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The Mechanoreceptive Origin of Insect Tympanal Organs: A Comparative Study of Similar Nerves in Tympanate and Atympanate Moths

J.E. YACK AND J.H. FULLARD
Department of Zoology, Erindale College, University of Toronto, Mississauga, Ontario, Canada, L5L 1C6

ABSTRACT

A chordotonal organ occurring in the posterior metathorax of an atympanate moth, *Actias luna* (L.) (Bombycoidea: Saturniidae), appears to be homologous to the tympanal organ of the noctuoid moth. The peripheral anatomy of the metathoracic nerve branch, IIIN1b1 was examined in *Actias luna* with cobalt-lysine and Janus Green B, and compared to its counterpart, IIIN1b (the tympanal branch), in *Feltia heralis* (Grt.) (Noctuoidea: Noctuidae). The peripheral projections of IIIN1b1 were found to be similar in both species, dividing into three branches, the second (IIIN1b1b) ending as a chordotonal organ. The atympanate organ possesses three sensory cell bodies and three scolopales, and is anchored peripherally via an attachment strand to the undifferentiated membranous region underlying the hindwing alula, which corresponds to the tympanal region of the noctuoid metathorax. Extracellular recordings of the IIIN1b1 nerve in *Actias luna* revealed a large spontaneously active unit which fired in a regular pattern (corresponding to the noctuoid B cell) and smaller units (corresponding to the noctuoid acoustic A cells) which responded phasically to low frequency sounds (2 kHz) played at high intensities (83–96 dB, SPL) and also responded phasically to raising and lowering movements of the hindwing.

We suggest that the chordotonal organ in *Actias luna* represents the evolutionary prototype to the noctuoid tympanal organ, and that it acts as a proprioceptor monitoring hindwing movements. This system, in its simplicity (consisting of only a few neurons) could be a useful model for examining the changes to the nervous system (both central and peripheral) that accompanied the evolutionary development of insect tympanal organs.

Key words: auditory, evolution, chordotonal organ, homology, Lepidoptera

Tympanal organs (ears) are known to occur in at least five insect orders and have evolved independently in different taxa within the same order. In the Orthoptera, ears are found on the first abdominal segment (Acrididae) or on the tibiae of the forelegs (Ensifera); in the Neuroptera, on the base of the wing (Lacewings: Chrysopidae); in the Hemiptera, on the second abdominal segment (Cicadidae), or on the meso and metathorax (Corixidae and Notonectidae); and in the Lepidoptera, on the metathorax (Noctuoidea), the first abdominal segment (Geometridae and Pyralidae), or the second abdominal segment (Uraniidae) (see Michelson and Larsen, '85, for review). Most recently tympanal organs have been identified on the ventral surface of the thorax in some Dictyoptera (Mantidae) (Yager and Hoy, '86, '87). The different types of tympanal organs range widely in complexity: from that of the Notodontid moth, which has only one auditory receptor cell (Eggers, '19; Surlykke, '84) to that of the cicada with more than one thousand (Michel, '75; Young and Hill, '77).

Despite such variations in distribution and complexity, all tympanal organs are fundamentally alike: they are characterized by the presence of a thinned region of exoskeleton (the tympanic membrane), exposed on one side to the external air, and having associated with its inner surface, a closely appressed air sac [or a fluid filled sac, as in lacewings (Miller, '70) and a genus of waterbug (Arntz, 1975)] and a chordotonal organ (CO).

Several authors have speculated that the primitive, undifferentiated form of the insect tympanal CO was a proprioceptor, which detected the displacement of one part of the

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exoskeleton with respect to another (von Kennel and Eggers, '33; Pumphrey, '40; Haskell, '61; Dethier, '63; Roeder, '67; Elsner and Popov, '78). This theory is feasible for two reasons: First, proprioceptive chordotonal organs and tympanal organs share both structural and physiological similarities. Briefly, all CO's consist of one or more structural units called scolopidia. Each scolopidium consists of one or more sensory neurones (bipolar Type 1), a scolopale cell, and an attachment (or cap) cell. The cells are arranged in a linear manner, the sensory cell body at one end and the attachment cell at the other, with the scolopale and scolopale cap in between enveloping the distal tip of the neurone's dendrite. Typically, both the cap cell and the axonal end of the receptor are anchored, either directly or indirectly to two points of the body wall. Proprioceptive CO's are widely dispersed throughout the body cavity, often occurring at the articulation points of appendages, or spanning between the intersegmental membranes. In the insect ear, the CO attaches at one end (generally the end of the attachment cells) onto the tympanic membrane or to the trachea associated with the membrane, while the other end attaches to the body wall (see Howse, '68; Finlayson, '76; and Moulins, '76 for reviews of CO's).

Both proprioceptors and auditory receptors are mechanoreceptors which respond to mechanical deformation of the cuticle: Proprioceptors respond to changes in tension caused by movements of the body (e.g., Burke, '54; Finlayson and Lowenstein, '58; Young, '70; Burns, '74; Orchard, '75) while acoustically transmitted vibrations of the tympanic membrane elicit a response in the auditory receptor cells associated with the membrane (see Michelson and Larson, '85). Indeed, several cases have indicated the absence of a clear distinction between the "proprioceptive" and "acoustic" stimulus: Chordotonal receptors which appear to be normally adapted for detecting proprioceptive stimuli have been shown to respond to substrate or airborne vibrations (e.g., Hughes, '52; Kehler et al., '70; Orchard, '75). Similarly, tympanal organs have been shown to respond to touch, or movements of the body (e.g., Roeder and Treat, '57; Ball and Hill, '78; Hedwig et al., '88; Schildberger et al., '88; Hedwig, '88, '89).

The second reason why the proprioceptive theory is feasible is related to the concept of neural parsimony (Dethier, '63), which describes the conservative design of insect nervous systems. It appears that evolutionary changes in neuronal architecture are few compared to changes that take place in peripheral structures, and that pre-existing neural elements are modified to serve new functions. This correspondence of neural elements in insects is seen within the same species, as in serially homologous neurones (Wilson and Hoyle, '78; Robertson et al., '82; Davis, '83; Pearson et al., '85) and between taxonomic groups (Goodman, '76; Goodman and Williams, '76; Altman and Tyrer, '77; Bacon, '80; Wilson et al., '82; Arbas, '83; King and Valentino, '83).

Considering these points, therefore, the chordotonal proprioceptor might be considered a good "candidate" to become a tympanal receptor, as the progressive evolution of the tympanal organ may have involved the reduction of an area of cuticle to which a proprioceptive sensillum was attached, to the extreme thinness of a tympanic membrane.

At present, there is no direct evidence concerning the evolutionary origin of insect tympanal organs. Paleontological evidence is of little assistance since neural tissue does not leave fossil records, and developmental studies related to this subject have been few (Young and Ball, '74; Ball and Hill, '78; Reichert and Meier, '88). In the absence of these methods, an examination of evolutionary trends can be made indirectly through comparative studies of extant forms (e.g., Edwards and Reddy, '86).

Moths offer a special opportunity to examine the changes to the nervous system which accompanied the evolutionary development of the tympanum. Moths of the superfamily Noctuoidea possess a pair of metathoracic tympana (Fig. 1). These ears are unique compared to those of other insects in their simplicity. Each tympanal organ has only two auditory sensillae [cells A_1 and A_2 (Eggers, '19; Roeder and Treat, '57; Ghiradella, '71) with the exception of the Notodontid family which has only one A cell (Eggers, '19; Surlykke, '84)] in addition to a larger, multipolar neuron (the B cell) which is spontaneously active and non-auditory (Roeder and Treat, '57; Treat and Roeder, '59). In all other Lepidopterans, a metathoracic tympanal organ is absent (Eggers, '19). We suggest that moths which lack a metathoracic tympanal organ possess neural homologies to the auditory sensillae of the noctuid moth, and that these homologies represent the ancestral form.

