Published in final edited form as:

Microsc Res Tech. 2004 April 15; 63(6): 388–399.

Anatomical and Molecular Design of the *Drosophila* Antenna as a Flagellar Auditory Organ

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Abstract

The molecular basis of hearing is less well understood than many other senses. However, recent studies in *Drosophila* have provided some important steps towards a molecular understanding of hearing. In this report, we summarize these findings and their implications on the relationship between hearing and touch. In *Drosophila*, hearing is accomplished by Johnston's Organ, a chordotonal organ containing over 150 scolopidia within the second antennal segment. We will discuss anatomical features of the antenna and how they contribute to the function of this flagellar auditory receptor. The effects of several mutants, identified through mutagenesis screens or as homologues of vertebrate auditory genes, will be summarized. Based on evidence gathered from these studies, we propose a speculative model for how the chordotonal organ might function.

Keywords

hearing; mechanosensation; Johnston's organ; chordotonal organ; axoneme; cilia

INTRODUCTION

Mechanosensation is intricately linked to an organism's survival. The "transduction of mechanical forces (stimuli) into a cellular electrochemical signal" (García-Añoveros and Corey, 1997) allows animals to interact with their environment as they feed, mate, and fend off prey. Research is beginning to shed light on some of the molecular mechanisms of mechanotransduction. The mechanosensory organs of the fly are easily accessible, thereby allowing mechanotransduction to be observed behaviorally and assayed electrophysiologically. Touch and hearing are two mechanotransduction processes that are now being characterized with the genetic tools available in *Drosophila*.

DROSOPHILA AUDITORY ORGAN

A pair of antennae is located on the front of the fly's head, between the eyes. Here they serve as near-field sound receptors that initiate transduction to convert a mechanical signal to an electrochemical response in the peripheral nervous system. The precise biomechanical movements in the antennal auditory mechanism have recently been described (Göpfert and Robert, 2001, 2002). The mechanisms of how acoustic vibrations are delivered to the sensory organs are extremely varied, but the sense organs for all insect auditory organs, whether flagellar or tympanal, are of the chordotonal type (Eberl, 1999).

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Contract grant sponsor: Whitehall Foundation; Contract grant sponsor: National Institutes of Health; Contract grant number: DC04848.

As near-field receptors, the antennae function best when within one wavelength of a sound stimulus (Bennet-Clark, 1971). Each antenna consists of a thin arista and three large proximal segments (a1–a3, also known as the scape, pedicel, and funiculus, respectively). The arista is the main receptor of sound and has recently been characterized as analogous to hairs and sensory bristles (He and Adler, 2001). In the presence of sound stimuli, bulk displacement of air particles cause the arista to vibrate, thereby transforming the sound energy into a mechanical signal. This signal activates the third antennal segment (a3), which then twists relative to the second segment (a2). A thin stalk connects the second and third segments (a2/a3 joint). The antennal nerve descending from the third segment is housed in this stalk. Johnston's Organ (JO), in a2, detects the sound signal and serves as the transducer of the sound stimulus.

Electron microscopy reveals over 150 chordotonal units (ch), or scolopidia, within JO. A schematic of the structure of the scolopodial units is shown in Figure 1. The chordotonal organs are Type I sensory neurons that are basally attached to the cuticle of the second antennal segment. All of the scolopidia in JO appear to insert tangentially on the articulating membrane so as to be stretched, and thus stimulated, when the arista is rotated posteriorly. The axons of the chordotonal neurons accumulate at the antennal nerve and project to the antennal mechanosensory region of the brain (Fig. 2).

Each chordotonal unit consists of two sensory neurons and support cells that form a unit known as the scolopidium. The main feature of the scolopidium is the scolopale space, a fluid-filled, isolated, spindle-shaped cavity, which encloses the ciliated dendrites of the neurons (Fig. 1). The space may be K^+ -rich in analogy to the environment observed in external sensory (es) organs (Küppers, 1974; Thurm and Küppers, 1980) and similar to the K^+ -rich scala media of the vertebrate cochlea (Corey and Hudspeth, 1979). The scolopale is produced internally by the scolopale cell (Moulins, 1976; Thurm, 1994; Uga and Kuwabara, 1965) and consists of bundles of actin microfilaments as well as scattered microtubules (our observations and Wolfrum, 1997). Scolopale cells in other insects, and probably in *Drosophila* as well, also contain tropomyosin and microtubule-associated protein 2 (Wolfrum, 1997). The scolopale encloses only the outer dendritic segment of the ciliated dendrite. This segment consists of an axoneme with the canonical $9 \times 2 + 0$ microtubule arrangement, as observed frequently in sensory cilia. An enlargement consisting of paracrystalline inclusions is observed at about three-fourths of the length of the cilium, distally (Fig. 3A). This is known as the ciliary dilation. More proximally, at the lower limit of the scolopale, the cilium transitions through the basal body (Fig. 3C) at the zone between the inner and outer dendritic segments, to form the ciliary rootlets that continue past the nucleus, often into the axon (Fig. 1). The scolopale is attached to the outer dendritic segment through septate junctions (Baumgartner et al., 1996; Carlson et al., 1997), sealing the scolopale space and maintaining its ion concentrations. As the ciliary structure crosses through the basal body, it often makes a sharp turn of approximately 60° (Fig. 3C and D). The two ciliary rootlets have been shown in other insects to contain centrin, a Ca^{2+} binding protein (Wolfrum, 1997).

