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Unique Transgenic Animal Model for Hereditary Hearing Loss

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Abstract

Objectives—This study capitalizes on the unique molecular and developmental similarities between the auditory organs of *Drosophila* and mammals, to investigate genes implicated in human syndromic and nonsyndromic hearing loss in a genetically tractable experimental animal model, the fruit fly *Drosophila*.

Methods—The *Drosophila* counterparts of 3 human deafness genes (DIAPH1/DFNA1, ESPN/DFNB36, and TMHS/DFNB67) were identified by sequence similarity. An electrophysiological assay was used to record sound-evoked potentials in response to an acoustic stimulus, the *Drosophila* courtship song.

Results—Flies with mutations affecting the *diaphanous, forked*, and *CG12026/TMHS* genes displayed significant reductions in the amplitude of sound-evoked potentials compared to wild-type flies ($p < 0.05$ to $p < 0.005$). The mean responses were reduced from approximately 500 to 600 μV in wild-type flies to approximately 100 to 300 μV in most mutant flies.

Conclusions—The identification of significant auditory dysfunction in *Drosophila* orthologs of human deafness genes will facilitate exploration of the molecular biochemistry of auditory mechanosensation. This may eventually allow for novel diagnostic and therapeutic approaches to human hereditary hearing loss.

Keywords

auditory evoked response; deafness mutant; *Drosophila*; microfilament protein; tetraspan membrane protein

INTRODUCTION

Congenital hearing disorders affect 1 in 1,000 children, and approximately half of these cases are hereditary.[1] Currently, more than 100 genes related to either syndromic or nonsyndromic hearing loss have been identified, as reported on the Hereditary Hearing Loss Homepage (http://webh01.ua.ac.be/hhh). In many cases, however, the underlying molecular function of these genes, as well as their role in auditory development and physiology, remains unknown. The auditory apparatus of the fruit fly, *Drosophila melanogaster*, shares

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both functional and developmental similarity with mammalian auditory organs.[2-5] Although the actual details of these functional and developmental mechanisms may differ, the involvement of many homologous genes in insect and vertebrate hearing has been documented.[6-8] The essential molecular details of mechanotransduction, including the channel identities, have not yet been elucidated in any of these systems, so the degree of molecular conservation at this level is unknown.[6-8] The fact that these auditory systems arose from a common ancestral mechanosensor, together with the availability of a reliable assay for testing the flies' response to sound,[9] makes *Drosophila* a powerful animal model with which to investigate the role of genes in audition. As with other sensory systems, the comparative approach is valuable, and often the differences between organisms are not only interesting but also revealing. In this case, the *Drosophila* auditory system, seated in the antenna, is optimized mechanically for sensitivity to near-field sound[10] (see below), but the chordotonal mechano-receptors responsible for transduction are basically the same as those that innervate the tympanal auditory organs in other insects, which are optimized in various ways for far-field sound detection, as is the typical vertebrate auditory organ.[1-8]

In *Drosophila*, reception of auditory stimuli, or hearing, is behaviorally relevant for courtship. The male fly uses unilateral wing vibration to stimulate species-specific copulation with female flies. The wing vibration or "courtship song" is structured into 2 alternating components: a sine song of approximately 140 to 170 Hz and a pulse song consisting of 5- to 10-ms pulses.[11] The interpulse interval (IPI) or length of time between pulses is unique to individual species of flies; for example, the IPI is 28 to 40 ms in *Drosophila melanogaster*, whereas it is approximately 45 ms in *Drosophila simulans*.[11] Thus, this courtship song communicates species-specific information that is utilized in the context of mating and courtship.

