THE JOURNAL OF COMPARATIVE NEUROLOGY 409:1–12 (1999)

Temporal Tuning of Odor Responses in Pheromone-Responsive Projection Neurons in the Brain of the Sphinx Moth *Manduca sexta*

THOMAS HEINBOCKEL, THOMAS A. CHRISTENSEN, AND JOHN G. HILDEBRAND*
Arizona Research Laboratories, Division of Neurobiology, University of Arizona, Tucson, Arizona 85721–0077

ABSTRACT

By means of intracellular recording and staining, we studied the ability of several distinct classes of projection (output) neurons, which innervate the sexually dimorphic macroglomerular complex (MGC-PNs) in the antennal lobe of the male moth *Manduca sexta*, to encode naturally intermittent sex pheromonal stimuli. In many MGC-PNs, antennal stimulation with a blend of the two essential pheromone components evoked a characteristic triphasic response consisting of a brief, hyperpolarizing inhibitory potential (I_1) followed by depolarization with firing of action potentials and then a delayed period of hyperpolarization (I_2). MGC-PNs described in this study resolved pulsed pheromonal stimuli, up to about five pulses/second, with a distinct burst of action potentials for each pulse of odor. The larger the amplitude of I_1, the higher the pulse rate an MGC-PN could follow, illustrating the importance of inhibitory synaptic input in shaping the temporal firing properties of these glomerular output neurons. In some MGC-PNs, triphasic responses were evoked by antennal stimulation with only one of the two key pheromone components. Again, the maximal pulse rate that an MGC-PN could follow with that pheromone component as sole stimulus was high in MGC-PNs that responded with a strong I_1. These component-specific MGC-PNs innervated only one of the two principal glomeruli of the MGC, while MGC-PNs that were primarily excited by antennal stimulation with either key pheromone component had arborizations in both major MGC glomeruli. These observations therefore suggest that the population of antennal olfactory receptor cells responding to a single pheromone component is functionally heterogeneous: a subset of these sensory cells activates the excitatory drive to many uniglomerular MGC-PNs, while others feed onto inhibitory circuits that hyperpolarize the same PNs. This convergence of opposing inputs is a circuit property common to uniglomerular MGC-PNs branching in either of the major MGC glomeruli, and it enhances the ability of these glomerular output neurons to resolve intermittent olfactory input. Synaptic integration at the uniglomerular PN level thus contributes to the transmission of behaviorally important temporal information about each key pheromone component to higher centers in the brain. J. Comp. Neurol. 409:1–12, 1999.

Indexing terms: deutocerebrum; glomerulus; insect; pheromone; temporal tuning

In order to locate a conspecific female releasing sex pheromone, a male moth must recognize and encode certain features of the pheromonal signal (Hildebrand, 1995, 1996). These include qualitative (chemical composition) and quantitative (relative levels of components) features of the pheromone. Moreover, behavioral studies have shown that spatial and temporal properties of the pheromonal stimulus also contribute to orientation and mate-finding (Christensen et al., 1996). The coding of the temporal structure of the signal by morphologically characterized, pheromone-sensitive projection neurons (PNs) in the antennal lobes (ALs) of male moths, *Manduca sexta*, is the focus of this study.

Grant sponsor: Flinn Foundation; Grant sponsor: NIH; Grant number: AI23253.

Thomas Heinbockel's present address: Institut für Physiologie, Otto-von-Guericke-Universität, Leipziger Str. 44, D-39120 Magdeburg, Germany.

*Correspondence to: Dr. John G. Hildebrand, ARL Division of Neurobiology, University of Arizona, P.O. Box 210077, Tucson, AZ 85721–0077. E-mail: jgh@neurobio.arizona.edu

Received 21 August 1998; Revised 8 February 1999; Accepted 8 March 1999

Many factors can affect the spatiotemporal character of an odor stimulus. Owing to the presence of physical obstacles in the environment, movement of the animal, wind turbulence, and sometimes the pulsatile release of the sex pheromone (e.g., in the Arctiid moth *Utetheisa ornatrix*; Connor et al., 1980), the pheromonal signal is virtually never a continuous stream of odor (Murlis et al., 1992). Instead, it occurs as filaments or blobs of odor of varying concentration which, on average, diminish in concentration with increasing distance from the odor source (Murlis et al., 1992). The intermittency of the signal (i.e., the time during which it is absent) increases further downwind from the odor source, whereas closer to the source, both the spatiotemporal frequency of odor pulses and the concentration of pheromone within the pulse are elevated (Murlis et al., 1992). It is likely that these basic physical principles apply to nonpheromonal odors as well.

In behavioral experiments using female sex pheromones to attract male moths, continuous plumes have been shown to be inferior to intermittent pheromonal stimuli in eliciting odor-modulated upwind movement of male moths toward the source (Baker et al., 1985, 1988; Kramer, 1986, 1992; Kaissling, 1987; Baker, 1989; Kaissling and Kramer, 1990). The behavioral response to changes of pheromone concentration occurs within about 100 milliseconds (Kaissling and Kramer, 1990; Mafra-Neto and Cardé, 1994; Vickers and Baker, 1994), and neural correlates to this rapid response have been found at the level of antennal olfactory receptor cells (ORCs; Rumbo and Kaissling, 1989; Almaas et al., 1991; Marion-Poll and Tobin, 1992) and in the ALs (Christensen and Hildebrand, 1988, 1997; Christensen et al., 1989a, 1998; Vickers et al., 1998). Some ORCs and central olfactory interneurons can follow stimulus pulses up to about 12/second (Christensen and Hildebrand, 1988, 1997; Christensen et al., 1989a, 1996; Rumbo and Kaissling, 1989; Almaas et al., 1991), but most of these neurons follow pulse rates in the range of three to five/second. Studies in the brain have focused on the functional characterization of PNs because these neurons, through their long axons, represent the essential route for information flow between the olfactory glomeruli and higher brain centers (Christensen et al., 1996). These neurons are convergence stations for synaptic input from pheromone-responsive sensory cells in the antennae and project to distinct targets in the protocerebrum such as the mushroom bodies and the lateral protocerebrum (Homberg et al., 1989).

The insect macroglomerular complex (MGC), the dedicated site of first-order processing of pheromonal information in the male brain, is a useful model for studying the functional organization of olfactory glomeruli and the mapping of olfactory information in the brain (Hildebrand, 1995, 1996). In the present study, using intracellular recording and staining, we focused on the pulse-following ability of MGC-PNs that were shown to have arborizations confined to one or both of the two main glomeruli (the cumulus and toroid) of the MGC. MGC-PNs with arborizations limited to the toroid respond preferentially to antennal stimulation with the predominant essential pheromone component, *E,Z*-10,12-hexadecadienal (bombykal, Bal). In contrast, MGC-PNs with arborizations confined to the cumulus respond preferentially to *E,Z*-11,13-pentadecadienal ("C15"), a relatively stable mimic of the second key pheromone component, *E,E,Z*-10,12,14-hexadecatrienal (E10,E12,14Z-16:Al; Kaissling et al., 1989; Hansson et al., 1991). C15 is electrophysiologically equivalent to the second key pheromone component because C15 stimulates the same type of olfactory receptor cell in the pheromone-responsive trichoid sensilla on the antennae and evokes the same temporal response pattern in these cells (Kaissling et al., 1989). The two key pheromone components are necessary and sufficient to evoke male-specific pheromone-guided behavior in male *M. sexta* (Tumlinson et al., 1989). Thus, MGC-PNs provide information about the pheromone blend that is essential for initiating pheromone-guided behavior.

In a previous study, we examined the ability of MGC-PNs to encode information about the natural intermittency of pheromonal stimuli (Christensen and Hildebrand, 1997). Some of the MGC-PNs could follow pulsed stimuli more accurately if a blend of the two key pheromone components was used as the stimulus. For other MGC-PNs, addition of the second component had no detectable effect on the response to the major component presented alone, showing that single-component information is preserved in some glomerular output pathways. The possible relationship between temporal response properties of these MGC-PNs and their patterns of arborization in the MGC, however, has not been investigated.

One aim of the present study was to determine if the ability to encode stimulus intermittency was a function of intra- or interglomerular cellular interactions, or both. Pulse following of MGC-PNs was used as a criterion for cell responsiveness to pheromone stimulation. We report here that, for some MGC-PNs with arborizations confined to the cumulus, the single pheromone component C15 can evoke a triphasic (inhibitory/excitatory/inhibitory) response similar to that evoked by the pheromone blend; and, likewise, for some MGC-PNs with arborizations confined to the toroid, Bal can evoke a similar 'mixed' response. The maximal pulse frequency encoded by these uniglomerular PNs is not increased in the presence of the pheromone blend, but seems to arise through the convergence of 2 parallel pathways—one excitatory and one inhibitory—that target the same glomerulus, and are both activated by the same olfactory stimulus.

