MECHANISMS OF FREQUENCY-SPECIFIC RESPONSES OF OMEGA NEURON 1 IN CRICKETS (*TELEOGRYLLUS OCEANICUS*): A POLYSYNAPTIC PATHWAY FOR SONG?

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Summary

In crickets (Teleogryllus oceanicus), the auditory interneuron omega neuron 1 (ON1) responds to sounds over a wide range of frequencies but is most sensitive to the frequency of conspecific songs (4.5 kHz). Response latency is longest for this same frequency. We investigate the mechanisms that might account for the longer latency of ON1 to cricket-like sounds. Intracellular recordings revealed no evidence for appropriately timed postsynaptic inhibition of ON1 that might increase its latency, nor was latency affected by picrotoxin. The onset of excitatory postsynaptic potentials (EPSPs) was delayed for 4.5 kHz stimuli compared with ultrasound stimuli, pointing to a presynaptic locus for the latency difference. When ON1 is stimulated with high frequencies, discrete, apparently unitary EPSPs can be recorded in its dendrite, and these are latency-locked to spikes recorded simultaneously in the auditory nerve. This suggests that input to ON1 from high-frequency-tuned auditory receptor neurons is monosynaptic. In agreement with this, brief ultrasound stimuli evoke a single, short-latency EPSP in ON1. In contrast, the EPSP evoked by a brief 4.5 kHz stimulus consists of an early component, similar in latency to that evoked by ultrasound and possibly evoked by ultrasoundtuned receptors, and a later, dominant component. We interpret the early peak as arising from a monosynaptic afferent pathway and the late peak from a polysynaptic afferent pathway. Multiple-peak EPSPs, with timing similar to those evoked by sound stimuli, were also evoked by electrical stimulation of the auditory nerve.

Key words: cricket, *Teleogryllus oceanicus*, auditory interneuron, omega neuron 1 (ON1), audition, insect, song, excitatory postsynaptic potential, monosynaptic pathway, polysynaptic pathway.

Introduction

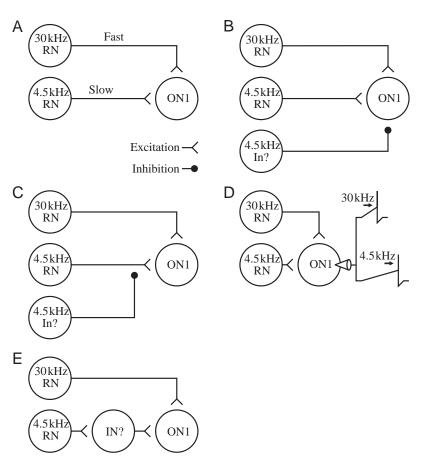
Crickets use sound to find mates (Alexander, 1960), to compete with rivals (Alexander, 1961) and to detect echolocating bats (Moiseff et al., 1978; Nolen and Hoy, 1986). Because of the importance of sound to the biology of crickets and the well-known advantages of insects for neurobiological studies, crickets have long served as subjects for the study of auditory processing (for a review, see Hoy et al., 1998). A prominent bilateral pair of auditory interneurons in crickets are the omega neurons 1 (ON1) (Casaday and Hoy, 1977; Popov et al., 1978; Wohlers and Huber, 1982). Each ON1 receives excitatory input mainly from one ear (the ear ipsilateral to its soma) and inhibits several auditory interneurons that receive their input from the opposite ear, including the contralateral ON1 and ascending neurons 1 and 2 (AN1, AN2) (Selverston et al., 1985; Faulkes and Pollack, 2000). The resulting inhibition enhances binaural contrast in the auditory pathway, thereby improving the ability to localize sounds (Atkins et al., 1984; Horseman and Huber, 1994; Schildberger and Hörner, 1988).

The neuron ON1 responds to sound over a wide frequency range, but it is most sensitive to the carrier frequency of conspecific calling song (Popov et al., 1978; Wohlers and Huber, 1982; Atkins and Pollack, 1986), which is approximately 4.5 kHz in *Teleogryllus oceanicus* (Hill et al., 1972; Balakrishnan and Pollack, 1996). Previously, we showed that the latency of ON1 is longer, by up to 10 ms, for 4.5 kHz compared with other frequencies, including ultrasound (Pollack, 1994; Faulkes and Pollack, 1997; Faulkes and Pollack, 2000). We have reported on the functional consequences of this elsewhere (Faulkes and Pollack, 2000), and in the present paper we focus on the mechanisms that might be responsible for the delayed response to cricket-like frequencies.

Fig. 1 illustrates several candidate mechanisms. Pollack (Pollack, 1994) suggested that the conduction velocities of auditory receptors might differ in a frequency-specific manner (Fig. 1A), but Pollack and Faulkes (Pollack and Faulkes, 1998) found no evidence for this. The remaining hypotheses are,

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Fig. 1. Hypotheses (not mutually exclusive) that could explain frequency-specific differences in the latency of auditory interneuron omega neuron 1 (ON1). (A) Subpopulations of receptor neurons (RNs) could have different conduction velocities. Although extracellular recordings show no difference in the mean conduction velocity of low- and high-frequency RN populations (Pollack and Faulkes, 1998), more recent work indicates that a sub-population of low-frequency RNs has lower conduction velocities than other RNs (K. Imaizumi and G. S. Pollack, in preparation). (B) There is frequency-specific postsynaptic inhibition (In) of ON1. ON1 is inhibited by several sources (Wiese, 1981; Selverston et al., 1985; Pollack, 1988; Schildberger et al., 1988), but none is known that is specific to 4.5 kHz and arrives before excitatory auditory input. (C) There is frequency-specific presynaptic inhibition of auditory receptors, similar to that found in insect proprioceptive systems (Sauer et al., 1997; Burrows and Laurent, 1993). Some receptors do receive synapses within the prothoracic ganglion (Hirtz and Wiese, 1997; Watson and Hardt, 1999), although their source is not known. (D) Intrinsic properties of ON1 cause differences in integration properties. The shapes of ON1 excitatory postsynaptic potentials (EPSPs) differ depending on the frequency that evokes them: low-frequency sounds generate smooth, graded EPSPs, whereas high-frequency sounds generate large, discrete EPSPs (Pollack, 1994). The resulting difference in EPSP rise times could, in turn, create different spike latencies. (E) Interneurons (IN)



are interposed between the majority of low-frequency sensory neurons and ON1, but high-frequency sensory neurons connect directly to ON1. Some receptors do synapse directly with ON1 (Hirtz and Wiese, 1997; also see Fig. 5).

briefly, that there is frequency-specific inhibition, either postsynaptic (Fig. 1B) or presynaptic (Fig. 1C), that the integrative properties of ON1 create differences in excitatory postsynaptic potential (EPSP) shape and thus in the timing of spike onset (Fig. 1D) and that there are frequency-specific afferent pathways to ON1 (Fig. 1E).