The *Drosophila* auditory organ, called the Johnston's organ (JO), is housed within the second segment (a2) of the flies' antennae (Fig 1).[2,4-7,9-13] The *Drosophila* antennae consist of 3 segments (al, a2, and a3) and a feathery, hair-like extension, called the arista, firmly attached to the third, most distal antennal segment (Fig 1B). The JO consists of more than 200 stretch receptor units called scolopidia, arranged radially around the a2-a3 joint of the antenna (Fig 1C). Each scolopidium encloses the ciliated sensory dendrites of 2 or 3 mechanosensory neurons (Fig 1D), whose axons coalesce to form the antennal nerve and ultimately carry peripheral sound information to the auditory center of the brain.[13] As in mammals, these auditory structures initiate conversion of sound energy to mechanical signals and, ultimately, to an electrochemical response that is interpreted by the central nervous system. Signal transduction is initiated when near-field sound (particle movement), typically the courtship song produced by the wing of a nearby male fly, is received by the arista.[10] Deflection of the arista causes rotation of the a2-a3 joint, leading to activation of the stretch receptors (the scolopidia) within the JO.[10] Research into the ultrastructure of scolopidia has shown some organizational similarity with mammalian cells. Each scolopidium consists of monodendritic ciliated neurons, actin- and microtubule-rich scolopale cells, and associated supportive cells (Fig 1D). The neurons are sealed within the scolopale space by the septate junctions of the scolopale cells. A key feature is the potassium ion–rich or "endolymph-like" composition of this fluid[2]; much like the scala media of the vertebrate cochlea, this ionic compartment is believed to drive receptor potentials.[3,14]

Despite the large evolutionary distance between humans and flies, the development and physiology of the respective auditory organs has remarkable parallels and common signaling and cell fate molecules, probably reflecting independent adaptations of ancestral metazoan mechanosensors to receive auditory stimuli.[8] Proneural genes such as *atonal* and its vertebrate homolog *Atohl* (formerly *Mathl* in the mouse) are required for specification of

peripheral sensory cells in flies,[15] and for development of auditory hair cells in mammals.[16,17] In addition, the basic mechanism of lateral inhibition and the role of Notch and its ligands are also conserved across species,[16] although an additional role in the formation of prosensory patches has been described in chicks.[18,19] Using a *Drosophila* model, previous research has provided insight into the underlying molecular mechanism for a number of human deafness disorders that cause progressive nonsyndromic hearing loss, such as Usher 1B syndrome.[12] Finally, microRNAs in the miR-183 family are expressed in vertebrate sensory hair cells, as well as in the *Drosophila* auditory organs, and in mechanosensory organs in other invertebrates.[20]

This study focuses on 3 genes believed to be involved in nonsyndromic hearing loss in humans: DIAPH1 (DFNA1), ESPN (DFNB36), and TMHS (DFNB67). Thus far, each gene has been characterized only by familial linkage studies in humans. Both mouse and fly orthologs have been identified (Table 1[21]). Mutations in the DIAPH1 gene cause autosomal dominant, progressive nonsyndromic low-frequency hearing loss in humans (DFNA1).[22] Originally identified as an ortholog of the *Drosophila diaphanous* gene (*dia*), it is thought to be involved in regulation of actin polymerization in auditory hair cells.[22] Next, mutations of ESPN (encoding the Espin actin-bundling protein) cause recessively inherited nonsyndromic deafness and vestibular dysfunction in humans (DFNB36)[23] and *jerker* mutant mice.[24] Last, TMHS (tetraspan membrane protein in hair cell stereocilia) was characterized by a spontaneous mutation causing deafness and vestibular dysfunction in mice named *hurry-scurry* (*hscy*)[25] and nonsyndromic hearing loss in humans (DFNB67).[26] TMHS encodes a tetraspan protein involved in the development of mouse inner ear hair cells.[25] On the basis of their apparent role in mammalian audition, we hypothesized that mutations in the fruit fly orthologs of these 3 genes will lead to abnormal hearing in *Drosophila*. Understanding of how these genes affect audition in flies will inform and stimulate further basic and translational research in flies and mammals, ultimately leading to human health benefits in the form of novel diagnostic approaches and therapies for these devastating hereditary hearing loss disorders. Preliminary findings from this study were presented at the 2007 meeting of the Academy of Otolaryngology–Head and Neck Surgery.[27]