In the present study we describe some anatomical and physiological characteristics of the tympanal nerve counterpart (IIIN1b) in an atympanate moth, *Actias luna* (L.) (Bombycoidea: Saturniidae).

MATERIALS AND METHODS

General

A. luna were obtained as pupae during the winter months of 1988 and 1989. Tympanate noctuoid moths, *Feltia heralis* (Grt.) (Noctuidae) that were used for comparative morphological purposes were provided by the Agriculture Canada Research Branch in Lethbridge, Alberta. *Halysi-*

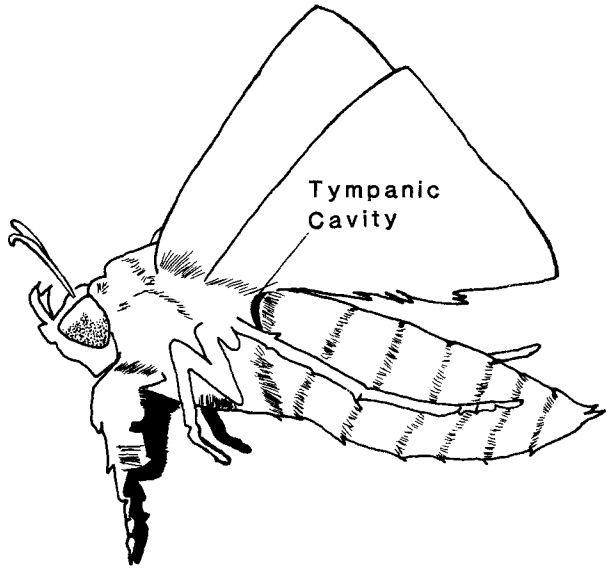


Fig. 1. Lateral view of a typical noctuoid moth, indicating the location of the exterior opening to the tympanic cavity. The tympanic membrane is situated deeply within the cavity, set in the skeletal framework of the posterior metathorax, ventral to the hindwing alula.

dota tessellaris[1] (J.E. Sm.) (Arctiidae), a different species of noctuoid moth used for extracellular nerve recordings, was reared from wild captured eggs. All pupae were stored at 5°C for 1 to 3 months and allowed to emerge in an environmental chamber maintained at 28°C and on a 12:12 light cycle as the adults were needed. Both male and female moths were used, as there were no apparent differences between the two in either tympanate or atympanate species with respect to the characteristics examined.

The thoracic nervous system was exposed using a modified dorsal approach (cf. Roeder, '66; Fullard, '84) for both anatomical and neurophysiological experiments. The head and legs were amputated and the unanaesthetized moth was mounted, dorsal side up, on a block of modeling clay into which had been impressed a narrow, shallow groove to accommodate the body. The wings and abdomen were secured into position with wire staples. With a camel-hair brush the dorsal thoracic scales were brushed off in order to facilitate the dissection. A shallow cut was made around the perimeter of the mesonotum (scutum and scutellum), followed by two more shallow cuts made on each side just medial to the forewing alula. Having made these incisions, the mesonotum, along with the attached flight musculature and alimentary canal, was lifted out, exposing the thoracic ganglia and their nerve branches.

Anatomy

The thoracic nerve IIIN1b (tympanal branch of noctuoids) was followed out to its peripheral innervation sites in 36 *A. luna* and 15 *F. heralis*. Following the standard dorsal dissection, the thorax was sectioned in a sagittal plane (but leaving the ganglia intact on one side) and one-half was pinned to Sylgard (Dow Corning Corporation, Midland, MI) in a petri dish. To the fresh preparation, a few drops of 0.5–1% Janus Green B (Miller, '79) were applied and replaced after 2 to 3 minutes with saline (Paul, '74). More stain was applied throughout the dissection as nerve branches were exposed. The branches of IIIN1b were traced as closely as possible to their target muscles or tissues and drawn with the aid of a Wild (M5) dissecting microscope and camera lucida attachment. Photographs were taken using a Leitz Laborlux K compound microscope with a Wild MPS 05 camera attachment.

Anatomical nomenclature used for muscles and nerves follows that of Nüesch ('53, '57). The musculature was identified according to the Nüesch scheme of classification, which is based upon position and skeletal insertion points. Nomenclature relating to tympanal structures was derived from Eggers ('19). Structural details of the metathorax have been introduced only as they relate to the branches of the metathoracic IIIN1 nerve.

The discovery of previously undescribed fine branches of the penultimate branch in both the noctuid and saturniid moths, necessitated that their peripheral projections be stained in vivo using cobalt-lysine (Gallyas et al., '78; Arbas, personal communication). Moths were dissected in the manner previously described to expose the thoracic nervous system. The IIIN1b branch was severed from the main nerve and its cut end draped over the rim of vaseline into 10–15% cobaltic lysine. The boat and any exposed tissue was carefully covered over with petroleum jelly in order to prevent dehydration. The moth then was placed in a partially sealed container (lined with moistened paper towels in order to maintain a high humidity) and placed in the refrigerator at 5–7°C for 12–180 hours (see Results). Following this period, only moths still alive (i.e., showing signs of life after being brought back to room temperature) were processed so as to minimize the possibility of artifactual results due to the leakage of cobalt from the nerves into surrounding tissues. After the petroleum jelly, the boat, and the cobalt-lysine were removed, the thoracic cavity was rinsed well with saline. The IIIN1b nerve, along with that half of the metathorax, was then dissected out, the cobalt precipitated with ammonium sulphide, and intensified (Davis, '82). The preparations were cleared and stored in methyl salicylate until further dissection was performed in order to isolate individual neural elements for examination. Permanent mounts were then made in Canada Balsam and photographed.

For scanning micrographs of the noctuid tympanal region in *F. heralis* and the hindwing area in *A. luna*, specimens were critical point dried, secured with silver paint, then sputter-coated with gold-palladium. Observations and photographs of the noctuid tympanic membrane and saturniid hindwing alula area were made with a Cambridge S 180 scanning electron microscope.

Extracellular physiological recordings

Modifications of techniques described by Fullard ('84) were used for recording units from IIIN1b. The nerve was exposed using the dorsal approach, and an insulated stainless steel hook electrode was used as the recording electrode. The IIIN1b branch was hooked onto the electrode and drawn up slightly out of the saline. We then routinely deefferented the IIIN1 nerve, by severing it at as it joins the ganglion, in order to ensure that we would record only centripedally conducted impulses. A tapered stainless steel wire was inserted into the exposed thoracic muscle as a reference electrode. Once the electrodes were situated, saline was drawn out of the cavity and replaced with petroleum jelly applied with a syringe.

Extracellular impulses were amplified with a Grass Instruments P15 preamplifier and monitored with a Tetronix storage oscilloscope and an audio speaker. Spikes were recorded onto a Hewlett-Packard 3968A Instrumentation Recorder. All neurophysiological preparations were situated inside a Faraday cage open to one side and lined with 4 cm of sound-attenuating foam.

Acoustic stimuli were generated by an Exact VCF/sweep 126 Signal Generator and amplified by an Epitek 1270 amplifier. Low frequency (500 Hz to 20 kHz) sounds were produced by a low frequency audiospeaker. High frequency (20–100 kHz) sounds were produced by a high frequency Panasonic (EAS-10TH 400B) speaker. Stimulus frequencies were monitored with a Non-linear Systems FM-7 frequency counter. The speaker was placed 1 m away from the preparation and mounted onto a cardboard platform outside the Faraday cage ipsilateral to the recording electrode. In an attempt to isolate vibration, the cardboard platform was not in contact with the cage or the preparation. Sound pressure levels were simultaneously measured with a 0.5 in (1.35 cm) Brüel and Kjaer microphone coupled

[1] *H. tessellaris* was a convenient species to use at the time physiological recordings were performed. No apparent differences exist in either the morphology or physiology of the tympanal nerve between the Noctuoid subfamilies Noctuidae and Arctiidae (Eggers, '19; Roeder, '74), although slight differences do exist in the skeletal structures surrounding the tympanum (Forbes, '16; Eggers, '19; Richards, '32).

to a Brüel and Kjaer Type 2203 sound level meter which was positioned directly beside the preparation [(all dB's reported are SPL) (r.m.s. re 20 μPa.)]. Sensitivity to both high and low frequency sounds was tested in both tympanate and atympanate moths.

