J Comp Physiol A (1998) 182: 703–714

ORIGINAL PAPER

T. Heinbockel · P. Kloppenburg · J. G. Hildebrand

Pheromone-evoked potentials and oscillations in the antennal lobes of the sphinx moth *Manduca sexta*

Accepted: 19 December 1997

Abstract Using intra- and extracellular recording methods, we studied the activity of pheromone-responsive projection neurons in the antennal lobe of the moth *Manduca sexta*. Intracellularly recorded responses of neurons to antennal stimulation with the pheromone blend characteristically included both inhibitory and excitatory stages of various strengths. To observe the activity of larger groups of neurons, we recorded responses extracellularly in the macroglomerular complex of the antennal lobe. The macroglomerular complex is part of a specialized olfactory subsystem and the site of first-order central processing of sex-pheromonal information. Odors such as the pheromone blend and host-plant (tobacco) volatiles gave rise to evoked potentials that were reproducible upon repeated antennal stimulation. Evoked potentials showed overriding high-frequency oscillations when the antenna was stimulated with the pheromone blend or with either one of the two key pheromone components. The frequency of the oscillations was in the range of 30–50 Hz. Amplitude and frequency of the oscillations varied during the response to pheromonal stimulation. Recording intracellular and extracellular activity simultaneously revealed phase-locking of action potentials to potential oscillations. The results suggest that the activity of neurons of the macroglomerular complex was temporally synchronized, potentially to strengthen the pheromone signal and to improve olfactory perception.

T. Heinbockel[1] · P. Kloppenburg[2] · J.G. Hildebrand (✉)
Arizona Research Laboratories Division of Neurobiology, University of Arizona, Gould-Simpson 611, P.O. Box 210077, Tucson, AZ 85721-0077, USA
Tel.: +1-520 621-6626; Fax: +1-520 621-8282
e-mail: jgh@neurobio.arizona.edu

Present addresses:
[1] Otto-von-Guericke-Universität, Institut für Physiologie, Leipziger Str. 44, D-39120 Magdeburg, Germany

[2] Section of Neurobiology and Behavior, Cornell University, Seeley G. Mudd Hall, Ithaca, NY 14853, USA

Key words Glomerulus · Evoked potential · Olfaction · Oscillation · Pheromone

Abbreviations *AL* antennal lobe · *Bal* bombykal · *C-15* (E,Z)-11,13-pentadecadienal · *CSD* current source density · *MGC* macroglomerular complex · *MGC-PN* projection neuron of the MGC · *PN* projection neuron

Introduction

Coherent rhythmic activity patterns of neurons have been observed in various regions of the vertebrate brain (Singer 1993; Gray 1994; Singer and Gray 1995). Rhythmic activities measured extracellularly as field potential oscillations vary in form and frequency. These oscillations are thought to arise from temporal synchrony of neuronal discharge or synaptic potentials in groups of neurons (Gray 1994). Possible functions of these activities include the integration of distributed processes in the nervous system and temporal coordination of activity within and between subsystems in the CNS (Gelperin 1989; Singer 1993; Gray 1994; Singer and Gray 1995; Gelperin et al. 1996). Adrian (1942) described a rhythmic fluctuation of voltage in the olfactory bulb, now known as the induced wave (reviewed in Gray 1994). Evoked by odor stimulation of the olfactory receptor sheet, the induced wave has been observed in the olfactory bulbs of amphibia, fish, and mammals.

The anatomical similarity of the primary olfactory centers in the brains of diverse animals, such as the antennal lobes (ALs) of insects and the olfactory bulbs of vertebrates, has led several authors to speculate that mechanisms to encode olfactory information also might be comparable. Such similarity of organization and function of olfactory circuitry might have resulted in the operation of common computational rules in the olfactory systems of phylogenetically distant animal groups (Shepherd 1992; Hildebrand 1995; Laurent 1996a, b; Gelperin et al. 1996).

Although synchronous rhythmic activity has been investigated in the olfactory systems of vertebrates for many years, studies of the central olfactory pathway in insects have emphasized morphological and physiological investigations of individual olfactory interneurons or interacting pairs of neurons (e.g., Boeckh et al. 1990; Hansson 1995; Christensen et al. 1996; Hildebrand 1996). Recent evidence suggests, however, that brain oscillations occur in the olfactory pathways of invertebrates, including the procerebrum of a snail (Gelperin 1989; Gelperin and Tank 1990), the hemi-ellipsoid neuropil in the lateral protocerebrum of a crayfish (Mellon et al. 1992), the ALs and mushroom bodies of a locust (Laurent and Davidowitz 1994; Laurent and Naraghi 1994; Laurent et al. 1996), and the ALs of a moth (Wu et al. 1995). Potential oscillations of assemblies of neurons have been implicated in coding information about odorants (Hopfield 1991; Laurent and Davidowitz 1994; Laurent 1996a, b; Laurent et al. 1996).

Work on locusts and snails has dealt with general olfactory systems that detect and process information about a large variety of odors. We focus in this study on a highly specialized olfactory subsystem, the macroglomerular complex (MGC) in the AL of the male sphinx moth *Manduca sexta* (Hildebrand 1996). The AL of male *M. sexta* contains 64 ± 1 "ordinary" spheroidal glomeruli and the sexually dimorphic MGC, which is the first-order site of synaptic processing of sex-pheromonal information (Christensen and Hildebrand 1987; Christensen et al. 1989; Rospars and Hildebrand 1992). The MGC comprises three glomeruli (Hansson et al. 1991; Heinbockel et al. 1995, 1996; Homberg et al. 1995; N.J. Strausfeld, personal communication). The two major glomeruli of the MGC, which have been called the "cumulus" and the "toroid" based on their shapes, each receive input from antennal olfactory receptor cells specifically responsive to one of the two essential components of the sex pheromone (Kaissling et al. 1989; Hansson et al. 1991; Christensen et al. 1995). Projection neurons (PNs) with axons projecting from the MGC to the protocerebrum respond to either one or both of the two key pheromone components, and these PNs have arborizations in the toroid or cumulus or both, correlating with their pheromonal responsiveness (Hansson et al. 1991). The function of the third glomerulus of the MGC is still unknown (Heinbockel et al. 1996).

Here, we demonstrate that slow changes and fast oscillations of the extracellular potential can be induced in this specialized olfactory subsystem by stimulation of the ipsilateral antenna with the pheromone blend or individual pheromone components. Oscillations appear to indicate synchrony of larger groups of PNs and may facilitate olfactory coding.

Materials and methods

Preparation of animals

M. sexta (Lepidoptera: Sphingidae) were reared on an artificial diet (modified from that of Bell and Joachim 1976) at 25 °C and 50–60% relative humidity under a long-day photoperiod regimen (17 h:7 h light:dark) as described previously (Christensen and Hildebrand 1987). Adult male moths (1–3 days post-eclosion) were used for all experiments. Details of the preparation have been described elsewhere (Christensen and Hildebrand 1987). With the antennae and their innervation intact, the head was separated from the rest of the body and pinned in a Sylgard-coated (Dow Corning) recording chamber (volume < 0.5 ml) (Fig. 1). To facilitate insertion of the recording electrode into the tissue, part of the AL was desheathed manually. The brain was superfused constantly with physiological saline solution (ca. 2 ml min^{-1}), modified from that of Pichon et al. (1972) and containing ($mmol \cdot l^{-1}$): 149.9 NaCl, 3.0 KCl, 3.0 $CaCl_2$, 10.0 TES (pH 6.9), and 25.0 sucrose to balance osmolarity with that of the extracellular fluid.

Intracellular recording and staining

Borosilicate glass capillary electrodes (o.d. 1.0 mm, i.d. 0.5 mm; World Precision Instruments) were produced with a Flaming-Brown Puller (P-2000, Sutter Instrument). The tip of each glass electrode was filled with dye solution (see below), and the electrode shaft was filled with filtered (0.2 μm pore size) 2.5 $mol \cdot l^{-1}$ KCl solution. Electrodes had resistances of 60–100 MΩ, measured in tissue. Intracellular recordings were made from neurites in the neuropil of the MGC. Intracellular recordings and current injections were carried out in bridge mode with an Axoclamp-2A amplifier (Axon Instruments). Data were digitized at 5–10 kHz with a TL1 A/D board (Axon Instruments) and pCLAMP6 software (Axon Instruments) run on an i30386 microcomputer and stored on disk. The software programs pCLAMP6, Axograph2 (Axon Instruments), and Origin 3.7 (Microcal Software) were used for data analysis.