Previous investigations of *Drosophila* chordotonal organs describe two neurons for each scolopidium. However, we report here that three-neuron scolopidia are not infrequent in wild-type flies (Fig. 3B). As can be observed in Figure 3B, cross-sections from the second antennal segments of wild-type flies show several scolopidia with three ciliary profiles. These organs appear to constitute approximately 10% of the scolopidia in JO. Their arrangement appears scattered throughout a2. Furthermore, it appears that one of the ciliary profiles in the triply innervated scolopidia does not have a clear axonemal arrangement, compared to the axonemal organization of the adjacent dendrites (Fig. 3B). We do not know if these scolopidia serve a different role from the ones consisting of two neurons, or if one of the ciliated dendrites serves a function different from the other two in each scolopidium. In any case, such morphologically

mixed scolopidia are common in other insects as well (Field and Matheson, 1998; Moulins, 1976).

Apically, the cilia are attached to the dendritic cap (Fig. 3B and E), an extracellular matrix produced by the scolopale cell (Keil, 1997b) and ultimately through the cap cell to points of attachment at the a2/a3 joint (Fig. 1). Mutations that lead to the detachment of the cilia from the dendritic cap, or of the cap cell from the cuticle, have been recovered and will be discussed below. Whether the chordotonal cilia in the *Drosophila* JO generate active movements has not been determined. However, ciliary movements have been reported previously in the chordotonal organs of the grasshopper (Moran et al., 1977). Furthermore, motile cilia lacking the central microtubule pair have been reported in the sperm flagella of horseshoe crabs (Ishijima et al., 1988) and eels (Gibbons et al., 1985; Wooley, 1997), as well as in the nodal area of developing mice (Nonaka et al., 1998). In addition, the finding of oto-acoustic emissions in insects, as we discussed earlier (Eberl, 1999), is also consistent with active ciliary movements. Finally, the non-linearity and spontaneous activity of the *Drosophila* antennal mechanics can be explained by active movements generated by JO neurons, most likely by ciliary activity (Göpfert and Robert, 2002, 2003).

Basally, the scolopidia are heavily anchored to the proximal a2 cuticle by ligaments (Fig. 3F), presumably assembled by the equivalent of ligament cells found in embryonic chordotonal organs. These ligaments insert into the cuticle via apodemes that resemble those of muscle attachments. Whether all basal attachments are of the same nature is not clear. We have seen some variations anecdotally, but have not studied this variation enough to be confident that it is real; this question clearly needs a more careful study. It is clear that ligaments can be stained with phalloidin, indicating that a significant component of ligaments is actin.

Within a2, scolopidia are organized radially, attaching apically to the a2/a3 joint (Figs. 1, 2, 3E; Eberl, 1999; Moulins, 1976; Uga and Kuwabara, 1965). As such, when the third antennal segment rotates due to particle displacement, the scolopidia are stretched and relaxed. This causes putative mechanosensitive gates, not yet isolated and whose position along the cilium is still unknown, to open and allow influx of positive ions from the scolopale space into the neuron, generating a receptor potential. This may lead to action potentials that are conducted by axons leaving JO and joining the antennal nerve.

The dendritic cilium may have one or more functions during transduction. First, it may serve as a rigid framework to propagate the physical stimulus from the a2/a3 joint to the ion channels. Second, it may act as a tension-generator, amplifying the propagation process by active contractile strokes throughout its length. Finally, it may serve as some sort of adjustable tensioning device to offset cuticular elasticity, and optimize the propagation process through the apical chordotonal attachments by keeping them taut at all times (see review by Eberl, 1999).

Electrophysiological Response to Acoustic Stimuli

Electrophysiological recordings are conducted to determine whether or not a fly can detect a sound stimulus at the level of the chordotonal organ. These sound-evoked compound potentials are recorded from the antennal nerve, by placing an electrode between a1 and a2 (Eberl et al., 2000). The antennal nerve, which includes axons from ch, es, and olfactory organs, passes through this joint. The sine and pulse components of the *Drosophila* courtship song are used in electrophysiological testing, as the sound stimuli. When the sine wave is played, at its normal song frequency of around 160 Hz (see Wheeler et al., 1988), wild-type flies produce a sustained response, with multiple peaks corresponding to each stimulus cycle (Eberl et al., 2000). When the pulse song is administered as the sound stimulus, the pulse pattern is reflected in the response (Eberl et al., 2000).

Flies mutant for *atonal* completely lack chordotonal organs (Eberl et al., 2000; Jarman et al., 1995). Therefore, these flies were used to identify the source of the electrophysiological signals. When presented with the sine and pulse songs, *atonal* flies produced no sound-evoked potential (Eberl et al., 2000). This result demonstrates that JO is the source of the sound-evoked potentials in wild-type flies. Based on the mechanics of the antennal vibrations, it is unlikely that other sensory cells lost in the *atonal* mutant, namely a subset of antennal olfactory sensilla (Gupta and Rodrigues, 1997), contribute to the sound-evoked potentials. The responses from wild-type and *atonal* flies are used as the standard when measuring the sound-evoked potentials of other mutants.

Genetic Approach to Hearing and Touch in *Drosophila*

Extensive work on the gentle body wall touch mechanotransduction system of *C. elegans* resulted in the genetic and molecular characterization of several transduction proteins involved in touch (Chalfie and Au, 1989, reviewed by García-Añoveros and Corey, 1997; Gillespie and Walker, 2001). In *Drosophila*, Kernan and colleagues undertook a genetic approach to identify components of the *Drosophila* mechanosensory transduction pathway (Kernan et al., 1994; Kernan and Zuker, 1995), with a focus on touch-sensitivity and bristle organ function. A later genetic screen based on hearing was conducted by Eberl et al. (1997) in *Drosophila*. A genetic screen was most favorable for these undertakings as it allows for easy identification of mutants with a phenotype, and has no prerequisites regarding expression level or pattern.