MATERIALS AND METHODS

Experimental preparation

Adult, laboratory-reared *M. sexta* (Lepidoptera: Sphingidae), 1–3 days post-eclosion, were anesthetized on ice and dissected as described by Christensen and Hildebrand (1987). Briefly, the head capsule was opened by removing the proboscis and palps and cutting frontally along the compound eyes to the bases of the antennae. The head was isolated from the thorax, which eliminated mechanical problems due to movement of the animal but did not affect neural responses (Christensen and Hildebrand, 1987). The head was fixed with four insect pins to a Sylgard-coated platform, and one antenna was immobilized with two additional pins. The antennal lobe (AL) was mechanically desheathed to facilitate electrode penetration and continuously superfused with saline solution (150 mM NaCl, 3 mM $CaCl_2$, 3 mM KCl, 25 mM sucrose, 10 mM TES buffer, pH 6.9; modified from Pichon et al., 1972).

Intracellular recording

Glass-capillary micropipettes were prepared and used for intracellular recording as described before (Heinbockel

et al., 1998). Forward and backward movements of microelectrodes were controlled by a Burleigh Inchworm (Model 6000/ULN; Burleigh Instruments Inc., Fishers, NY) attached to a Leitz micromanipulator. Because the site of the electrode impalement in a neuron can affect the amplitude of postsynaptic potentials, impalements targeted the same area of neuropil in all preparations. Intracellularly recorded signals were amplified with an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) and stored on FM tape. Records were then transferred to a computer hard disk and analyzed by means of Experimenter's Workbench (Data Wave Technologies, Longmont, CO) or Autospike (Syntech, Hilversum, The Netherlands) software and finally stored on removable data cartridges. The data were analyzed for differences using one-way analysis of variance (Kruskal-Wallis One-Way Analysis of Variance on Ranks, ANOVA) and a multiple comparison procedure to isolate groups from each other (All Pairwise Multiple Comparison Procedures, $P < 0.05$).

Intracellular staining

Neurons were stained intracellularly, through intracellular recording electrodes, with Neurobiotin (Vector Laboratories, Burlingame, CA; 3–5% in 2 M KCl with 0.05 M Tris buffer, pH 7.4), biocytin (Sigma, St. Louis, MO; 3–5% in 2 M KCl with 0.05 M Tris buffer, pH 7.4), or Lucifer Yellow CH (Aldrich Chemical Co., Milwaukee, WI; 4% in distilled water). The electrode shaft was filled with 2.5 M potassium acetate solution in the case of biocytin and Neurobiotin electrodes or with 2 M LiCl solution for Lucifer Yellow electrodes. Injection of these markers was achieved by means of: 1-nA hyperpolarizing current for 10 minutes for Lucifer Yellow as described previously (Christensen and Hildebrand, 1987); alternating hyperpolarizing and depolarizing 1-minute, 1-nA current pulses for a total of 10 minutes for biocytin; or 1-nA depolarizing current for 10 minutes for Neurobiotin. Brains containing neurons stained with Neurobiotin or biocytin were dissected, fixed overnight, and incubated with Cy3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, PA), diluted 1:100 with 0.2 M phosphate buffer containing 0.3% Triton X-100) for 3 days on a shaker at 4°C. After incubation with Cy3-streptavidin, the brains were dehydrated through a graded ethanol series, cleared in methyl salicylate, and examined by either fluorescence or laser-scanning confocal microscopy (see below). Two PNs were labeled in the same AL by staining one with biocytin and one with Lucifer Yellow. Pseudocolors were applied to both MGC-PNs using computer software (Confocal Assistant, Adobe Photoshop, Adobe Systems Inc., Mountain View, CA).

Confocal microscopy

Intracellularly stained neurons were viewed with a laser-scanning confocal microscope (BioRad MRC-600, Bio-Rad, Cambridge, MA, with a Nikon Optiphot-2 microscope and a 100-mW Argon or 15-mW Krypton/Argon laser and appropriate dichromatic filter cubes; Mesce et al., 1993; Sun et al., 1993). Serial, 2-μm optical sections were imaged through the depth of whole mounts and saved as a series of images on a rewritable optical disk.

Olfactory stimulation

Techniques for preparation and delivery of odor stimuli were essentially as described previously (Christensen and Hildebrand, 1987) and are described here only briefly. In each preparation, the antenna ipsilateral to the impaled AL was stimulated using glass odor cartridges, each containing a piece of filter paper bearing 10 ng of a single compound, applied in *n*-hexane solution, or 10 ng each of compounds blended in mixtures. Odor stimuli were delivered by diverting charcoal-filtered, humidified air (1,000 ml/minute) through the odor cartridge directed at the basal region of the antenna. The odor stimuli used in these experiments were: 1) *E*,*Z*-10,12-hexadecadienal (bombykal, Bal), the predominant essential component of the female's sex pheromone (Starratt et al., 1979; Tumlinson et al., 1989; Kaissling et al., 1989; Christensen et al., 1989b); 2) *E*,*Z*-11,13-pentadecadienal ("C15"), a mimic of the second key component of the female's sex pheromone (*E*,*E*,*Z*-10,12,14-hexadecatrienal) that is chemically more stable than the trienal (Kaissling et al., 1989); 3) a mixture of Bal and C15 ("Bal+C15"); and 4) the complete, natural sex pheromone, a 60-second *n*-hexane wash of one female pheromone gland (1 FE) (Christensen and Hildebrand, 1987). When we obtained a stable recording, the neuron was challenged by antennal stimulation with Bal+C15 in a series of 50-millisecond pulses at five/second. The aim was to determine the maximal pulse rate an MGC-PN could follow and how this relates to the presence of one or both key pheromone components, to the amount of inhibitory and excitatory input, and to the dendritic arborizations of a neuron in the MGC. To characterize the pulse-following ability of MGC-PNs, the stimulus frequency was lowered until the MGC-PN could resolve each odor pulse with a distinct burst of at least five action potentials clearly separated by a period of 50 milliseconds from the burst of action potentials in response to the next odor pulse. This was done for the pheromone blend first and repeated subsequently for the individual components, Bal and C15. The IPSP (inhibitory postsynaptic potential) and EPSP (excitatory postsynaptic potential) amplitudes were determined from the first response of a cell to stimulation at a particular frequency. The time interval between stimulations was at least 1 minute to prevent sensory adaptation.

RESULTS

General physiological characteristics of MGC projection neurons

As described previously, stimulation of the antenna with the odor blend, Bal+C15, elicits a response that in many MGC-PNs comprises three distinct response phases (Christensen et al., 1998). Figure 1A shows an example (one of 96 such neurons from a total of 117 neurons mentioned in this study) of this triphasic postsynaptic response, consisting of an initial, rapid inhibitory phase (I_1), an excitatory phase with spiking (E), and an interval without spiking but often with hyperpolarization (referred to as delayed hyperpolarization, I_2). The MGC-PN in Figure 1A responded to stimulation of the antenna with C15 with inhibition lasting longer than the stimulating odor pulse. In contrast, the response to stimulation with Bal alone comprised three phases similar to those observed with Bal+C15. Thus, one blend component alone evoked a triphasic response in this neuron (see also Figs. 2A, 5A,B).

