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Proprioceptive activity of the wing-hinge stretch receptor in *Manduca sexta* and other atympanate moths: a study of the noctuoid moth ear B cell homologue

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Abstract. A multiterminal neurone, recently identified at the wing-hinge of the atympanate moth *Manduca sexta*, is shown to respond as a proprioceptor monitoring elevatory movements of the hind wing. Extracellular recordings from the individual receptor axon confirm this cell to be the source of the spontaneous and regular discharge observed in previous recordings of peripheral nerve 3N1b1. When the wing is raised, this tonic discharge rate increases proportionally with the angle of elevation. When the wing is displaced sinusoidally at a low frequency, the receptor discharge is modulated throughout the wing beat, increasing steadily to a maximum at the top of the upstroke, then slowly decreasing to a minimum at the bottom of the downstroke. At higher wing-beat frequencies, a phasic burst of activity occurs near the top of the upstroke, followed by a silent period during the downstroke. Video-microscopic observations of the wing-hinge during active, stationary flight suggest that the receptor is stimulated by the stretching of its peripheral attachment, the subalar membrane. Stretch receptor sensitivity to wing movement is demonstrated in representatives of 4 lepidopteran families, suggesting that the proprioceptive response is widespread among the Lepidoptera. The functional role of the wing-hinge receptor, and its proposed homologous relationship to both the B cell of the noctuoid moth ear, and the locust wing-hinge stretch receptor are discussed.

Key words: Moth – Flight – Proprioceptor – Sensory – Homology

Introduction

Recently, a group of mechanoreceptors supplied by peripheral nerve branch 3N1b1, were identified at the posterior edge of each metathoracic wing in the tobacco hornworm moth, *Manduca sexta* (Yack 1992). This group comprises a single multiterminal stretch receptor (SR) which attaches to the non-sclerotized membrane ventral to the hind wing axillary cord; a simple chordotonal organ (CO), which is suspended between the dorsal scutum and the posterior sclerotized epimeron; and a hair plate (HP), located on the dorso-lateral metathoracic scutum. The atympanate moth SR and CO sensilla are considered to be homologues and evolutionary prototypes of the noctuoid moth ear (tympanal) sensilla, and as such, predicted to function as proprioceptors monitoring movements of the hind wing (Roeder 1967; Yack and Fullard 1990; Yack 1992; Yack and Roots 1992).

Previous extracellular recordings from the 3N1b1 branch (tympanal nerve homologue) in *M. sexta* and several other atympanate moths have indicated the presence of a large, spontaneously active sensory unit (Yack 1988; Yack and Fullard 1990; Yack 1992). Similar activity has been shown in tympanal nerve (3N1b1) recordings of noctuoid moths, and the source of this activity is known to be the tympanal B cell, a large multiterminal neurone of unknown function (Treat and Roeder 1959), and presumed homologue of the atympanate SR.

In the present study, two questions were of interest: Does the spontaneous activity recorded from the atympanate moth 3N1b1 branch originate from the multiterminal SR unit? Does this unit act as a wing proprioceptor, contributing information concerning movement or position of the wing? The answers to these questions were investigated in *M. sexta* and 3 other distantly related species of atympanate moth.

Materials and methods

Animals. Adult *Manduca sexta* L. (Sphingoidea: Sphingidae) were reared from pupae purchased from a commercial supplier (North

Abbreviations: CO, chordotonal organ; EGAA, Enhanced Graphics Acquisition and Analysis System; HP, hair plate; 3N1b1, tympanal nerve; SR, stretch receptor

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Carolina Biological Supply Company). Pupae were placed in an environmental chamber maintained at 26 to 28°C on a 12 h light/12 h dark cycle. Both male and female moths were examined, there being no apparent differences between the two in the characters examined. Three other species of atympanate moth [*Olceclostera angelica* (Bombycoidea: Apatelodidae); *Prionoxystus robiniae* (Cossoidea: Cossidae); *Phyllodesma americana* (Bombycoidea: Lasiocampidae)] were collected from the wild at the Queen's University Biology Station, near Chaffey's Lock, Leeds County, Ontario, Canada.