Auditory threshold curves (audiograms) for *A. luna* were determined by playing continual sinusoidal sounds at frequencies between 500 Hz and 20 kHz at 500 Hz increments. Threshold curves for *H. tessellaris* were determined by playing frequencies from 5 to 100 kHz in 5 kHz increments. The stimulus intensity for each test frequency (frequencies were delivered either randomly or alternated from low to high frequency) was increased until the first detectable neural response was attained.

To determine if the sensory units were responsive to hindwing movements, the left wings were positioned so that their movement was not restricted by the modelling clay and they could be moved freely. A small protractor was embedded into the modelling clay anterior to the base of the hindwing so that the angle of wing elevation could be recorded. Following recording of spontaneous activity, the wing was artificially elevated from 0° through to 90° using a glass pipette held by a micromanipulator and then allowed to fall back to its horizontal position.

RESULTS

Branching patterns of the IIIN1b nerve were determined using both Janus Green B, and cobalt-lysine retrograde fills. Janus Green B allowed us to identify chordotonal organs by heavily staining the caps and lightly staining the scolopales of the scolopale cells. The cobalt fills provided us with information on the number of axons leading into the CO and some details of the sensory receptor cells. Thus, using both methods, we were able to derive information concerning the number of scolopalial units associated with each sensory neuron.

Due to the long filling times required to fill small branches in *A. luna* out to the periphery (24–180 hours at 5–7°C) with cobalt-lysine, only moths which had recently eclosed could be used, and of these, only a small percentage resulted in complete fills (determined by the presence of peripheral cell bodies): Of 26 fills we obtained 7 complete fills, 10 partial fills, and 9 were unsuccessful. Noctuid moth filling times were much shorter (12–68 hours), and a higher percentage of complete fills were obtained: Of the 13 fills, we obtained 6 complete fills, 5 partial fills, and 2 were unsuccessful.

IIIN1 branches of *A. luna* and *F. heralis*

The peripheral projection patterns of the IIIN1 nerve with respect to musculature and peripheral structures are basically similar in both the tympanate and atympanate moth. The origin of the main nerve, IIIN1, is on the lateral surface of the pterothoracic ganglion posterior to the junction of the meso and metathoracic components (Fig. 2a). The nerve follows a lateral and posterior course and sends its first branch (IIIN1a) to the lateral intersegmental muscle (IIis). The main peripheral nerve trunk (IIIN1c) runs dorsally and laterally between the meso- and metathoracic segments, where it innervates the base of the hindwing. The second branch (IIIN1b) is posteriorly directed, running across the medial surface of the metathoracic dorsoventral flight muscles (dv 1–5), at which point it bifurcates. The larger of the two branches continues posteriorly and divides into branches which innervate the muscles dI2, and dI1a, and b (Fig. 2b,c). The smaller of the two branches (which we named IIIN1b1) disappears between dl2 and dv1–5, where it continues toward the periphery (and to the tympanum in the noctuid). In a comparison of the thoracic musculature associated with the IIIN1b nerve in both *F. heralis* and *A. luna*, only minor differences were encountered. In *F. heralis*, dv2 lies lateral to dv1 instead of posterior to it as is seen in the *A. luna*. Also, dv4 and 5 appear as one muscle and are not obviously subdivided as they are in *A. luna*. In agreement with Treat ('59), who compared the thoracic musculature of the noctuid *Crymodes devastator*, with that of Nüesch's ('53) examination of the thoracic musculature in *Telea polyphemus* (Cr.), we found that all muscles of *F. heralis* could be homologized to those in *A. luna*. Because the branches of the IIIN1 nerve follow consistent courses in both the noctuid and saturniid, we propose that the IIIN1b1 branch of the saturniid is homologous to the tympanic branch of the noctuid. The remainder of this description will focus on the peripheral destination of the IIIN1b1 nerve.

Along its course towards the periphery in both tympanate and atympanate moths, the IIIN1b1 nerve divides into 3 branches (Figs. 3, 4). The first branch (IIIN1b1a) runs dorsally between the dorsoventral and dorsolongitudinal musculature, ending beneath the dorsal rim of the metathoracic scutum. The main branch (IIIN1b1c) continues posteriorly and then medially behind dv2, where it progresses dorsally and branches extensively directly beneath the cuticle of the metathoracic scutellum. This nerve was examined in several cobalt-lysine and Janus Green preparations, and no cell bodies were detected. We suspect that this branch innervates the third dorsolongitudinal muscle (dl 3), described by Nüesch ('53) as a very thin flat muscle lying directly beneath and contiguous with the hypodermis of the scutellum. The following will provide a detailed description of the second branch of IIIN1b1 (IIIN1b1b).

The penultimate branch and tympanum of the noctuid

The course of the second branch of IIIN1b1 [described by Paul ('73) as the penultimate branch, which contains only those fibers leaving the tympanic cavity] has been described in detail by other authors (e.g., Eggers, '19; Roeder and Treat, '57; Treat, '59; Ghiradella, '71) and will only be outlined briefly here for comparative purposes. This branch passes through an expanded air-filled cavity encased in the tracheal epithelium of the tympanic air sac which leads to the tympanic membrane. Before reaching the tympanum, it briefly runs along an apodemal infold [known as the Bügel (Eggers, '19)] ventral to the hindwing alula which comprises part of the skeletal framework of the tympanic membrane. At this attachment site a large multipolar neuron occurs (the B cell). The nerve continues towards the tympanic membrane, ending in two bipolar (Type I) sensory cells (A_1 and A_2), each of which is associated with a scolopalial unit. The CO attaches to the inner surface of the tympanic membrane and is suspended by a short ligament extending dorsally from the scutum.

The tympanum itself (Fig. 3) faces posteriorly and sits in a cleft covered by the abdominal hood which consists of a fold of the first abdominal pleuron. The tympanic membrane is separated from another lateral membrane (the conjunctiva) by a strip of thick cuticle, the nodular sclerite or epaulette (Eggers, '19). Both membranes are roofed by