METHODS

Fly Strains and Target Genes

The amino acid sequence of each human target gene identified on the Hereditary Hearing Loss Homepage (http://webh01.ua.ac.be/hhh) was obtained from the Genbank database (http://www.ncbi.nlm.nih.gov/Genbank) and used to locate the orthologous *Drosophila* gene by use of the FlyBase database (http://www.flybase.org), an online searchable database containing the entire *Drosophila* genome. Percent sequence similarity was determined with the BLASTP alignment tool.[21] Each human gene chosen for this study shared an extremely high degree of sequence similarity with its respective *Drosophila* gene, as listed in Table 1. For DFNA1, the orthologous gene in *Drosophila* is *diaphanous* (*dia*). The dia^5 mutant used in this study is a recessive lethal null allele that was generated by excision of a P-element from the dia^1 mutant, but the exact molecular nature of the lesion is unknown.[28] The *Drosophila* ortholog of ESPN (DFNB36) is the *forked* (*f*) gene, which causes defects in bristle morphology. We used the f^1 mutant allele, a recessive viable spontaneous mutant caused by insertion of a *gypsy* transposable element into the *f* gene coding region.[29] For TMHS, our search for orthologous genes revealed *CG12026*, a *Drosophila* gene of unknown function identified by gene prediction. We used flies with deletions that span the 62B9 chromosome region in which *CG12026* is located. We labeled these mutant lines $TMHS^{\Delta 1}$ (*del62B7-12*) and $TMHS^{\Delta 2}$ (*del62B4-12*). We also used flies with a transposable element insertion that is predicted to disrupt expression of the *CG12026* gene, which we labeled $TMHS^{P1}$ (*P{hsneo} l(3)62Be*). Neither the dia^5 nor the *TMHS* mutants exhibited any gross

defects in external bristle morphology. Stocks of flies with mutations in each target gene were obtained from the *Drosophila* Stock Center (Bloomington, Indiana). Known auditory mutant flies, *atonal*, were used as the deaf control, having been established in previous studies as lacking all sense organs of the chordotonal type, including those in the JO.[15] Wild-type flies from a stock commonly used for behavioral and transgenic experiments, $white^{118isoCJ}$,[30] were used as normal-hearing controls. The animals were raised on a cornmeal medium and housed in standard vials at either 18°C or 25°C. All flies were tested 3 to 7 days after eclosion, and were subjected to the same environmental testing conditions.

Electrophysiological Assay

As described previously,[9,31] recordings of sound-evoked potentials (SEPs) were made from the antennae of mutant and control flies in a standardized fashion. Un-anesthetized flies were immobilized in a 200-μL micropipette tip with the head exposed and supported by modeling wax, leaving the antenna free to vibrate (Fig 1A). Sharp tungsten electrodes were placed into the joint between the first and second antennal segments (recording electrode) and into the head near the dorsal orbital bristles edge (reference electrode), as shown in Fig 1A. To reduce variation due to electrode placement, we used the position of characteristic bristles on the antenna and head as a guide for insertion sites. The electrodes were connected to a DP-301 differential amplifier (Warner Instruments, Hamden, Connecticut) with gain set at 1,000, a low-pass filter at 10 kHz, and a high-pass filter at 10 Hz. Signals were acquired and digitized with a PCI-6023E data acquisition board and Labview 8.0 software (National Instruments, Austin, Texas). The apparatus was placed within a Faraday cage, and all external sources of electrical noise were eliminated. Acoustic stimuli consisting of 5 pulses with a 35-ms IPI (to mimic the pulse song component of the fly's courtship song[11]) were generated with Labview and sent to a loudspeaker unit. The resulting acoustic stimulus was delivered directly to the fly's antenna via a 1.5-m length of 6.35–mm inner diameter tubing, one end of which was positioned inside (but not touching) the speaker cone. The fly's head was positioned close to the other end of the tube, within the hemisphere circumscribed by the tube opening, to ensure complete near-field acoustic conditions. The length of the tubing has no impact on the variation of the peak amplitude of the response (data not shown). These sound waves caused mechanical deflection of the arista and the third antennal segment. The responses to 10 presentations of the stimulus were averaged in each trial, and the maximum amplitude of the peak responses was calculated (peak amplitude). About 20 flies from each mutant stock were subjected to auditory electrophysiological recording. Wild-type flies were routinely alternated with mutant flies to ensure that valid comparisons could be made and that the equipment was functioning. Testing was performed in a semiblind fashion such that the experimenter did not know which particular mutant genotype was being assayed. Statistical analyses were performed with GraphPad Prism v4.0c/v5.00 (GraphPad Software, San Diego, California). Statistical significance was calculated with a nonparametric Mann-Whitney analysis of median peak SEP amplitude in wild-type versus mutant flies, with a sample size of 10 to 30.