Scanning electron microscopy. For anatomical examinations, the CO and SR branches of 8 *M. sexta* were exposed by removing a small region of the metathoracic scutum anterior to the base of the hind wing axillary cord. While the wing was maintained in a horizontal position, the CO and SR were flooded with buffered fixative (0.25% glutaraldehyde and 4% formaldehyde), and left for 2 to 4 h at 5°C. Following fixation, half of the metathorax was dissected out of the animal, rinsed thoroughly in distilled water, and prepared for examination with hexamethyldisilazane, according to Nation (1983). The tissue was then air dried, sputter coated with gold, and examined with an Hitachi S2500 scanning electron microscope. The external metathoracic wing-hinge was prepared for examination by removing the posterior metathoracic scales, and following the procedure for tissue preparation described above.

Videotaping. Movements of the posterior metathoracic wing-hinge during stationary flight were videotaped in 11 *M. sexta.* Moths were temporarily cooled and the scales were removed from the posterior wing-hinge and ventral portion of the thorax. The ventral surface of the thorax was waxed to one end of a wooden dowel (6 mm in diameter) with low melt-point wax. The wings were then temporarily clipped together in a dorsal position with a paper clip, and a speck of black oil paint was applied, with a 000-gauge insect pin, to the external subalar membrane, where the SR attachment site was estimated to be attached internally. The other end of the dowel was secured in a retort stand, and the moth positioned such that the wing-hinge could be viewed through a dissecting microscope. The wings were then un-clipped, and movements of the wing-hinge during flight were filmed with a Panasonic WV 1850 videocamera. Following the videorecordings, post-mortem dissections were performed to determine if the black paint marked the SR attachment site accurately. Recordings of individuals with accurately marked SR attachment sites (8 out of 11 individuals) were played back at low speed, and still images of the attachment site at different angles of wing elevation were photographed directly from the video-monitor.

Electrophysiological recordings. Direct recordings of SR branch activity in 4 *M. sexta* were carried out by exposing the SR branch through a small window in the dorsal metathoracic wing base, as described above for anatomical examination of the CO and SR (see Figs. 1, 4), and then recording directly from the SR branch using a hook electrode, as described below. This direct approach was used only to confirm the pattern of the single neurone's activity.

Examinations of SR activity in response to wing movements in all species were performed by recording from the peripheral nerve branch 3N1b1. For extracellular nerve recordings of 3N1b1, the metathoracic nervous system was exposed using the dorsal dissection approach (Roeder 1966; Yack and Fullard 1990; see Fig. 4). Briefly, this involved removing the mesonotum (scutum and scutellum) and its attached flight musculature, exposing the thoracic ganglia and their peripheral nerve branches. The nerve was hooked onto an insulated stainless steel recording electrode, and a stainless steel reference electrode was inserted into the abdomen. The thoracic nerve roots were routinely severed close to the ganglion, to ensure that only afferent activity was recorded from 3N1b1, and to prevent interference from active muscle movements. Once the electrodes were placed, they were insulated with silicon grease (Dow Corning) applied with a syringe. Extracellular impulses were amplified with a Grass Instruments P15 preamplifier and monitored with a Tektronix storage oscilloscope and an audio amplifier. Spikes were recorded onto a Racal (Store 4DS) instrumentation tape recorder.

Changes in SR activity in response to different positional angles of the hind wing were examined in 3 individuals each, of the 4 moth species. While recording from the 3N1b1 nerve branch, the ipsilateral hind wing (attached to a plexiglass rod held in a micromanipulator) was raised to 4 different degrees of elevation between 0° and 90° (0° being horizontal, and 90° being perpendicular to the body). Frequency discharges were measured after the wing was held stationary for 60 s at each position.

Responses of the SR to sinusoidal wing movements were observed in 10 *M. sexta.* The preparations were mounted on an elevated, narrow platform so that the left wing could be moved throughout its normal range. A plastic pin constructed with a wide base was inserted through the bottom of the wing, and fixed in place with a small amount of low melt-point wax. The pin was attached with an articulating pin to a Plexiglass arm, which in turn was driven up and down by the arm of a pen motor. This mechanical device, driven by a function generator (Hewlett Packard 3311A) moved the wing up and down in a sinusoidal pattern through an amplitude of 110° of arc (55° above and 55° below horizontal) at frequencies of 0.1, 0.4, and 0.8 Hz. Stretch receptor responses were recorded from the dorsally exposed 3N1b1 branch, and the stimulus output from the function generator was recorded simultaneously on tape and monitored with the oscilloscope.