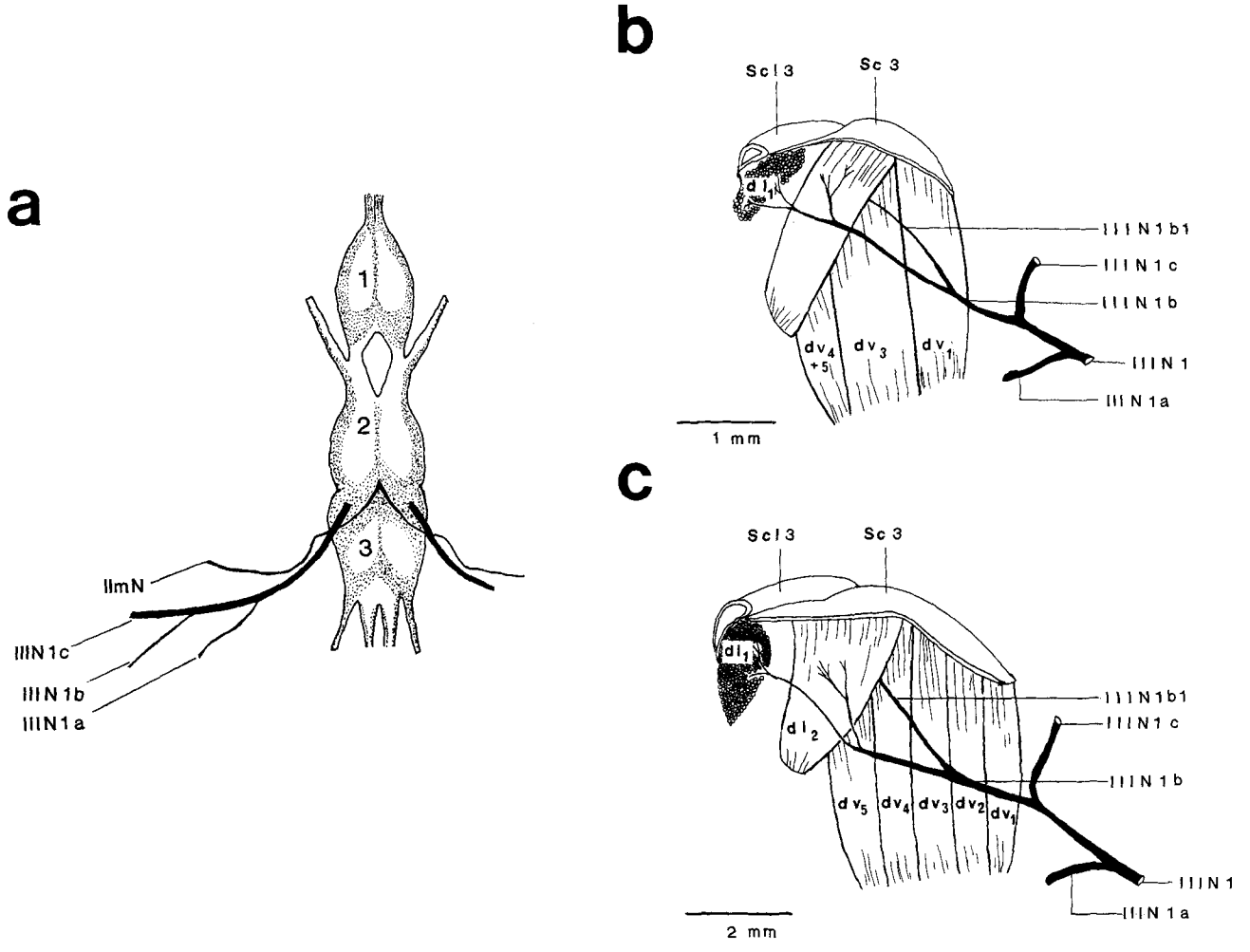


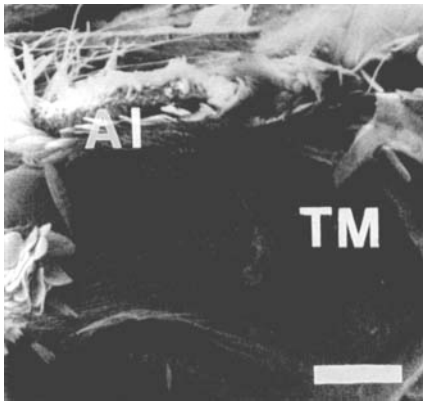
Fig. 2. **a:** Dorsal view of the 1) pro-, 2) meso-, and 3) metathoracic ganglia of a moth (representative of both the tympanate noctuid and atympanate saturniid) indicating the position of peripheral nerve roots IImN and IIIN1, and further illustrating the main branches of the latter; IIIN1a, b, and c. IIIN1b contains the tympanic sensilla in the noctuid moth. **b:** Left lateral metathorax of the noctuid (*F. heralis*), viewed from the midline showing branches of the IIIN1 nerve. IIIN1a eventually innervates the intersegmental muscle (IIis) and IIIN1c continues laterally to supply the base of the hindwing. The first branch of IIIN1b (IIIN1b1) follows a lateral and posterior course between a dorsoventral muscle (dv3) and a dorsolongitudinal muscle (dl2) peripherally towards the tympanic membrane (not shown). The main branch of IIIN1b supplies the dl1 and dl2 muscles. The dorsoventral muscle dv2 is not shown, as it lies lateral to dv1 in the noctuid moth. **c:** Metathoracic nerves and musculature of the saturniid (*A. luna*) indicating proposed homologies to those of the noctuid in b above.

the alula of the hindwing (which is expanded to form a flap over the tympanic cavity) and are set in a framework of sclerotized cuticle which also serves in joining the thorax and abdomen.

The penultimate branch and its peripheral innervation site in *A. luna*

In *A. luna*, this previously undescribed IIIN1b1b branch (the penultimate branch) of the IIIN1b1 nerve follows a short (approximately 180 μm) ventral and posterior course through a non-expanded region of tracheal tissue before it peripherally terminates in three bipolar (Type 1) sensory cell bodies, associated with a CO (Figs. 4–6). At the distal tip of each dendrite is a scolopale cap, an extracellular component of the scolopale cell which is characteristic of chordotonal organs (Fig. 6a,b). The entire CO [the swollen area (65–75 μm across) containing the three sensory cells and the three scolopale cells] is anchored at its proximal end to the IIIN1b1 nerve and to surrounding tracheal tissue by support strands. Distally, the CO attaches to a long strand of tissue (approximately 800 μm) which travels in a posterior and lateral direction towards the periphery (Fig. 5). Once it reaches the periphery, the attachment tissue is anchored to the internal surface of the membranous region lying ventral to the hindwing alula, at a point where the membrane meets the ridge of sclerotized cuticle of the epimeron (Fig. 4). The structural components of the attachment structure were not identified in the present study. By direct observation it was seen that movement of the wing base induces stretching of the CO and its attachment strand.

The external skeletal morphology surrounding the region to which the organ attaches is relatively simple compared to that of the noctuid. Directly ventral to the hindwing alula, a membranous region extends medially from the subalar plate to the sclerotized region of the epimeron. There