This work has been published previously in abstract form (Faulkes and Pollack, 1997).

Materials and methods

Animals

Field crickets, *Teleogryllus oceanicus* (Le Guillou), were raised in laboratory colonies where food (Purina Cat Chow) and water were available continuously. Unmated female crickets, 1–3 weeks of age after the final moult, were used in all experiments.

Recordings

Animals were anaesthetised by chilling on ice. The metaand mesothoracic legs were autotomised, and the wings and antennae were removed. We affixed the animals, ventral side up, to a magnetic base with wax, and then used wax to immobilise the tibia and femur of the prothoracic legs, with the femur positioned horizontally at right angles to the body axis. Two fine Teflon-coated silver wires were inserted into the femur of the leg ipsilateral to the sound source to record sensory compound action potentials (Pollack and Faulkes, 1998). We removed the ventral thoracic cuticle to expose the prothoracic ganglion, which was supported on a metal platform. The ganglion was submerged in physiological saline (140 mmol1⁻¹ NaCl, 10 mmol1⁻¹ KCl, 7 mmol1⁻¹ CaCl₂, 4 mmol1⁻¹ NaHCO₃, 1 mmol1⁻¹ MgCl₂, 5 mmol1⁻¹ Tes, 5 mmol1⁻¹ trehalose; modified from Strausfeld et al., 1983).

We made extracellular recordings of ON1 in the hemiganglion contralateral to the main dendrite receiving auditory input (hereafter designated 'contralateral', after Schildberger and Hörner, 1988; the laterality of all auditory interneurons will be described relative to their main source of auditory input) using low-resistance microelectrodes (<10M Ω) filled with 2 mol l⁻¹ NaCl. ON1 was identified by its greater sensitivity to sounds contralateral to the recording electrode (i.e. ipsilateral to input) (Pollack, 1986). We made intracellular recordings from the main auditory dendrite of ON1 using high-resistance (>30M Ω) microelectrodes filled with 2 mol l⁻¹ potassium acetate, 2 mol l⁻¹ lithium chloride or 3 mol l⁻¹ potassium chloride. A chlorided silver-wire indifferent electrode was placed in the abdomen. ON1 was identified by the correspondence between intracellularly recorded spikes and simultaneously recorded extracellular spikes. Intra- and extracellular recordings were amplified using a microelectrode amplifier (Getting 5A or WPI M-707). For some experiments, we made extracellular recordings of ascending neuron 2 (AN2; 'Int-1' in Casaday and Hoy, 1977; Wohlers and Huber, 1978) with a polyethylene suction electrode attached to the cervical connective. We identified AN2 by the laterality of its auditory input (i.e. greater sensitivity to sound presented ipsilateral to the recorded connective), large-amplitude spikes and strong response to ultrasound (Wohlers and Huber, 1978; Moiseff and Hoy, 1983; Faulkes and Pollack, 2000).

Sound stimuli

Stimuli were produced by an analog/digital board (National Instruments AT-MIO-64-F5; 12 bits, update rate 250 kHz) and relayed through a programmable attenuator (Tucker-Davis Technologies PA4) and amplifier (Amcron D150A). The sound stimuli were played through piezoelectric loudspeakers situated to the left and right of the cricket, perpendicular to its longitudinal axis, at a distance of 35 cm. Experiments were performed in a chamber lined with echo-attenuating mineral-wool wedges.

Sound stimuli were played at 2 pulses s⁻¹. The pulse envelope was trapezoidal in shape and, in most experiments, had a duration of 20.4 ms (including 0.2 ms rise and fall times). In some experiments, we used 0.4 ms sound pulses (0.2 ms rise and fall times). As reported previously, the latency of ON1 varies with sound frequency (Faulkes and Pollack, 2000) and is longest at approximately 4.5 kHz. On the basis of this finding, we used 3 kHz, 4.5 kHz ('lowfrequency'), 10 kHz and 30 kHz ('high-frequency') stimuli to characterise the response of ON1 in these experiments. Stimulus intensity ranged from 25 to 100 dB SPL (re $2 \times 10^{-5} \,\mathrm{N \, m^{-2}}$). Data and event markers were recorded on a frequency-modulated tape recorder (Vetter; model D) and then digitised through an analog/digital board (National Instruments; AT-MIO-64-F5) using the program SWEEPS (Pollack, 1997), at a sampling rate of 10 kHz per channel with 12 bits of analog to digital resolution. The same program was also used for analysing data off-line.

In some experiments, we deafened crickets unilaterally by cutting or crushing the leg nerve contralateral to the sound source. For experiments in which we used $0.1 \text{ mmol } 1^{-1}$ picrotoxin (PTX) (Sigma), we cut the anterior and posterior connectives of the prothoracic ganglion to ensure that the drug entered the ganglion quickly. The ganglion remained bathed in PTX for 10 min before data collection commenced.

Data analysis

The latency of ON1 spikes was measured from extracellular recordings as the time between stimulus onset and the succeeding ON1 spike. In most cases, threshold was defined as the lowest sound intensity ($\pm 2.5 \text{ dB SPL}$) that generated a mean response of one or more spikes per 100 ms sampling window. In the PTX experiments, threshold was defined in a different

manner because PTX tended to increase background activity. Here, spontaneous ON1 activity (spikes per 100 ms in the absence of stimulation) was measured for 3 s to provide a baseline; threshold was defined as the lowest intensity at which the mean spike count per 100 ms sampling window exceeded the baseline count by one or more spikes.

Statistical analyses were performed using the software package Statistica for Windows 5.1 (StatSoft, Inc.). The results for 3 kHz were similar to those for 4.5 kHz, and the results for 10 kHz were similar to those for 30 kHz. The data for 3 and 10 kHz were, therefore, omitted from the figures for clarity and brevity but were included in the statistical analyses.