If the quality of the electrode impalement after physiological experimentation allowed, neurons were injected iontophoretically with either neurobiotin (Vector Laboratories, 3–5% in 2 $mol \cdot l^{-1}$ KCl with 0.05 $mol \cdot l^{-1}$ TRIS buffer, pH 7.4) or biocytin (Sigma, 3–

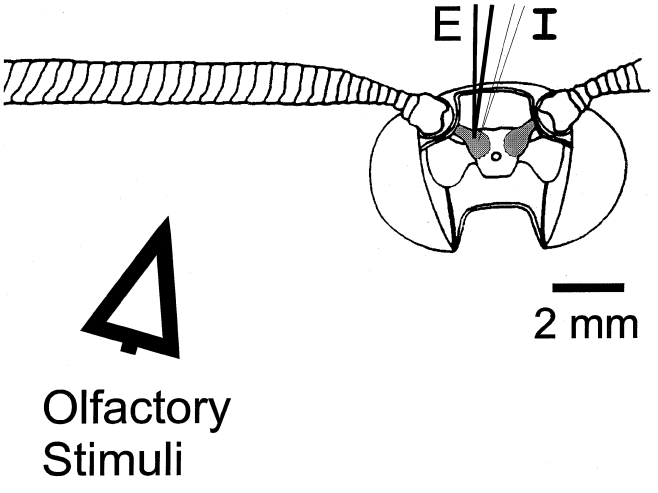


Fig. 1 Schematic representation of the experimental set-up. Neuronal activity was recorded with an extracellular (*E*) and/or intracellular (*I*) electrode in one antennal lobe (*antennal lobes are shaded*). The basal region of the ipsilateral antenna was stimulated with olfactory stimuli (pheromone or tobacco volatiles)

5% in 2 mol·l^{-1} KCl with 0.05 mol·l^{-1} TRIS buffer, pH 7.4). Alternating hyperpolarizing and depolarizing 30-s, 1-nA current pulses for about 10 min were used to inject dye. Brains were dissected and fixed overnight in 2.5% formaldehyde with 3% sucrose in 0.1 mol·l^{-1} phosphate buffer. Neurons stained with neurobiotin or biocytin were visualized by incubating brains with Cy3-conjugated streptavidin (Jackson Immunologicals, diluted 1:100 with 0.2 mol·l^{-1} phosphate buffer containing 0.3% Triton X-100) for 3 days on a shaker at 4 °C. The brains were dehydrated in increasing concentrations of alcohol and cleared in methylsalicylate. Brains were first inspected as whole mounts with a fluorescence microscope (Nikon), and those with successfully stained neurons were further investigated with a laser scanning confocal microscope (BioRad MRC 600).

Recording of evoked potentials

Methods of recording evoked potentials in the insect brain have been described elsewhere (Kaulen et al. 1984; Laurent and Naraghi 1994; Kloppenburg and Erber 1995). For our studies borosilicate glass electrodes (patch pipettes) were produced from 100-µl capillary micropipettes (o.d. 1.71 mm, i.d. 1.32 mm, VWR Scientific) with a Flaming-Brown Puller (P-87, Sutter Instrument). During recording the electrodes were placed in the MGC region of the AL about 100 µm below the surface of the AL (Burleigh Inchworm), thus being in the central region of the MGC and not in the afferent fiber bundle. The MGC can be distinguished from the rest of the AL because of different opacity. Electrode position was approximately the same among different preparations. The electrodes were filled with filtered (0.2 µm pore size) extracellular saline solution (see above) and had resistances of 1–5 MΩ, measured in saline. The signals were amplified using an Axoclamp-2A amplifier (Axon Instruments) in bridge mode, digitized (5–10 kHz), and stored on a computer hard disk with the aid of the software program pCLAMP6 (Axon Instruments) and the TL1 A/D board (Axon Instruments). The software programs pCLAMP6, Axograph2 (Axon Instruments), and Origin 3.7 (Microcal Software) were used for data analysis. For filtering and smoothing we used the Savitzky-Golay method (see Press et al. 1988, implemented in Origin). To control for phase shifts caused by filtering, we usually displayed both the original and the filtered trace. To determine the oscillation frequency, potential changes were considered as oscillations when the peak shifts in potential were larger than twice the noise of the filtered trace and when the peaks were separated by potentials crossing the baseline.

Odor stimulation

The ipsilateral antenna was stimulated in the basal region with one of the following: (1) E,Z-10,12-hexadecadienal, (bombykal, Bal), one of the two essential components of the female sex pheromone, 10 ng (Starratt et al. 1979; Christensen et al. 1989; Kaissling et al. 1989; Tumlinson et al. 1989), (2) E,Z-11,13-pentadecadienal (''C-15''), a relatively stable mimic of the second essential pheromone component (E,E,Z-10,12,14-hexadecatrienal), 10 ng (Kaissling et al. 1989), (3) a mixture of both (Bal + C-15), and (4) crushed tobacco leaves (2 cm^2 leaf area). The odor components were dissolved in hexane and applied to a piece of filter paper (1 cm × 2 cm), which was inserted into a glass cartridge (acid-cleaned glass syringe barrels). A pulse of air, controlled by a computer-activated (pCLAMP, TL1 interface) solenoid-driven valve (General Valve), moving through the cartridge carried the odor stimulus to the antenna.

To test if the elicited responses were due to mechanical stimulation, we stimulated the antenna with clean air (cartridges containing clean filter paper, blank control). Because odorants were dissolved in hexane, we also tested all preparations with cartridges carrying hexane-loaded filter paper (solvent control). The responses observed with solvent control were not different from the responses seen with blank control.