The magnitude of I_1 varied among different MGC-PNs from 0 to approximately 14 mV. Injection of depolarizing current into another MGC-PN (Fig. 1B) increased the size

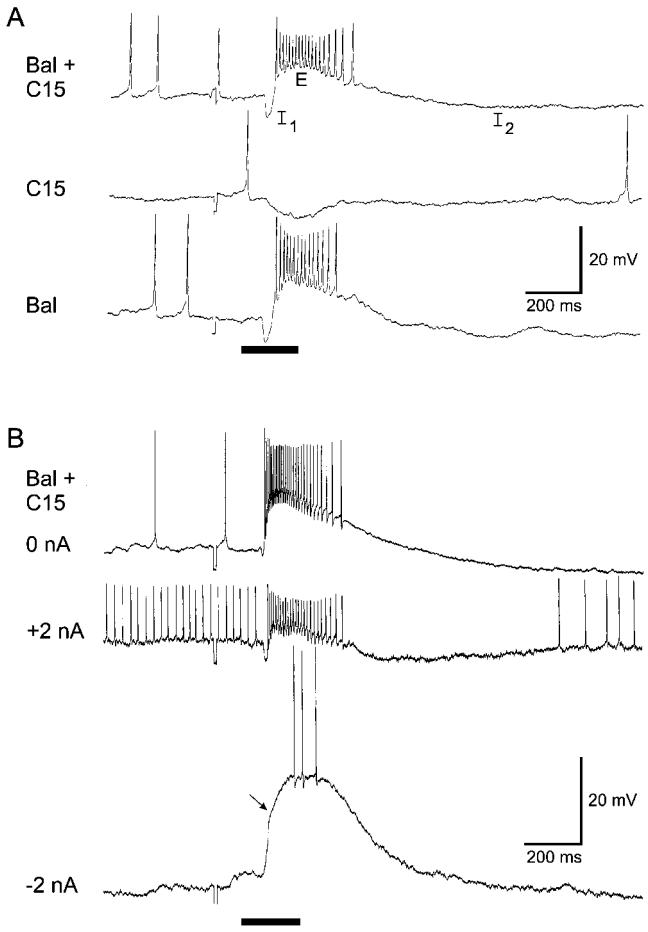


Fig. 1. **A:** MGC-PN (macroglomerular complex) (a Bal [bombykal] specialist) responding to antennal stimulation with the two-component blend or with Bal alone with an initial IPSP (inhibitory postsynaptic potential) (I_1), followed by an EPSP (excitatory postsynaptic potential) (E) with spiking and an interval of after-hyperpolarization and absence of spikes (I_2). C15 (*E*,*Z*-11,13-pentadecadienal) evoked only an IPSP, with a time course that reflected the duration of the stimulus, and did not affect the response to Bal when the blend was used as the stimulus. **B:** Responses to the blend are altered by injected current. In another MGC-PN, the amplitude of I_1 increased and that of E decreased upon injection of depolarizing current. The polarity of I_1 was reversed upon injection of hyperpolarizing current (indicated by arrow). All responses (Fig. 1A,B) were preceded by a calibration pulse (5 mV). The time course of the odor stimulus is indicated by the bar beneath the records.

of I_1, while injection of hyperpolarizing current reversed I_1. This is one of 23 neurons in which such current-injection was performed, with similar results. Previous experiments indicated that I_1 can be blocked reversibly by picrotoxin or bicuculline and reversed by low-chloride saline, and that the excitatory response phase and I_2 persist under these conditions (Waldrop et al., 1987; Christensen et al., 1998). Many AL neurons are hyperpolarized by GABA, and muscimol (a GABA mimic) inhibits activity in many AL neurons (Waldrop et al., 1987; Christensen et al., 1998). Current-injection protocols (see also Christensen et al., 1993) and pharmacological experiments suggest that I_1 is mediated by chemical synaptic transmission. Thus, the amplitude of I_1 is at least in part a function of the membrane potential of the cell. In addition, the amount of inhibitory input that a given cell receives is likely to influence the amplitude of I_1. Injection of depolarizing current into the neuron of Figure 1B reduced the size of E and increased the frequency of spontaneous spiking, whereas injection of hyperpolarizing current greatly increased the size of E and substantially reduced the number of action potentials. Taken together, these findings indicate that I_1 and E are inhibitory and excitatory postsynaptic potentials (PSPs), respectively, and that in most cases, the recording site was electrically close to the sites of synaptic input mediating these PSPs.

Morphological types of MGC projection neurons

The MGC-PNs reported in this study fell into three anatomical classes: neurons with arborizations in the cumulus (n = 44, with a total of 117 neurons mentioned in this study), the toroid (n = 57), or both glomeruli (n = 12), as reported previously (Hansson et al., 1991). MGC-PNs belonging to other anatomical classes have been encountered as well but at a lower frequency. The functional role of those MGC-PNs is not known. For instance, MGC-PNs were observed with arborizations restricted to the recently discovered third glomerulus of the MGC (n = 4), which has been given the anatomical name "horseshoe" or toroid II (Heinbockel et al., 1994, 1995, 1996; Homberg et al., 1995).

MGC-PNs with arborizations confined to either the cumulus or the toroid. Figures 2 and 3 show the responses and branching patterns of a pair of MGC-PNs in one AL. One MGC-PN (Fig. 2A,dxB) gave a triphasic response to antennal stimulation with Bal+C15. Stimulation with either Bal or the complete pheromone blend (wash of one pheromone gland that contains key components as well as other components of the pheromone blend) evoked a response from this neuron that was similar to its response to Bal+C15 (with respect to the presence and amplitude of I_1 and E and the numbers of action potentials associated with the E phase of the response), but neither clean air (a gentle mechanical stimulus) nor C15 alone evoked a response.

Since natural odor stimuli are intermittent sensory signals, pulse following of MGC-PNs was used as a criterion for cell responsiveness to pheromone stimulation. In order to characterize the pulse-following ability of an MGC-PN we determined the cut-off frequency, i.e., the highest frequency that a cell was able to follow. This was done by using a range of stimulation frequencies which were adjusted until the MGC-PN followed each of five pheromone pulses at a given pulse rate with a distinct burst of action potentials (≥five spikes) clearly separated from the next burst (≥50 milliseconds). At first the antenna was stimulated with five pulses/second. The "Bal specialist" neuron in Figure 2 could not resolve the individual odor pulses delivered at five pulses/second; instead of firing a clearly separated burst of action potentials in response to each odor pulse, the neuron fired irregularly but continuously for approximately 1 second. Stimulation of the antenna with four pulses/second (Fig. 2B) evoked a strong response to the first pulse and distinct bursts to the fourth and fifth pulses. When the stimulation frequency was reduced to three pulses/second, this neuron responded to each odor pulse with a distinct burst of action potentials, although these bursts did not accurately reflect the duration of the stimuli. Detectable hyperpolarization preceded the burst only in response to the first odor pulse. With each

successive odor pulse, the number of action potentials, size of the EPSP, and degree of spike adaptation decreased.

The second MGC-PN studied in the same AL exhibited a pattern of responses that contrasted with that of the first one (compare Fig. 2C with 2A,B). Although Bal+C15 again evoked a triphasic response, with an IPSP, a burst of action potentials, and delayed hyperpolarization, stimulation with Bal appeared to hyperpolarize the neuron slightly and to inhibit background spiking. The response to C15 was similar to the response to Bal+C15, but C15 did not depolarize the neuron as much as the two-component blend. This might be explained by rundown of the preparation or long-lasting adaptation of the neuron. Mechanical stimulation (clean-air blank) evoked no response in this second MGC-PN ("C15 specialist").