Since touch and hearing both involve the processing of mechanical stimuli into an electrochemical output with ciliated sense organs, it is possible that they act in a common pathway and utilize the same elements. This sharing of transduction components has been confirmed to a large extent, at least with the genes identified to date (Eberl et al., 2000). In this report, we will review the known genes from the touch and hearing screens, and those implicated from homology to vertebrate auditory genes, and we will examine whether their roles in the mechanotransduction systems are functionally similar.

Touch Mutants

Previous findings indicated that failure of the external mechanosensory organs (bristles) to develop results in reduced viability of adults (Kernan et al., 1994; Villares and Cabrera, 1987). Therefore, Kernan et al. screened for defects in larval mechanosensory behavior. This screen specifically targeted the X chromosome. The three genes associated with the most striking touch-insensitivity were mapped to 19E-20, on the X chromosome. Two were mutations in previously identified genes *uncoordinated* (*unc*) and *uncoordinated-like* (*uncl*) (Lifschytz and Falk, 1968; Perrimon et al., 1989; Schalet and Lefevre, 1976), while the third was in a new gene, *touch-insensitive larvae B* (*tilB*). Immunostaining of the entire embryonic nervous system with monoclonal antibodies indicated that the morphology of *unc*, *uncl*, and *tilB* are similar to wild-type animals (Kernan et al., 1994).

The bristle scratch reflex assay, along with electro-physiological recordings, were used to characterize the *unc*, *uncl*, and *tilB* mutants (Kernan et al., 1994). Mechanically evoked changes in the transepithelial potential (TEP) were measured to determine if the mutations affect bristle transduction. The TEP is the voltage difference between the apical and basal sides of the sensory epithelium characteristic of insect external sensory organs at rest. When the bristle is mechanically stimulated, a mechanoreceptor potential (MRP) is generated by cation flow from the receptor lymph into the neuron, creating a negative potential or depolarizing receptor current.

Since most of the isolated mutants are lethal as pharate adults, Kernan and colleagues created gynandromorph mosaics to show that while bristle morphology appears normal in the *unc* and

uncl mutant patches, these mutations act at the sense organ rather than the CNS, by reducing the MRP, while not affecting the TEP. On the other hand, *tilB* mutants showed normal bristle functions by these assays (Kernan et al., 1994).

Another screen targeting the second chromosome was designed with a pre-screen for pupal lethality (Kernan et al., 1994). Mutations in four complementation groups generated almost no MRP in most or all bristle groups and were named *no mechanoreceptor potential* mutants *nompA*, *nompB*, *nompC*, and *nompD*. A reduction in the MRP and TEP recordings was observed for a fifth locus, *remp* (*reduced mechanoreceptor potential*). Overall, these results indicate that mechanosensitive sense organs are disrupted by these mutations.

Mutations of Auditory Behavior

The *Drosophila* auditory system plays a significant role in courtship behavior. Through wing vibration, the male generates a courtship song with pulse song and sine song components, which are important for communicating species information and stimulating courtship (Hall, 1994). Eberl et al. (1997) undertook a genetic screen to identify components of auditory mechanosensation and auditory behavior. Because female receptivity is difficult to measure, making it prohibitive as an assay for mutagenesis, an alternate auditory response was used. Specifically, males will respond to the pulse song by initiating courtship directed at any nearby fly, resulting in chaining behavior among a group of males. Therefore, the mutagenesis screen targeting the second chromosome consisted of presenting male flies with the pulse component of the courtship song and selecting mutants with subnormal response. Fifteen mutant lines were recovered (Eberl et al., 1997).

To classify these mutants, a behavioral audiogram was measured. Because the frequency components of the song are species-specific signals, intensity gradients were used. Of the 15 lines, seven showed no response at any sound intensity, seven displayed a reduced response at all intensities, and one line (*5L3*) only responded at high sound intensities (Eberl et al., 1997).

To test which of these mutants affects sensory function rather than central or motor function, the sound-evoked potentials were measured (Eberl et al., 2000). One mutant line of particular interest, *5PI*, exhibited a strong reduction in the sound-evoked response to the pulse song stimulus; only a small response was elicited, primarily when the intensity of the stimulus was very high. Genetic and cytological mapping placed the *5PI* locus at polytene chromosome interval 36E1-3, and it was renamed *beethoven* (*btv*) (Eberl et al., 2000). Adult flies, heterozygous for chromosomal deletions that overlap at the *btv* gene, show reduced sound-evoked potentials similar to that of homozygous *btv*^{*5PI*} mutants, suggesting that this mutant may be a null. However, the deficiency combination has a male sterility phenotype in addition; we think that this is a separate gene function, but have not firmly ruled out the possibility that male sterility is related to the *btv* locus.

Similarities and Differences Between Touch and Hearing

Touch and hearing are mediated by external sensory organs and chordotonal organs, respectively. These are both Type I sensory organs, predicting some functional similarity. To test the degree of sharing of mechanosensory components between these two classes, the touch mutants were tested for auditory function (Eberl et al., 2000).

Most mutants in which bristle MRPs were reduced or absent showed corresponding reductions in sound-evoked potentials. Thus, there is extensive sharing of mechanotransduction machinery between touch and hearing. However, one prominent exception was *nompC*, which showed significant auditory function despite complete lack of MRPs (Eberl et al., 2000). In

the other direction, *tilB* and *biv*, which have strong effects on sound-evoked potentials, have normal bristle function (Eberl et al., 2000). Thus, while many components are shared, there are elements that function primarily in one class or the other of these Type I sense organs.