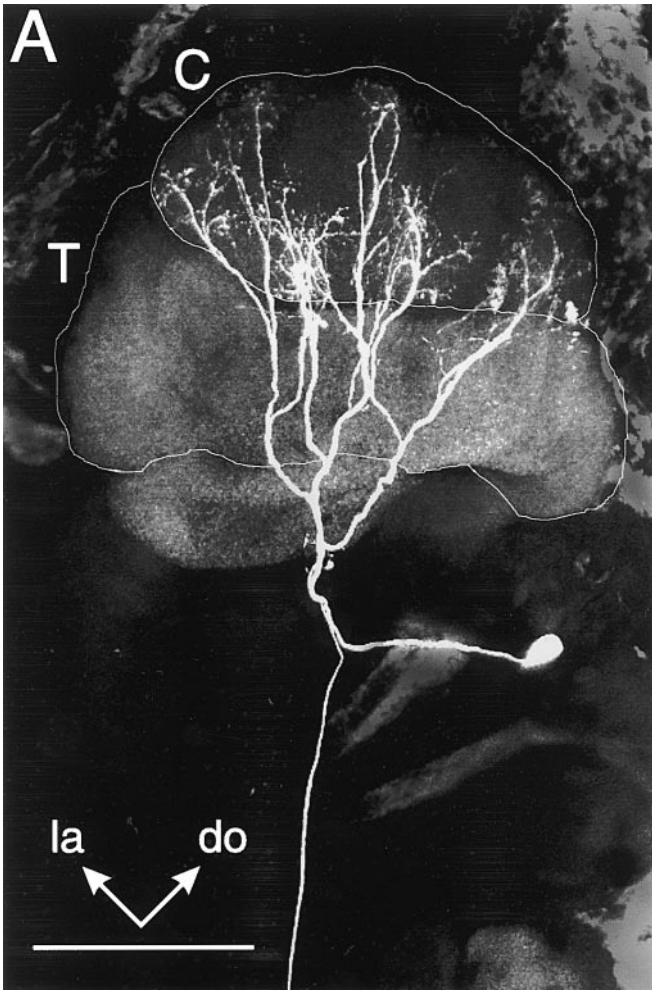
Results

Morphology and physiology of pheromone-responsive MGC projection neurons

Pheromone-responsive projection neurons of the MGC (MGC-PNs) encode pheromonal information (Christensen and Hildebrand 1987; Christensen et al. 1989; Hansson et al. 1991) and form the principal output pathway from the MGC to higher brain centers such as the mushroom bodies and the lateral horn of the protocerebrum (Homberg et al. 1988, 1989). The activity of MGC-PNs is a reflection of the processes that take place in the MGC circuitry. MGC-PNs gave an excitatory response with a burst of action potentials followed by an afterhyperpolarization to stimulation of the ipsilateral antenna with the pheromone blend (Figs. 2–4). The EPSP was often very small, probably due to the position of the recording site relative to the site of synaptic input. The excitatory response often was preceded by an initial hyperpolarization (Fig. 2B) of variable amplitude (Christensen et al. 1996; Christensen and Hildebrand 1997; T. Heinbockel, unpublished results). PNs with arborizations in the cumulus typically gave an excitatory response, or a mixed inhibitory/excitatory response followed by an afterhyperpolarization, to antennal stimulation with the pheromone mimic C-15 (Fig. 2). Some of these PNs were inhibited by Bal. PNs arborizing in the toroid showed the inverse response pattern to stimulation with Bal and C-15 (Fig. 3). These PNs gave primarily excitatory responses to antennal stimulation with the pheromone blend or Bal, and some were inhibited by C-15. PNs innervating both toroid and cumulus responded with excitation or mixed inhibition/excitation to both Bal and C-15 (Fig. 4). The response to the blend was stronger than the responses to the individual components, but it was substantially weaker than the sum of the responses to the individual components.

Odor-evoked field potentials and oscillations

Pheromonal stimulation of the antenna evoked extracellular potential changes in the MGC region. Examples of such responses from four different animals are presented in Fig. 5A–D. The form and polarity of the evoked potentials were different among different ALs and varied with the location of the recording site in the AL. In some preparations antennal stimulation with pheromone resulted in positive deflections of the potential (Fig. 5A, B), and in others, the potential change was in the negative direction (Fig. 5C, D). Pheromonal stimuli induced oscillations and always resulted in evoked potentials of larger amplitude than control stimulations with clean air. The potential change typically showed a short initial rising or falling phase until it reached a plateau. In some cases the plateau was maintained during stimulation with pheromone. After stimulation the potential slowly returned to the pre-



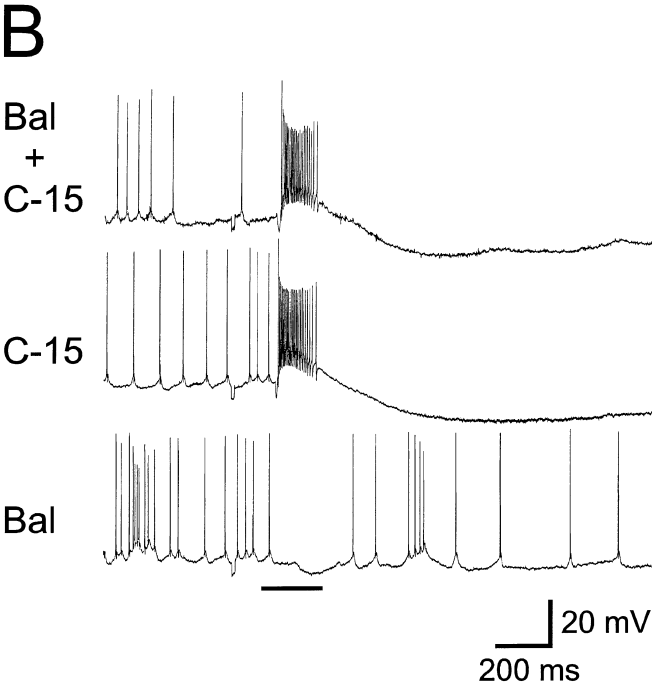


Fig. 2A, B Projection neuron with arborizations in the cumulus (*C*). Laser scanning confocal image **A** and intracellular responses to antennal stimulation with pheromone **B**. The dendritic branches of the neuron spanned the cumulus but did not reach into the toroid (*T*). *Scale bar*: 100 μm; *do* dorsal; *la* lateral. The responses consisted of mixed inhibition/excitation/inhibition to stimulation with the pheromone blend and C-15. No response, or possibly suppression of firing, was observed upon stimulation with bombykal (Bal). The responses were preceded by a calibration pulse. *Stimulus bar* (200 ms) beneath the record

◀

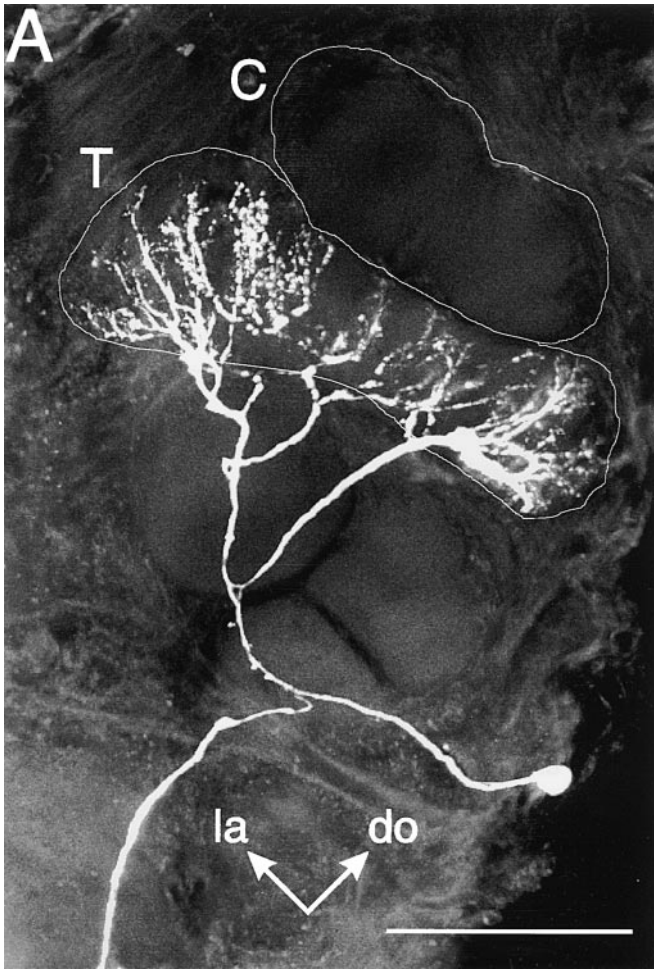
stimulus level. Often fast potential oscillations with a mean frequency (determined as the mean frequency during the entire response) of 30–50 Hz were superimposed on the evoked potentials. Peak frequencies of the oscillations during short periods of the response exceeded 50 Hz (Fig. 6). While the form and amplitude of the oscillations varied among different preparations, their occurrence was observed repeatedly. In some preparations the first pheromonal stimulus did not evoke oscillations but subsequent stimuli did (see Discussion).

Effect of different odors on potentials and oscillations

The quality of the stimulus affected the evoked potentials and oscillations (Fig. 7A–F). In this experiment different stimuli were tested on one animal with the extracellular electrode in the same position in the MGC throughout the experiment. Each stimulus was tested two (blank) or three (odorants) times. The response to the pheromone blend was tested at the beginning and end of the stimulus series (Fig. 7B, F). Olfactory stimulation resulted in strong evoked potentials, whereas clean air elicited small changes of the extracellular potential. A response to clean air was not seen in all preparations. Potentially this was a mechanosensitive response, as mechanical responses were sometimes seen in intracellular recordings from central olfactory neurons (unpublished results). Oscillations occurred only in response to pheromonal stimulation and not to stimulation with tobacco (hostplant) volatiles (Fig. 7B, C). Antennal stimulation with individual pheromone components also resulted in potential oscillations. The frequency and amplitude of the oscillation changed during the response to pheromonal stimulation (Fig. 7B) and, in response to the blend, were different (Fig. 7B, F) from those evoked by stimulation with individual components (Fig. 7D, E). The observed responses were highly reproducible upon repeated stimulation with a given stimulus and stimulation with the pheromone blend using a different odor cartridge at the end of the experiment (Fig. 7F).

