action of the mutant gene. Electron microscopy on the original *shaker-1* mutant showed that there is little evidence of early histological abnormality of the inner-ear. However, 12 days after birth, the hair cells, both inner and outer, can be seen to degenerate. More telling is the morphology in newer and more severe *shaker-1* mutant alleles. Additional alleles were derived by ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis; including the original *shaker-1*, there are now seven mutant alleles, of which one other spontaneously arose at the Jackson Laboratories. Each of the six new mutations displays a more severe phenotype than the original and all are characterised by an early and marked effect on the stereocilia bundles of both the OHC's and IHC's. The stereocilia grow to form the usual graduated-height ranks but are disorganised into clumps atop the hair cells. In the OHC's the usual 'W' configuration never develops. All this can be seen as early as 18 days into gestation, and, soon after, the hair cells themselves start to degenerate [20; K.P. Steel, T. Self, M. Mahony and J. Cable, unpublished observations and on-going work].

Gene hunting

Gates studied the segregation of the *shl* gene and reported its linkage to albinism in 1931 [as cited in ref. 3]. This placed *shl* on chromosome 7. To delimit the physical boundaries of the candidate gene, an intra-specific backcross was performed between CBA/Ca *shl/shl* mice and C57BL/10. The F₁ progeny were back-crossed to the CBA/Ca *shl/shl* mice [4, 18] to yield 1,066 progeny segregating *shl*. RFLP analysis of the back-cross showed that *shl* was non-recombinant to the gene *Omp* (olfactory marker protein) and potentially narrowed the region in which the gene resides to 0.1 centimorgan (about 200 kilobases). *Omp* was used to recover YAC's from the Princeton, MIT and St. Mary's Hospital Medical School (London) mouse YAC libraries via PCR screening. Microsatellites were isolated from the first YACs recovered and the libraries were re-screened with these markers. Ultimately, a 1.5 Mb YAC contig was constructed across the *shl* locus region. Genetic analysis of

DNA markers within the contig identified a non-recombinant region to *shl* extending to over 500 kb. Exon-trapping in the non-recombinant region recovered several products which were used to isolate clones from a cochlea-derived cDNA library; a gene encoding myosin VII was recovered and its candidacy status was elevated to causative agent when mutations in it were found in three of the seven *shl* alleles [18].

Usher in Usher

The conserved-syntenic region to which the mouse myosin VII gene maps in the human genome is 11q13. Previously two deafness syndromes have been mapped to that region, vis à vis Usher Syndrome type 1B [25] and DFNB2 [19]. Myosin VIIa immediately became a candidate gene for both diseases.

Along with Waardenberg and Alport's syndromes, Usher is one of the commonest of the syndromal deafnesses in humans [36]. Its prevalence is estimated to range from 0.6% to 28% among the deaf population [26]. The disease expression is heterogeneous with both ophthalmic and neurologic components [15]. The trait is autosomal-recessively inherited and combines both congenital and progressive bilateral sensorineural hearing impairment with vestibular dysfunction, and progressive retinitis pigmentosa (RP) [15]. Type 1 encompasses congenital deafness coupled to progressive RP with onset as early as 2 years and achieving full penetrance by the second decade of life [34]. The subdivision B indicates that the causative gene is linked to 11q13.5 as was initially demonstrated in a study on British pedigrees [25, 39].

Nine unrelated families demonstrating Usher 1B in their ranks were screened by cloning and sequencing the 5' end of their myosin VIIa gene. Five different mutations were found and the gene was concluded to be the causative agent of Usher 1B [46].

There, however, remains the question of retinitis pigmentosa. Unlike all Usher 1B patients, *shl* mice do not show any sign of RP throughout their adult lives [J. Cable, M. Mahony and K.P. Steel, unpublished observation]. Recent immunocytological work indicates that myosin VIIa is expressed at high levels in the pigmented epithelium of the eye of both mouse and man [21] but only in humans does it also occur in the rods and cones [13]. If the latter finding is accurate, then it

could be explain the RP discrepancy. Nevertheless, it is still possible that since primates are precocial (hearing begins at the late prenatal stage) whereas murine species are altricial (hearing does not begin until at least 10 days post-natal) [12], and RP in Usher 1B patients starts developing a few years after birth, this symptom may never have time to express itself within the natural life-span of the mouse.