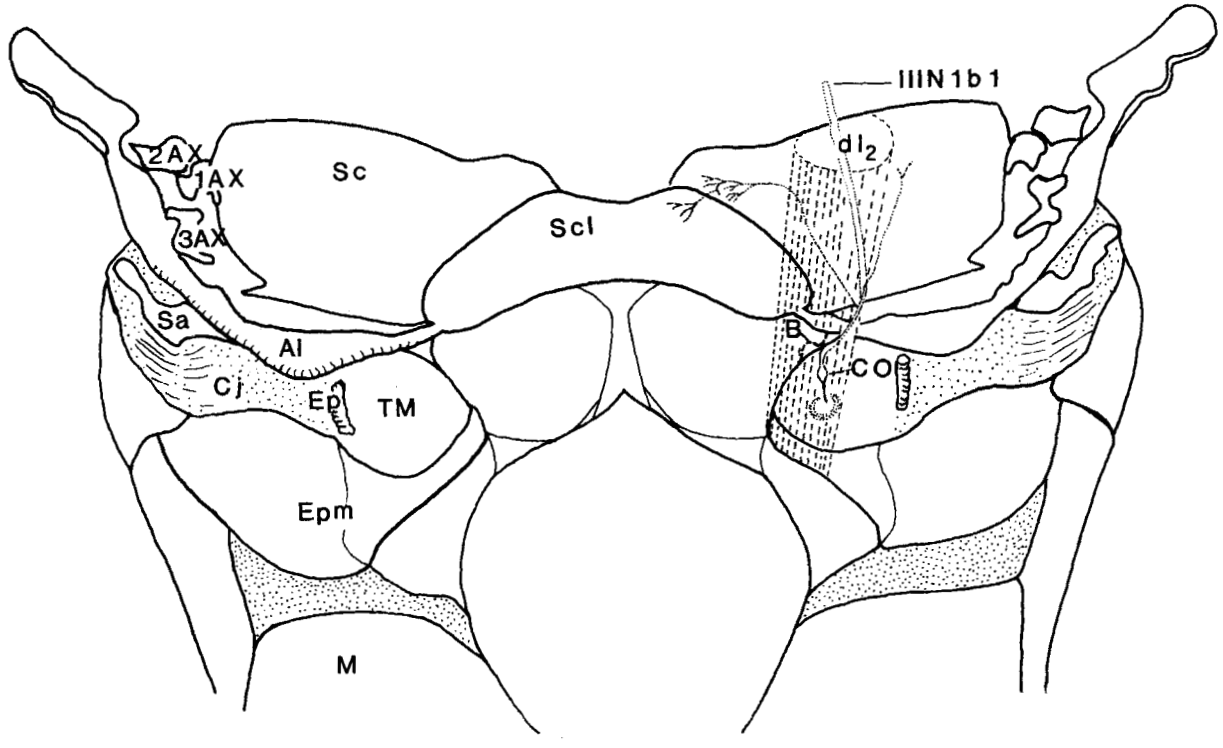


Fig. 3. A dorsal and posterior diagram of the noctuid metathorax, with thoracic scales and abdomen (including abdominal hood) removed for clarity. Left half of drawing shows external skeletal morphology: Scl, scutellum; Sc, scutum; AX 1, 2, and 3, articular sclerites of the hindwing; Al, hindwing alula; Sa, subalar plate; Cj, conjunctiva; Ep, epaulette; TM, tympanic membrane; Epm, epimeron; M, meron. Stippled regions indicate areas of membranous cuticle. Right half is drawn as though the external cuticle were transparent in order to illustrate the branches of the IIIN1b1 nerve (based upon camera lucida drawings of cobalt fills): dl2, dorsolongitudinal muscle; B, Bügel; CO, chordotonal organ. Broken lines indicate location of the dl2 muscle; dotted lines, the branches of IIIN1b1. Scanning electron micrograph in top left corner shows the tympanic membrane and some surrounding skeletal structures in *F. heralis*. Scale bar: 300 μm.

appeared to be no external signs marking the attachment site of the CO (Fig. 4).

Extracellular recordings of IIIN1b in *H. tessellaris* and of IIIN1b1 in *A. luna*

Spontaneous activity. Extracellular recordings of the IIIN1b nerve in *H. tessellaris* revealed a B cell, described by Treat and Roeder ('59) as a large non-auditory multipolar cell which fires spontaneously at common rates between 10 and 20 Hz (Fig. 7). Also present in the resting discharge was an occasional spike of smaller amplitude originating from the auditory sensilla (not shown in Fig. 7).

When we recorded from the same branch in *A. luna*, we observed a cell similar in activity to the B cell in a small number of preparations only, and such recordings were difficult to observe, since the spike amplitudes were not much larger than the noise level of the system. We then moved the electrode towards the periphery to record from only the IIIN1b1 branch, where we consistently recorded from a cell of similar activity to the noctuid B cell in the deefferented preparation (Fig. 7). The discharge rates of this cell varied from 8 to 38 Hz in 14 different preparations (with a mean discharge rate of 12 Hz), but tended to remain constant throughout the life of any one preparation, which often lasted up to 3 or 4 hours. Also present in the resting preparation were at least two cells (based on different spike heights) of smaller amplitude which fired in a more irregular pattern than the larger cell.

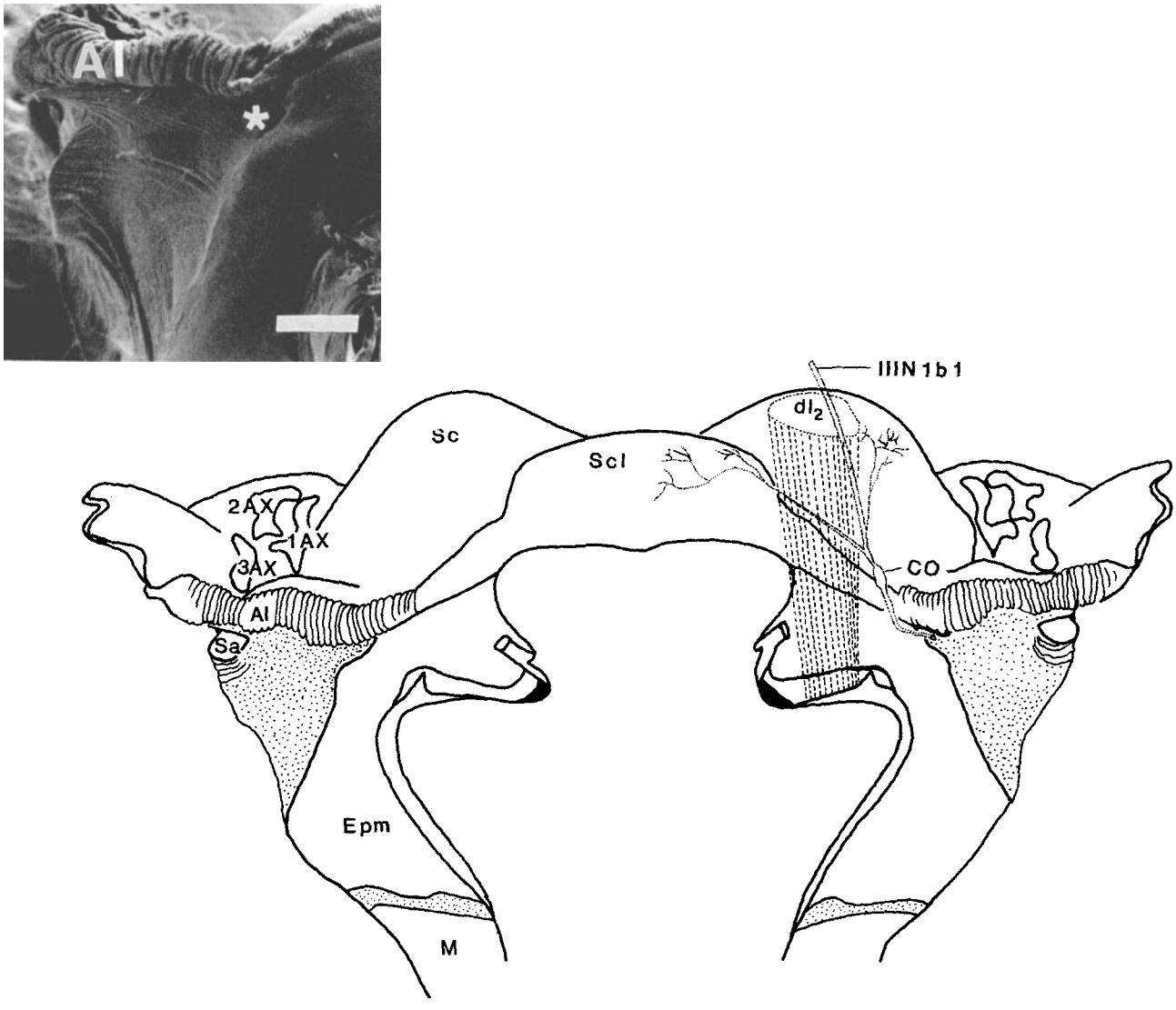


Fig. 4. Dorsal and posterior view of the atympanate (saturniid) metathorax with thoracic scales and abdomen removed for clarity. The structural details are directly comparable with those of the noctuid (illustrated in Fig. 3) except for the skeletal structures of the latter which are associated with the tympanic membrane. The scanning electron micrograph in the top left corner shows the region of membranous cuticle underlying the hindwing alula in *A. luna*. An asterisk marks the area to which the attachment strand of the CO inserts on the internal surface of the membranous cuticle. Scale bar: 600 μm.