Oscillations phase-locked to action potentials

Paired recordings of evoked potentials and intracellular activity of MGC-PNs often but not always revealed



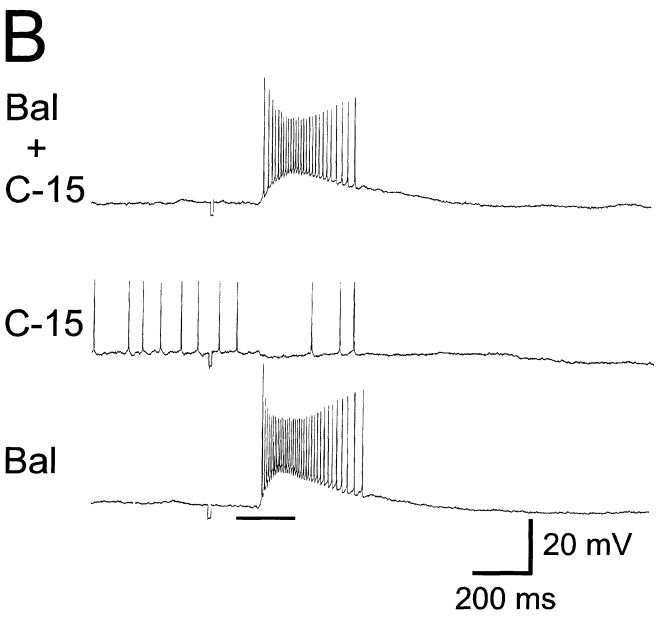


Fig. 3A, B Projection neuron with arborizations in the toroid (*T*) but not the cumulus (*C*). Laser scanning confocal image **A** and intracellular responses to antennal stimulation with pheromone **B**. The responses consisted of mixed inhibition/excitation/inhibition to the pheromone blend and Bal but no response (or possibly inhibition) to C-15. The responses were preceded by a calibration pulse. *Stimulus bar* (200 ms) beneath the record; scale bar **A**: 100 μm; *do* dorsal; *la* lateral

◀

phase-locking of the oscillations to action potentials (Fig. 8A, B). In recordings where phase locking was observed, each peak of the oscillations was in phase with an action potential recorded intracellularly (Fig. 8A). The temporal relationship between peaks of the oscillations and action potentials was the same for a given paired recording but could be different in other paired recordings.

The instantaneous frequency of firing in an MGC-PN could be much higher (> 100 Hz) than the frequency of the fast extracellular oscillations (30–50 Hz), and not every action potential was phase locked to an extracellular potential oscillation. Phase locking suggests synchrony of firing of the MGC-PN with activity of many other neurons in the population.

Discussion

The MGC and the PNs innervating it are prominent parts of a specialized olfactory subsystem, the sex-pheromone processing system in male sphinx moths. The MGC is innervated by local interneurons (Matsumoto and Hildebrand 1981) as well as PNs that respond to antennal stimulation with the pheromone blend or individual pheromone components. Based on counts of PN somata in the medial cell group in ALs of male and female *M. sexta*, it has been estimated that 30–40 PNs innervate the MGC (Homberg et al. 1988). Some additional PNs with somata in the lateral cell group of the AL innervate the MGC as well (Heinbockel et al. 1996). Comparing the number of MGC-PNs with somata in the lateral cell group to the number of MGC-PNs with somata in the medial cell group suggests that there are fewer than 50 MGC-PNs in an AL. These neurons are the primary output elements of the MGC, comparable with mitral/tufted cells in the vertebrate olfactory bulb.

The response pattern of an MGC-PN to pheromonal stimulation is correlated with its dendritic arborization in the cumulus, toroid, or both (Hansson et al. 1991). Whereas antennal stimulation with the pheromone blend typically gives rise to a mixed response pattern (Christensen and Hildebrand 1987; Heinbockel et al. 1995), stimulation with individual pheromone components elicited opposite response patterns in PNs innervating the cumulus or toroid (Figs. 1, 2), and similar responses to each essential pheromone component in PNs arborizing in both cumulus and toroid (Fig. 3). Responses of MGC-PNs to pheromonal stimulation were generally characterized by an interplay of inhibition and excitation. Typically, early hyperpolarization was followed by excitation and then long-lasting afterhyperpolarization. No MGC-PN was observed that showed responses comprising several excitatory response phases interrupted by

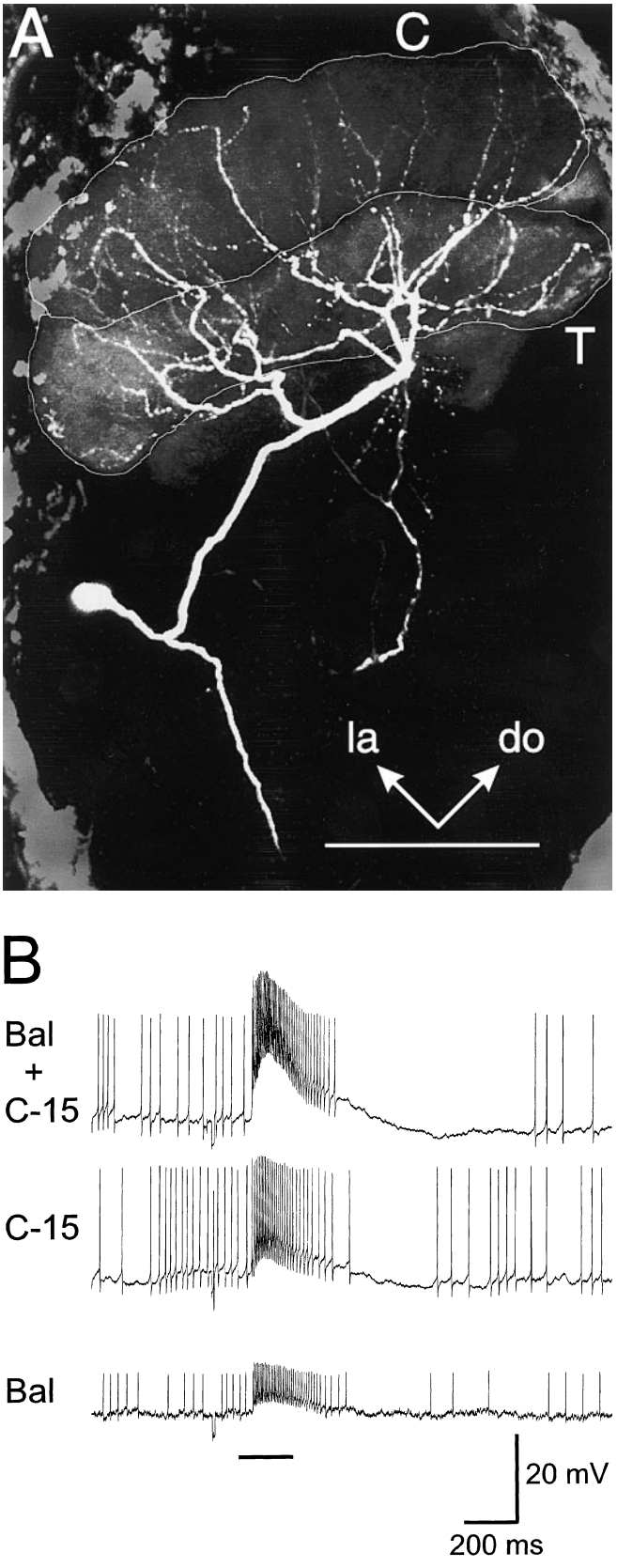


Fig. 4A, B Projection neuron with arborizations in both the cumulus (*C*) and the toroid (*T*). Laser scanning confocal image **A** and intracellular responses to antennal stimulation with pheromone **B**. The responses consisted of excitation/inhibition to either the pheromone blend or the individual pheromone components. The amplitude of the action potentials during the response to Bal was smaller probably due to run-down of the recording at the end of the experiment. The responses were preceded by a calibration pulse. *Stimulus bar* (200 ms) beneath the record; *scale bar* **A**: 100 μm; *do* dorsal; *la* lateral

◀

inhibitory ones as has been described for generalist odor responsive PNs in the AL of the locust (Laurent and Davidowitz 1994; Laurent et al. 1996). In *M. sexta* no PNs innervating ordinary glomeruli have been found to be excited in response to antennal stimulation with pheromone (Christensen and Hildebrand 1987), but some PNs with arborizations in ordinary glomeruli close to the MGC were inhibited by pheromonal stimulation (T. Heinbockel, unpublished results).