RESULTS

Representative auditory traces for wild-type and mutant flies are shown in Fig 2. Wild-type males and females have similar robust responses to the pulse song stimulus; their traces demonstrate well-defined sharp peaks, followed by 3 or 4 secondary peaks that are probably due to poststimulus deflection of the arista. The time lag between the auditory stimulus (the courtship song) and the peak response is directly related to the length of tubing used to direct the stimulus to the fly's head.[9] In contrast, *atonal* mutants lacking JO neurons[15] showed no response to the stimulus. Although their aristae were free to vibrate, these mutants uniformly demonstrated no detectable response, as previously reported.[15] Overall,

flies with mutations in the genes of interest demonstrated reduced peak amplitudes compared to wild-type flies (Fig 2). Unlike *atonal*, all flies with mutations in the target genes demonstrated some response to the courtship song (Fig 2). Although often markedly reduced, the response was clearly related to the stimulus presentation, with a lag time mimicking that of wild-type flies, as indicated by the dashed line showing the onset of stimulus presentation (Fig 2). The dia^5 and *TMHS* mutations are homozygous lethal and therefore were maintained and tested as heterozygotes over balancer chromosomes (dia^5/*CyO, TMHS*$^{\delta 1}$/*TM6, TMHS*$^{\Delta 2}$/*TM6, TMHS*^{P1}/*TM3*). The f^1 mutant is homozygous or hemizygous viable. As a control for the effect of balancer chromosomes, we also tested SEPs in flies carrying 1 copy of the balancer chromosome in a wild-type background (+/CyO, +/TM6, +/TM3). These flies had normal SEP responses (data not shown).

The graph in Fig 3 shows the peak amplitudes of the SEPs recorded from wild-type and mutant flies. Statistical evaluations of the data are presented in Table 2. The responses of wild-type males and females consistently exceeded 100 μV, with mean responses in the range of approximately 500 to 600 μV (Fig 3 and Table 2). A large range of peak amplitudes is commonly observed with this assay[9,10]; however, there are clear differences in the responses of flies with mutations affecting the *diaphanous, forked*, and *CG12026* genes (Fig 3), which are statistically significant ($p < 0.05$ to $p < 0.005$; Fig 3). The most obvious qualitative difference is that many of the mutants exhibit SEPs that are below 100 μV, whereas none of the wild-type flies have SEPs below 100 μV. The mean response was reduced to approximately 150 to 300 μV in dia^5 males and females and to approximately 100 to 300 μV in *TMHS* mutant males and females (Table 2). The mean response of f^1 females was also reduced (approximately 190 μV); however, the f^1 males' mean response was indistinguishable from that of wild-type flies (approximately 500 μV; Table 2).

DISCUSSION

The auditory response to the courtship song was found to be both robust and reliable in wild-type male and female *Drosophila*. As expected, *atonal* flies, which lack JO neurons,[15] showed no response to the acoustic stimuli. Significantly reduced auditory responses were demonstrated in flies with mutations affecting each target gene: *diaphanous* (DIAPH1), *forked* (ESPN), and *CG12026* (TMHS). The fact that SEP defects were observed in heterozygous mutant flies (dia^5/*CyO, TMHS*$^{\Delta 1}$/*TM6, TMHS*$^{\Delta 2}$/*TM6*, and *TMHS*^{P1}/*TM3*) suggests that 50% reduction of these genes is sufficient to substantially disrupt auditory function. Further experiments will be needed to confirm the effect of these 3 target genes on audition, including testing of additional mutant lines and generation of new mutants, using RNA interference and the Gal4-UAS system to knock out these genes specifically in the JO. This will be particularly important for *CG12026* (TMHS), because the deletion mutants each encompass a large region surrounding the *CG12026* transcript. The interval in common between the 2 deletions, 62B7-12, contains 27 predicted genes, only 6 of which have known biological functions (Fly-Base). Our data, showing that the transposon insertion mutant *TMHS*^{P1} also disrupts hearing, are encouraging, but further experimentation will be necessary to prove the cause of the disruption, including precise excision of the insertion to restore hearing, and rescue of hearing by expression of the wild-type gene product in the JO of mutant flies.