Acoustic response. Records of IIIN1b1 in *A. luna* indicated that at least two cells responded phasically to low frequency (1,600 to 2,200 Hz) sounds of high intensity (83 to 96 dB), being most sensitive to 2 kHz (Figs. 7, 8). Figure 7 (top) shows a phasic response to a 2 kHz sound burst played at 84 dB. Cells also responded readily to slight tapping on the recording table, and showed no response to high frequency sounds. The two acoustic cells of the IIIN1b nerve in the tympanate moth, *H. tessellaris*, respond to substantially higher frequencies, with a peak sensitivity of 25 to 40 kHz at intensities between 38 to 42 dB (Figs. 7, 8). They did not respond to tapping on the recording table or to low frequency sounds. The large, spontaneously active cell in both the *A. luna* and *H. tessellaris* showed no response to sound.

Proprioceptive response. The possibility of either the large spontaneously firing cell or the smaller units acting as proprioceptors monitoring hindwing movements in *A. luna* were tested by artificially raising and lowering the wing. Phasic bursts of activity, lasting approximately 180 msec, were observed in the smaller cells when the wing was initially raised and then again when the wing was released from its raised position (Fig. 9). No changes were detected in the firing rate of the large spontaneous cell during wing movements.

DISCUSSION

The purpose of this study was to experimentally test the hypothesis that insect tympanal organs are derived from proprioceptive CO's. In our examination of the atympanate metathoracic condition of the saturniid moth *A. luna*, we expected to find proprioceptors which are homologous to the noctuid tympanal receptors. If our data is to be considered supportive of this theory, it is necessary to discuss the answers to three questions: 1) Are the sensory

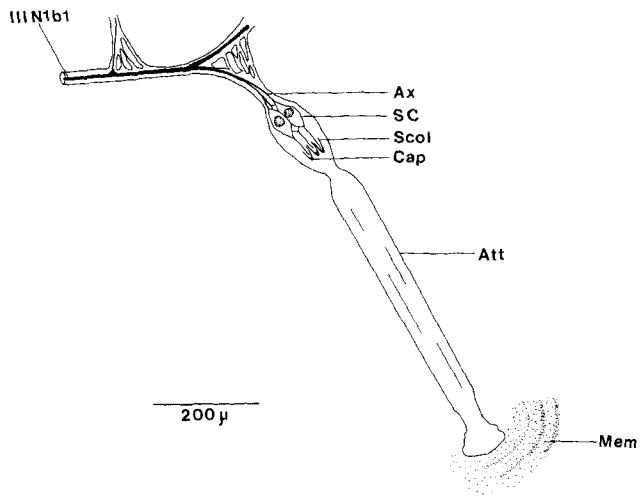


Fig. 5. Illustrative reconstruction of the CO in *A. luna* as determined by cobalt fills and Janus Green B dissections. The second branch of the IIIN1b1 nerve ends in three bipolar sensory cells, forming part of a CO (see text for description of CO). Peripherally, the organ attaches to the membranous region lying ventral to the hindwing alula by means of an attachment strand. Ax, axon; SC, sensory cell body; Scol, scolopale; Cap, scolopale cap; Att, attachment strand; Mem, internal portion of attachment membrane.

units found in the atympanate moth homologous to those of the tympanal receptors of the noctuid moth? 2) Can the chordotonal sensillae of the saturniid moth be considered to represent the evolutionary antecedents to those of the noctuid moth? 3) If the saturniid receptors *are* homologous and evolutionary antecedents, can they be demonstrated to be proprioceptors? Each of these questions shall be considered in the following.

Homology

The concept of homology implies more than anatomical resemblance. By definition, neurons of different species are homologous if they can be traced back through a genealogical series to a common ancestral precursor (Campbell and Hodos, '70). Fossil records provide the most direct evidence for establishing phylogenetic relationships, but unfortunately this method is not applicable to neural tissue. Developmental studies can identify homologies by an examination of nerve cells descended from homologous neuroblasts, although such studies are rare (Bate et al., '81; Pearson et al., '85; Reichert and Meier, '88). In the absence of fossil records or developmental studies, similarities in morphology such as axon course, soma locations, dendritic patterns, and anatomical relationships with other homologous structures such as identified interneurons or muscles have formed the best evidence for homology (King and Valentino, '83).

Considering the skeletal differences imposed by the presence or absence of a tympanal organ, the small number of differences in the peripheral branching patterns of the IIIN1b nerve between tympanate and atympanate moths is surprising. The branching patterns of primary nerves leaving the ganglia correspond closely in both species. A comparison of the main IIIN1b branches with respect to the innervation of peripheral sites revealed no differences between the two moths. IIIN1a consistently innervates the IIis muscle and the two medial branches of IIIN1b innervate dl2 and dl1, while the main branch (IIIN1c) supplies the base of the hind wing.

A comparison of the smaller branches of IIIN1b1 also showed resemblance between both species, each having one branch reaching the scutum, another ending in a CO, and another following a medial course to the scutellum (IIIN1b1a, b, and c, respectively). The significance of the first and third of these branches remains unknown. Paul ('73) describes only one branch (xn) (in addition to the tympanal CO) in the IIIN1b1 nerve of the noctuid *Prodenia eridania* (Cramer). This branch runs proximally to the anterior wall of the tympanic cavity, which, according to her drawing, appears to climb dorsally and branch just beneath either the scutum or scutellum, possibly resembling IIIN1b1a which we have described here. Also, Treat and Roeder ('59) have drawn the stump of a nerve branch which leaves the tympanic nerve before it enters the tympanic air sac. However, there are no accompanying descriptions for these branches in either paper suggesting their functions. Nüesch ('57), in his description of the thoracic nervous system of the atympanate saturniid *T. polyphemus*, describes two branches which resemble IIIN1b1a and IIIN1b1c. He describes IIIN1b1 as a fine branch which climbs up laterally on the dl2 muscle to the epidermis of the scutum and scutellum (presumably our IIIN1b1a) and then innervates the flat IIIdl3 muscle (presumably our IIIN1b1c).

The discovery of a previously undescribed branch bearing a CO in the atympanate saturniid moth was of greatest significance. Anatomically, this organ matches the noctuid tympanal CO in at least two ways: First, both organs attach peripherally to a membranous region underlying the hindwing alula (Figs. 3, 4). If the two structures were homologous, the transition from proprioceptor to tympanal receptor must have involved both a medial and inward shift of the proprioceptive attachment site and a reduction of either the subalar membrane or the adjacent sclerotized cuticle of the epimeron to the extreme thinness of a tympanic membrane. Richards ('32) came to similar conclusions in his examination of the sclerite modifications associated with the progressive evolutionary development of the noctuid ear.

A second feature shared by both CO's is that each scolopalial unit is associated with only one neuron. Interestingly, this feature is shared with all insect tympanal organs, but not all proprioceptive chordotonal organs, which may have two or three neurons associated with each scolopalial unit (Moulins, '76). Perhaps this suggests that the optimal tympanal precursor has only one sensory cell associated with each scolopidium.

One difference that does exist between the noctuid and saturniid is the number of sensory cells in the CO; the saturniid having three, and the noctuid having only two. Interestingly, one noctuoid family, the Notodontidae, has only one auditory cell (Eggers, '19) and this cell is apparently homologous to the noctiud A_2 cell (Surlykke, '84). Yack ('87) has observed signs of CO's (swellings in the same region of the IIIN1b1) in other families within the superfamily Bombycoidea, including other species of Saturniidae [*Automeris io* (F.), *Eacles imperialis* (Dru.), *Dryocampa rubicunda* (F.)], one species of Lasiocampidae [*Malacosoma americanum* (F.)], one species of Apatelodidae [*Olceclostera angelica* (Grt.)], and one species belonging to the primitive superfamily Cossoidea [*Prionoxystus robiniae* (Peck)]. A comparison of different taxa could provide insights into how conservative this organ is with respect to cell numbers.