The olfactory systems of invertebrates and vertebrates are similar with respect to transduction processes in receptor cells of the olfactory epithelium, glomerular architecture and synaptic circuitry of the primary olfactory centers in the CNS, physiological responses of the principal output neurons in antennal lobe and olfactory bulb (Boeckh et al. 1990; Hildebrand 1996; Laurent 1996a, b), and network properties such as oscillations and firing synchronization of AL or olfactory-bulb output neurons (Tank et al. 1994; Laurent 1996a, b; Gelperin et al. 1996). The role of oscillations in odor processing is still under investigation (Gray 1994; Laurent 1996a, b; Gelperin et al. 1996). In *Limax*, oscillations and activity waves occur in the procerebrum in the resting state. The phase gradient of the waves collapses in response to odor stimulation (Gelperin et al. 1996). In the AL of the locust, a variety of general odorants can induce oscillations that have the same frequency (Laurent and Davidowitz 1994; Laurent et al. 1996). By recording extracellularly in the MGC region of the AL of *M. sexta*, we observed evoked potentials in response to antennal stimulation with pheromone, consisting of a sustained component overlaid by faster oscillatory potential changes. The occurrence of odor-induced potential oscillations in the AL of *M. sexta* supports the idea that oscillations could be a general phenomenon in insects as well as in other invertebrates and vertebrates (Laurent 1996b; Laurent et al. 1996). As has been observed previously in the mushroom bodies of the locust with some odorants the first odor stimulus did not always evoke oscillations. This might indicate rapid plastic changes in the brain (Laurent and Naraghi 1994).

The mean frequencies of the oscillations in the MGC were in the range of 30–50 Hz, which is comparable to the frequencies observed in the AL of the locust (20–30 Hz) (Laurent 1996a, b) and substantially higher than the 0.7-Hz oscillations found in *Limax* (Gelperin et al. 1996). In the MGC, no oscillations were recorded in response to antennal stimulation with tobacco-leaf volatiles. Extracellular potential changes in response to such hostplant odors might reflect the activity of PNs in other regions of the AL or unsynchronized activity in

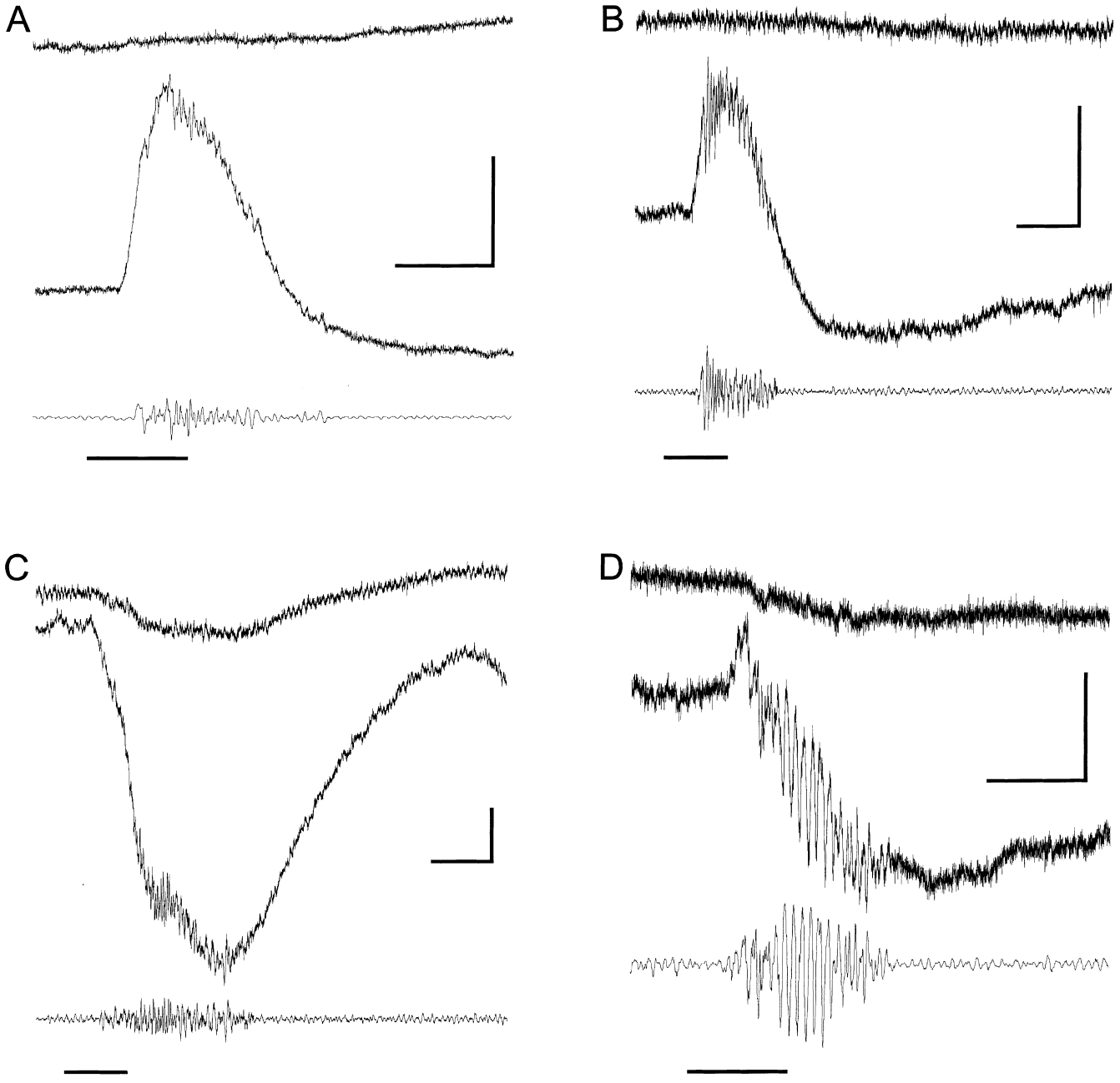


Fig. 5A–D Extracellular potentials recorded in the MGC of four different preparations. The first record in each of **A–D** shows the response to clean air; the second record depicts the response to the pheromone blend; and the third record is a filtered version of the second record after low-pass (ca. 90 Hz) and high-pass (ca. 3 Hz) filtering to improve visualization of high-frequency potential oscillations. In **A** and **B**, antennal stimulation with the pheromone blend resulted in an upward deflection of the extracellularly measured potential, whereas in **C** and **D**, the result was a downward deflection of the potential. In all four cases filtering of the responses revealed high-frequency potential oscillations. *Stimulus bar* (300 ms) beneath the record; *scale bars*: 300 ms, 0.2 mV

the MGC. The amplitude and frequency of the oscillations in the MGC were not constant throughout the response to pheromonal stimulation, and for short periods the frequency exceeded 50 Hz. As in the locust (Laurent and Davidowitz 1994; Laurent et al. 1996), intracellular recordings from MGC-PNs revealed that the neuronal discharge in individual PNs can be phase locked to the oscillatory activity measured extracellularly and therefore possibly synchronized with the responses of many other neurons. The temporal relationship between oscillatory and intracellular activity was the same for a given paired recording but can be different in other paired recordings as has also been observed in the locust olfactory system (Laurent and Naraghi 1994; MacLeod and Laurent 1996).