Molecules Required for Transduction in JO

As described above, a number of mutations affecting touch and hearing were identified by screens conducted by Kernan et al. (1994) and Eberl et al. (1997). Furthermore, functional genomic approaches have identified auditory genes in the fly by homology to vertebrate genes that affect hearing. Here we outline the results of new investigations regarding molecular mechanisms revealed by several of these mutations, and their relevance to hearing in the fly.

nompA is located on the second chromosome, at cytogenetic position 47F1-5 (Chung et al., 2001). *nompA* mutant bristles show TEP within normal range; however, these mutants do not respond to mechanical or auditory stimulation (Eberl et al., 2000; Kernan et al., 1994).

nompA encodes a protein predicted to have several distinct domains, including an N-terminal sequence, hydrophobic regions, ZP modules, tetrabasic motifs where cleavage occurs in the extracellular domain, and PAN modules (Chung et al., 2001). Bristle organs, like chordotonal organs, consist of three support cells and bipolar neurons. The distal segment of the sensory process, a modified cilium, is the probable site of transduction (Thurm, 1964). The support cells surround the modified cilium concentrically. The outer two cells construct the bristle socket and shaft, and they generate the K⁺-rich receptor lymph (Thurm and Küppers, 1980). The inner cell, the thecogen (homologous to the scolopale cell in scolopidia), is penetrated by the ciliated dendrite (Keil, 1997a). The cap is directly attached to external cuticular structures, unlike the cap in scolopidia, where a cap cell is also present.

In pharate adults, *nompA* is strongly expressed in JO, bristle organs, and other sensory organs (Chung et al., 2001). Immunostaining and in situ hybridization indicated that *nompA* transcription does not take place in the neuron, but in one of the support cells. In es organs it appears to take place in the thecogen cell, while in chordotonal organs it occurs in the scolopale cell. GFP-*nompA* fusion proteins were used to show that *nompA* is synthesized and released by the thecogen/scolopale cell (Chung et al., 2001). Bristle shaft and socket in the *nompA* mutants do not show morphological defects (Chung et al., 2001). Since TEP appears normal, the defect would not appear to disrupt the outer support cell function, which establishes this potential through active extrusion of K⁺ ions into the receptor lymph. Instead, neurons are detached from the cap in *nompA* bristle organs and campaniform organs (Chung et al., 2001). In JO, the scolopidial array is somewhat disorganized with extensive detachment from the a2/a3 joint (Chung et al., 2001). Individual scolopidia show normal structure, except that the dendritic cap frequently fails to enclose the tip of the dendritic cilia.

nompA is a component of the dendritic cap. In the mutants, the cap is still present, though its structure is somewhat disorganized. The ZP-domains may be responsible for the organizational properties of *nompA* because ZP domains are the putative filament-forming module of extracellular fibrillar matrices (Killick et al., 1995). Thus, defective *nompA* may lead to a disruption of signal propagation from the cuticle or bristle to the transducing portion of the sensory cilium. The identity of the ciliary component in the interaction between cilium and dendritic cap is not yet known (Chung et al., 2001).

Another mutation that affects both mechanosensation and audition is *nompB* (Eberl et al., 2000; Han and Kernan, 2001; Kernan et al., 1994). Bristle recordings from *nompB* flies show normal TEP, but no MRP (Kernan et al., 1994). Recordings from the antennal nerve also show that sound-evoked potentials are absent (Eberl et al., 2000). Recently, Han and Kernan (2001) reported that *nompB* encodes a homolog of the mouse *Tg737/orpk*, mutations in which lead to a variety of anomalies, including polycystic kidney disease as well as random left-right

asymmetries (Pazour et al., 2000). These are also homologues of the nematode *osm-5* and *Chlamydomonas* *IFT88* (Haycraft et al., 2001; Pazour et al., 2000), members of the intraflagellar transport complex that constructs and maintains cilia. Consistent with this, *nompB* mutations have malformed or missing ciliary outer dendritic segments in sense organs (Han and Kernan, 2001). Thus *nompB*, like *nompA*, disrupts the propagation of the mechanical signal, though here the defect is in the cilium rather than the dendritic cap.

Mutations in *nompC*, which maps to polytene chromosome position 25D7, also lead to the absence of bristle response to mechanical stimulation, as well as complete deafness (Eberl et al., 2000; Kernan et al., 1994). Recently, Walker et al. (2000) provided evidence that *nompC* encodes a transducing component, a mechanically-sensitive ion channel, in the *Drosophila* bristle organs. The NompC protein is predicted to have six transmembrane domains, similar to many other ion channels (Walker et al., 2000). Unlike other channels, however, it has an extremely long cytoplasmic N-terminal extension, which includes 29 ankyrin repeats that could mediate interaction with a variety of cellular components, perhaps for the purpose of anchoring the channel (Sedgwick and Smerdon, 1999; Walker et al., 2000).

nompC is expressed in ciliated sensory organs including bristles and chordotonal organs (Walker et al., 2000). The authors provide the following evidence that NompC functions as the primary, if not the only, transduction channel in bristle organs. First, there is sequence similarity between NompC and ion channels. Second, *nompC* expression appears restricted to mechanosensory organs. Third, *nompC* loss-of-function mutations eliminate almost completely the bristle response to mechanical stimulation. However, a residual gating current is observed, and may indicate the presence of another transduction channel member in the bristle organ. Fourth, a *nompC* mutation that changes residue 4820 from C to Y alters the behavior of the induced current without eliminating the response. Furthermore, since channels are expected to connect to the cytoskeleton, the presence of ankyrin repeats would presumably serve this function. The authors propose NompC may act either singly, or as a multimeric protein, tethered to the cytoskeleton and extracellular matrix (Walker et al., 2000).

NompC may also serve as a transduction channel in chordotonal organs. Studies by Eberl et al. (2000) indicate that *nompC* mutants show some deafness electro-physiologically. Compared to control flies, the response from *nompC* mutant flies is reduced by almost 50%, indicating that *nompC* serves a function in JO, but is not the only ion gate/subunit operating in chordotonal organs. This non-absolute requirement of NompC in auditory cells contrasts with the recent finding that a zebrafish NompC loss of function leads to loss of hair cell transduction (Sidi et al., 2003).