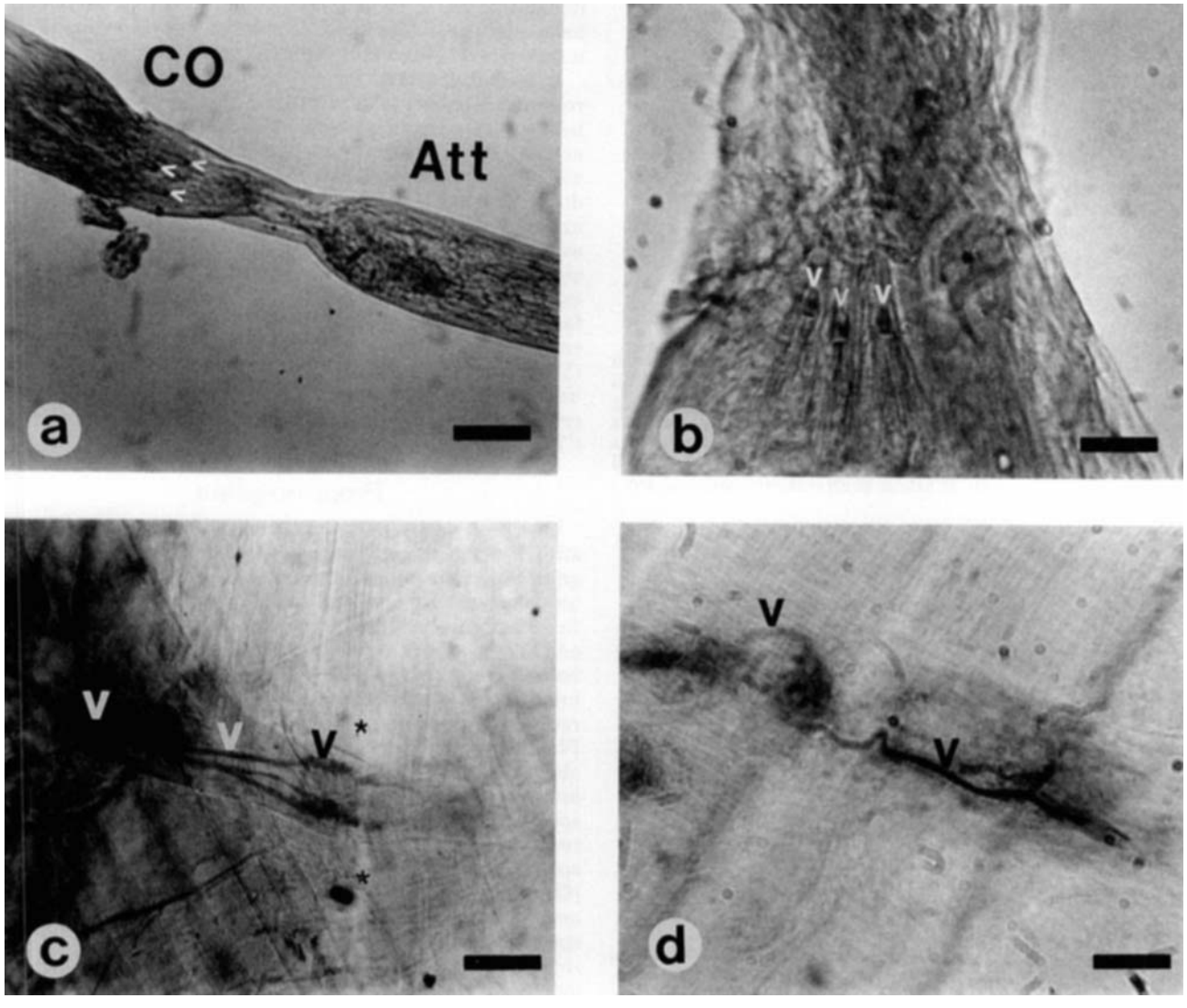


Fig. 6. Whole-mount, light micrographs showing features of the IIIN1b1 CO in *A. luna*. **a:** Distal end of the CO (left of restricted region) and proximal end of the attachment strand (right of the restricted region). Arrows mark locations of the three scolopale caps (scale bar: 10 μm). **b:** Higher magnification of scolopale caps (indicated by arrows) which were stained with Janus Green B (scale bar: 45 μm). **c:** Cobalt fill of IIIN1b1b leading peripherally to the CO. Arrows from left to right point out IIIN1b1b, one of its three sensory cell axons, and one of the three cell bodies respectively. Asterisks mark a fracture in the cell bodies and surrounding tissues which occurred during processing of the tissue (scale bar: 10 μm). **d:** Cobalt fill of a single CO sensillum: Left arrow points to region of sensory cell body and right arrow points to cell's dendrite (scale bar: 45 μm).

Extracellular recordings of the deefferented IIIN1b1 nerve in *A. luna* and the IIIN1b of the noctuid also suggest that the two nerves bear homologous sensory neurons. Each contains a spontaneously active single cell which fires at a regular rate and appears to be unresponsive to acoustic stimulation. Additionally, both recordings indicate the presence of one or more cells of smaller amplitude which will respond phasically to acoustic stimulation.

Further investigations into the ultrastructure of this organ and the central projections of its sensory neurons will provide more conclusive evidence for homology.

Is the atympanate condition a prototype to the noctuid tympanum?

According to those phylogenies of the Lepidoptera which we have examined, the superfamily Noctuoidea belongs to a separate lineage derived from the ancestral atympanate form (Hampson, 1898; Forbes, '23; Brock, '71). Robbins ('87) demonstrates the derived state of the noctuid tympanum using modern means of constructing phylogenies. Considering that neither a tympanal organ, nor a vestige of one, has ever been reported in any member of the large and cosmopolitan atympanate groups, and that the external metathoracic structures of the atympanate form appear unmodified and uniform throughout (Forbes, '16; Eggers, '19; Richards, '32), we hold that the noctuoid metathoracic tympanum is evolutionarily derived from the atympanate metathoracic condition.

What then, is the significance of our observed "acoustic" response, and how can we discard the possibility that we may be observing an adaptively functional ear? One criterion for calling a mechanoreceptor a hearing organ (i.e., an

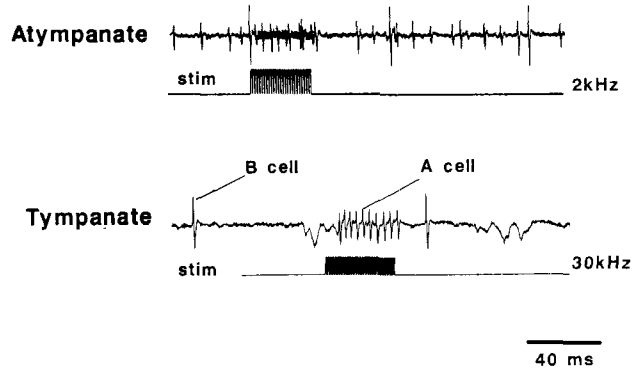


Fig. 7. Extracellular hook electrode recordings of the IIIN1b branch in a tympanate moth (*H. tessellaris*) and the IIIN1b1 branch in an atympanate moth (*A. luna*). Both traces reveal a large, regularly firing, spontaneously active cell (B cell in the tympanate) which does not respond to a sound stimulus, and one (or more) smaller cells (A cell in the tympanate) responding phasically to a sound stimulus (2 kHz at 84 dB in the atympanate moth; 30 kHz at 45 dB in the tympanate moth).