Data analysis for the SR response to sinusoidal wing movements was performed on an RC-electronics, Enhanced Graphics Acquisition and Analysis system (EGAA). Spikes over a certain amplitude were selected using the EGAA's waveshape recognition software, and spike frequency/time histograms of these large spikes were generated with the use of EGAA's Rate Meter Histogram software.

Results

Anatomical observations

The SR and CO lie in a small cavity near the posterior edge of the hind wing, and both the SR branch and CO strand are tightly suspended between their 2 attachment sites when the wing is maintained in a horizontal position (Figs. 1, 4). Both sensory organs arise from the same peripheral nerve branch (one of the two 3N1b1 branches), which bifurcates between the outer edges of the dorsolongitudinal and dorsoventral muscle bundles. One branch of this bifurcation gives rise to the CO sensilla, which are attached to the sclerotized epimeron by an attachment strand. The SR cell body lies at the peripheral end of the SR branch, and attaches to the soft membrane underlying the hind wing axillary cord, in a position slightly ventral and medial to the subalar plate (Figs. 2, 3). Although not confirmed histologically, it appears that the SR attaches directly to the epidermis, as there were no obvious signs of accessory structures (i.e. muscle or connective tissue strands) in the vicinity of its 'tentacular processes' (i.e. dendritic region), which could be visualized when the SR branch was pulled slightly from the membrane.

Video-recordings of the subalar membrane during flight demonstrates that the SR attachment site undergoes observable changes in position and tension during one wingbeat cycle. When the wing is in a downward position, the membranous region to which the SR attach-

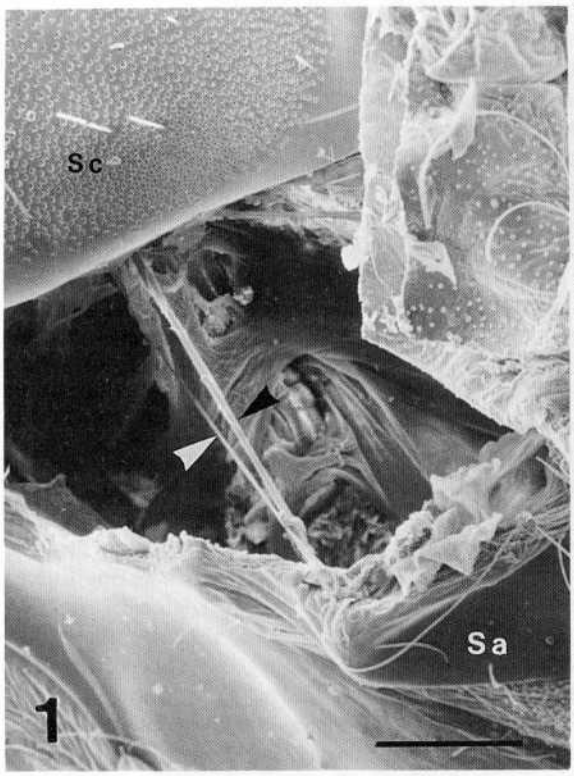

Fig. 1. Scanning electron micrograph (dorsal view) of the right metathoracic SR nerve branch (*white arrowhead*) and CO attachment strand (*black arrowhead*) in an adult *M. sexta*. The SR and CO have been exposed by the removal of a small section of the axillary cord slightly posterior and lateral to the metathoracic scutum (*Sc*), and underlying tracheal tissue (see also Fig. 4). The external attachment site of the SR is obscured by the subalar plate (*Sa*) in this micrograph. Scale bar = 280 μm