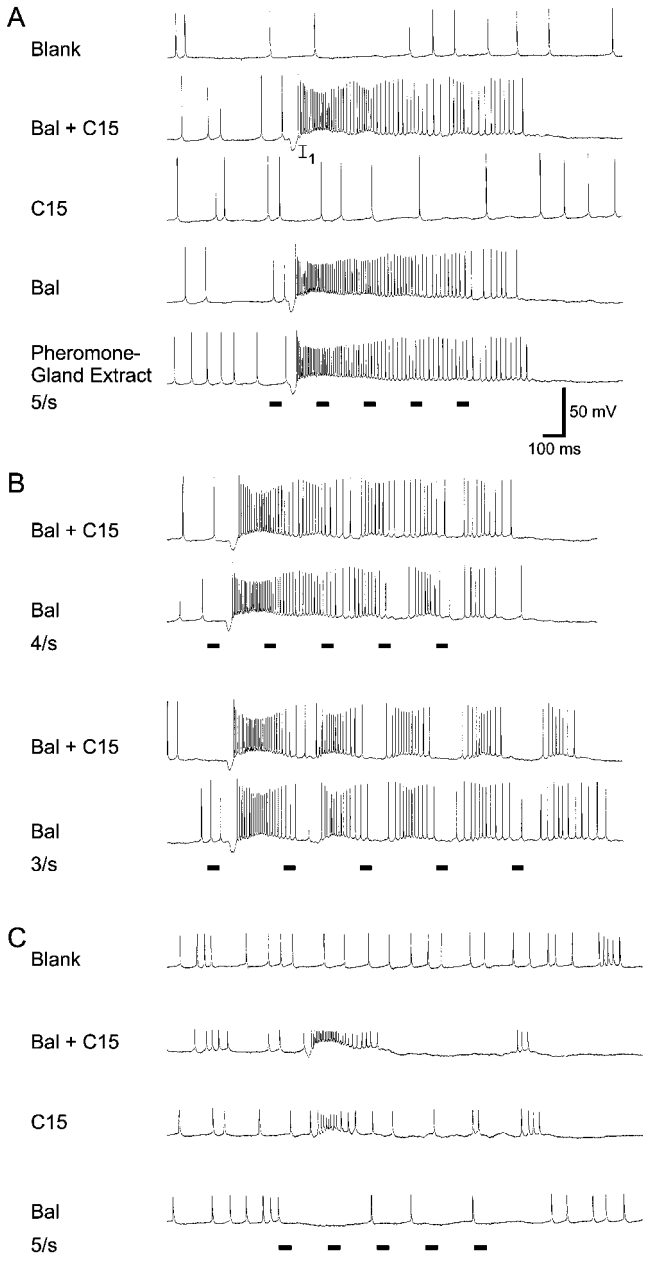
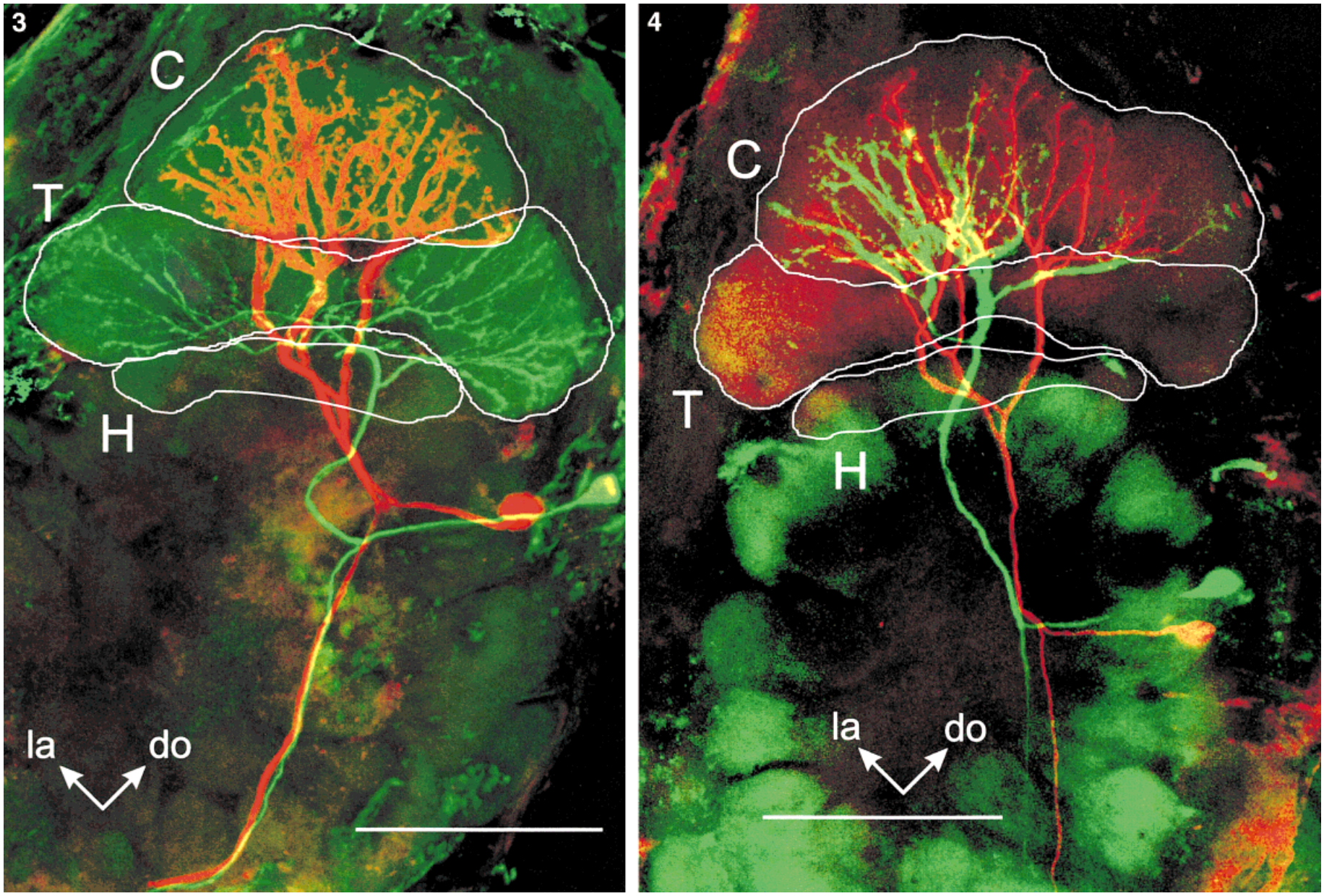


Fig. 2. Responses of two specialist MGC-PNs (macroglomerular complex) to pulsed antennal stimulation with Bal (bombykal) and C15 (*E*,*Z*-11,13-pentadecadienal). **A:** Antennal stimulation with the two-component blend, Bal, and a solvent wash of the female's sex-pheromone gland elicited a strong IPSP (inhibitory postsynaptic potential) (I_1) followed by a burst of action potentials in one MGC-PN (green neuron in Fig. 3), whereas there was no response to stimulation with C15. The neuron did not resolve the five 50-millisecond odor pulses delivered at five/second. **B:** The same neuron could follow pulsed antennal stimulation with Bal or the two-component blend up to three pulses/second. **C:** Another specialist MGC-PN (red neuron in Fig. 3) gave a primarily excitatory response to antennal stimulation with the two-component blend or with C15.

The Bal specialist PN in this AL (green neuron in Fig. 3) started to branch only as it entered the toroid, where its arborizations were found mainly in the medial and lateral regions. By contrast, the C15 specialist PN (red neuron in Fig. 3) started to branch outside the MGC, gave off several side branches as it passed through the holes of the horseshoe and toroid, but had dendritic arborizations only in the cumulus. The cell bodies of these MGC-PNs were situated close to each other in the medial group of AL neuronal somata. Thus, these two functionally different MGC-PNs also had distinctly different structures, each innervating only one of the two major glomeruli of the MGC.

Figure 4 shows the branching patterns in the AL of two additional C15 specialist PNs that had arborizations in the cumulus. One cell (green neuron) extended several branches in the lateral half of the cumulus and one major branch in the medial half, whereas the other MGC-PN (red neuron) had branches in the lateral region of the cumulus and even more branches in its medial half. No overlap of branches of these two neurons was apparent by looking through the entire set of serial 2-μm optical sections, indicating that these neurons occupied different regions of the cumulus. Nevertheless, the physiological responses of these MGC-PNs (Fig. 5) were remarkably similar. Both neurons gave mixed inhibitory/excitatory responses to antennal stimulation with Bal+C15 or C15 alone but did not respond to the clean-air blank. The response to Bal appeared to be weak inhibition. During some of the tests with Bal, the red MGC-PN exhibited weak hyperpolarization (Fig. 5B). Neither of the two MGC-PNs could resolve five odor pulses/second, but each neuron responded with one strong burst to the first odor pulse. The response to the subsequent odor pulses consisted of one or a few additional spikes or, in some cases, depolarization that did not reach spike threshold (asterisks).

MGC projection neurons with arborizations in both cumulus and toroid. Although most MGC-PNs (>80%) gave brief responses to pheromonal stimulation of the antenna, in some cases the response outlasted the stimulation by several hundred milliseconds. The difference in response duration was particularly obvious in MGC-PNs that responded with long-lasting excitation to one component and brief excitation to the other component ("pheromone generalists") (Fig. 6). The MGC-PN in Figure 6 responded to antennal stimulation with Bal+C15 with early hyperpolarization followed by depolarization but failed to respond clearly to each successive pulse of the blend. Likewise this neuron could not follow pulses of C15 delivered to the antenna, but instead gave a burst of spikes only to the first pulse and a few additional spikes to subsequent pulses. This response resembled those of C15 specialist PNs described above (Figs. 2C, 5A,B). Upon antennal stimulation with Bal, this neuron gave a mixed



Figures 3 and 4

response with a long-lasting, irregular burst of spikes that continued far beyond the end of the stimulation period. Thus, the responses to the two components were quantitatively as well as qualitatively different. This became even more evident when the stimulus frequency was reduced to three or four pulses/second. In response to antennal stimulation with C15, at all frequencies tested, this MGC-PN fired a burst of spikes after the first pulse. At three and four pulses/second, an initial hyperpolarization preceded the burst. Subsequent bursts were weak and separated from each other by inhibitory phases. When Bal and C15 were combined, the response at three and four pulses/second was a sequence of strong bursts that were clearly separated from each other and phase-locked to the stimulus. In contrast, the neuron could not distinctly resolve each odor pulse when the antenna was stimulated with Bal alone at three or four pulses/second.