Results

The long latency of ON1 is not due to inhibition

Postsynaptic inhibition could explain the sound-frequencyspecific differences in latency of ON1 if it were appropriately timed, i.e. either preceding sound-evoked excitation or simultaneous with it. Earlier intracellular recordings (e.g. Pollack, 1994) did not reveal inhibitory postsynaptic potentials (IPSPs) preceding excitation, but those recordings were made using microelectrodes containing Cl⁻, while ON1 was strongly hyperpolarised. Such conditions could mask IPSPs, which are typically mediated by Cl⁻ channels and have a reversal potential near the resting potential. We made intracellular recordings using electrodes filled with potassium acetate. No early IPSPs were seen in response to ipsilateral sound stimuli (Fig. 2A,B), either at the normal resting potential of the cell or when ON1 was depolarised with a current of 2-4 nA, which should enhance IPSPs. When depolarised by injected current, ON1 spikes tonically. If there were cryptic inhibition preceding excitation not visible as an IPSP, one might expect (i) that the firing frequency of spontaneous ON1 spiking would be reduced, and (ii) that the height of these current-evoked spikes would be reduced prior to the onset of the auditory-evoked response, as a result of shunting by the conductance increase associated with the IPSP. No reduction in firing rate (Fig. 2C) or in spike height (Fig. 2D) was observed before the soundevoked response. Previous experiments have shown that ON1 experiences post-excitatory inhibition, which is most prevalent at higher sound intensities and persists after the offset of excitation (Pollack, 1988). This serves as a positive control for the utility of firing frequency and spike-attenuation measurements as a means of revealing inhibition. Currentevoked spikes occurring during the post-excitatory period of inhibition occur at lower frequency (Fig. 2C) and are attenuated (Fig. 2Dii).

Inhibition of ON1 by contralateral sound is well documented and is caused largely, if not exclusively, by the contralateral ON1 (Wiese and Eilts, 1985; Horseman and Huber, 1994; Selverston et al., 1985). We checked for any effect of contralateral inhibition on the latency of ON1 by cutting the leg nerve contralateral to the sound source, thereby eliminating any sound-evoked contralateral influences. Contralateral deafening did not affect the latency of ON1 (repeated-measures

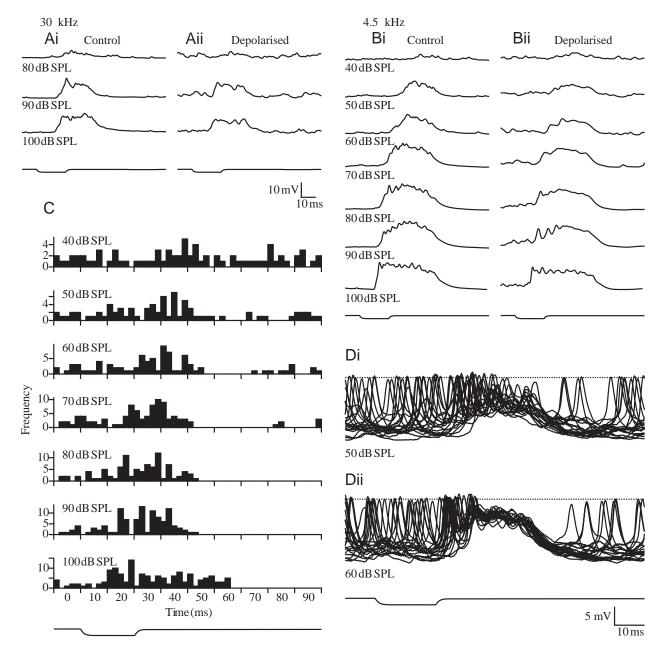
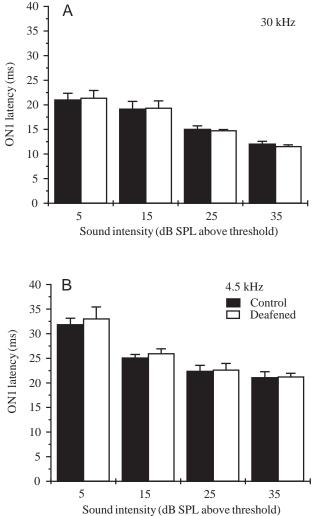


Fig. 2. Postsynaptic inhibition cannot explain the long latency of auditory interneuron omega neuron 1 (ON1) to low-frequency sound stimuli. No inhibitory postsynaptic potentials (IPSPs) are evident preceding sound-evoked excitatory postsynaptic potentials (EPSPs) in response to ipsilateral (A) 30 kHz or (B) 4.5 kHz sound stimuli. Furthermore, injecting depolarising current (approximately 2–4 nA) does not reveal IPSPs (also see D). The top traces in A and B are at threshold intensity for each frequency. Traces in A and B are averages of 20 recordings; scale bars for B as in A. (C) Firing rate of continuous ON1 spiking (caused by injecting approximately 3 nA depolarising current) does not decrease prior to the increase in spiking caused by auditory stimuli, as would be expected if cryptic inhibition were occurring. Post-stimulus inhibition is evident. Histograms (2 ms bin width) of ON1 spikes are compiled from responses to 20 presentations of 4.5 kHz stimuli; bottom trace, stimulus marker. (D) Spike height is not reduced prior to the onset of auditory-evoked spiking, as might be expected from any shunting caused by cryptic IPSPs. In contrast, spikes occurring on the falling phase of the EPSP, when conductance may still be high, or during post-stimulus inhibition (ii) are shunted. Stimuli: 4.5 kHz, 50 dB SPL (i), 60 dB SPL (ii). Continuous spiking caused by injecting approximately 3 nA depolarising current. Twenty superimposed traces are shown.

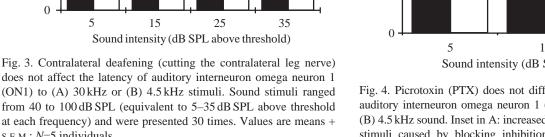
analysis of variance, ANOVA, N=5; $F_{1,125}=0.37$, P>0.5) (Fig. 3).

 γ -Aminobutyric acid (GABA), a common inhibitory neurotransmitter in arthropods, is responsible for presynaptic

inhibition in many invertebrates and vertebrates (Watson, 1992), and GABA-immunoreactive profiles terminate both on ON1 (Watson and Hardt, 1996) and on terminals of auditory receptors (Watson and Hardt, 1999). Thus, GABA is a good



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at each frequency) and were presented 30 times. Values are means + S.E.M.; N=5 individuals. candidate to mediate presynaptic inhibition of auditory receptors (Fig. 1C) or postsynaptic inhibition of ON1 itself (Fig. 1B). Picrotoxin (PTX) blocks GABA receptors in many invertebrates (Sattelle et al., 1991) and has been shown to block inhibition in the auditory systems of crickets and bush

crickets (Harrison et al., 1988; Stumpner, 1998). If GABAmediated inhibition were responsible for the frequencyspecific responses of ON1, PTX should shorten the latency of ON1 specifically to low-frequency sounds. Treatment with 0.1 mmol l⁻¹ PTX caused a small, but statistically significant, reduction in ON1 latency across all frequencies (control, 25.7±4.63 ms; PTX, 24.88±4.52 ms; repeated-measures ANOVA; N=5; $F_{1,119}=20.01$, P<0.01; means \pm s.D.). Nevertheless, there was no specific effect on responses to low frequencies (Fig. 4; no significant interaction between PTX