Evoked potentials have been recorded extracellularly in different neuropils of the insect nervous system to stimuli of various modalities (e.g., Kaulen 1982; Mercer and Erber 1983; Kaulen et al. 1984; Kloppenburg 1990;

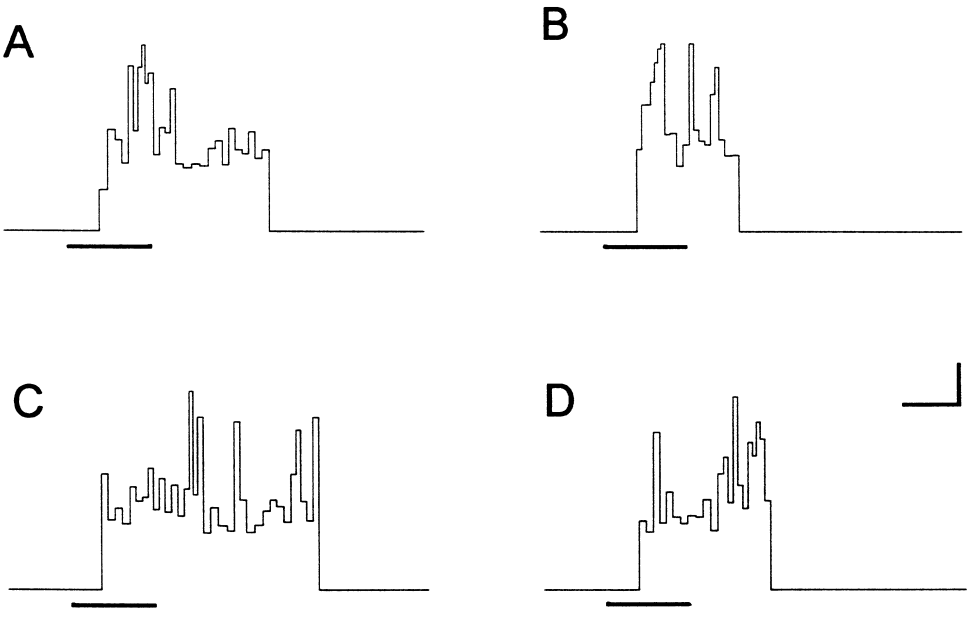


Fig. 6A–D Instantaneous frequency of the oscillations from four different preparations during and after stimulation with the pheromone blend (Bal + C-15). *Stimulus bar* (300 ms) beneath the trace; *scale bars*: 200 ms, 20 Hz; *baseline* in all four traces: 0 Hz

Rössler et al. 1990; Laurent and Naraghi 1994; Laurent and Davidowitz 1994). Evoked potentials are thought to result from summed postsynaptic membrane currents of many neurons (Freeman and Nicholson 1975; Nicholson and Freeman 1975; Nicholson and Llinas 1975; Mitzdorf and Singer 1977; Zimmerman 1978; Lai-Wo Stan Leung 1979; Habets et al. 1980). Net inward and outward currents of action potentials are believed to be too small to contribute significantly to these potentials. In the insect AL potentials evoked by stimulating the ipsilateral antenna with odors can be recorded extracellularly (Laurent and Naraghi 1994; Laurent and Davidowitz 1994; this paper). Evoked potentials can be generated by near and distant current sources (Nunez 1981). To determine the precise location of current sources and sinks, it is necessary to perform a three-dimensional current source density analysis (CSD). To our knowledge this type of analysis has not been performed in the ALs of an insect. One reason for this lack of information might be the inhomogeneous structure of the AL, which makes a reasonably precise CSD very challenging. Despite the lack of a precise CSD, recordings at different sites in the of an insect suggest that the odor-evoked potentials including the oscillations are indeed generated in the AL (Laurent and Davidowitz 1994; this paper). In *M. sexta*, the region where the oscillatory component of the pheromone-evoked potential is generated can be narrowed down further, namely to a specific region of the AL, the MGC.

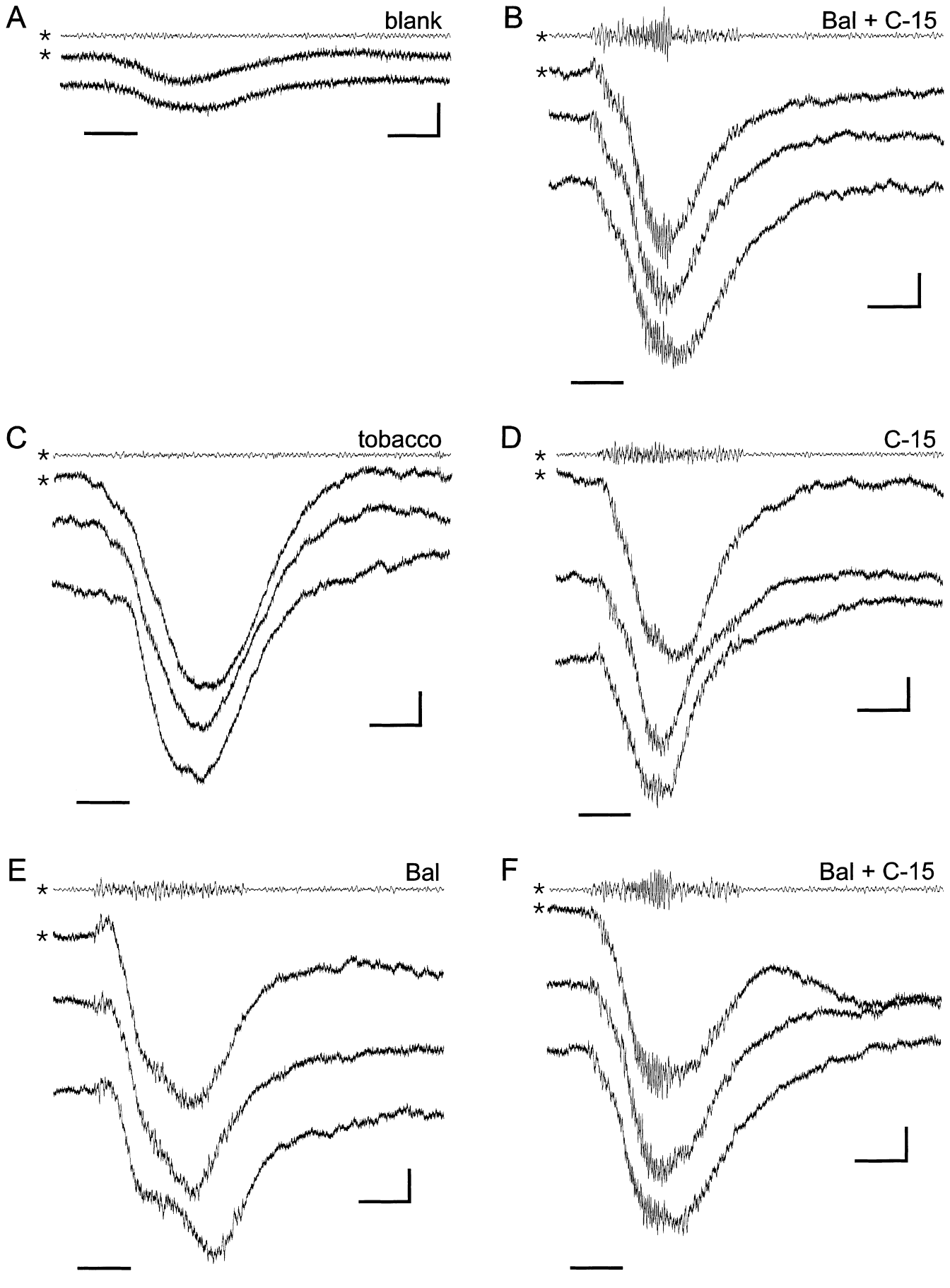
The extracellular potentials evoked by pheromonal stimulation often changed shape and/or polarity when the position of the electrode was altered and often were different among several preparations. This was the case even though we attempted to obtain the same electrode position in different preparations and thus to maintain high reproducibility of the recording site. Potentially the differences in polarity reflected differences of the recording site in relation to current sources and sinks, correlated with the presence of layers and tracts of neurons as described in the mushroom bodies of the bee (Kaulen et al. 1984). Similarly, the MGC is a complex neuropil with several distinct glomeruli, each with a lobular structure and fascicles of output neurons (Homberg et al. 1988, 1995; Strausfeld 1989; Hansson et al. 1991; Heinbockel et al. 1994, 1995). The observation that small changes of the recording site can have drastic effects on the recorded signal suggests a high density of current sources and sinks in the AL.

It is generally thought that the olfactory system of male moths consists of two subsystems (Hildebrand 1995), one that processes plant odors encoded in across-fiber patterns of activity, and the other a sexually dimorphic system that processes sex-pheromonal information in a labeled-line pathway. The occurrence of oscillations and phase-locking of intracellular activity to population activity in the ALs of *M. sexta* and the locust, suggests a basic function for oscillations in synaptic processing.