Because several neurons were studied in this preparation and stained intracellularly (not shown), we could not discern the branching pattern of each individual neuron. MGC-PNs that are excited by both Bal and C15 (pheromone generalists), however, have been described earlier and have arborizations in both the cumulus and the toroid (Hansson et al., 1991; Heinbockel et al., 1998).

Pulse following during stimulation with the pheromone blend

From the preceding description of the physiological responses of MGC-PNs, it is clear that not all neurons could resolve several odor pulses per second. Previous observations (Christensen and Hildebrand, 1988, 1997) suggested that the amplitude of the early inhibitory potential (I_1) is important for the ability of some MGC-PNs to follow higher stimulus frequencies and to copy stimulus durations. In accordance with these data, the IPSP amplitudes of all MGC-PNs that followed a certain pulse rate were plotted in Figure 7A. MGC-PNs that followed a higher pulse rate (five pulses/second) responded with a stronger initial inhibition than neurons that showed weak pulse following (<1 pulse/second) (regression analysis for plotting pulse following over IPSP amplitude: n = 101; F = 135.73; r^2 = 0.58; P = 2.88E-20; y = 0.97 + 0.56x.) Such a relationship was not found for pulse following and EPSP amplitude (Fig. 7B) (regression analysis for plotting pulse following over EPSP amplitude: n = 101; F = 2.52; r^2 = 0.02; P = 0.12; y = 1.97 + 0.04x). Some MGC-PNs that responded with EPSPs of more than 20 mV could not resolve pulses of higher frequencies than those neurons that responded with very small EPSPs.

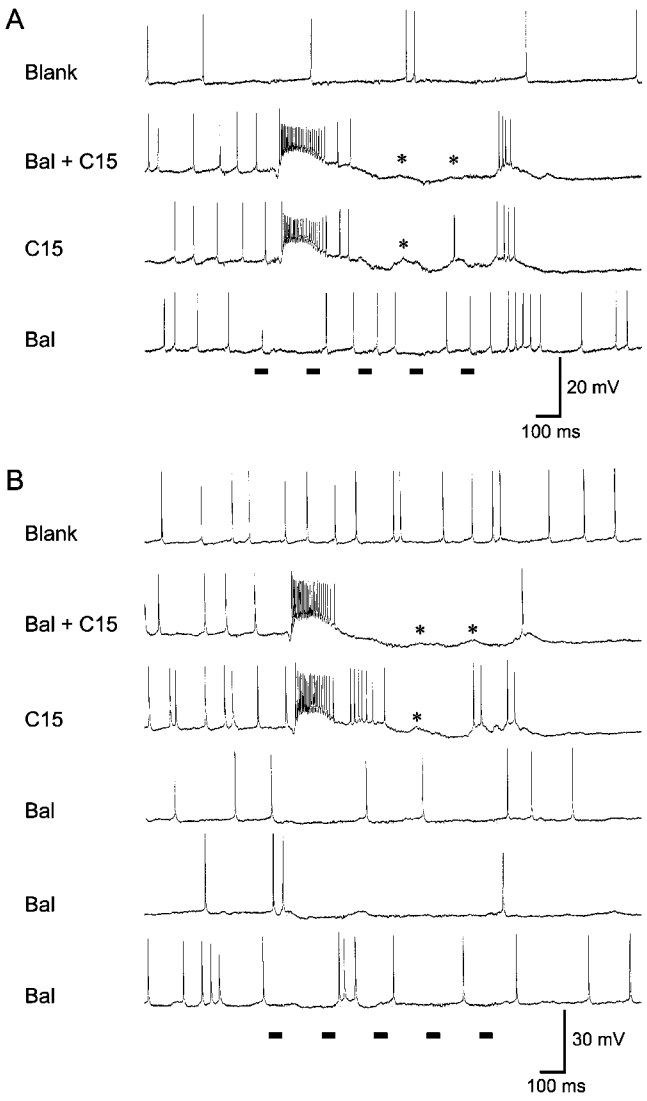


Fig. 5. Physiological responses of the 2 C15 (*E,Z*-11,13-pentadecadienal) -specialist MGC-PNs (macroglomerular complex) shown in Figure 4 (**A:** green neuron; **B:** red neuron). In spite of their morphological differences, these two neurons gave similar responses to odor stimuli. Both MGC-PNs gave a mixed response to the two-component blend and to C15. In both neurons, although Bal (bombykal) appeared to be slightly inhibitory by itself, when presented in the binary mixtures, it did not greatly influence the neurons' responses to C15. The first of the five vertical lines indicates the onset of the response to the Bal+C15 blend; the other four vertical lines are drawn at 200-millisecond intervals after the first line. The horizontal lines in the last three records of B indicate the lowest membrane potential observed prior to stimulation. Asterisks indicate depolarizations without action potentials in response to pheromonal stimuli.

Pulse-following during stimulation with individual components

As shown above, antennal stimulation with Bal or C15 alone elicits in some MGC-PNs a triphasic response comprising an early IPSP (I_1), followed by depolarization and delayed hyperpolarization (Figs. 2, 5). In Figure 8, the I_1

Fig. 3. Laser-scanning confocal micrograph showing the two specialist MGC-PNs (macroglomerular complex) of Figure 2. One neuron, stained with Lucifer Yellow (colored red here), had arborizations confined to the cumulus (C), and the other neuron, stained with biocytin (colored green here) had arborizations confined to the toroid (T). Areas of apparent overlap between the two neurons are shown in yellow and are possible sites of synaptic contact. H, horseshoe; do, dorsal; la, lateral. Scale bar = 100 μm.

Fig. 4. Morphological diversity in cumulus neurons. Laser-scanning confocal images of two C15 (*E,Z*-11,13-pentadecadienal) -specialist MGC-PNs (macroglomerular complex) with arborizations confined to the cumulus. While the branches of the two neurons apparently overlapped in certain parts of the cumulus (indicated in yellow), other parts were innervated by just one of the two neurons. The physiological responses of the neurons are shown in Figure 5. The green neuron was stained with Lucifer Yellow and the red neuron with biocytin. C, cumulus; H, horseshoe; T, toroid; do, dorsal; la, lateral. Scale bar = 100 μm.

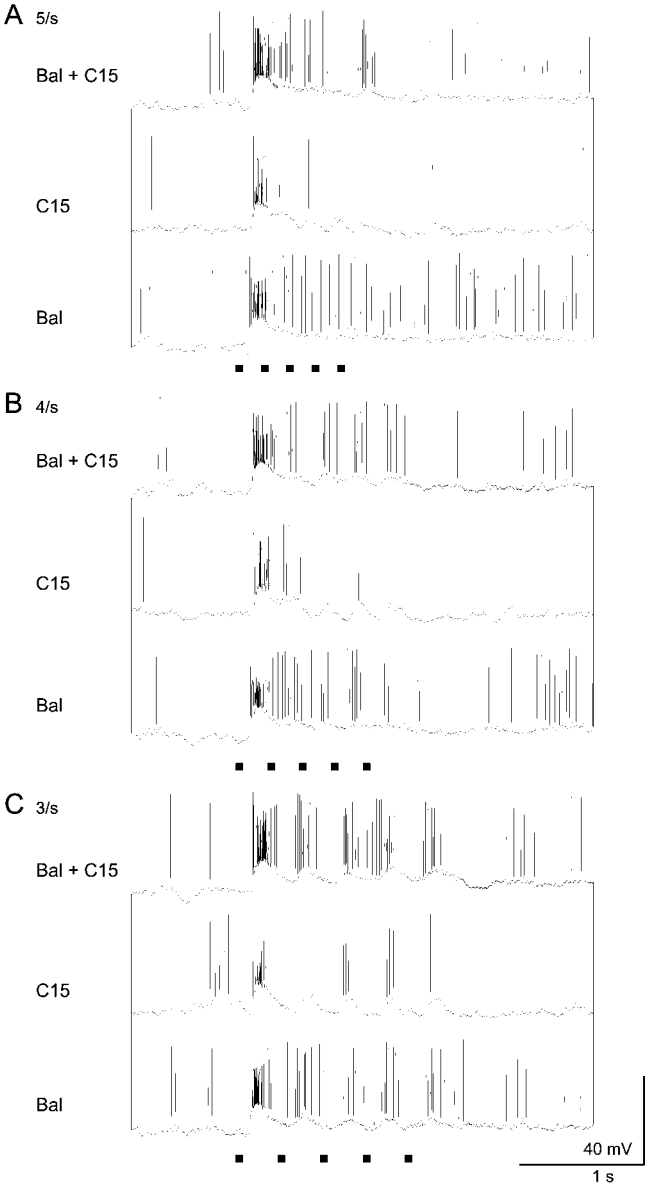


Fig. 6. Responses of a generalist MGC-PN (macroglomerular complex), which had arborizations in both the cumulus and toroid (not shown), to pulsed stimulation with Bal (bombykal) and C15 (*E*,*Z*-11,13-pentadecadienal) at different frequencies (**A:** Five/second; **B:** four/second; **C:** three/second). Antennal stimulation with Bal, C15, or the two-component blend elicited a primarily excitatory response in this type of MGC-PN. The responses to the binary blend (Bal+C15) were intermediate in terms of length and strength of after-hyperpolarization compared to the responses evoked by stimulation with either C15 or Bal alone. This neuron resolved higher pulse frequencies when stimulated with the binary blend.