Recent evidence presented by Kim et al. (2003) implicates the Nanchung (Nan) protein as a primary transducer channel in chordotonal organs. Nan is an ion channel subunit similar to vanilloid-receptor-related (TRPV) channels of the TRP superfamily. One of the two predicted TRPV genes in *Drosophila*, CG5842, encodes Nan. The predicted sequence consists of 833 amino acids and displays the domain structure expected for the TRPV family. This includes five cytoplasmic ankyrin repeats preceded by six transmembrane domains and a pore region (Kim et al., 2003). Kim et al. (2003) found Nan expression specifically in chordotonal neurons, localized to the sensory cilia. Consistent with a role in JO, no sound-evoked potentials were observed in *nan* homozygous mutants, but they were restored by a *nan* cDNA transgene expressed from the *nan* promoter (Kim et al., 2003). The activation of Nan by hypo-osmotic stress suggests that this molecule may be sensitive to the membrane stretch stimulation believed to trigger sound transduction in JO.

Mutants of the *uncoordinated* (*unc*) gene, at position 19E on the X chromosome, show severe uncoordination due to loss of proprioception (Kernan et al., 1994). Moreover, a recent study

by Baker et al. (2001) indicates that *unc* males do not produce mature sperm. The *unc* gene encodes a coiled-coil protein, expressed in testes, localized in the centrioles of the spermatids, and basal bodies of ciliated sensory neurons (Baker et al., 2001). The sperm defects appear to stem from anomalous flagella in the testes; these are often disrupted with split axonemes, or just missing. The deafness in *unc* flies (Eberl et al., 2000) and proprioceptive defects causing severe uncoordination (Kernan et al., 1994) appear to stem from similar defects in the cilia of mechanosensory cells. Ultrastructurally, the axonemes of chordotonal organs are missing, split, or truncated. Thus *unc* is necessary for the organization and function of ciliogenic centrioles (Baker et al., 2001). Deafness may arise at least from non-propagation of the physical stimulus from the a2/a3 joint to the transduction channel, because of the absence or truncation of the cilium.

One mutant that affects hearing without affecting bristle MRPs is *beethoven* (*btv*). *btv* affects the integrity of chordotonal neurons; this is particularly obvious in the cilia, as determined through ultrastructural and electrophysiological techniques (Eberl et al., 2000). The auditory response recorded from the antennal nerve in *btv* flies is virtually lost. A residual response is observed only at very high sound intensities (Eberl et al., 2000). We have mapped *btv* to polytene chromosome region 36E1-3, where two major candidate genes are predicted from the sequence. One candidate is a dynein heavy chain gene, specifically the 1b isoform, which could lead to the observed ciliary abnormalities through defective intraflagellar transport (Cole et al., 1998; Pazour et al., 1999; Porter et al., 1999; Signor et al., 1999; Wicks et al., 2000). The other candidate is a new cadherin gene related to N-cadherin. The cadherin could affect neuronal structure by mediating important cell contacts required for assembly or maintenance of the neuron, or for generating or maintaining the specialized receptor lymph in the scolopale space.

The ciliary defects of *btv* together with the residual auditory response may suggest a possible role of the cilia in regulating tensile stretch in the scolopale, optimizing or facilitating the detection of the physical stimulus. Therefore, in the mutant, only when the physical stimulus is strong enough, as in high-intensity sounds, would the cilia bend enough to lead to an activation of the ion channels, and allow transduction to occur. In this case, at normal intensities, the cilia are not pulled taut enough to permit the propagation of the stimulus (Eberl, 1999). In the end, the axoneme and the ciliary dilation may play an active role in the transduction process, acting as more than just physical anchors of the ion channels to the cuticular elements.

Another mutation that affects hearing in the fly is touch-insensitive larva B (*tilB*). *tilB* maps to cytogenetic position 20A4. Similar to *btv*, *tilB* mutants affect only chordotonal organs, while leaving the bristle response intact (Eberl et al., 2000; Kernan et al., 1994). *tilB* flies are both deaf and male sterile. Ultrastructurally, *tilB* affects the sperm tail axonemes which appear to lack the inner and outer dynein arms and perhaps also the nexin bridges (Eberl et al., 2000). The effects of *tilB* on ciliary ultrastructure in scolopidia may be more subtle, though the sperm tail defect would strongly implicate the sensory cilia to be where *tilB* functions. Since the cilium still appears to be firmly attached to the dendritic cap, the lack of auditory response suggests that the cilium does more than passively transmit the physical signal.

Genes important for hearing in the fly have been discovered not only directly through genetic screens, but also through comparative genomics. The gene to be discussed in most detail here is a homolog of a well-known gene responsible for hereditary deafness in humans, mice, and zebrafish, namely myosin VIIA (Ernest et al., 2000; Gibson et al., 1995; Hasson, 1997; Keats and Corey, 1999; Petit, 2001; Weil et al., 1995). Myosin VIIA is an unconventional myosin; it is unable to assemble into filaments like myosin II (conventional myosin). Myosin VIIA, along with other unconventional myosins, is believed to act as an intracellular molecular motor, possibly carrying cargoes along actin filaments (Hasson, 1999; Mermall et al., 1998; Petit,

2001). Mutations in myosin VIIA are responsible for syndromic (Usher 1B) and non-syndromic (DFNB2, DFNA11) deafness in humans, *shaker1* in mice and *mariner* in zebrafish, associated with sensorineural deafness and balance anomalies (Ernest et al., 2000; Hasson, 1999; Liu et al., 1997a,b; Petit, 2001; Weil et al., 1995, 1997). Structurally, myosin VIIA is able to dimerize through a coiled-coil domain. The motor domain is situated on the amino-terminus. Distal from the amino-terminus are five light chain-binding domains, MyTH4, FERM, and SH3 domains, which might provide the protein with the ability to bind a number of other molecules and cargoes (Hasson, 1999; Petit, 2001).