It is likely that the odors tested in the locust (Laurent and Davidowitz 1994; Laurent et al. 1996) are processed

►

Fig. 7A–F Extracellular potentials in response to odor measured at the same recording site in the MGC of one preparation. The ipsilateral antenna was stimulated consecutively with air (blank) **A**, the pheromone blend **B**, tobacco volatiles **C**, C-15 **D**, Bal **E**, and again with the pheromone blend **F**. Each odor stimulus was tested three times with 1-min intervals between stimuli. The blank stimulus was tested twice. In each of **A–F**, the first trace (marked by an *asterisk*) shows the response to the first stimulation after low- and high-pass filtering to visualize potential oscillations. The second trace (also marked by an *asterisk*) illustrates the same response, unfiltered. The third and fourth traces depict repeated stimulations with the same stimulus to verify the reproducibility of the response. Clear potential deflections were seen in response to each odor stimulus, and potential oscillations were observed during stimulation with the pheromone blend and individual pheromone components. *Stimulus bar* (300 ms) beneath the record; *scale bars*: 300 ms, 0.2 mV


A
blank
B
Bal + C-15
C
tobacco
D
C-15
E
Bal
F
Bal + C-15

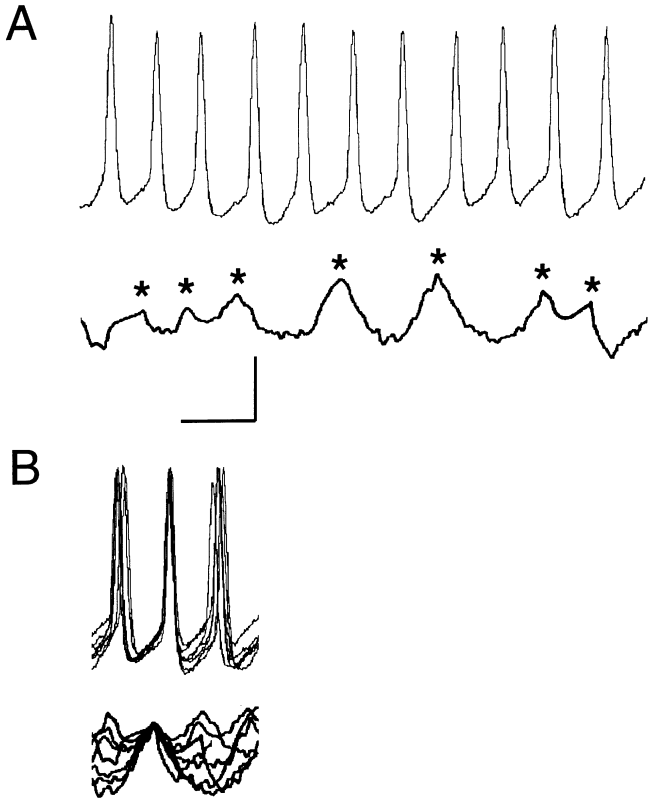


Fig. 8A, B Paired extra- and intracellular recordings in one AL. **A** During antennal stimulation with the pheromone blend, each peak of the potential oscillations (*asterisk*) measured extracellularly (*lower trace*) was in phase with an action potential measured intracellularly in a PN (*upper trace*). **B** Overlying the peaks of the extracellular potential oscillations from Fig. 8A revealed phase-locking of oscillations and action potentials. *Scale bars*: 30 ms, 15 mV (*upper trace*), 0.2 mV (*lower trace*)

through ensemble coding involving output neurons innervating a number of glomeruli. In the locust AL it has been proposed that odors are represented by a dynamic representation in transiently overlapping assemblies of neurons (Laurent and Davidowitz 1994; Laurent 1996; Laurent et al. 1996). Activity is thought to be distributed over time and among different groups of neurons, such that individual neurons participate in the oscillating population during odor-specific time windows (Laurent 1996). In *M. sexta*, pheromonal information is provided to the MGC by highly tuned receptor cells specific for each of the two essential pheromone components. The information is processed by a relatively small population of neurons innervating the cumulus and toroid of the MGC. The responses of MGC-PNs to pheromonal stimulation vary in the duration and strength of their inhibitory and excitatory response phases (Christensen et al. 1996) but generally occur in the same time window. From wind-tunnel experiments it is clear that only a blend of both essential pheromone components elicits the characteristic behavioral responses shown by male *M. sexta* in response to the natural pheromone blend (Tumlinson et al. 1989). In another moth species it was shown that the complete pheromone blend has to arrive at the antenna simultaneously to evoke significant levels of sustained upwind flight (Vickers and Baker 1992). Both studies indicate the importance of simultaneous processes in different information pathways, suggesting that the input on the labelled-line pathways for the essential pheromone components needs to be synchronized to influence neuronal target populations. MGC-PNs could act as coincidence detectors for temporal coincidence of synaptic input as has been proposed for the cortex (reviewed by König et al. 1996). Temporal synchrony might help to integrate information about the labeled lines into coherent representational patterns and could be a requirement to evoke behavioral responses.

Oscillations have been interpreted as the temporal synchronization of activity in a population of neurons (Singer 1993; Gray 1994; Singer and Gray 1995). While the oscillatory structure of activity appears to be an important parameter, the oscillations per se and their frequency are not thought to be relevant for olfactory coding (Gray 1994; Laurent 1996). As discussed by Singer (1993) and Singer and Gray (1995), summation of activity by synchronizing inputs may be a more effective means of enhancing synaptic transmission than increasing neuronal firing rates, and synchronization indicates which neurons belong together. They suggest that synchronous responses are likely to result in very effective responses in neuronal target populations. The result could be signal enhancement by means of synaptic strengthening and potentially could improve olfactory perception.

Acknowledgments We are grateful to Drs. T. A. Christensen, A. Gelperin, and G. Laurent for encouragement and discussions. We greatly appreciate helpful suggestions and comments on the manuscript by Dr. G. Laurent and anonymous reviewers. We thank Peggy Randolph for general laboratory assistance, Dr. A. A. Osman for insect rearing, and Drs. J. Buckner and J. Svoboda of the US Department of Agriculture for supplying *M. sexta* eggs. This study was supported by a Flinn Biology Graduate Fellowship and a Sensory Science Scholarship to T.H., by DFG Kl 762/1-1 to P.K. and by NIH grant AI-23253 to J.G.H.

References

Adrian ED (1942) Olfactory reactions in the brain of the hedgehog. J Physiol (Lond) 100: 459–473

Bell RA, Joachim FA (1976) Techniques for rearing laboratory colonies of tobacco hornworms and pink boll worms. Ann Ent Soc Am 69: 365–373

Boeckh J, Distler P, Ernst K-D, Hösl M, Malun D (1990) Olfactory bulb and antennal lobe. In: Schild D (ed) Chemosensory information processing. NATO ASI Series, vol 39. Springer, Berlin Heidelberg New York, pp 201–227

Christensen TA, Hildebrand JG (1987) Male-specific, sex-pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta*. J Comp Physiol A 160: 553–569

Christensen TA, Hildebrand JG (1997) Coincident stimulation with pheromone components improves temporal pattern resolution in central olfactory neurons. J Neurophysiol 77: 775–781

Christensen TA, Hildebrand JG, Tumlinson JH, Doolittle RE (1989) Sex pheromone blend of *Manduca sexta*: responses of central olfactory interneurons to antennal stimulation in male moths. Arch Insect Biochem Physiol 10: 281–291