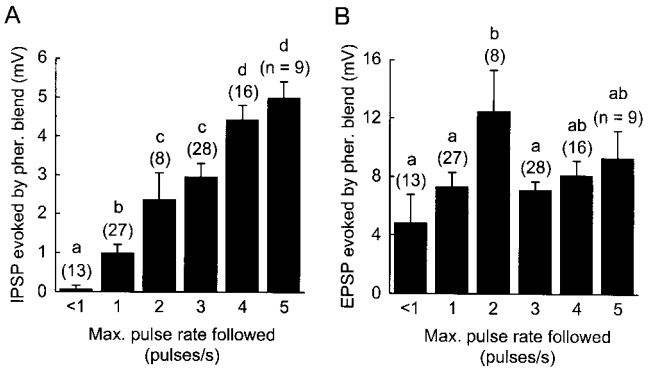


Fig. 7. **A:** Relationship between maximal pulse rate followed by MGC-PNs (macroglomerular complex) and the amplitude of the initial IPSP (inhibitory postsynaptic potential) (I_1) elicited by antennal stimulation with the two-component pheromone blend (mean IPSP amplitude ± SEM; numbers above columns indicate the number of neurons in a given group; columns with different letters are significantly different from each other; ANOVA (Kruskal-Wallis One-Way Analysis of Variance on Ranks) and All Pairwise Multiple Comparison Procedures, significance level $P < 0.05$). MGC-PNs resolving higher pulse rates showed a stronger IPSP than neurons with weak pulse following abilities. **B:** Relationship between maximal pulse rate followed by MGC-PNs and the amplitude of the EPSP (excitatory postsynaptic potential) elicited by antennal stimulation with the two-component pheromone blend. MGC-PNs that followed different maximal pulse rate had similar EPSP amplitudes.

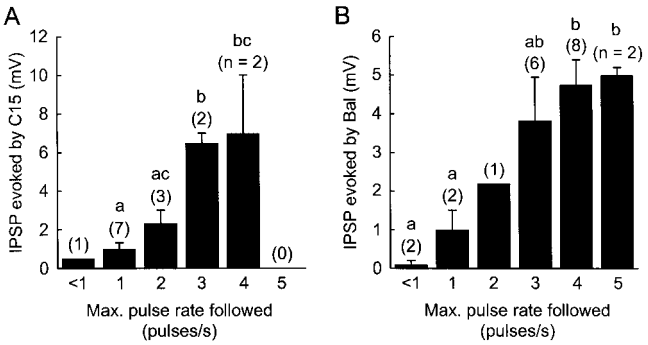


Fig. 8. **A:** Pulse following and IPSP (inhibitory postsynaptic potential) amplitude of MGC-PNs (macroglomerular complex) that gave a primarily excitatory response to antennal stimulation with C15 (*E*,*Z*-11,13-pentadecadienal) (mean IPSP amplitude ± SEM; numbers above columns indicate the number of neurons in a given group; columns with different letters are significantly different from each other; ANOVA (Kruskal-Wallis One-Way Analysis of Variance on Ranks) and All Pairwise Multiple Comparison Procedures for groups with $n \geq 2$, significance level $P < 0.05$). MGC-PNs resolving higher pulse rates showed a stronger IPSP in response to antennal stimulation with C15 than neurons with weak pulse following abilities. **B:** Likewise, MGC-PNs that showed a primarily excitatory response to stimulation with Bal (bombykal) and followed high pulse rates, had a strong IPSP in response to Bal.

evoked by antennal stimulation with one pheromone component was plotted for MGC-PNs with different maximal pulse rate followed. For C15 specialist PNs that showed a triphasic response to stimulation with C15 and followed a high maximal pulse rate, the I_1 was higher than in C15 specialist PNs that only followed lower pulse rates (Fig. 8A) (regression analysis for plotting pulse following over IPSP amplitude: $n = 15$; $F = 30.24$; $r^2 = 0.70$; $P = 0.0001$; $y = 0.81 + 0.35x$). Likewise, for Bal specialist PNs that responded with a mixed response to Bal, the amplitude of the IPSP evoked by Bal was higher in neurons that followed higher pulse rates (Fig. 8B) (regression analysis for plotting pulse following over IPSP amplitude: $n = 21$; $F = 16.07$; $r^2 = 0.46$; $P = 0.0008$; $y = 1.59 + 0.41x$).

The responses of three MGC-PNs to pheromonal stimulation are shown in terms of three key response param-

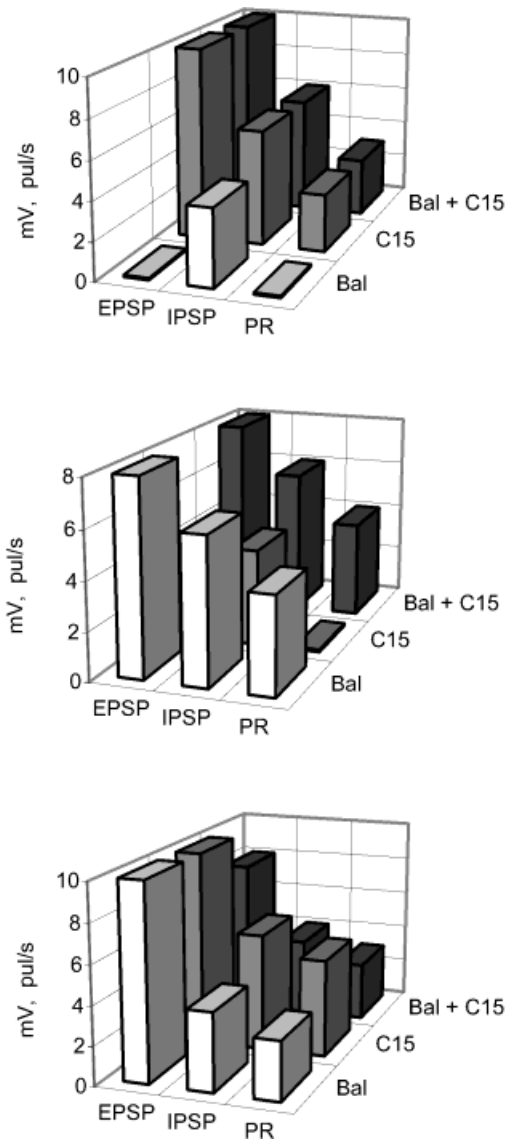


Fig. 9. Response parameters IPSP (inhibitory postsynaptic potential) (mV), EPSP (excitatory postsynaptic potential) (mV), and maximal pulse rate followed (PR in pulses per second) for a C15 (*E*,*Z*-11,13-pentadecadienal) specialist MGC-PNs (macroglomerular complex), a Bal (bombykal) specialist PN, and a pheromone generalist PN. These neurons were tested with C15, Bal, and the two-component blend and were from different animals. The C15 specialist PN was inhibited/excited by C15 and responded with inhibition to stimulation with Bal. The responses to antennal stimulation with the blend and with C15 were indistinguishable from each other (see also Figure 5). The ordinate gives the values for EPSP and IPSP in mV and for pulse rate in pulses/second, respectively. The Bal specialist PN was inhibited/excited by Bal and responded with an inhibition to stimulation with C15. The responses to antennal stimulation with Bal+C15 and with Bal were indistinguishable from each other (see also Figures 1A, 2A,B). The pheromone generalist neuron responded with inhibition/excitation to stimulation with C15, Bal, or Bal+C15, even though the responses were different from each other.

eters in Figure 9. These MGC-PNs were tested with all three stimuli: Bal, C15, and Bal+C15. The columns indicate the amplitudes of the EPSP (E) and the IPSP (I_1), as well as the maximal pulse rate followed by an MGC-PN (PR in pulses/second) for stimulation with Bal+C15 as well as both individual components. In both "specialist" MGC-PNs, the C15 specialist cell and the Bal specialist cell, the response parameters (EPSP, IPSP, and maximal pulse rate followed) were identical for antennal stimulation with Bal+C15, and for stimulation with Bal or C15, respectively. This was the case even though the other component clearly inhibited the MGC-PN.