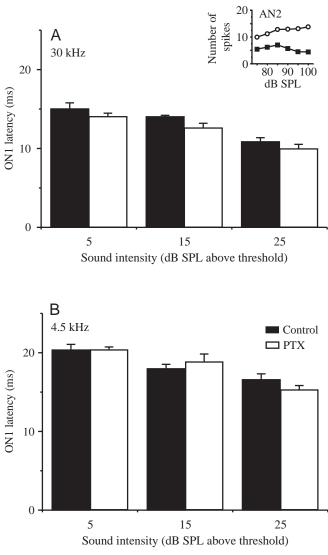


Fig. 4. Picrotoxin (PTX) does not differentially affect the latency of auditory interneuron omega neuron 1 (ON1) to (A) 30 kHz sound or (B) 4.5 kHz sound. Inset in A: increased spike count of AN2 to 4.5 kHz stimuli caused by blocking inhibition with PTX (see text). Filled squares, control; open circles, PTX-treated. Sound stimuli ranged from 40 to 100 dB SPL (equivalent to 5-25 dB SPL above threshold at each frequency) and were presented 30 times. Values are means + s.E.M.; *N*=5 individuals (error bars are not shown in the inset).

and sound frequency; repeated-measures ANOVA, N=5; $F_{3,119}=2.02, P=0.12$).

As a positive control for the effectiveness of PTX, we also recorded from ascending neuron 2 (AN2). This neuron is strongly excited by ultrasound and receives mixed excitation/ inhibition in response to 4.5 kHz stimuli (Nolen and Hoy, 1987). Previous experiments showed that the low-frequency inhibitory input is PTX-sensitive (Harrison et al., 1988). As expected from the foregoing, PTX treatment enhanced the response of AN2 to 4.5 kHz (Fig. 4A, inset), but had no effect on the response to ultrasound (not shown; interaction between PTX treatment and stimulus frequency on the spike count of

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AN2, repeated-measures ANOVA: N=8; $F_{3,168}=45.67$; P<0.01). Treatment with PTX also tended to increase spike counts in ON1 (control, 6.71 ± 3.40 ; PTX, 6.90 ± 3.58 ; repeated-measures ANOVA: N=5, $F_{1,134}=3.91$; P=0.05; means \pm s.D.). This increase, however, could be attributed largely to an increase in spontaneous activity (see Materials and methods). When spontaneous activity was subtracted, PTX caused a small, but statistically significant, decrease in spike number (control, 6.23 ± 3.33 ; PTX, 5.95 ± 3.56 ; repeated-measures ANOVA: N=5, $F_{1,134}=5.47$; P<0.05; means \pm s.D.) but, as for latency, this effect was not frequency-specific (repeated-measures ANOVA: N=5, $F_{3,134}=0.88$, P>0.05).

EPSPs differ in onset times

The EPSPs evoked by ultrasonic stimuli rise more rapidly than those evoked by low frequencies (Pollack, 1994). Intracellular recordings, however, indicated that the difference

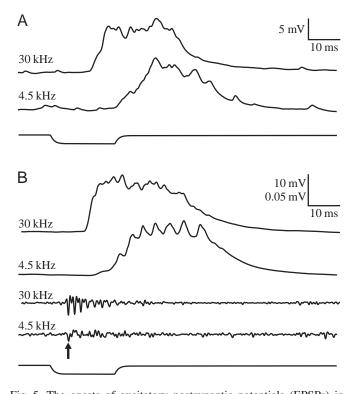


Fig. 5. The onsets of excitatory postsynaptic potentials (EPSPs) in response to 4.5 kHz sounds are delayed compared with those evoked by 30 kHz sound. (A) Intracellular recordings of auditory interneuron omega neuron 1 (ON1) in response to ipsilateral sound stimuli show a difference in EPSP onset latency, even when sound intensities are equivalent relative to threshold for each frequency. No inhibitory postsynaptic potentials (IPSPs) were obvious (see also Fig. 2); 20 dB SPL above threshold; absolute intensity 100 dB SPL for 30 kHz, 60 dB SPL for 4.5 kHz. (B) Differences in EPSP onset are not explained by differences in the onset of receptor firing. The first compound action potentials (arrow) have been aligned for both frequencies, but the onsets of EPSPs (top two traces) are not synchronous; 30 dB SPL above threshold; absolute intensity 70 dB SPL for 4.5 kHz, 90 dB SPL for 30 kHz. Different animals were used in in A and B. Average of 20 traces in A and B.

in rise times could not be the sole explanation for the longer latency of ON1 to low-frequency sounds. The EPSPs evoked by low-frequency sound also had longer onset latencies (Fig. 5A). The EPSPs also differed in onset latency relative to the first receptor compound action potential (Fig. 5B), demonstrating that the long latency to low-frequency sound is not a reflection of different latencies of receptor neurons.

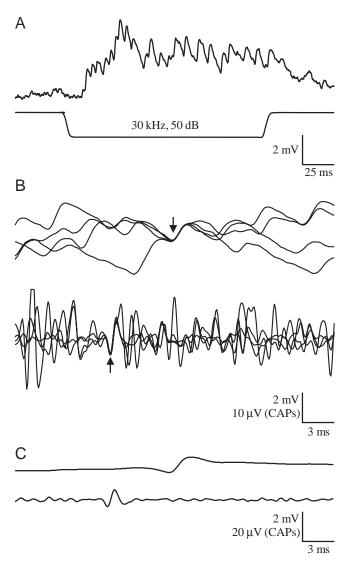


Fig. 6. Evidence for a monosynaptic connection between an ultrasound receptor and auditory interneuron omega neuron 1 (ON1). (A) Intracellular recording of ON1, showing discrete excitatory postsynaptic potentials (EPSPs) in response to low-intensity, high-frequency stimulus. (B) Top traces: four traces from ON1, with EPSPs aligned (arrow). Bottom traces: corresponding traces from an extracellular recording from the leg nerve, recorded simultaneously, showing a spike that precedes the EPSP with constant latency (arrow). Similar results were obtained in a second individual (not shown). (C) Top trace: average of 770 EPSPs from ON1, accumulated over several responses similar to that shown in A. Bottom trace: averaged extracellular recordings of the leg nerve reveal a single unit, latency-locked to the EPSP in ON1. CAPs, compound action potentials.