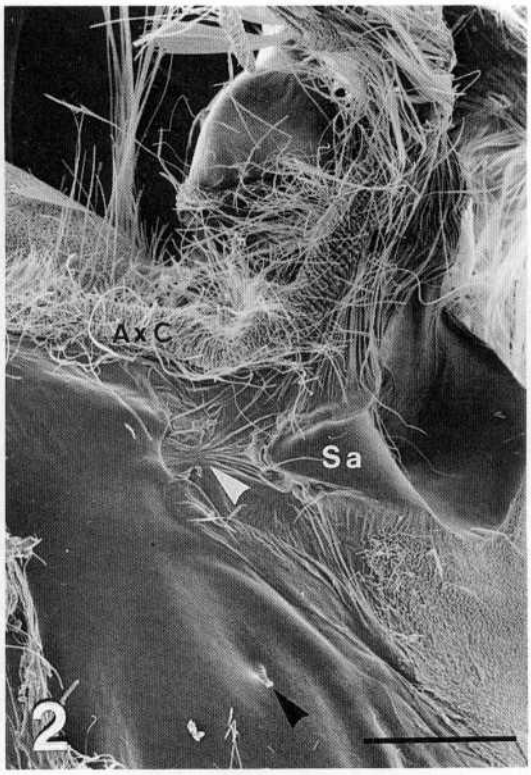

Fig. 2. Scanning electron micrograph of the right metathorax (posterior view) in an adult *M. sexta* with the wing fixed in an upward position. The attachment of the SR (*white arrowhead*) is located on the soft membrane medial to the subalar plate (*Sa*), and ventral to the axillary cord (*AxC*) of the wing. The attachment site of the CO strand (*black arrowhead*) is located on the sclerotized epimeron, and is manifested externally by a small, peg-like structure. Scale bar = 600 μm

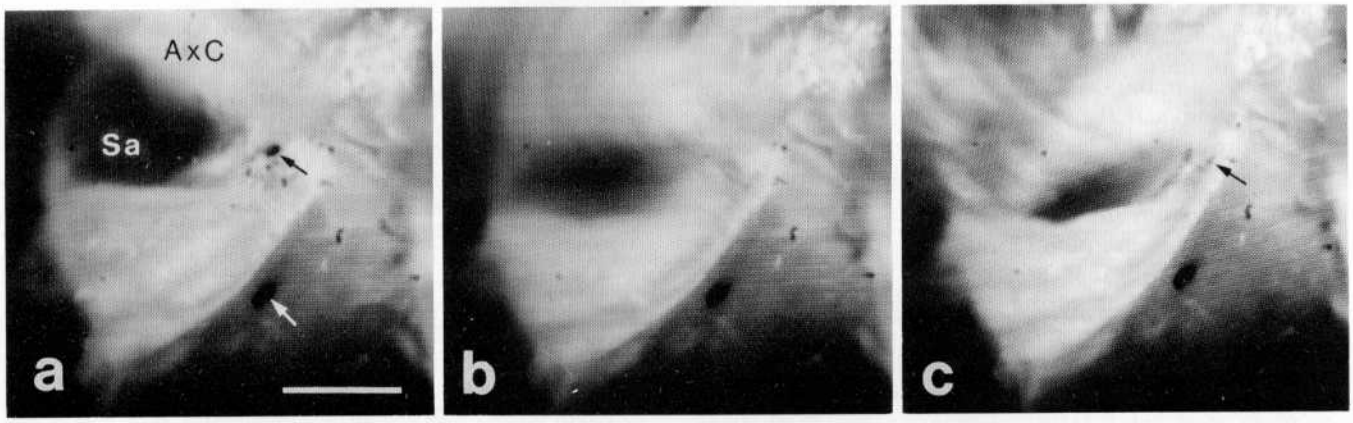

Fig. 3a–c. Still image photographs of the left, posterior wing-hinge in *M. sexta* during stationary flight. **a** The wing is in an upward position (+55° from horizontal) and the membrane to which the SR is attached (*black arrow* marks attachment site) is stretched. The CO attachment site (*white arrow*) is located on the sclerotized epimeron. **b** The wing is descending, and the membrane to which the SR attached begins to relax. **c** The wing has descended to a −55° position below horizontal. The membrane is completely folded, and the SR attachment site (*arrow*) is found within one of these folds. *Sa* subalar plate; *AxC* Axillary cord. Scale bar = 1000 μm

es is relaxed and folded. As the wing is raised, the membrane unfolds and stretches, reaching a maximum degree of stretch at the top of the upstroke. In contrast, the sclerotized cuticular attachment site of the CO strand shows no movement during the wing-beat cycle (Fig. 3).