Based on intracellular recording and staining, the number of MGC-PNs with arborizations in both cumulus and toroid and which are excited by both Bal and C15 (pheromone generalist PNs) apparently is relatively small (Hansson et al., 1991; this study), perhaps fewer than 20% of all MGC-PNs. The relationship between the amplitudes of the IPSP and the EPSP and the maximal pulse rate followed appeared to be similar to the relationships described for Bal cells and C15 cells, i.e., the larger the I_1 phase evoked by one component or the blend, the greater the pulse rate the MGC-PN could follow (Fig. 9). The response to antennal stimulation with Bal+C15, however, did not necessarily reflect the response to either component. In some cases presenting both components together elicited a response that was temporally more accurate than the response to either component alone, as described above (Fig. 6).

DISCUSSION

In *M. sexta*, the MGC-PNs that encode pheromonal information are physiologically and morphologically diverse. Some MGC-PNs respond specifically to antennal stimulation with only one of the two key pheromone components (specialist MGC-PNs; Hansson et al., 1991). In other neurons ("blend MGC-PNs"), antennal stimulation with the pheromone blend evokes a characteristic, multiphasic response consisting of early inhibition, excitation, and delayed hyperpolarization (Christensen and Hildebrand, 1987, 1988, 1997; Christensen et al., 1998). The findings from this study show that there are also specialist MGC-PNs associated with a single MGC glomerulus for which stimulation with one of the two key pheromone components is sufficient to elicit such a multiphasic response. This had been observed previously in only one MGC-PN, which exhibited a multiphasic response to Bal (Hansson et al., 1991), but we have found that the multiphasic response can be elicited in some specialist MGC-PNs by stimulation with C15 as well.

An MGC-PN with arborizations confined to the cumulus (Fig. 4) responds preferentially to antennal stimulation with the second essential pheromone component (E10,E12,14Z-16:Al) or its synthetic mimic, C15 (Hansson et al., 1991). Some such neurons give a purely excitatory response (Hansson et al., 1991), whereas others give a mixed response with both inhibitory and excitatory phases (Fig. 5A,B). Moreover, some MGC-PNs that innervate the cumulus exhibit hyperpolarization and/or reduction of background firing activity in response to antennal stimulation with Bal. This suggests that sensory afferents tuned to Bal stimulate either local interneurons or MGC-PNs that have arborizations in the toroid, which in turn inhibit C15 specialist PNs with arborizations in the cumulus. The hole in the toroid, through which the large-diameter neurites of cumulus neurons pass, is a likely site for these synaptic interactions. In that region, at the light-microscopic level, thick branches of cumulus and toroid neurons appear to be in close apposition (Fig. 3; Heinbockel et al.,

1996; unpublished observations) and thus may form synaptic contacts.

The responses of specialist MGC-PNs to antennal stimulation with the two-component blend or to stimulation with just one of its components were similar in terms of the response phases present and the amplitudes of the evoked IPSPs and EPSPs. Furthermore, in most cases, the maximal pulse rate followed by a specialist MGC-PN was the same for stimulation with the two-component blend or with one of its components (Figs. 2, 5, 9). This was the case even when stimulation with the other component evoked hyperpolarization in the same neuron. These neurons are therefore functionally distinct from the blend MGC-PNs that are primarily excited by one component and inhibited by the other (Christensen and Hildebrand, 1987, 1997; Christensen et al., 1998). For that type of neuron, with Bal alone as the stimulus, the dynamic range for encoding stimulus intermittency was reduced in nearly 60% of the neurons tested (Christensen and Hildebrand, 1997).

MGC-PNs appear not to be tuned to a specific pulse rate, i.e., they did not resolve odor pulses at only one particular pulse rate and not at higher or lower rates. Instead, MGC-PNs act as low-pass filters, following pulses up to a maximal pulse rate or cut-off frequency. All pulse rates below the cut-off frequency were resolved, whereas the temporal resolution of each odor pulse was impaired at higher pulse rates.

A small group of MGC-PNs, which we have called generalist MGC-PNs, innervates both principal glomeruli (cumulus and toroid) of the MGC (Hansson et al., 1991; this study). The responses of these MGC-PNs to antennal stimulation with Bal, C15, or the two-component blend were primarily excitatory. The responses of generalist MGC-PNs to the blend, however, were not necessarily similar to their responses to stimulation with either Bal or C15 alone. The difference of response often was evident in the number of action potentials and the maximal pulse rate that was followed by that MGC-PN. This was particularly obvious when one component evoked a brief, excitatory response and the other component evoked a continuous burst lasting several seconds. Generalist neurons of this type, and probably other multiglomerular AL neurons as well, are not simple convergence stations for afferent input of the same type, which then relay this information to higher centers in the protocerebrum. Instead, these MGC-PNs integrate information from both Bal and C15 primary-afferent input channels at the level of the AL. In contrast, specialist MGC-PNs that innervate only the toroid or the cumulus give excitatory responses preferentially or exclusively to antennal stimulation with only one component, Bal or C15 (Christensen and Hildebrand, 1987; Christensen et al., 1989b; Hansson et al., 1991). These MGC-PNs send information from one input channel to the protocerebrum, and integration of the two parallel pathways for Bal and C15 takes place in higher brain centers (Kanzaki et al., 1991a,b; Hildebrand, 1996).

When the depolarization in response to pheromonal stimulation is preceded by an IPSP, most MGC-PNs can follow odor pulses up to several per second (Christensen and Hildebrand, 1988, 1997). In the present study, we explored this relationship further, asking whether the amplitude of the IPSP is correlated with the maximal pulse rate followed by specialist MGC-PNs when the antenna was stimulated with: a) the blend of Bal and C15, or b) either Bal or C15 alone. In both cases, the size of the IPSP but not the EPSP was clearly correlated with the ability of an MGC-PN to follow higher odor pulse rates. For example, when the antenna was stimulated with C15 and a strong IPSP of several mV preceded the excitatory response, the MGC-PN typically could resolve pulse rates of three to five/second (Figs. 2B, 9). This relationship was observed in Bal specialist PNs for stimulation with Bal and in C15 specialist PNs for stimulation with C15. These findings, coupled with the knowledge that these neurons are uniglomerular, indicate that the antennal ORCs responding to a single pheromone component are a functionally diverse population. Some afferent axons terminate on excitatory elements that in turn depolarize the PNs, while others invading the same glomerulus terminate on inhibitory elements that provide feed-forward inhibition to PNs (Christensen et al., 1993). Thus, although these afferent neurons are activated by a single odorant, they specifically target different types of MGC neurons. These data reinforce our view that even within highly specified "labelled line" systems like the moth pheromone-processing MGC, neurons that belong to such "dedicated" pathways (as defined by their odor specificities) may nevertheless be heterogeneous in other respects, and serve different functional roles in shaping glomerular output.

In previous studies of *M. sexta*, five blend MGC-PNs receiving strong inhibitory and excitatory inputs were observed that followed a pulse rate up to about 10/second with depolarizing potentials and time-locked bursts of one or several action potentials (Christensen and Hildebrand, 1988, 1997). Most MGC-PNs (≥90%) in those studies, however, followed pheromone pulses only up to three to four/second, similar to the present findings. The maximal odor-pulse rates followed by MGC-PNs therefore cover well the range of stimulus frequencies for which behavioral changes have been observed in moths (Kaissling and Kramer, 1990; Mafra-Neto and Cardé, 1994; Vickers and Baker, 1994, 1996). Pheromone-responsive ORCs in the antennae of male *M. sexta* also differ in their responsiveness to pulsed stimulation (Marion-Poll and Tobin, 1992), and thus low- and high-frequency pulse-processing pathways probably exist throughout the olfactory system, from receptor neurons to motor output. In another moth, *Antheraea polyphemus*, pheromone-responsive ORCs in a single sensillum differ in terms of their molecular receptive ranges, their relative sensitivities, and their temporal filtering properties (Meng et al., 1989). In this species, two of three types of pheromone-responsive ORCs can resolve at least five stimulus pulses/second, whereas the third cell is slower, resolving only about two pulses/second (Rumbo and Kaissling, 1989). Thus, different pathways in the moth brain might register the structure of the odor plume in a different manner, as suggested by Murlis et al. (1992).