Christensen TA, Harrow ID, Cuzzocrea C, Randolph PW, Hildebrand JG (1995) Distinct projections of two populations of

olfactory receptor axons in the antennal lobe of the sphinx moth *Manduca sexta*. Chem Senses 20: 313–323
Christensen TA, Heinbockel T, Hildebrand JG (1996) Olfactory information processing in the brain: encoding chemical and temporal features of odors. J Neurobiol 30: 82–91
Freeman JA, Nicholson C (1975) Experimental optimization of current source density technique for anuran cerebellum. J Neurophysiol 38: 369–382
Gelperin A (1989) Neurons and networks for learning about odors. In: Carew TJ, Kelley DB (eds) Perspectives in neural systems and behavior. MBL lectures in biology, vol 10. Liss, New York, pp 121–136
Gelperin A, Tank DW (1990) Odour-modulated collective network oscillations of olfactory interneurons in a terrestrial mollusc. Nature (Lond) 345: 437–440
Gelperin A, Kleinfeld D, Denk W, Cooke IRC (1996) Oscillations and gaseous oxides in invertebrate olfaction. J Neurobiol 30: 110–12
Gray CM (1994) Synchronous oscillations in neuronal systems: mechanisms and functions. J Computational Neurosci 1: 11–38
Habets A, Lopes Da Silva FH, Mollevanger WJ (1980) An olfactory input to the hippocampus of the cat: field potential analysis. Brain Res 182: 47–64
Hansson BS (1995) Olfaction in Lepidoptera. Experientia 51: 1003–1027
Hansson BS, Christensen TA, Hildebrand JG (1991) Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta*. J Comp Neurol 312: 264–278
Heinbockel T, Christensen TA, Hildebrand JG (1994) Functional organization of the macroglomerular complex in the antennal lobe of male *Manduca sexta*. Soc Neurosci Abstr 20: 139
Heinbockel T, Christensen TA, Hildebrand JG (1995) Subunit architecture of the macroglomerular complex and the integrative function of output neurons in the moth, *Manduca sexta*. In: Elsner N, Menzel R (eds) Göttingen Neurobiol Report 1995. Proc 23rd Göttingen Neurobiol Conf 1995, vol II. Thieme, Stuttgart, p 364
Heinbockel T, Christensen TA, Hildebrand JG (1996) Encoding features of a pheromone signal by selective output pathways in the antennal lobes of the moth, *Manduca sexta*. In: Elsner N, Schnitzler H-U (eds) Brain and evolution. Proc 24th Göttingen Neurobiol Conf 1996, vol I. Thieme, Stuttgart, p 69
Hildebrand JG (1995) Analysis of chemical signals by nervous systems. Proc Natl Acad Sci USA 92: 67–74
Hildebrand JG (1996) Olfactory control of behavior in moths: central processing of odor information and the functional significance of olfactory glomeruli. J Comp Physiol A 178: 5–19
Homberg U, Montague RA, Hildebrand JG (1988) Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta*. Cell Tissue Res 254: 255–281
Homberg U, Christensen TA, Hildebrand JG (1989) Structure and function of the deutocerebrum in insects. Annu Rev Entomol 34: 477–501
Homberg U, Hoskins SG, Hildebrand JG (1995) Distribution of acetylcholinesterase activity in the deutocerebrum of the sphinx moth *Manduca sexta*. Cell Tissue Res 279: 249–259
Hopfield J (1991) Olfactory computation and object perception. Proc Natl Acad Sci USA 88: 6462–6466
Kaissling K-E, Hildebrand JG, Tumlinson JH (1989) Pheromone receptor cells in the male moth *Manduca sexta*. Arch Insect Biochem Physiol 10: 273–279
Kaulen P (1982) Feldpotentiale und eine Analyse der Stromquellendichte in der Corpora Pedunculata von *Apis mellifera*. Diplomarbeit, Freie Universität Berlin
Kaulen P, Erber J, Mobbs P (1984) Current source-density analysis in the mushroom bodies of the honeybee (*Apis mellifera carnica*). J Comp Physiol A 154: 569–582
Kloppenburg P (1990) Neuroanatomische Charakterisierung der antennalen Motoneuronen und elektrophysiologische Untersuchungen zur aminergen Modulation des visuellen Antennenreflexes der Honigbiene. Dissertation, Technische Universität Berlin, Fachbereich 14
Kloppenburg P, Erber J (1995) The modulatory effects of serotonin and octopamine in the visual system of the honey bee (*Apis mellifera* L.). II Electrophysiological analysis of motion-sensitive neurons in the lobula. J Comp Physiol A 176: 119–129
König P, Engel AK, Singer W (1996) Integrator or coincidence detector? The role of the cortical neuron revisited. Trends Neurosci 19: 130–137
Lai-Wo Stan Leung (1979) Potentials evoked by alvear tract in hippocampal CA1 region of rats. II. Spatial field analysis. J Neurophysiol 42: 1571–1589
Laurent G (1996a) Odor images and tunes. Neuron 16: 473–476
Laurent G (1996b) Dynamical representation of odors by oscillating and evolving neural assemblies. Trends Neurosci 19: 489–496
Laurent G, Davidowitz H (1994) Encoding of olfactory information with oscillating assemblies. Science 265: 1872–1875
Laurent G, Naraghi M (1994) Odorant-induced oscillations in the mushroom bodies of the locust. J Neurosci 14, 5: 2993–3004
Laurent G, Wehr M, Davidowitz H (1996) Temporal representations of odors in an olfactory network. J Neurosci 16: 3837–3847
MacLeod K, Laurent G (1996) Distinct mechanisms for synchronization and temporal patterning of odor-encoding neural assemblies. Science 274: 976–979
Matsumoto SG, Hildebrand JG (1981) Olfactory mechanisms in the moth *Manduca sexta*: response characteristics and morphology of central neurons in the antennal lobes. Proc R Soc Lond B 213: 249–277
Mellon D, Sandeman DC, Sandeman RE (1992) Characterization of oscillatory olfactory interneurons in the protocerebrum of the crayfish. J Exp Biol 167: 15–38
Mercer AR, Erber J (1983) The effects of amines on evoked potentials recorded in the mushroom bodies of the bee brain. J Comp Physiol 151: 469–476
Mitzdorf U, Singer W (1977) Laminar segregation of afferents to lateral geniculate nucleus of the cat: an analysis of current source density. J Neurophysiol 40: 1227–1244
Nicholson C, Freeman J (1975) Theory of current source-density analysis and determination of conductivity tensor for anuran cerebellum. J Neurophysiol 38: 356–368
Nicholson C, Llinas R (1975) Real time current source-density analysis using multielectrode array in cat cerebellum. Brain Res 100: 418–424
Nunez PL (1981) Electric fields of the brain. Oxford University Press, New York
Pichon Y, Sattelle DB, Lane NJ (1972) Conduction processes in the nerve cord of the moth *Manduca sexta* in relation to its ultrastructure and haemolymph ionic composition. J Exp Biol 56: 717–734
Press WH, Flannery BP, Teukolsky SA, Vetterling WT (1988) Numerical recipes in C, the art of scientific computing. Cambridge University Press, New York
Ropars JP, Hildebrand JG (1992) Anatomical identification of glomeruli in the antennal lobes of the male sphinx moth *Manduca sexta*. Cell Tissue Res 270: 205–227
Rössler W, Bailey WJ, Schröder J, Kalmring K (1990) Resolution of time and frequency patterns in the tympanal organs of tettigoniids. I. Synchronization and oscillation in the activity of receptor populations. Zool Jb Physiol 94: 83–99
Shepherd GM (1992) Modules for molecules. Nature (Lond) 358: 457–458
Singer W (1993) Synchronization of cortical activity and its putative role in information processing and learning. Annu Rev Physiol 55: 349–374
Singer W, Gray CM (1995) Visual feature integration and the temporal correlation hypothesis. Annu Rev Neurosci 18: 555–586

Starratt AN, Dahm KH, Allen N, Hildebrand JG, Payne TL, Röller H (1979) Bombykal, a sex pheromone of the sphinx moth *Manduca sexta*. Z Naturforsch 34C: 9–12

Strausfeld NJ (1989) Cellular organization in male-specific olfactory neuropil in the moth *Manduca sexta*. In: Elsner N, Singer W (eds) Dynamics and plasticity in neuronal systems. Thieme, Stuttgart, p 79

Tank DW, Gelperin A, Kleinfeld D (1994) Odors, oscillations, and waves: does it all compute? Science 265: 1819–1820

Tumlinson JH, Brennan MM, Doolittle RE, Mitchell ER, Brabham A, Mazomenos BE, Baumhover AH, Jackson DM (1989) Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. Arch Insect Biochem Physiol 10: 255–271

Vickers NJ, Baker TC (1992) Male *Heliothis virescens* maintain upwind flight in response to experimentally pulsed filaments of their sex pheromone (Lepidoptera: Noctuidae). J Insect Behav 5: 669–687

Wu JY, Wu CH, Guan L, Cohen AH (1995) Local field potential in the antennal lobe of the Antheraea moth (*A. pernyi*): pheromone related oscillations? Soc Neurosci Abstr 21: 134

Zimmerman RP (1978) Field potential analysis and the physiology of second-order neurons in the visual system of the fly. J Comp Physiol 126: 297–316