Myosin VIIA is expressed by cells that contain microvillar or ciliary projections and the molecules are primarily concentrated along the projections themselves (Sahly et al., 1997; Wolfrum et al., 1998). In the vertebrate inner ear, myosin VIIA is localized exclusively in the sensory hair cells of the cochlea and vestibular apparatus (Ernest et al., 2000; Hasson et al., 1997; Self et al., 1998). Studies in mice defective for myosin VIIA indicate an important role for myosin VIIA in stereocilia arrangement. The stereocilia, rather than being organized in the usual V shape, are splayed out (Self et al., 1998). At least one function of myosin VIIA in the sensory cells of the inner ear is the organization of stereocilia. The exact role myosin VIIA plays in this aspect is not known, but it is suspected to act in cooperation with vezatin, an adherens junction molecule (see below), in establishing lateral links that bring together neighboring stereocilia, as well as affecting stereocilia anchoring (Küssel-Andermann et al., 2000; Petit, 2001). Mice exist with less severe specific mutations in myosin VIIA whose stereocilia do not appear disorganized (Self et al., 1998). Nevertheless, these mice still display some deafness (Self et al., 1998), indicating the role of myosin VIIA in more than just organization and development in the vertebrate cochlea. Corroborating this are recent findings by Kros et al. (2002) that myosin VIIA acts in transduction in inner ear hair cells by anchoring the transducing channels to the actin core of stereocilia. Without proper anchorage, tension on the channel gates is too slack; therefore, the mechanosensitive channels cannot respond to normal-range deflection of stereocilia (Kros et al., 2002). Furthermore, the adaptation properties of the channels are also affected in mice mutant for myosin VIIA (Kros et al., 2002). These conclusions are also supported by evidence from zebrafish studies (Ernest et al., 2000).

The *Drosophila* homologue of myosin VIIA is *crinkled* (*ck*). It is located in the second chromosome at polytene position 35C1. The fly homologue, 2,167 amino acids long (Ashburner et al., 1999; Chen et al., 1991), shows structure similar to the vertebrate myosin VIIA. Mutations in *crinkled* have been studied for their effects on wing prehair formation and elongation (Turner and Adler, 1998). *ck* mutations lead to branching and or bending, as well as slower prehair elongation (Turner and Adler, 1998; Winter et al., 2001). They also affect morphology of the bristle shafts in the wing margin (Turner and Adler, 1998).

Our ongoing investigations indicate that strong *ck* mutations completely block fly hearing as determined electrophysiologically. Preliminary ultrastructural analyses reveal no major morphological defects in the scolopidium structure. Occasionally, we have observed what appears to be incomplete encapsulation of the ciliary structures by the cap. However, we found strong effects on the overall organization of the scolopidial array inside a2, including frequent apical detachments from a2/a3 joint. Often adjacent scolopidia were arranged perpendicularly to one another, a phenomenon unobserved in wild-type flies. The detachment appears to take place at the level of the cap cell, rather than between the cilium and the dendritic cap.

There is evidence from studies in vertebrates where myosin VIIA has been implicated as a member of adherens junctions (Küssel-Andermann et al., 2000). The tail of myosin VIIA is able to interact with a molecule called vezatin, a ubiquitous protein of adherens junctions. Vezatin is able to interact with both myosin VIIA and cadherin-catenin complexes (Küssel-

Andermann et al., 2000). Moreover, Tuxworth et al. (2001) indicate that the sole myosin VII in *Dictyostelium* may play a role in mediating initial binding of cells to extracellular matrixes by organizing adhesion proteins in the plane of the membrane. In this case, myosin VII may not only be a component of the junction complex, but may also help establish it. We do not know whether scolopodial detachment results from an inability to initiate the attachment, or from a failure to maintain it. In conclusion, at least one reason that *ck* mutants lead to deafness appears to reside in the disorganization and detachment of the auditory units from external cuticular elements, similar to the role it plays in stereocilia arrangement in the vertebrate inner ear. However, aside from ensuring continuity of physical stimulus propagation, an active role for *Drosophila* myosin VIIA in transduction or adaptation is also possible, as in the vertebrate system.

The effects of myosin VIIA in fly sensory function are not limited to hearing. We find that the most severe mutants show not only gross morphological defects, as described previously (Turner and Adler, 1998), but functional defects in bristle organs as well. Bristle shafts are shorter, often bent, and do not show TEP (resting potential) in the normal range. The bristle MRP is also drastically reduced; this may be solely an effect of the reduced TEP, or an additional effect of myosin VIIA in the transduction process. The reduction or loss of TEP may be due to a passive leakage of the receptor lymph, and, in addition, myosin VIIA may also have an active involvement. It is curious that severe *ck* mutants show significant lethality at the embryonic stage, but the mechanism of this lethality is not yet understood (Gubb et al., 1984, Kiehart et al., 2004). Further experiments will be required to decipher the precise roles for myosin VIIA.

The final molecule to be discussed here is prestin. In mammals, prestin, an integral membrane protein thought to change its conformation in a voltage-dependent manner, mediates active mechanical amplification of sound stimuli, allowing the cochlea to achieve its frequency selectivity and sensitivity (Dallos and Fakler, 2002; Ludwig et al., 2001; Zheng et al., 2000). Prestin is expressed by the outer hair cells of the inner ear, and it is a member of the SLC26 solute carrier anion transport family (Zheng et al., 2000).