Our results have some interesting implications for the synaptic organization of the glomerular neuropil in the moth MGC. Similar studies in heliothine moths have suggested that the tuning characteristics of different classes of MGC-PNs may reflect specific input from physiologically distinct populations of ORCs (Christensen et al., 1995: Vickers et al., 1998), and these new data agree with those findings. The exceptional ability of blend MGC-PNs to follow stimulus pulses up to about 10/second may also have to do with the spatial arrangement of inhibitory inputs relative to the spike-initiation zone in these neurons, thus providing a more precise regulation of spiking output that closely reflects the intermittent input (Chris-

tensen and Hildebrand, 1997). The specialist MGC-PNs, on the other hand, may receive greater numbers or stronger excitatory synapses that would more effectively counterbalance the inhibitory input. Specialists may also have longer integration times, such that even a brief stimulus would lead to prolonged firing, even after the stimulus had been discontinued (Fig. 2). Whatever the causes, it is clear that different types of neurons possess differing abilities to encode temporally varying olfactory stimuli, and it is tempting to speculate that these differences are related to the specific roles played by each of types of neurons at different stages in mate-seeking behaviors. Only a subpopulation of MGC-PNs, the blend neurons, can resolve the fastest rates of pulsing, and these pathways could therefore signal that the male is in close proximity to the pheromone source (Christensen and Hildebrand, 1997). Specialist neurons, having a slower resolving power in the range of three to five/second, may be specifically involved with identifying the correct components of the species-specific blend. Still other ouput pathways may serve another specific function: blend neurons and some generalist neurons respond differently to the complete blend and the individual components of the blend (Fig. 6), and thus could signal the presence of the correct ratio of components in the blend (Christensen and Hildebrand, 1997). These results therefore provide further support for the hypothesis that the moth MGC (and probably other arrays of glomeruli) is organized to process in parallel the many different features of an airborne odor stimulus.

ACKNOWLEDGMENTS

We thank Drs. P. Kloppenburg, V.D.C. Shields, and L.P. Tolbert for encouragement and valuable discussions, Drs. G.D. Burd and L.P. Tolbert for helpful comments on the manuscript, Peggy Randolph for general laboratory assistance, Dr. A.A. Osman for insect rearing, and Dr. J.H. Tumlinson for providing the odorants used in this study.

LITERATURE CITED

Almaas TJ, Christensen TA, Mustaparta H. 1991. Chemical communication in heliothine moths. I. Antennal receptor neurons encode several features of intra- and interspecific odorants in the male corn earworm moth *Helicoverpa zea*. J Comp Physiol A 169:249–258.

Baker TC. 1989. Sex pheromone communication in the Lepidoptera: new research progress. Experientia 45:248–262.

Baker TC, Willis MA, Haynes KF, Phelan PL. 1985. A pulsed cloud of sex pheromone elicits upwind flight in male moths. Physiol Entomol 10:257–265.

Baker TC, Hansson BS, Löfstedt C, Löfqvist J. 1988. Adaptation of antennal neurons in moths is associated with cessation of pheromone-mediated upwind flight. Proc Natl Acad Sci USA 85:9826–9830.

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. 1988. Frequency coding by central olfactory neurons in the sphinx moth *Manduca sexta*. Chem Senses 13:123–130.

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

Christensen TA, Mustaparta H, Hildebrand JG. 1989a. Discrimination of sex pheromone blends in the olfactory system of the moth. Chem Senses 14:463–477.

Christensen TA, Hildebrand JG, Tumlinson JH, Doolittle RE. 1989b. 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, Waldrop B, Harrow ID, Hildebrand JG. 1993. Local interneurons and information processing in the olfactory glomeruli in the moth *Manduca sexta*. J Comp Physiol A 173:385–399.

Christensen TA, Mustaparta H, Hildebrand JG. 1995. Chemical communication in heliothine moths. VI. Parallel pathways for information processing in the macroglomerular complex of the male tobacco budworm moth *Heliothis virescens*. J Comp Physiol A 177:545–557.

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.

Christensen TA, Waldrop BR, Hildebrand JG. 1998. Multitasking in the olfactory system: context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. J Neurosci 18:5999–6008.

Connor WE, Eisner T, Vander Meer RK, Guerrero A, Ghiringelli D, Meinwald J. 1980. Sex attractant of an Arctiid moth (*Utetheisa ornatrix*): a pulsed chemical signal. Behav Ecol Sociobiol 7:55–63.

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*. Chem Senses 20:707.

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, editors. Brain and evolution, Proceedings of the 24th Göttingen Neurobiology Conference 1996, Vol I. Stuttgart, New York: Thieme, p 69.

Heinbockel T, Kloppenburg P, Hildebrand JG. 1998. Pheromone-evoked potentials and oscillations in the antennal lobes of the sphinx moth *Manduca sexta*. J Comp Physiol A 182:703–714.

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, Christensen TA, Hildebrand JG. 1989. Structure and function of the deutocerebrum in insects. Ann 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.

Kaissling K-E. 1987. R.H. Wright lectures on insect olfaction. In: Colbow K, editor. Burnaby, BC: Simon Fraser University.

Kaissling K-E, Kramer E. 1990. Sensory basis of pheromone-mediated orientation in moths. Verh Dtsch Zool Ges 83:109–131.

Kaissling K-E, Hildebrand JG, Tumlinson JH. 1989. Pheromone receptor cells in the male moth *Manduca sexta*. Arch Insect Biochem Physiol 10:273–279.

Kanzaki R, Arbas EA, Hildebrand JG. 1991a. Physiology and morphology of protocerebral olfactory neurons in the male moth *Manduca sexta*. J Comp Physiol A 168:281–298.

Kanzaki R, Arbas EA, Hildebrand JG. 1991b. Physiology and morphology of descending neurons in pheromone-processing olfactory pathways in the male moth *Manduca sexta*. J Comp Physiol A 169:1–14.

Kramer E. 1986. Turbulent diffusion and pheromone triggered anemotaxis. In: Payne TL, Birch MC, Kennedy CEJ, editors. Mechanisms in insect olfaction. Oxford: Oxford University Press. p 59–67.

Kramer E. 1992. Attractivity of pheromone surpassed by time-patterned application of two nonpheromone compounds. J Insect Behav 5:83–97.

Mafra-Neto A, Cardé RT. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. Nature 369:142–144.

Marion-Poll F, Tobin TR. 1992. Temporal coding of pheromone pulses and trains in Manduca sexta. J Comp Physiol A 171:505–512.

Meng LZ, Wu CH, Wicklein M, Kaissling K-E, Bestmann HJ. 1989. Number and sensitivity of three types of pheromone receptor cells in *Antheraea pernyi* and *A. polyphemus*. J Comp Physiol A 165:139–146.

Mesce KA, Klukas KA, Brelje TC. 1993. Improvements for the anatomical characterization of insect neurons in whole mount: the use of cyanine-derived fluorophores and laser scanning confocal microscopy. Cell Tiss Res 271:381–397.

Murlis J, Elkinton JS, Cardé RT. 1992. Odor plumes and how insects use them. Ann Rev Entomol 37:505–532.

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.

Rumbo ER, Kaissling K-E. 1989. Temporal resolution of odour pulses by three types of pheromone receptor cells in *Antheraea polyphemus*. J Comp Physiol A 165:281–291.

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.

Sun XJ, Tolbert LP, Hildebrand JG. 1993. Ramification pattern and ultrastructural characteristics of the serotonin immunoreactive neuron in the antennal lobe of the moth *Manduca sexta*: a laser scanning confocal and electron microscopic study. J Comp Neurol 338:5–16.

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. 1994. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. Proc Natl Acad Sci USA 91:5756–5760.

Vickers NJ, Baker TC. 1996. Latencies of behavioral response to interception of filaments of sex pheromone and clean air influence flight track shape in *Heliothis virescens* (F.) males. J Comp Physiol A 178:831–847.

Vickers NJ, Christensen TA, Hildebrand JG. 1998. Combinatorial odor discrimination in the brain: attractive and antagonist odor blends are represented in distinct combinations of uniquely identifiable glomeruli. J Comp Neurol 400:35–56.

Waldrop B, Christensen TA, Hildebrand JG. 1987. GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, *Manduca sexta*. J Comp Physiol A 161:23–32.