Monosynaptic and putative polysynaptic pathways to ON1

The last hypothesis (Fig. 1E) makes two separate claims: (i) that there are monosynaptic connections to ON1, predominantly for high frequencies, and (ii) that there is a polysynaptic pathway to ON1, mainly for low frequencies. There is anatomical evidence (Hirtz and Wiese, 1997) for direct input from receptor neurons to ON1, but the frequencysensitivity of these receptors was not determined. To provide physiological evidence for monosynaptic input from highfrequency receptors, we made simultaneous recordings of EPSPs in ON1 and extracellular spikes in the leg nerve. Ultrasound stimuli evoke trains of discrete EPSPs in ON1 (Pollack, 1994). Aligning many of these EPSPs in digitised recordings revealed that each was preceded, with constant latency, by a spike in the leg nerve (Fig. 6). The one-to-one, fixed-latency correspondence between EPSPs and receptor spikes is most readily explained by a monosynaptic connection between the receptor and ON1. Low-frequency stimuli, unlike ultrasound stimuli, do not evoke large, discrete EPSPs (Pollack, 1994), so this technique cannot clarify the nature of the connections between low-frequency receptors and ON1.

We sought evidence for a polysynaptic pathway in the shape of compound EPSP waveforms. If all the presynaptic neurons fire once, and simultaneously, they should generate a single EPSP peak if only monosynaptic connections are present. Conversely, the same single, tightly synchronised volley of presynaptic spikes should generate multiple EPSP peaks if a mixture of mono- and polysynaptic connections is present. This assumes that auditory receptors have similar conduction velocities, and we have shown previously that this is the case for the bulk of the population of high- and low-frequency receptors (Pollack and Faulkes, 1998). More recent studies show that the minimum latencies of two different subgroups of low-frequency receptors differ by 2-3 ms, as measured at the entry of the auditory nerve into the prothoracic ganglion (Imaizumi and Pollack, 2001). We applied short (0.1 ms) electrical stimuli to the leg nerve while recording intracellularly from ON1 and extracellularly from the leg nerve. Electrical stimuli evoked compound EPSPs with

Fig. 7. Short (0.01 ms) electrical stimuli given to the leg nerve generate a multiple-peaked compound excitatory postsynaptic potential (EPSP) in auditory interneuron omega neuron 1 (ON1). (A,B) Recordings from two individuals. Top traces: compound **EPSPs** recorded intracellularly from ON1; ON1 was hyperpolarised to prevent spiking; 10 representative traces from 100 stimulus presentations. Second trace: average of 100 traces. Third trace: compound action potential (CAP) from the leg nerve (average of 100 traces). Although the short electrical stimulus evokes multiple CAPs, the interval between them is shorter than the interval between the two main peaks in the compound EPSPs. Bottom trace: stimulus marker. The arrows in B show the onset of the second peak in the compound EPSP.

multiple peaks (Fig. 7). The compound action potentials recorded in the leg nerve also showed multiple peaks, reflecting multiple firings of receptors and/or successive volleys of receptor groups with slightly different conduction velocities. However, successive compound action potentials were separated by only 2–3 ms, which is too short to account for the difference in onsets of approximately 10 ms of the successive compound EPSPs.

Interpreting the results from electrical stimulation is difficult, however. First, electrical stimulation of the entire nerve cannot selectively stimulate different groups of auditory receptors tuned to different frequencies. Second, the 'auditory' nerve also carries the axons of non-auditory sensory neurons and of motor neurons (Eibl and Huber, 1979), and additional peaks in the compound EPSP of ON1 might be generated by ortho- or antidromic stimulation of these non-auditory axons. To restrict our stimuli more selectively to auditory receptors, we used short (0.4 ms) sound stimuli to create brief, tightly synchronised receptor responses. In an effort selectively to excite different populations of receptors, we generated these sound pulses at both low and high sound frequencies. These stimuli were amplitude-modulated throughout their entire durations (see Materials and methods) and thus had broader spectra than our standard stimuli, which consisted mainly of a constant-amplitude sine wave. Spectral analysis confirmed, however, that the 'intended' frequency (either 5 kHz or 30 kHz; see Materials and methods) dominated the spectra of these brief stimuli, with other components being less intense by at least 30 dB. Nevertheless, each of these stimuli probably excited both high- and low-frequency-tuned receptors when presented at high intensities, both because of their broad spectra and because individual receptors often have additional sensitivity peaks at frequencies other than that to which they are most sensitive (Imaizumi and Pollack, 1999).

Brief ultrasound stimuli always generated a single EPSP peak, regardless of intensity (N=7 individuals; Fig. 8A). Equally brief, low-frequency sounds generated more complex waveforms. At high intensities, these stimuli produced EPSPs with two main peaks (Fig. 8), approximately 10–12 ms apart (Table 1). At lower intensities, the early EPSP became less prominent (often visible as a change in slope in the compound EPSP, rather than as a true peak that rose and fell before rising

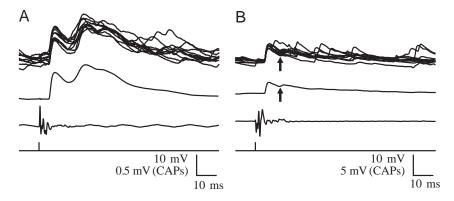


Fig. 8. Short, low-frequency stimuli generate two excitatory postsynaptic potentials (EPSPs) (arrows in Aii and Bii) in auditory interneuron omega neuron 1 (ON1); (Ai) 30 kHz stimuli; (Aii) 5 kHz stimuli (used instead of 4.5 kHz, because 5 kHz generates an integer number of sound cycles with 0.4 ms stimuli). Top traces: intracellular ON1 recordings, showing six representative single traces. Middle traces: average of 30 traces. Bottom traces: receptor compound action potentials (CAPs) recorded from the leg nerve; average of 30 traces. Below: stimulus markers. In Ai,ii and Fig. 9, the stimuli were shorter than indicated by the marker, because the stimulus monitoring circuit includes a low-pass filter that distorts short stimuli. (B) Example from another individual (same individual as shown in Fig. 7A; note the similarity in timing of the successive peaks in the compound EPSP evoked by low-frequency sound in this figure and by electrical stimulation of the afferent nerve in Fig. 7A). (Bi) Response to 0.4 ms, 30 kHz, 80 dB SPL stimuli. (Bii) Response to 0.4 ms, 5 kHz, 80 dB SPL stimuli. Top traces: 10 representative traces. Middle traces: average of 50 traces. Bottom traces: stimulus markers.