DFNB2 was localised to 11q13 in a consanguineous South Tunisian family [19]. Apart from four members who complained of vertigo, the neurosensory deafness appears to be non-syndromal. It is this apparent lack of vertigo that has led investigators to disregard myosin VIIa as a candidate gene for this disease. However, it is well documented that patients with vestibular dysfunction may have normal gait by simple observation. Visual- and proprio-stimuli can compensate for the lack of inertial guidance [41]. The litmus test would be a Bruininks-Oseretsky balance-function protocol. It is unclear whether the patients in the above study [19] have been so examined and whether there still is a possibility of the occurrence of some vestibular dysfunction. If indeed the undetected syndrome does exist, then myosin VIIa is an even better candidate for this disease than Usher 1B since no RP is involved.

Genotype and Phenotype

Gene structure

Myosin VIIa is a non-muscle (unconventional) myosin. Five out of the eighty-odd myosins discovered

thus far belong to the myosin VII class of which two are from human, one from mouse, one from *Drosophila melanogaster* [10] and one from pig [2]. The full length transcript in humans, spliced from 49 exons, is 7,466 base pairs (bp) long of which 272 bp belong to the 5'UTR, 544 bp the 3'UTR and 6,647 bp the coding sequence proper [46; GenBank accession no. U17180]. This translates to a peptide 2,216 amino-acids (aa) long with a predicted mass of 254 kDa [47] and an isoelectric point of 8.5. (Another group describes their myosin VIIa to have an open reading frame of 6,525 bp translating to 2,175 aa [8].) The Usher 1B gene and the *shaker-1* gene show 88% identity at the nucleic-acid level and 95% at the protein level (unpublished data). Recently, a smaller transcript with an open reading frame (ORF) 3,609 bp long has been discovered. A read-through of a splice site signal at nucleotide 3,512 (of the ORF) causes a stretch of an (otherwise) intron to be transcribed with a stop codon 96 nucleotides (nt) downstream. This novel transcript is thought to encode a 138 kDa protein that is expressed at extremely low levels compared to the longer transcript, since it is not detectable through immunoblots but only by RT-PCR on human testis and lymphocytes. This shorter transcript still has not been detected in various frog or rodent tissues [8].

Expression pattern

Northern blots have shown that myosin VIIa—in humans, mice and rats—are expressed in the testis, lung, kidney, inner-ear and retina [18]. In addition,

Tissue/organ	Localisation
Inner-ear	Hair cells of cochlea and vestibular utricula and macula ¹⁻³ , both in cell body and stereocilia ¹
Retina	Pigmented epithelium ¹⁻⁴ ; labelling pronounced near apical and basal membranes with punctate staining in cytoplasm ⁴ . Photoreceptor cells in humans—not detectable in mouse—localised in the tip of up-to-28-weeks-old embryos and the inner-segment, base of outer segment and synaptic endings in adults ³ .
Testis	Sertoli cells including ectoplasmic specialisations but not in mature spermatozoa ⁴ .
Lung	No localisation, but a smaller product on Western blots at 220 kDa detected ^{1, 4} .
Others	Renal cells ⁴ , hepatocytes, choroidal plexus, epithelial cells of small intestines ² .

¹[21]. ²[46]. ³[13]. ⁴[22]

part of the gene was first cloned from a cell line derived from intestinal epithelium [2]. Finer localisation via immunostaining techniques on both mouse- and human histological sections show the following:

If the enigma of RP occurring only in humans could be explained by the fact that the photoreceptor cell in mice do not express myosin VIIa [47], it leaves us with the even more perplexing question of the reason for the difference of expression between the species.

In the testis, although the Sertoli cells seem to express a large amount of the protein, spermatogenesis and the mature spermatozoa do not seem to be affected [22]. Indeed the fertility of *shaker-1* mice appears normal [K.P. Steel, personal communication]. In humans the effect of a mutation in the gene on fertility is not known since the deafness and blindness of Usher 1B patients places them in a special social niche which excludes them from direct comparison with the population at large [X. Liu, personal communication].

The initial discovery that the hair-cells in the inner-ear express the protein and are affected by a mutation in the gene prompted the question as to whether ciliated epithelia in general were affected. In mice, the nasal epithelium appears to be normal in several mutant alleles [J. Cable and K.P. Steel, unpublished observation] and in humans, although there is an early report of abnormal nasal cilia with Usher patients [1] it is unclear whether any of the patients was of subtype 1B. Regardless, the question still remains open. Contrariwise, two factors seem to indicate that ciliated epithelia in general may not be affected to a degree considered important: a) none of the Usher 1B patients nor the mouse models shows symptoms similar to Kartagener's Syndrome; b) myosin VIIa is actin-, and hence is unlikely to be microtubule-, associated.