It is unclear why f^1 females were more severely affected than f^1 male flies. This trend is also observed for the other 2 genes, in which the mean response of dia^5, *TMHS*$^{\Delta 1}$, *TMHS*$^{\Delta 2}$, and *TMHS*^{P1} mutant females is lower than that of males (Fig 3 and Table 2). Responses of wild-type flies show that both male and female flies can exhibit SEPs of similar magnitude (Fig 3). The *diaphanous* and *CG12026* genes are located on chromosomes II and III, respectively; however, the *forked* gene is located on the X chromosome, so there may be an effect of

dosage compensation in the f^1 mutants. In female humans, one X chromosome is inactivated; in flies, however, genes on the male X chromosome are expressed at approximately twice the level of those on each of the female X chromosomes.[32] These expression levels may differ slightly from gene to gene; however, it is possible that the levels of the mutant Forked protein may be high enough in f^1 males to overcome the auditory deficit. Additional studies using quantitative polymerase chain reaction or anti-Forked antibodies may be able to resolve this issue; however, the *forked* gene encodes multiple isoforms and is not expressed at high levels. Further, the differences between males and females could be restricted to a small number of cells that are crucial for sex-specific auditory functionality.

CONCLUSIONS

Despite the evolutionary distance between *Drosophila* and humans, the remarkable genetic and molecular similarities in auditory organ systems provide a unique opportunity to study hearing. Previous research has supported a common genetic origin for the auditory mechanosensory cells in the *Drosophila* JO and mammalian cochlea, suggesting a conservation of molecular and cellular mechanisms in hearing across species.[3,6-8] More than 100 different nonsyndromic deafness disorders have been described in humans; however, the genetic defect has only been identified in 45 of these disorders (Hereditary Hearing Loss Homepage). Mutations in many of these genes also lead to various phenotypes of vestibulocochlear dysfunction in mice. When utilized, these animal models have facilitated the identification of the underlying molecular cause for a number of auditory disorders. Additional deafness genes, however, are currently identified only by consanguineous human linkage studies; the mouse or fly orthologs of these genes have not yet been identified or targeted. The present study identified *Drosophila* orthologs of 3 target genes: DIAPH1, ESPN, and TMHS. The demonstration of significant auditory dysfunction in these mutants will provide the basis for exploring the molecular biochemistry of auditory mechanosensation and may eventually allow for novel diagnostic and therapeutic approaches to human hereditary hearing loss.

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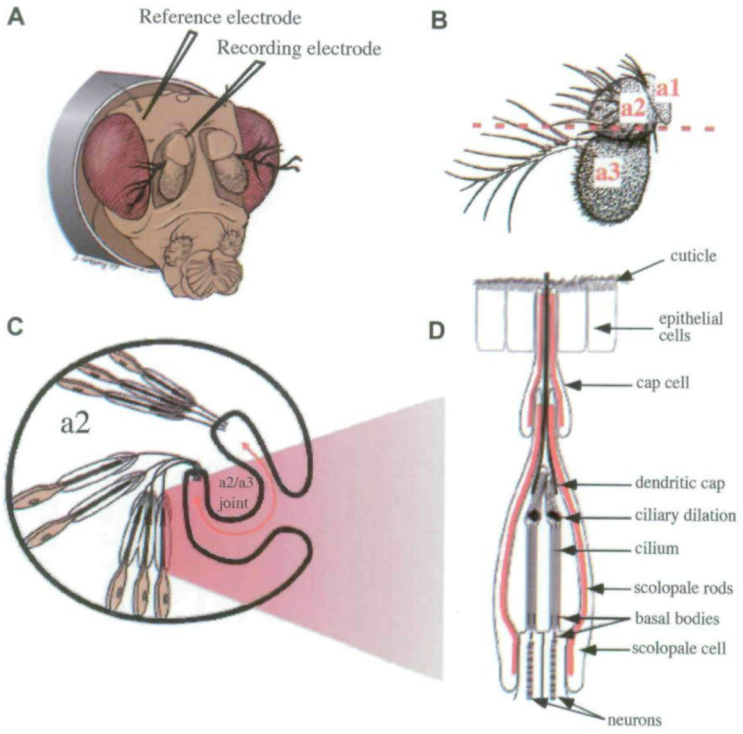

Fig 1.