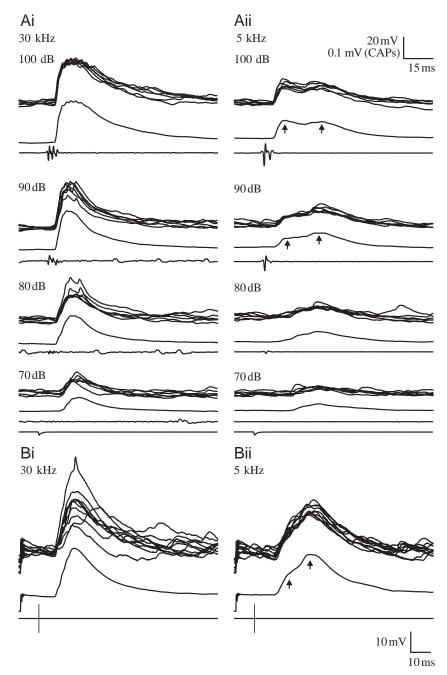


Table 1. Properties of ON1 compound EPSP peaks evoked by short, low-frequency stimuli

Sound intensity (dB SPL above threshold)	Late EPSP onset (ms)	Early EPSP onset (ms)	Difference peak-to-peak (ms)	Early EPSP amplitude (% late EPSP amplitude)	Ν
0	24.87±3.62	14.78±3.82	10.09±0.21	52.57±15.64	2
10	23.55±1.16	12.04±1.03	11.51±0.77	53.06±6.71	4
20	22.34±2.45	10.15 ± 1.02	12.19±2.66	67.99±25.85	5
30	22.21±3.95	9.47±0.95	12.74±4.55	85.87±30.22	6

Sound intensity is relative to the threshold for the late peak EPSP. Only cases with two peaks are shown (see text).

Values for onsets and delays are means \pm standard deviation.

The amplitude of late peak is measured relative to baseline.

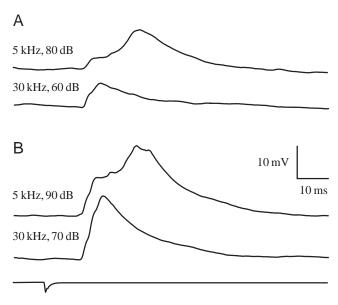


Fig. 9. Early, low-frequency-evoked compound excitatory postsynaptic potentials (EPSPs) have the same latency as ultrasoundevoked compound EPSPs. (A) Recordings made at threshold intensities for early, low-frequency-evoked compound EPSP and ultrasound-evoked compound EPSP. (B) Recordings made at 10 dB SPL above threshold for both EPSPs. Bottom trace: stimulus marker. Average of 30 traces. The cell was injected with approximately 3 nA hyperpolarising current to reduce spiking (see also Table 2).

again) or only a single peak was visible (Fig. 8Aii; Table 1). The mean threshold for any visible EPSP was 66.7 dB SPL (N=6), and the timing of these single EPSP peaks indicates that they correspond to the late EPSPs seen at higher intensities. Two peaks in the EPSP were visible at an average of 78.3 dB SPL (N=6); this should represent the threshold for the early EPSP seen at higher intensities. The latency of the early, low-frequency-evoked EPSP corresponded well with the latency of the ultrasound-evoked EPSP (Fig. 9; Table 2), suggesting that this early peak may result from stimulation, by low-frequency sound, of ultrasound-tuned receptors.

Despite the short duration of the sound stimuli, the recordings of receptor compound action potentials exhibit multiple peaks, indicating repetitive firing of receptor neurons and/or successive volleys from receptors with different conduction velocities or response latencies (Fig. 8A). As in

the experiments with electrical stimuli, however, the delay between the compound action potentials, 2–3 ms, is too short to account for the multiple peaks in the compound EPSP, which are separated by approximately 12 ms. Indeed, there is no indication in the EPSP waveform of the arrival of successive, closely spaced waves of afferent input, perhaps because the strong initial depolarisation from the first volley of receptor spikes masks additional EPSPs evoked by subsequent firings of receptors.

Discussion

We can eliminate several of the proposed hypotheses as mechanisms for the frequency-specific response latency of ON1 (Fig. 1). The conduction velocities of the majority of lowand high-frequency-tuned receptors are not significantly different (Pollack and Faulkes, 1998). More recent work shows that spikes from one subset of low-frequency-tuned receptors arrive at the central nervous system 2-3 ms later, on average, than those of other receptors, including those tuned to ultrasound (Imaizumi and Pollack, 2001), but the magnitude of the delay is too short to account for the difference in the latency of ON1, which may be as much as 10 ms. The longer latency of ON1 to low-frequency sound stimuli is evident not only at the level of spike timing, but also in the onset of EPSPs (Figs 2, 3). We found no evidence for appropriately timed postsynaptic inhibition that might explain the delay in EPSP onset (Fig. 2). Thus, the locus of the delay in the response of ON1 is, at least in part, presynaptic to ON1. PTX-resistant presynaptic inhibition (Fig. 1D) and frequency-specific polysynaptic pathways (Fig. 1E) remain the two most likely possibilities.

It is known that GABA-immunoreactive processes are present in the cricket auditory system (Watson and Hardt, 1996; Watson and Hardt, 1999). Given that PTX is generally an effective blocker of GABA receptors (Sattelle et al., 1991), our PTX experiments (Fig. 4) should have revealed any GABA-mediated inhibition of ON1, whether pre- or postsynaptic. Nonetheless, we cannot rule out the possibility that the long latency of ON1 to low-frequency sound is caused by PTX-resistant inhibition. First, some GABA receptors in insects are PTX-resistant (Amat and Hue, 1997). Second, there is evidence for non-GABAergic inhibition in the cricket auditory system. Specifically, although ON1 is known to be an inhibitory interneuron, it is not immunoreactive for GABA nor

 Table 2. Timing of ON1 EPSPs generated by short ultrasound stimuli and early peak EPSPs generated by short, low-frequency stimuli

Sound intensity	Ultrasound EPSP onset		Low-frequency early	
(dB SPL above threshold)	(ms)	Ν	EPSP onset (ms)	Ν
0	11.08±1.64	5	12.61±2.71	6
10	9.50±1.35	5	10.70±1.10	5
20	7.08 ± 2.44	5	9.36±0.82	5
30	6.48 ± 2.82	4	8.91±0.75	3

Onset values are shown as means ± standard deviations.

Sound intensity relative is to the threshold for the EPSP.

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does it contain typical GABA synaptic vesicles (Watson and Hardt, 1996). Skiebe et al. (Skiebe et al., 1990) proposed that histamine is the inhibitory neurotransmitter of ON1 because histamine mimicked the inhibitory effects of ON1 on AN2, and histamine antagonists blocked inhibition of AN2. The latency of ON1 (Fig. 4) and the spike count of ON1 in response to sound are affected only slightly by PTX, suggesting that most of its abundant GABA-immunoreactive input synapses (Watson and Hardt, 1996) are activated by non-auditory input (e.g. subgenual organ, Wiese, 1981; walking motor system, Schildberger et al., 1988).