Protein structure

Myosin VIIa, like all myosins, is biochemically an Mg^{2+} -ATPase that is actin-activated. This function resides in the, approximately, 80 kDa head region (N-terminal) that is known as the "motor-domain", and is conserved in many species [10]. ATP co-ordinates with most of the residues in the conserved sequence G₁₅₈ESGAGKT [14]. The solved 3-dimensional structure of myosin II head in chicken shows a 50 kDa cleft

which is interior to a 50–20 junction (so called because proteolytic cleavage there results in a 50 kDa and a 20 kDa domain). The actin-binding interface is thought to be near this junction since it is protected from trypsin cleavage when bound to actin (in early experiments on myosin II this junction corresponds to residues 635–641) [35]. Whilst the actual events that result in movement of the myosin head along actin are still unknown, a conserved [10] run of residues in myosin II, corresponding to L₄₃₆ to F₄₆₄ in mouse myosin VII, has been implicated in a 35 Å movement [48].

Following the head domain from D₇₃₁ to V₈₆₃ in mouse myosin VII is the regulatory domain; the signature of which is an 'IQ' motif made up of mostly hydrophobic residues [33, 50]. This region is thought to bind to calmodulin or calmodulin-like proteins [9]. Immediately following this is a 70 residue long region that is predicted to form a coiled-coil structure that has been implicated in molecule dimerisation in myosins and kinesins. Although this structure in myosin VII compared to other motor molecules is very short (unpublished observation), it is thought to be long enough to dimerise adequately [M.J.T.V. Cope, personal communication].

Spanning the rest of the molecule is the tail region. It is this region that is singular to the myosin VII class and probably defines its unique molecular function *in vivo*. Nothing is known of the functional domains of the tail region but there are several regions of interest:

- a 460 aa internal repeat corresponding to residues 1,016–1,568 and 1,708–2,167 with approximately 25% identity [8; unpublished observations];
- regions of similarity to *Acanthamoeba* myosin IV tail at residues P₁₁₆₇–I₁₂₁₈ and N₁₇₅₆–R₁₈₂₄. Both are similar to the same region in myosin IV at residues approximately 1,805–1,872 with identities of 51% and 36% respectively [8; unpublished observations];
- a short region between the two myosin IV-like domains from Q₁₄₅₆–R₁₆₁₀ that is similar to a few uncharacterised *Caenorhabditis elegans* cDNA clones (yk54f6 and yk9c2) [unpublished observation];
- a large region of similarity averaging out to an identity of 20% with two plant kinesins from *Arabidopsis thaliana* and *Solanum tuberosum*.

This region spans residues 1,166 to the end of the molecule but is interrupted at residues 1,403–1,626 [unpublished observation]. The *A. thaliana* kinesin is a calmodulin binding protein [37]; unfortunately the corresponding similarity domain falls into the uncharacterised tail region;

- similarity to the band 4.1 superfamily: the last 300 residues appear to be 20% identical to the ezrin/radixin/moesin (ERM) families [8, 47; unpublished observations];
- a computer-predicted transmembrane region spanning residues 2,063–2,077 [unpublished observation].

Of interest is the kinesin similarity. Kinesins are known to be associated with, and are involved in the intracellular trafficking of, cellular vesicles [24]. It is quite conceivable that sub-cellular vesicles thought to be handled specifically by kinesins may also be transported along actin by other kinds of motor molecules like myosin. This may be a necessity considering that the cells or sub-cellular regions (e.g. the stereocilia and cuticular plate) within which myosin VIIa is expressed are actin-rich. This possible interaction with sub-cellular vesicles could explain the region of similarity to the

band 4.1 superfamily as talin & ERM's are associated with membranes via integral membrane proteins [38]. However, without biochemical assays little more can be concluded from these similarities, and even less from the calculated transmembrane region. Our group is currently engaged in studying some of the intra-cellular interactions of myosin VII.