Johnston's organ (JO), ie, *Drosophila* auditory organ. **A)** Schematic of electrophysiological preparation. Flies are immobilized in micropipette tips with head exposed and antennae free to vibrate. Tungsten electrodes are placed into joint between first and second antennal segments (recording electrode) and into head, near dorsal orbital bristles (reference electrode). **B)** Representation of single *Drosophila* antenna with segments a1, a2, and a3. Feathery arista attached to third antennal segment vibrates in presence of "near-field" sound waves, causing rotation of a3 segment relative to a2. (Modified with permission.[9]) **C)** Diagram of cross section through antenna at junction of a2 and a3 segments. JO is seen as radially oriented scolopidial units in a2 antennal segment. Movement of a3 segment stretches mechanosensory units in a2, resulting in signal to central nervous system via antennal nerve. (Modified with permission.[12]) **D)** Individual scolopidia contain 2 or 3 neurons whose axons join antennal nerve proximally in a2 segment. Dendrites of neurons are surrounded by scolopale cell, and their ciliary tips are connected to epithelium of a2-a3 joint by dendritic cap and supporting cells. (Modified with permission.[12])

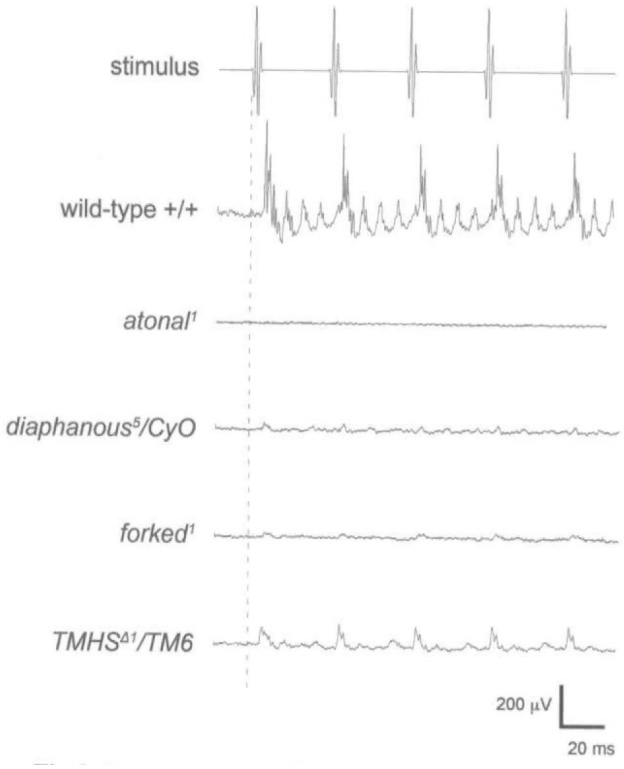

Fig 2.

Representative auditory traces of wild-type and mutant flies in response to computer-synthesized auditory stimulus mimicking flies' courtship song. Wild-type flies exhibit robust response to stimulus, whereas *atonal* flies lacking JO neurons show no response. Each of 5 mutant lines (*dia*^{5}, *f*^{1}, and *TMHS*$^{\Delta 1}$, as shown here; and *TMHS*$^{\Delta 2}$ and *TMHS*^{P1}. not shown) exhibit greatly reduced responses as compared to wild-type responses. Dashed line indicates time of onset of stimulus.