The balance of evidence suggests that, in addition to a monosynaptic afferent input to ON1 (Hirtz and Wiese, 1997; this study), there is also a polysynaptic path, specific to 4.5 kHz. The most telling evidence for a polysynaptic pathway is that very brief 5 kHz stimuli generate two EPSP peaks, approximately 12 ms apart (Figs 8, 9; Table 1), which is similar to the latency difference of ON1 spikes to low- and highfrequency stimuli (Pollack, 1994; Faulkes and Pollack, 2000). Multiple compound EPSP peaks are difficult to explain if only monosynaptic connections are present and receptor neurons have similar conduction velocities (Pollack and Faulkes, 1998). Brief ultrasound stimuli elicited only a single peak, even though, at high intensity, ultrasound stimuli may excite both low-frequency-tuned receptors and those tuned to ultrasound (Imaizumi and Pollack, 1999), which might be expected to evoke a second, delayed EPSP in ON1. Two factors might explain the absence of this second peak. First, the sensory input that ultrasound provides to low-frequency receptors is weak compared with that provided by low-frequency stimuli and, as a result, relatively few receptors might be expected to respond, causing a relatively small, delayed EPSP. Second, this delayed synaptic input would arrive on the falling phase of a much larger, ultrasound-induced EPSP, when it would be attenuated by both a decreased driving force and an increased membrane conductance. In contrast, the early EPSP elicited by intense low-frequency stimuli is relatively small and does not obscure the delayed peak. The relatively small size of this peak, at least at low-to-moderate sound intensities, may also explain why, despite the ability of low-frequency stimuli to activate a fast pathway to ON1, the latency of ON1 is long at these frequencies: the early EPSP may simply not be large enough to bring ON1 to threshold, at least over the intensity range that we have studied.

The possibility of a polysynaptic pathway to ON1 is consistent with recent findings on the anatomy of receptor neurons. The axon terminals of the most frequently sampled 4.5 kHz receptor neurons overlap little, if at all, with the dendrites of ON1 (Imaizumi and Pollack, 1996; Pollack and Imaizumi, 1999; Imaizumi and Pollack, 2001). If these receptor neurons provide input to ON1, then it seems probable that they do so *via* a polysynaptic pathway.

An implication of a polysynaptic pathway, of course, is that one or more low-frequency-tuned interneurons are interposed between low-frequency receptors and ON1. We speculate that these putative interneurons are nonspiking. If low-frequency input to ON1 were channelled through nonspiking interneurons, this might account for the difference in shapes of EPSPs evoked in ON1 by low and high sound frequencies. Graded transmitter release from nonspiking interneurons might produce the smooth, graded EPSPs that low-frequency stimuli elicit in ON1, whereas monosynaptic input from high-frequency-tuned receptors results in discrete, unitary EPSPs (Pollack, 1994; Fig. 6). Because they do not fire action potentials, nonspiking interneurons are inconspicuous in both extracellular and intracellular recordings, which might explain why few candidates for these intermediary neurons have yet been identified. Two nonspiking local auditory interneurons are known in the prothoracic ganglion of *Acheta domesticus*, and both are tuned to low-frequency stimuli (Stiedl et al., 1997).

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References

- Alexander, R. D. (1960). Sound communication in Orthoptera and Cicadidae. In Animal Sounds and Communication (ed. W. E. Layton and W. N. Tavolga), pp. 38–92. Washington: American Institute of Biological Sciences.
- Alexander, R. D. (1961). Aggressiveness, territoriality and sexual behavior in field crickets (Orthoptera: Gryllidae). *Behaviour* 17, 130–223.
- Amat, C. and Hue, B. (1997). Activation of picrotoxin-resistant GABA receptors and related compounds induces modulation of cockroach dorsal paired median (DPM) neuron firing. J. Insect Physiol. 43, 1125–1131.
- Atkins, G., Ligman, S., Burghardt, F. and Stout, J. F. (1984). Changes in phonotaxis by the female *Acheta domesticus* L. after killing identified acoustic interneurons. *J. Comp. Physiol.* A **154**, 795–804.
- Atkins, G. and Pollack, G. S. (1986). Age-dependent occurrence of an ascending axon on the omega neuron of the cricket, *Teleogryllus* oceanicus. J. Comp. Neurol. 243, 527–534.
- Balakrishnan, R. and Pollack, G. S. (1996). Recognition of courtship song in the field cricket, *Teleogryllus oceanicus*. Anim. Behav. 51, 353–366.
- Berry, M. S. and Pentreath, V. W. (1976). Criteria for distinguishing between monosynaptic and polysynaptic transmission. *Brain Res.* **105**, 1–20.
- Burrows, M. and Laurent, G. J. (1993). Synaptic potentials in the central terminals of locus proprioceptive afferents. *J. Neurosci.* 13, 808–819.
- **Casaday, G. B. and Hoy, R. R.** (1977). Auditory interneurons in the cricket *Teleogryllus oceanicus*: physiological and anatomical properties. *J. Comp. Physiol.* A **121**, 1–13.
- **Eibl, E. and Huber, F.** (1979). Central projections of tibial fibers within the three thoracic ganglia of crickets (*Gryllus campestris* L., *Gryllus bimaculatus* DeGeer). *Zoomorph.* **92**, 1–17.
- Faulkes, Z. and Pollack, G. S. (1997). Sound frequency-specific responses of cricket omega neuron. *Soc. Neurosci. Abstr.* 23, 1570.
- Faulkes, Z. and Pollack, G. S. (2000). The effects of omega neuron

1 (ON1) response delays in auditory circuits in crickets (*Teleogryllus oceanicus*). J. Neurophysiol. **84**, 1247–1255.