Recently Weber et al. (2003) used riboprobes generated from rat prestin cDNA for in situ hybridization to search for prestin homologues in JO of mosquitoes and *Drosophila*. Positive hybridization results in the auditory organs of these two insects led the investigators to search and identify, by using sequence alignment and gene cloning, prestin-homologous transcripts in non-mammalian vertebrates and insects. In *Drosophila*, the gene CG5485 encodes the prestin homologue. The authors showed the CG5485 transcript to be expressed within the second antennal segment, in the JO. The non-linear mechanics and motion generation by the *Drosophila* auditory system discussed above, together with the fact that vertebrate and *Drosophila* auditory sensorineural cells are developmentally specified by homologous genes, suggest that prestin-like proteins may contribute to an amplification mechanism in the auditory organs of non-mammalian vertebrates and insects as well (Weber et al., 2003).

SPECULATIONS

Model for Chordotonal Organ Function

As described above, recent progress on characterizing hearing and touch genes have now permitted a few brief snapshots of the molecular mechanisms of transduction in chordotonal organs. Based on these findings, we would like to indulge in some speculations by briefly sketching one possible model for a chordotonal mechanism that allows fly audition. In this “bow and string” model (Fig. 4), we propose four major forces. The central pair of opposing forces involves the scolopale rods and the cilium: the stiff scolopale rods, representing the bow, have a tendency to be straight (force 1 in Fig. 4), but they are bent into a curved configuration

by contraction of the cilium (force 2), which represents the string. Flanking this central pair of forces are another pair of opposing forces. The dendritic cap tends to pull (force 3) the apical end of the central pair toward the cuticle at the a2/a3 joint, while the basal ligaments tend to pull (force 4) the basal end of the central pair toward the basal cuticle. All these forces are linked in a dynamic mechanical system. During vibration, the dimensions of at least some of the structural components change and therefore some of the forces must change in magnitude.

Questions regarding two aspects for each of these forces must be considered. First, how is each of these forces generated? Second, what is the nature of the dynamic aspect of each force? Because all the apical ends of the radial array converge on a very small region of cuticle, the a2/a3 joint, this cuticle can move considerably. The joint is also shaped such that the force imposed on it from the twisting a3 allows it to flex. On the contrary, the basal attachments are spread across a large part of the a2 cuticle; thus the basal forces are distributed such that there will be little movement of basal cuticle.

Consider first the operation of the scolopale rods, which in the model correspond to the bow. These rods are large bundles of actin filaments with a few interspersed microtubules. The straightening force in the rods (force 1 in Fig. 4) is likely generated by sliding actin filaments. Alternatively, or in addition, the microtubules may also contribute to this force. This force could be rather static or it could be modulated during changes in the length of the entire scolopidium. We suggest that myosin VIIa is good candidate for generating, maintaining, or modulating this force by sliding actin filaments in the rods relative to each other, relative to membrane components, or relative to the interspersed microtubules. This would be consistent with our observation (and Wolfrum, 1997) that the scolopale rods contain scattered microtubules, and with the presence of tropomyosin and microtubule-associated protein 2 in scolopales (Wolfrum, 1997).

The cilium, which corresponds to the string in the model, is proposed to maintain some curvature in the scolopale rods by a contractile ciliary twist (force 2 in Fig. 4). This twisting would tend to shorten the cilium (as previously proposed by Crouau, 1980,1982,1997; Moran et al., 1977) and, because this is the sensory element, it is essential for it to be maintained taut. The twisting is often to such a degree as to leave a considerable bend in the cilium where it emerges from the basal body. The position of the mechanotransduction channels has remained elusive, but stretching the scolopidium could change the angle of this bend, a possibility that would predict the channels to reside at the base of the cilium and to be activated by a change in the angle. The Nan channel is localized along the length of the cilium, from the basal bodies to the ciliary dilation (Kim et al., 2003). Neuronal activation, by influx of cations from the K⁺-rich scolopale space to produce the transduction current, could also alter the contractile force exerted by the cilium, a mechanism that could either expedite a return to the resting condition or amplify the mechanical activation of the transduction channels.

The apical force (force 3 in Fig. 4) may result primarily from elasticity in the cuticle near the joint and perhaps elasticity in the matrix of the dendritic cap. The elasticity in this apical force could be entirely passive, but there may be some modulation in its tensile properties because the cap cell also contains scolopale-like rods and numerous microtubules. A microtubule motor such as dynein or kinesin, or an actin motor such as an unconventional myosin would be required to mediate such modulation.

Similar arguments can pertain to the basal force (force 4 in Fig. 4) mediated by the actin-containing ligaments. The anatomical nature of these attachments is not yet well understood. They may be static elements or elastic elements. Alternatively, they may be actively modulated, perhaps by an actin-based mechanism. If the apical or basal forces are actively modulated, it

is most likely done to bring the force toward an optimum (resting) level. Depending on the temporal properties of these modulations, they may have the potential to mediate adaptation.

Clearly this bow and string model is highly speculative and will require many kinds of experimentation to confirm or discard. However, it does allow formulation of predictions, in particular specific predictions on how the mutations in different genes should behave under laser vibrometry analysis, or where different gene products should be found.

Possible Roles of Triply Innervated Scolopidia

Previous descriptions of Johnston's organ in *Drosophila* have always maintained that there are two neurons per scolopidium based on Uga and Kuwabara's (1965) study. We have described here our finding that a significant subset of scolopidia have three neurons, a finding independently seen by Baker and Kernan (personal communication). Many other insects commonly have triply innervated scolopidia in JO (summarized by Moulins, 1976).