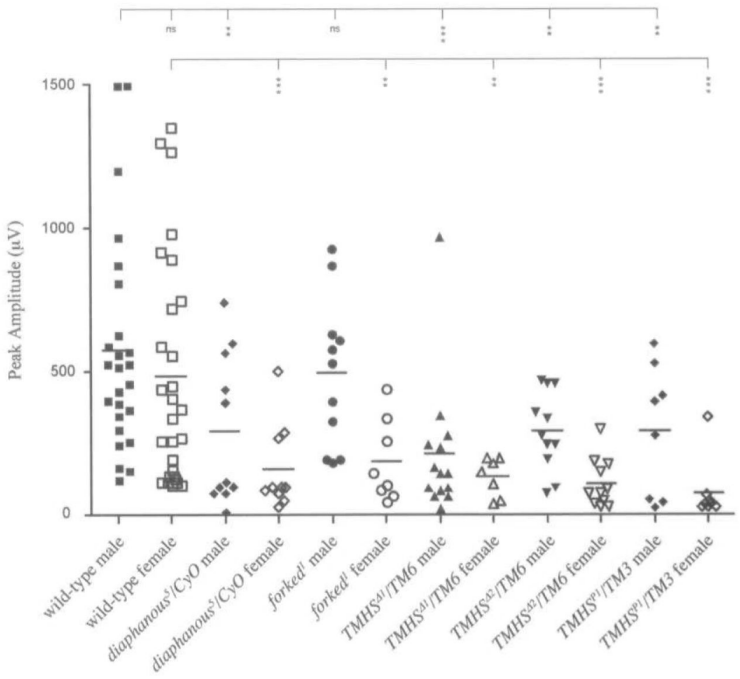

Fig 3.

Peak amplitudes of sound-evoked potentials (SEPs) in wild-type and mutant flies. Responses of wild-type males and females range from 100 to 1,500 μV. Majority of flies with mutations in *diaphanous* (*dia*^{5}), *forked* (*f*^{1}), and *TMHS* (*TMHS*$^{\Delta 1}$, *TMHS*$^{\Delta 2}$, *TMHS*^{P1}) genes exhibit reduced SEP responses, ranging from 10 to 500 μV in both males and females. Mean values for each experimental group are plotted as horizontal bars. Mutant means are significantly lower than wild-type means, except for *f*^{1} males (** – $p < 0.05$; *** – $p < 0.005$; ns – not significant; Mann-Whitney test). See Table 2 for detailed statistical analysis.

TABLE 1

TRANSGENIC ORTHOLOGY OF TARGET GENES

Human Gene/ Locus*	Human Phenotype	Mouse Gene*	Mouse Phenotype	Drosophila Gene†	Drosophila Mutant	Percent Similarity Fly-Human‡
DIAPH1/DFNA1	AD, nonsyndromic SNHL	*diap1*	Unknown	*diaphanous*	dia^{5}	65
ESPN/DFNB36	AR, nonsyndromic SNHL, vestibular defects	*espn*	"Jerker" deafness, vestibular dysfunction	*forked*	f^{1}	55
TMHS/DFNB67	AR, nonsyndromic SNHL	*hscy*	"Hurry-scurry" deafness, circling, vestibular dysfunction	*CGI2026*	$TMHS^{\Delta 1}$ $TMHS^{\Delta 2}$	58

AD – autosomal dominant; AR – autosomal recessive: SNHL – sensorineural hearing loss. Refer to text for other abbreviations.

*Entrez GeneID numbers: 1729 (DIAPH1), 13367 *(diapl)*, 83715 (ESPN), 56226 *(espn)*, 222662 (TMHS), 328789 *(hscy)*.

†Flybase ID numbers: FBgn0011202 *(dia)*, FGgn0000630 *(f)*, FBgn0035289 *(CGI2026)*.

‡Calculated by BLASTP alignment[21] of protein sequences.

TABLE 2

STATISTICAL ANALYSIS OF SOUND-EVOKED POTENTIAL DATA

Genotype	Sex	N	Minimum (μV)	Maximum (μV)	Median (μV)	Mean (μV)	SEM (μV)
wild-type	M	26	130	1,500	525	576	73
wild-type	F	28	110	1,350	355	481	74
diaphanous^{5}	M	11	10	740	100	292	78
diaphanous^{5}	F	10	30	500	100	161	47
forked^{1}	M	11	190	930	530	497	78
forked^{1}	F	8	50	440	130	189	50
TMHS$^{\Delta 1}$	M	14	30	970	150	216	63
TMHS$^{\Delta 1}$	F	7	40	200	150	133	26
TMHS$^{\Delta 2}$	M	11	80	470	280	296	41
TMHSΔ2	F	11	30	300	80	111	26
TMHS^{P1}	M	8	30	600	340	296	80
TMHS^{P1}	F	8	30	340	40	79	37

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