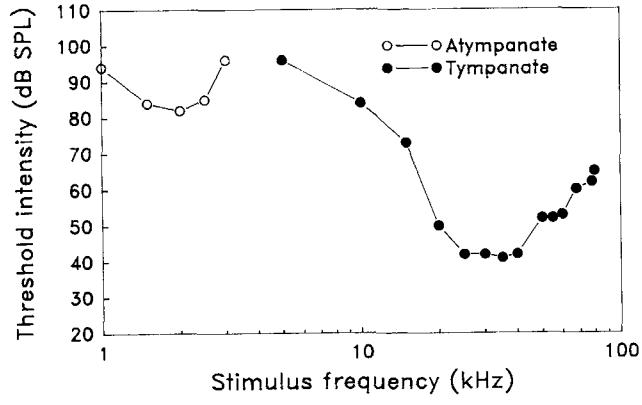


Fig. 8. Response threshold curves of acoustically sensitive neural units in both an atympanate (*A. luna*) and tympanate (*H. tessellaris*) moth. Peak sensitivity of the atympanate cells occurred at a frequency of 2 kHz at an intensity of 82 dB. Peak sensitivity of the tympanate moth occurred between 25 and 40 kHz at intensities between 41 and 42 dB.

auditory receptor) is that it be specifically tuned to biologically relevant sound frequencies and intensities, as in sounds made by predators or conspecific communication signals. The functional significance of these acoustically responsive sensilla in the atympanate moth as auditory receptors is questionable, due to the abnormally high intensities and low frequencies required to elicit a response. Compared to those of the tympanate moth, the sound frequencies to which the atympanate moths appeared sensitive were low, ranging between 1,800 and 2,200 Hz. The predominant frequencies to which the noctuid is most sensitive range between 15 and 60 kHz (Roeder and Treat, '57) which reflects those frequencies used by sympatric predatory bats (see Fullard, '88 for review). A few moths are known to produce sounds for communicative purposes, but these too are of much higher frequency than those seen here (Spangler et al., '84; Gwynne and Edwards, '86; Surlykke and Gogola, '86). Also, sound intensities required to elicit an acoustic response in *A. luna* were biologically irrelevant (82 to 96 dB). Such high response thresholds have also been observed in biologically non-functional insect ears (Ball and Hill, '78).

A second criterion for an auditory receptor is that its response is shown to be critical for the execution of evolved behavioural responses (Pumphrey, '40). Noctuid moths, for example, have been shown to avoid bat echolocation calls to which their ears are tuned (Roeder, '67; Fullard, '88). To date, there has been no positive behavioural evidence to our knowledge that atympanate species of moths respond to sound. Assuming that the feeding habits of bats impose a significant selection pressure on moths, it is worth considering how atympanate species *do* avoid predators. Some families may be temporally isolated from bats by emerging early or late in the season before bats become abundant (Yack, '88). Other families, such as the saturniids, may be unsuitable prey for small bats due to their tendency to fly erratically and close to the ground, as suggested by Roeder ('74).

Proprioception

Our data suggest that the saturniid CO could act as a stretch receptor monitoring position of the hindwing. Topographically, the organ is slung between the IIIN1b1 branch (and surrounding tracheal tissue) at its proximal end, and the membrane underlying the hindwing alula at its distal end. Thus when the wing is raised, the latter membrane becomes taught, appearing to stretch the CO. Also, extracellular recordings of the IIIN1b1 branch show receptors responding in a proprioceptive manner to wing movements: Phasic bursts of activity occur in the smaller cells (presumably CO receptor cells) when the wing was initially raised or lowered. No change of activity was recorded in the larger spontaneously active cell in this preparation, although recordings from the same nerve in two other atympanate species belonging to different families of the *Bombycoidea* [*Olceclostera angelica* (Apatelodidae) and *Phyllodesma americana* (Lasiocampidae)], and one species atympanate species belonging to a different superfamily [*Prionoxystus robiniae* (Cossoidea)] did show that when the hindwing was elevated there was a substantial increase in activity proportional to the degree of elevation (Yack, '87).

A similar organ has been described in the adult desert locust, *Schistocerca gregaria*. Gettrup ('62) reported a proprioceptor (which inserts onto the non-sclerotized cuticle posterior to the subalar sclerite) which is made up of two different sense organs: a large multipolar neuron responding tonically to wing elevation, and a smaller CO which possessed some tonic and some phasic receptors. Wilson and Gettrup ('63) have suggested that the B cell of the noctuid tympanum corresponds to the multipolar stretch receptor and that the A cells correspond to the chordotonal organ of the locust. Although the proprioceptive qualities of the multipolar stretch receptor have been established in wing pattern generation (e.g., Wilson and Gettrup, '63; Burrows, '75; Möhl, '85a,b; Wolf and Pearson, '88), the role of the CO remains uncertain. Recently Pearson et al. ('89) have shown that this CO in *Locusta migratoria* can be stimulated by low frequency (2–5 kHz) sounds at high intensities (intensities of 70 and 95 dB were reported, but sensitivity curves were not provided) and substrate vibrations, and that it may not contribute to wing pattern generation. It is possible that the wing hinge chordotonal organs of the locust and *A. luna* are involved in the detection of vibrations caused by the wings, such as during

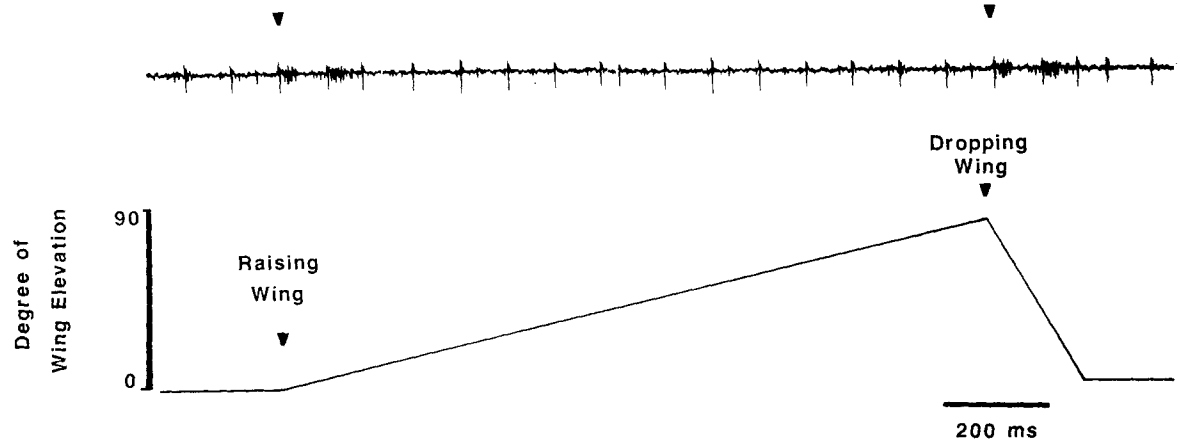


Fig. 9. Extracellular recording of the IIIN1b1 branch in *A. luna* showing response of smaller cells to artificial elevation and lowering of the hindwing. Arrows above trace indicate short bursts of activity which occurred when the wing was initially lifted or dropped to its resting position. No response to wing movement was observed in the larger spontaneously active cell.

warming before flight. There is also some evidence for a wing CO in belostomatid bugs (Heteroptera) which responds in a similar manner to vibrations and air borne sounds (Barber and Pringle, '66).

It is important to note that since the entire mesothoracic dorsum with its associated flight muscles have been removed from our preparations, it is impossible to draw any conclusions about what might be taking place during flight. Further investigations directed towards understanding the functional significance of this organ will be required before it can be identified as a proprioceptor.

In summary, the object of this study was to provide experimental evidence for the proprioceptive origin of insect tympanal organs. Using an atympanate moth as a model of the ancestral "earless" form, we have demonstrated the existence of a CO, which appears be homologous to the metathoracic tympanal CO of the noctuid moth. We have also shown that the atympanate CO responds to wing movements and possibly acts as a stretch receptor. Due to the simplicity of the CO (consisting of but a few neurons) in both tympanate and atympanate moths, we suggest that this system is a promising one for examining the changes to the nervous system, both the peripheral and central, which accompanied the evolution of insect tympanal organs.

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