Neural activity

The top trace of Fig. 4 shows the typical neural activity recorded from the 3N1b1 nerve of an adult *M. sexta* with the wings held in a horizontal position. A consistent fea-

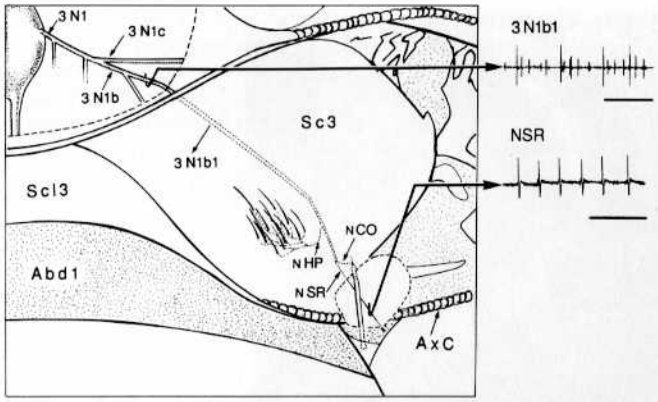

Fig. 4. Diagrammatic representation of the right metathorax (dorsal view) indicating where hook electrodes were positioned for extracellular recordings. Branch 3N1b1, which supplies the stretch receptor, chordotonal organ, and hair plate, was accessed by exposing the pterothoracic ganglia and its peripheral nerve roots via an opening in the dorsal mesothoracic scutum (*dashed line*). Recordings from 3N1b1 frequently exhibit spontaneous activity of a large, regularly firing unit, and several irregularly firing smaller units of various amplitudes, as indicated by the corresponding trace. The nerve branch supplying only the stretch receptor was exposed by removing a small region of the axillary cord and membrane near the posterior edge of the wing-hinge (*dashed line*). When recording directly from the SR branch, as indicated in the corresponding trace, only the larger, regularly firing unit was observed, confirming that the SR is the source of this activity. SR branch recordings often exhibited higher frequency spike rates of the SR, due to the upward pulling of the hook electrode. *Abd1* first abdominal segment; *AxC* axillary cord of right hind wing; *NCO* chordotonal organ nerve branch; *NHP* hair plate nerve branch; *NSR* stretch receptor nerve branch; *Sc3* metathoracic scutum; *Scl3* scutellum. Scale bars: 50 ms

ture of all 3N1b1 recordings is a large, spontaneously active unit which fires at regular intervals. In the present experiment, the resting discharge rates of individual SRs ranged between 25 and 36 pulses/s, and these rates could be altered by manually applying pressure to the base of the hind wing near the subalar membrane.

In addition to this large unit, there are several smaller units which fire irregularly, and are considerably smaller in amplitude (generally ranging between 5 and 45% of the larger spike's amplitude). Since the 3N1b1 branch supplies all of the wing-hinge mechanoreceptors (the CO, SR and HP), and therefore must contain at least 20 sensory axons, it was necessary to identify the source of the large unit. This problem was investigated by recording directly from the SR branch, known to contain only a single axon innervating the multiterminal cell (Yack 1992). Recordings from the SR branch always exhibited a single, large, spontaneously active spike, corresponding to the large unit observed in the 3N1b1 recordings (Fig. 4, lower trace). The discharge rate of this unit could be increased by pulling up slightly on the hook electrode, suggesting that the unit was sensitive to stretch. If the wing was moved up or down during direct SR branch recordings, the imposed thoracic movements resulted in spurious elevations in discharge frequency due to the electrode pulling on the branch. Therefore, SR responses to wing movements were observed by recording from the 3N1b1 (described below). Occasionally it was possible to hook an electrode onto the proximal region of the CO, where the sensory axons are located, but only irregularly firing spikes of small amplitude were observed. In addition, stimulation of the hair plate while recording from the 3N1b1 branch revealed phasic activity of the smaller units only. From these observations, we concluded that the multiterminal SR unit was the source of the large impulse.

When the wing is moved to a different stationary position above horizontal, the discharge rate of the SR increases proportionally to increasing the angle of the wing (Fig. 5). During the process of stretching, a phasic discharge is superimposed upon the tonic response, resulting in a higher overall frequency. When the stretching movement ceases, and the wing is maintained in this elevated position, the frequency drops slightly, and settles to a higher frequency than that observed at the lower angle of elevation. This new rate would continue while the wing was maintained in this elevated position. Similar responses of the SR to changes in wing elevation were observed in all of the species, although discharge rates at different degrees of elevation varied between individuals.