Mutations

Out of the seven mutant alleles in mouse, only three have thus far been discovered. On-going physiological and histological work by Steel's group and data from Hasson *et al.* [22] are summarised in the table below:

The first two mutants arose spontaneously whereas the last five are ENU-induced. The original *shaker-1* mouse has the mildest disease phenotype. This mutation actually occurs on the outer portion of the lower 50kDa domain of the head, diametrically opposite to the nucleotide binding site and physically at-a-distance from the actin-binding cleft; but it is near a residue (265) [unpublished data] thought to be involved in the closure of the ATP binding site when activated [10]. The mutated residue is not conserved between species and is likely that the mutation has resulted in a change in the kinetics

Allele	Mutation	mRNA levels ¹	Protein levels ²	Cochlear physiology ³	Cochlear structure ³	Visual physiology & retina structure ³
<i>shl</i>	R ₂₄₁ to P		75–87%	CAP abnormal SP poor CM poor	Normal initially	
<i>shl</i> ^{6J}	R ₅₀₂ to P		14–16%	Some SP	Abnormal	
<i>shl</i> ^{816SB}	10 aa in-frame deletion L ₆₄₆ -Q ₆₅₆	Wild-type levels	4%	No response	Abnormal	Normal
<i>shl</i> ^{26SB}	?		35–43%	No response	Abnormal	
<i>shl</i> ^{3336SB}	?		7–11%	No response	Abnormal	
<i>shl</i> ^{4494B}	?		0.4–0.8%	Some SP	Abnormal	
<i>shl</i> ^{4626SB}	?		0.4–0.7%	No response	Abnormal	

¹Via Northern blots on testis [22].

²Via Western blots in testis and kidney compared against wild-type CBA/Ca [22].

³K.P. Steel, unpublished data.

of the motor domain, hence the moderate phenotype.

The mutation in *shl*^{6J} affects an important residue located between the ATP-binding- and 50 kDa cleft. Computer solutions show it to be hydrogen-bonded to at least three other residues [unpublished data]. The severe phenotype that results comes as no surprise. Counter-intuitively, the protein levels are higher than the other mutations save the original and the 26SB mutants.

The 10 aa in-frame deletion of *shl*^{816SB} occurs within an extremely important region in the lower 50 kDa sub-domain. This region contains the largest number of conserved residues in the motor domain and is thought to maintain some structural integrity of the head. It may help transmit the actual structural deformation and/or the resultant forces which occur upon activation of the motor [10]. Indeed, the deletion occurs just upstream of a highly reactive cysteine (SH2) that is extremely conserved. It is not surprising that this mutant displays a more severe disease phenotype. The importance of the region to the structural integrity of myosin VII may explain the low protein levels associated with this allele.

All the mutations discovered to date occur in the motor domain of myosin VII. It is not inconceivable that some mutations yet undiscovered occur in the long tail domain. We are currently engaged in trawling for the remaining mutations.

All the mutations in Usher 1B patients have, to date, also been found to reside in the motor domain. Six mutations have been published, five of which were discovered soon after cloning the mouse myosin VII gene [46]. The mutations include:

- in two cases, cytosine to thymine substitutions resulting in a stop codon after residues S₁₄₉ and E₂₃₃;
- a C to T substitution resulting in a residue change of R₂₁₂ to C, and in another case, a G to A substitution one nucleotide downstream results in the same arginine residue to be substituted with histidine;
- a six base-pair in-frame deletion resulting in the deletion of D₂₁₈ & I₂₁₉;
- a deletion of cytosine at codon D₅₂₁ resulting in a stop codon after K₅₂₈ [30].

The arginine substituted in the second type of mutation occurs near the ATP binding pocket on the upper 50 kDa domain, and could have a deleterious effect on

motor function. Likewise for the nearby six-base pair deletion in case three. The other mutations result in severely truncated proteins.

Post Tenebras Lux

In all mutations in the mouse, development of the gross structure of the inner ear appears to be normal, but hair cell development and function is severely affected. Given, also that RP is progressive, it seems likely that myosin VIIa plays a more important role physiologically rather than structurally. However, we are still a long way from determining the function of this molecule. For the moment, clinically, identifying the gene has opened several doors. For instance, conventional audiological tests are notoriously unreliable [X. Liu, personal communication]. Already, definite diagnosis for Usher 1B can be made by screening patients for mutations. It is also a boon to the pathologist that the gene is expressed, albeit at low levels, in peripheral blood lymphocytes [2, 8]. In addition, Usher 1B patients can be put on the rapidly growing wish-list for gene-therapy.

More importantly, elucidation of the function of myosin VIIa will help us unravel the complex process of hearing. We have come a long way from Empedocle's gong, which he theorised to sit in the ear; and rings to transduce the air movements—caused by external sound—into the sensation we call hearing [17]. Despite this our understanding of this remarkable phenomenon is still far from sound [pun intended].

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