Triply innervated scolopidia may serve one or more of several possible functions in a2. First, they may act as detectors of specific sound frequencies, either higher or lower than the rest of JO. This could allow for some type of frequency discrimination. Alternatively, they may provide a tonic feedback for flight control, a function that has been ascribed to JO in other insects (summarized by McIver, 1985). A third possibility is suggested by the recent findings from the Beckingham lab for mutants with altered gravitaxic responses in *Drosophila*. The second antennal segment is indicated as a site of gene action for many of these mutants. In particular, a subset of the JO neurons appear to be specifically affected in one gravitaxic mutant named *yuri gagarin* (Armstrong et al., unpublished data). This leads to the intriguing possibility that the JO could be a gravity detector, with the triply innervated neurons perhaps providing tonic feedback when the orientation of the fly relative to gravity is altered. For each of these possible functions, one could hypothesize that this separate function is mediated specifically by the constitution of triple units (that is, it would be a scolopidial function), or alternatively, that the third, unusual, neuron in triply innervated scolopidia mediates the separate function independently from its two sister neurons.

Extensive studies need to be conducted to distinguish all these possibilities. First, the identity of the second order neurons contacted by these chordotonal organs needs to be established. It may be that the axons all terminate in the same brain structure. However, it may be that some of the axons from the newly identified chordotonal organs may terminate in CNS regions different from the other ones. Second, mutants affecting gravity sensation in the fly need to be correlated through electrophysiological, molecular, and imaging studies to specific neurons in JO.

Other future studies will need to address issues such as further identification and isolation of the mechano-sensitive gates, their position, the possibility of active contractions of the ciliated dendrite, as well the role of the ciliary dilation in the transduction process. Moreover, the identification and isolation of other molecules involved in positioning the scolopidia in the radial array and their attachment to the stalk and basal antennal segment need to be pursued.

Acknowledgements

We are grateful to Dean Abel for electron microscopy, to the Central Microscopy Research Facility at the University of Iowa for use of the confocal microscope, and to the Developmental Studies Hybridoma Bank at the University of Iowa for monoclonal antibody 22C10. We thank many people for stimulating discussions, including Kate Beckingham, Grace Boekhoff-Falk, Martin Göpfert, Maurice Kernan, Dan Kiehart, Beth Raff, Krishanu Ray, Daniel Robert, and members of our lab.

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ANATOMY AND MOLECULES IN *DROSOPHILA* HEARING **Fig. 1.**

Drawing of chordotonal structure in Johnston's organ. Chordotonal organs consist of two or three neurons and a number of support cells. The ciliated dendrite is divided into an inner and outer segment. The inner segment lies outside of the scolopale space. The outer dendritic segment is ensheathed by the K^+ -rich scolopale space. Apically, the dendritic cap encapsulates the sensory cilia. It is the cap and the cap cell that attach the scolopale to the external cuticle. Below the basal body, the axonemal arrangement of the cilium changes into a striated structure, the ciliary rootlet. This travels deep into the soma, sometimes passing the nucleus and entering the axon. The cellular composition of the scolopidia is not entirely clear. Uga and Kuwabara (1965) describe an envelope cell in addition to a scolopale cell, but the existence of the envelope cell has not been verified. Furthermore, the basal attachments are likely mediated by the equivalent of ligament cells in embryonic ch organs. Highly invaginated membranes near these basal attachments (see Fig. 3F) hampers interpretation of cell composition. (Drawing not to scale.)

Fig. 2. Fluorescent image of Johnston's organ. An adult antenna was stained with rhodamine-conjugated phalloidin (magenta) to label actin filaments and monoclonal antibody 22C10 (green) to stain neurons. The scolopale rods, consisting of actin filaments, are clearly delineated by phalloidin. The neuronal cell bodies are observed basally (arrow), closely positioned to the second antennal segment cuticle. Axons leaving the second segment, bypassing the antennal elevator muscle in the first segment en route to the CNS, are indicated by the block arrow.

Fig. 3.

Ultrastructure of Johnston's organ. **A:** Cross-section of a scolopidium shows the profile of the ciliary dilation (arrow). Unlike the rest of the cilium, here the axonemal arrangement is absent. Instead, a parallel tubular arrangement of what may be paracrystalline elements is evident. The role of the ciliary dilation remains unknown. Surrounding the scolopale space are scolopale rods (block arrow). **B:** This cross-section shows the presence of three ciliary profiles inside the scolopale space in each of several scolopidia. While two of the cilia show a clear axonemal arrangement, the third one shows a more disorganized arrangement of microtubular elements (arrows). The block arrow indicates the profile of a cap structure. **C,D:** Immediately after emerging from the basal body, the cilia are often observed to make a wide turn to create a ciliary angle, which is observed in both panels (arrows). The lower scolopidium in C is another view of the ciliary angle; the section shows both a longitudinal and cross-section of the same cilium, which can take place only if a wide angle is present (block arrow). D is a higher magnification of the region boxed in C. **E:** An oblique section through the apical region of some scolopidia. The dendritic caps (block arrows) taper down to fine cuticular strands that insert (arrows) at the a2/a3 joint. The strands have been severed by the oblique section. **F:** The basal attachments feature large ligaments surrounding the neuronal cell bodies (n) and inserting into the basal cuticle via apodemes (arrow). A bundle of axons (ax) projecting toward the antennal nerve is visible.

Fig. 4.

The “bow and string” model. A central pair of opposing forces, namely the scolopale rods representing the bow (force 1) and the contracting cilium representing the string (force 2), maintain tension against one another. The apical attachment (force 3) propagates the vibrations from the a2/a3 joint to the tips of the cilia, while the basal attachments (force 4) anchor the neuronal cell bodies to the cuticle. These 4 forces vary dynamically during the vibrations, and serve to keep the entire system taut. The extent to which the components passively comply to length changes or actively modulate their lengths (and thereby, the tensile force they confer to the system) is unknown. See text for speculations on these processes.