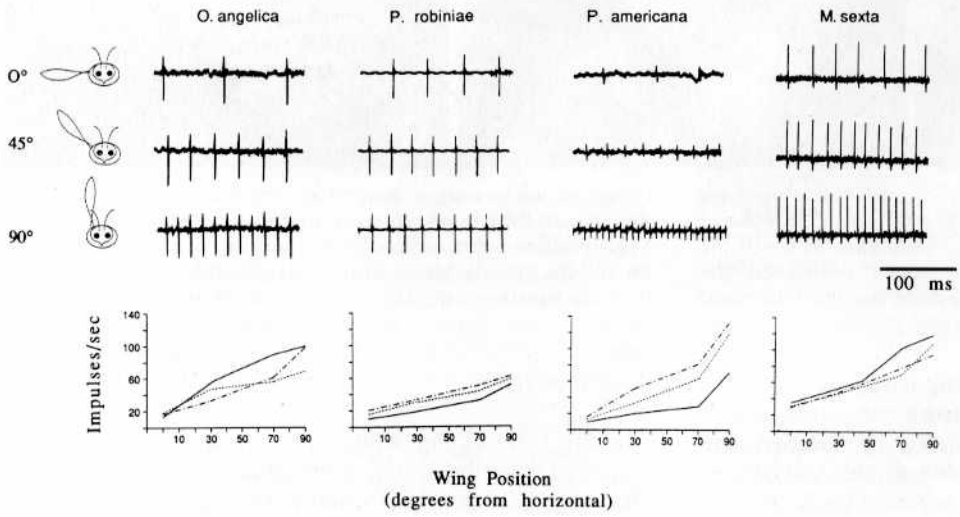

Fig. 5. Afferent spike activity recorded from the 3N1b1 branch of 4 atympanate moth species (representing 4 families) while the wing ipsilateral to the recording electrode is held at a constant position at increasing angles of wing elevation (0° = horizontal). At 0° the SR maintains a regular, spontaneous rate of discharge. This rate increases by increasing the positional angle of the wing, and at each angle, remains constant while the wing is held at this position. Each graph illustrates the changes in SR discharge rates in 3 individuals of each species

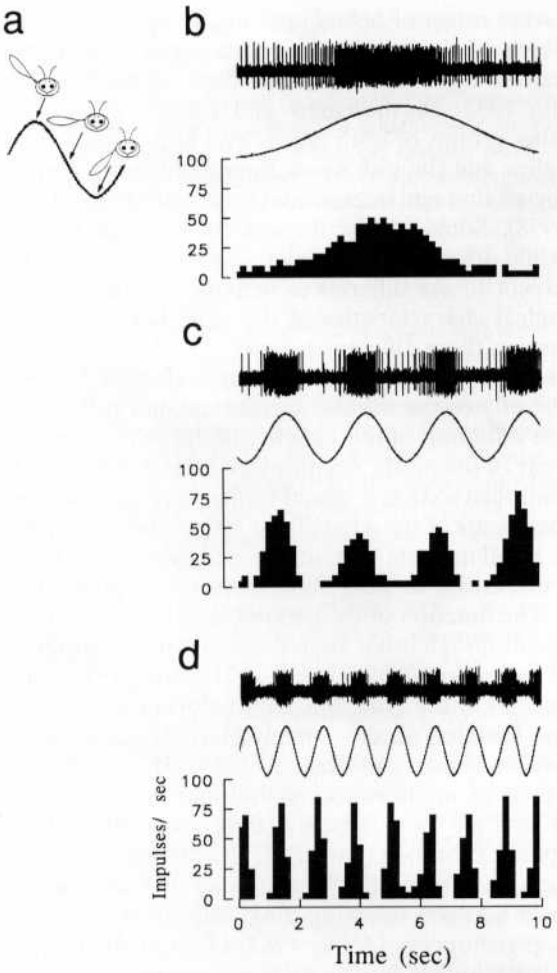

Fig. 6a–d. Afferent activity of the SR in *M. sexta* in response to sinusoidal movements of the wing at 3 different frequencies and constant displacement. **a** Diagram illustrating wing position in relation to the sinusoidal stimulus output of the pen motor. The top and bottom components of the sine wave correspond to the maximum upward displacement (+55°) and downward displacement (–55°) of the wing respectively. **b,c,d** Stimulation of the wing at frequencies of 0.1 Hz, 0.4 Hz, and 0.8 Hz respectively. The upper trace of each figure is an extracellular recording of 3N1b1, and the corresponding mechanical displacement of the wing is represented by the middle trace. Spike/frequency histograms (200 ms bins) for each trace illustrate changes in the discharge frequency of the SR during the course of wing displacement

This variation was attributed to slight differences in the strain on the subalar membrane due to the position of the wing. Qualitatively however, the relationship between wing elevation and SR discharge rate was similar.