- Harrison, L., Horseman, G. and Lewis, B. (1988). The coding of the courtship song by an auditory interneurone in the cricket *Teleogryllus oceanicus* (Le Guillou). J. Comp. Physiol. A 163, 215–225.
- Hill, K. G., Loftus-Hills, J. J. and Gartside, D. F. (1972). Premating isolation between the Australian field crickets *Teleogryllus commodus* and *T. oceanicus* (Orthoptera: Gryllidae). *Aust. J. Zool.* 20, 153–163.
- Hirtz, R. and Wiese, K. (1997). Ultrastructure of synaptic contacts between identified neurons of the auditory pathway in *Gryllus bimaculatus* DeGeer. J. Comp. Neurol. 386, 347–357.
- Horseman, G. and Huber, F. (1994). Sound localisation in crickets. II. Modelling the role of a simple neural network in the prothoracic ganglion. J. Comp. Physiol. A 175, 399–413.
- Hoy, R. R., Popper, A. N. and Fay, R. R. (1998). (eds.) Comparative Hearing: Insects. New York: Springer-Verlag.
- Imaizumi, K. and Pollack, G. S. (1996). Anatomy and physiology of auditory receptors in the Australian field cricket *Teleogryllus* oceanicus. Soc. Neurosci. Abstr. 22, 1082.
- Imaizumi, K. and Pollack, G. S. (1999). Neural coding of sound frequency by cricket auditory receptors. *J. Neurosci.* **19**, 1508–1516.
- Imaizumi, K. and Pollack, G. S. (2001). Neural representation of sound amplitude by functionally different auditory receptors in crickets. J. Acoust. Soc. Am., in press.
- Moiseff, A. and Hoy, R. R. (1983). Sensitivity to ultrasound in an identified auditory interneuron in the cricket: a possible neural link to phonotactic behavior. J. Comp. Physiol. A 152, 155–167.
- Moiseff, A., Pollack, G. S. and Hoy, R. R. (1978). Steering responses of flying crickets to ultrasound: mate attraction and predator avoidance. *Proc. Natl. Acad. Sci. USA* **75**, 4052–4056.
- Nolen, T. G. and Hoy, R. R. (1986). Phonotaxis in flying crickets. I. Attraction to the calling song and avoidance of bat-like ultrasound are discrete behaviors. J. Comp. Physiol. A 159, 441–456.
- Nolen, T. G. and Hoy, R. R. (1987). Postsynaptic inhibition mediates high-frequency selectivity in the cricket *Teleogryllus oceanicus*: implications for flight phonotaxis behavior. *J. Neurosci.* 7, 2081–2096.
- **Pollack, G. S.** (1986). Discrimination of calling song models by the cricket, *Teleogryllus oceanicus*: the influence of sound direction on neural encoding of the stimulus temporal patterns and on phonotactic behavior. *J. Comp. Physiol.* A **158**, 549–561.
- Pollack, G. S. (1988). Selective attention in an insect auditory neuron. J. Neurosci. 8, 2635–2639.
- **Pollack, G. S.** (1994). Synaptic inputs to the omega neuron of the cricket *Teleogryllus oceanicus*: differences in EPSP waveforms evoke by low and high sound frequencies. *J. Comp. Physiol.* A **174**, 83–89.
- Pollack, G. S. (1997). SWEEPS: a program for the acquisition and analysis of neurophysiological data. *Comput. Meth. Programs Biomed.* 53, 163–173.
- Pollack, G. S. and Faulkes, Z. (1998). Representation of behaviorally relevant sound frequencies by auditory receptors in the cricket *Teleogryllus oceanicus*. J. Exp. Biol. 201, 155–163.
- Pollack, G. S. and Imaizumi, K. (1999). Frequency analysis by insect auditory systems. *BioEssays* 21, 295–303.

- Popov, A. V., Markovich, A. M. and Andjan, A. S. (1978). Auditory interneurons in the prothoracic ganglion of the cricket, *Gryllus bimaculatus*. I. The large segmental auditory neurons (LSAN). J. Comp. Physiol. A **126**, 183–192.
- Sattelle, D. B., Lummis, S. C. R., Wong, J. F. H. and Rauth, J. J. (1991). Pharmacology of insect GABA receptors. *Neurochem. Res.* **16**, 363–374.
- Sauer, A. E., Büschges, A. and Stein, W. (1997). Role of presynaptic inputs to proprioceptive afferent in tuning sensorimotor pathways of an insect joint control network. J. *Neurobiol.* 32, 359–376.
- Schildberger, K. and Hörner, M. (1988). The function of auditory neurons in cricket phonotaxis. I. Influence of hyperpolarization of identified neurons on sound localization. *J. Comp. Physiol.* A 163, 621–631.
- Schildberger, K., Milde, J. J. and Hörner, M. (1988). The function of auditory neurons in cricket phonotaxis. II. Modulation of auditory responses during locomotion. J. Comp. Physiol. A 163, 633–640.
- Selverston, A. I., Kleindienst, H.-U. and Huber, F. (1985). Synaptic connectivity between cricket auditory interneurons as studied by selective photoinactivation. *J. Neurosci.* **5**, 1283–1292.
- Skiebe, P., Corrette, B. J. and Wiese, K. (1990). Evidence that histamine is the inhibitory transmitter of the auditory interneuron ON1 in crickets. *Neurosci. Lett.* **116**, 361–366.
- Stiedl, O., Stumpner, A., Mbungu, D. N., Atkins, G. and Stout, J.
 F. (1997). Morphology and physiology of local auditory interneurons in the prothoracic ganglion of the cricket *Acheta domesticus*. J. Exp. Zool. 279, 43–53.
- Strausfeld, N. J., Seyan, H. S., Wohlers, D. and Bacon, J. P. (1983). Lucifer yellow histology. In *Functional Neuroanatomy* (ed. N. J. Strausfeld), pp. 132–155. Berlin: Springer-Verlag.
- Stumpner, A. (1998). Picrotoxin eliminates frequency sensitivity of an auditory interneuron in a bushcricket. J. Neurophysiol. 79, 2408–2415.
- Watson, A. H. D. (1992). Presynaptic modulation of sensory afferent in the invertebrate and vertebrate nervous system. *Comp. Biochem. Physiol.* 103A, 227–239.
- Watson, A. H. D. and Hardt, M. (1996). Distribution of synapses on two local auditory interneurones, ON1 and ON2, in the prothoracic ganglion of the cricket: relationships with GABAimmunoreactive neurones. *Cell Tissue Res.* 283, 231–246.
- Watson, A. H. D. and Hardt, M. (1999). Distribution of input and output synapses on central branches of bushcricket and cricket auditory afferent neurons: immunocytochemical evidence for GABA and glutamate in different populations of presynaptic boutons. J. Comp. Neurol. 403, 281–294.
- Wiese, K. (1981). Influence of vibration on cricket hearing: interaction of low frequency vibration and acoustic stimuli in the omega neuron. J. Comp. Physiol. A 143, 135–142.
- Wiese, K. and Eilts, K. (1985). Evidence for matched frequency dependence of bilateral inhibition in the auditory pathway of *Gryllus bimaculatus. Zool. Jb. Physiol.* 89, 181–201.
- Wohlers, D. W. and Huber, F. (1978). Intracellular recording and staining of cricket auditory interneurons (*Gryllus campestris* L., *Gryllus bimaculatus* DeGeer). J. Comp. Physiol. 127, 11–28.
- Wohlers, D. W. and Huber, F. (1982). Processing of sound signals by six types of neurons in the prothoracic ganglion of the cricket, *Gryllus campestris* L. J. Comp. Physiol. A 146, 161–173.