When the wing is moved up and down sinusoidally at a fixed angle (110°) and low frequency (0.1 Hz), the SR spike frequency is active throughout the wing beat cycle, being lowest at the bottom, and highest at the top of the upstroke (Fig. 6b). An increase in frequency of the wing movement (hence an increase in the velocity of the upward stretch) to 0.4 Hz elicits a corresponding increase in spike frequency near the top of the upstroke, followed by a noticeable decrease of activity at the bottom of the downstroke (Fig. 6c). This phasic response is even more pronounced at a higher wing beat frequency (0.8 Hz), where the SR discharge rate is again increased during the upward phase, and the downward movement abolishes all response (Fig. 6d).

Discussion

Evidence of a large, spontaneously active sensory unit recorded from the metathoracic nerve 3N1b1 has been observed in all species of both tympanate (Treat and Roeder 1959; Lechtenberg 1971; Spangler 1988) and atympanate moths (Yack 1988; Yack and Fullard 1990; Yack 1992) examined to date. Although in the Noctuoidea this activity is known to originate from the multiterminal B cell located in the moth's ear (Treat and Roeder 1959), the source of such activity in moths lacking a metathoracic tympanum remained unknown. We have shown in this study that the multiterminal SR of the metathoracic wing-hinge in *M. sexta* is the source of this spontaneous discharge, and that it appears to act as a proprioceptor monitoring the position and movement of the hind wing.

Observations of the SR attachment site during stationary flight in *M. sexta* suggest that the SR is stimulated by the stretching and relaxation movements of the flexible subalar membrane which occur when the wing is moved up and down during flight. In support of this observation, we have shown that the firing rate of the atympanate moth SR is modulated by the stretching and relaxation movements of the subalar membrane imposed by elevatory movements of the wing.

In order to understand the functional role of the wing-hinge SR, ideally, one would record directly from the SR branch, or the 3N1b1 branch during natural wing movements. In the moth however, this is difficult, since the large movements of the thorax prohibit recording directly from the SR branch. Recording from the 3N1b1 branch in the intact animal has not yet been possible, as the branch runs between large flight muscles (Nüesch 1957; Yack 1992) and is inaccessible without substantial damage to metathoracic flight musculature. The 3N1b branch, which is longer and generally more accessible in the intact animal, contains a minimum of 5 motor axons which innervate dorsolongitudinal flight musculature (Surlykke and Miller 1982; Orona and Agee 1988; Yack, unpublished data) and if deefferented, would impair the function of these muscles. Therefore, we have stimulated the SR with passive sinusoidal wing movements, and made speculations on the natural function of these organs from our observations. However, we acknowledge that active, three dimensional thoracic movements may also influence sensory activity of the stretch receptor.

Our observations of the SR's responsiveness to both stationary and low frequency changes in the elevatory position of the wing, suggest that the SR may function as a wing proprioceptor during both flight and non-flight behaviours. In *M. sexta* and other Sphingoidea, the position of the wing changes in the vertical plane during the early stages of flight warm-up (Dorsett 1962), or when the moth is at rest (personal observations). Other groups,

such as the large saturniids and butterflies, commonly change the resting positions of their wings from vertical to horizontal, such as during wing eye-spot display, or for thermoregulation (eg. Klots 1958; Scott 1986). Low frequency movements of varying amplitudes are also common; such as those observed in some of the larger Lepidoptera during pheromone dispersal, flight preparation, and during gliding and soaring behaviour, where there may be only a single occasional wing beat (Klots 1958). During these low frequency movements, the SR could contribute information concerning the amplitude, frequency, and direction of the wing.

During flight, the wing beat frequencies in *M. sexta* are elevated to an average of 26 beats/s at total displacements of 112° (Heinrich 1971). Although the amplitudes used in our experiments reflected those of natural flight, we were unable to reach such high frequencies due to mechanical limitations. However, based on our observations of the wing-hinge SR response to low frequency wing movements, and its similarity to that of the well-known locust wing-hinge SR, we can predict how the lepidopteran SR would respond to high frequency movements such as those produced during flight. The wing-hinge SR in *M. sexta* responds to static changes in wing elevation, and to low frequency sinusoidal movements of the wing in a phasic-tonic manner very similar to that of the locust wing-hinge SR (Wilson 1961; Gettrup 1962; Pabst 1965). At much higher wing-beat frequencies than were observed in our experiments ($\sim$14 Hz), the locust SR gives from one to several action potentials just prior to, or at the top of the wing upstroke (Wilson and Gettrup 1963; Pabst 1965; Möhl 1985). This sensory information is known to be involved in modifying the frequency of the centrally generated motor pattern controlling wing movements during flight (Wilson and Gettrup 1963; Wendler 1974; Burrows 1975; Wolf and Pearson 1988). Therefore, we predict that during flight, similar activity to the locust will occur in the moth SR, and therefore be important in the regulation of the central flight motor pattern (Hanegan 1972; Kammer and Rheuben 1976; Kammer and Kinnamon 1979). The existence of such a proprioceptor, similar to that of the locust SR, has been suspected from previous studies of the effects of sensory input on flight in *Manduca* (cf. Kammer and Rheuben 1976). It will be interesting to look for central pathways between the metathoracic SR, or its mesothoracic serial homologue, and identified flight motoneurons (Casaday and Camhi 1976; Kondoh and Obara 1982; Claassen and Kammer 1986; Madsen and Miller 1987; Orona and Agee 1988), and to observe the effects of SR ablation on the output of the flight central pattern generator.

Due to the conservative nature of insect nervous systems (e.g. Bullock and Horridge 1965; Pearson et al. 1985; Dumont and Robertson 1986; Meier et al. 1991), and our observations of presumed SR activity in several distantly related moth and butterfly taxa (Yack 1988; this study; unpublished data), we suspect that the wing-hinge SR is found in all Lepidoptera. If this cell functions primarily as a proprioceptor monitoring wing movements, we might expect anatomical and physiological differences of the SR between groups. Within the Lepidoptera there exists a wide range of behaviours involving wing-movement. Some of the hawk moths are rapid and skillful flyers, exhibiting wing-beat frequencies around 70 Hz (Sotavalta 1952; Bartholemew and Casey 1978), while many other groups of both moths and butterflies are extremely slow and sluggish flyers, their flight being characterized by gliding and occassional fluttering (Kirby 1889; Klots 1958). Some moths do not fly but still possess wings, while others have completely lost their wings. We might expect to see differences in both anatomical and physiological characteristics of the wing-hinge SR neurone between these different groups.

In the Noctuidae, the SR homologue (B cell) does not attach directly to the subalar membrane, but rather, sits on a rigid infolding of the cuticle (the Bügel) located inside the ear of the moth. Anatomically the B cell appears to be positioned so that it would be mechanically isolated from movements of the wing (Treat 1959), and recordings from the B cell indicate that, unlike the atympanate moth SR, it is insensitive to wing movements (Treat and Roeder 1959). The function of the noctuid moth B cell remains unknown, although it has been proposed to be indirectly involved in hearing (Lechtenberg 1971), in detecting subtle distortions of the tympanic cavity during flight, or as having no function at all – merely existing as an evolutionary relict (Treat and Roeder 1959). If the latter is correct, then we might expect to find that the SR central arborizations are more extensive than those observed in the tympanal B branch (Paul 1973; Surlykke and Miller 1982; Agee and Orona 1988), corresponding to observations made between the wing-hinge SRs of winged and wingless grasshoppers (Arbas 1983, 1992). If the tympanal B cell no longer functions as a proprioceptor, it is possible that its proposed mesothoracic serial homologue compensates for the loss of sensory input from the metathoracic SR.

Other proposed homologues of the lepidopteran wing-hinge SR include the wing-hinge SRs of grasshoppers and crickets (Wilson and Gettrup 1963), which in turn have been proposed by these authors as serial homologues of the abdominal stretch receptors in larval Lepidoptera (Finlayson and Lowenstein 1958). Considering the potential wide distribution of SR homologues across a large number of both closely and distantly related taxa, this sensory receptor may be useful for examining changes to the nervous system that accompanied changes in SR function between groups.

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