Chem. Senses 28: 325–338, 2003

Functional Divergence of Spatially Conserved Olfactory Glomeruli in Two Related Moth Species

Neil J. Vickers[1] and Thomas A. Christensen[2]

[1]Department of Biology, University of Utah, Salt Lake City, UT 84112 and [2]Arizona Research Laboratories—Division of Neurobiology, University of Arizona, Tucson, AZ 85721, USA

Correspondence to be sent to: Neil J. Vickers, Department of Biology, University of Utah, 257 S. 1400 E., Rm 201, Salt Lake City, UT 84112, USA. e-mail: vickers@biology.utah.edu

Abstract

In different moth species, the number and spatial arrangement of olfactory glomeruli in the antennal lobe (AL) vary widely, but the spatial map within a species is thought to be invariant, making it possible to identify single glomeruli across individuals. We investigated the relationship between the physiological tuning of pheromone-selective interneurons and their association with specific, identified glomeruli in the macroglomerular complex (MGC) of the noctuid moth, *Heliothis subflexa*. Three odorants that are required for pheromone-source location in this species were tested individually and in blends. Recordings from 27 pheromone-specific projection neurons (PNs) indicated that the majority (48%) were selectively activated by the major pheromone component of this species, *Z*-11-hexadecenal (Z11–16:Ald), with 33% primarily tuned to *Z*-9-hexadecenal and 19% to *Z*-11-hexadecenol. Intracellular staining revealed that the dendrites of PNs tuned to Z11–16:Ald always branched within the largest glomerulus of the MGC, the cumulus. Similarly, each of the other two classes of PN was associated with a different 'satellite' glomerulus in the MGC. The spatial configuration of the four-glomerulus *H. subflexa* MGC was indistinguishable from that previously reported in the closely related species, *Heliothis virescens*. Hence, as these two species diverged, changes in the association of satellite MGC glomeruli with particular odorants have occurred without a measurable change in the anatomical arrangement of the glomerular array.

Key words: antennal lobe, chemotopy, glomerulus, olfactory coding, pheromone

Introduction

Numerous moth species have an acute sense of smell, as exemplified by the ability of males to correctly identify and locate conspecific females releasing sex pheromones from long distances upwind (David and Birch, 1989). Previous investigations in two species of noctuid moth, *Heliothis virescens* and *Helicoverpa zea*, have shown that a behavioral preference for the conspecific pheromone blend is reflected in the physiological responses of both olfactory receptor cells (ORCs) (Almaas and Mustaparta, 1990, 1991; Almaas *et al*., 1991; Hansson *et al*., 1995; Cossé *et al*., 1998) and central projection neurons (PNs) (Christensen *et al*., 1991, 1995; Berg *et al*., 1998; Vickers *et al*., 1998) innervating the glomeruli of the male-specific macroglomerular complex (MGC). It was shown that the number and spatial arrangement of the sexually dimorphic glomeruli that constitute the MGC were different in the two species (Christensen *et al*., 1991, 1995; Vickers *et al*., 1998). Within both species, however, the position of each glomerulus was invariant, thereby making it possible to identify all MGC glomeruli reliably and repeatedly across individuals. Using single-unit intracellular recording and staining methods, a precise chemotopic map was revealed across the different MGC glomeruli in *H. virescens* by showing that PNs with a similar physiological response profile always innervated the same glomerulus (Vickers *et al*., 1998). These results supported the hypothesis that odor-blend discrimination in these species occurs through the brain's recognition of different cross-glomerular (or combinatorial) activity patterns in the AL (Christensen and White, 2000).

Phylogenetic studies using genetic markers have shown that *Heliothis subflexa* is a close relative of *H. virescens* (Cho *et al*., 1995; Fang *et al*., 1997), confirming earlier studies using morphological characters (Mitter *et al*., 1993). Nevertheless, the pheromone blends produced and released by females of the two species are chemically distinct (Roelofs *et al*., 1974; Tumlinson *et al*., 1975; Klun *et al*., 1980, 1982; Teal *et al*., 1981, 1986; Vetter and Baker, 1983; Heath *et al*., 1991). Both species share the same primary pheromone component, (*Z*)-11-hexadecenal (Z11–16:Ald), but each requires the presence of a different set of secondary components to communicate with conspecifics. In a wind-tunnel bioassay, *H. virescens* males flew upwind when

a small amount of (*Z*)-9-tetradecenal (Z9–14:Ald) was combined with Z11–16:Ald (0.05:1 ratio), but their flight was arrested when a chemically similar odorant, (*Z*)-11-hexadecenyl acetate (Z11–16:Ac), was added to this binary blend (Vickers and Baker, 1997). Recent behavioral studies have revealed that (*Z*)-9-hexadecenal (Z9–16:Ald) and (*Z*)-11-hexadecenol (Z11–16:OH) are required in addition to Z11–16:Ald by *H. subflexa* males, but that upwind flight was unaffected by the addition of Z11–16:Ac to this blend (Vickers, 2002). All four of these odorants have been isolated from female *H. subflexa* pheromone glands (Teal *et al*., 1986; Heath *et al*., 1991; Teal and Tumlinson, 1997).

The MGC in *H. virescens* receives input from at least four different populations of ORCs on the antenna (Hansson *et al*., 1995; Berg *et al*., 1998). Two of these pathways represent the conspecific pheromone blend (Z11–16:Ald and Z9–14:Ald) and are mapped to two glomeruli (the cumulus and a dorso-medial glomerulus, DM, Figure 1A). Z11–16:Ac is not a component of the *H. virescens* pheromone blend, but it is produced and emitted by *H. subflexa* females (Teal *et al*., 1986; Heath *et al*., 1991) and it inhibits upwind flight in *H. virescens* (Vickers and Baker, 1997). This interspecific antagonist is represented by a third pathway that is mapped to a separate glomerulus situated in an anteromedial position relative to the cumulus (AM) (Vickers *et al*., 1998). These studies confirmed that the MGC in this insect, besides being involved in intraspecific communication, also plays a role in processing and discriminating chemically similar signals released by interspecific females. This chemotopic organization could serve as an important aid in maintaining reproductive isolation.

In this study, we test the hypothesis that glomeruli in the MGC of *H. subflexa* males (like their *H. virescens* congeners) are also organized chemotopically. We used intracellular recording and staining to match the odor-tuning properties of glomerular PNs with the spatial assignment of their dendritic arborizations in the MGC glomeruli. Based on results from other heliothine species, we predicted that neurons with similar physiological response profiles would show consistent patterns of dendritic arborization within specific identifiable glomeruli across individual males. In addition, by comparing the results of these studies with others on heliothine moths (particularly *H. virescens*), we gained insight into the divergence of male olfactory characteristics that have accompanied the evolution of these species.

Materials and methods

Insects

A series of *H. subflexa* colonies were established between 1997 and 1999 from pupae and eggs sent to the University of Arizona and the University of Utah from North Carolina State University. Neonate larvae were reared on either a pinto-bean diet (Shorey and Hale, 1965) in large ice-cream cups or a corn-soy blend diet (A. Sheck, personal communication) in individual 30 ml medicine cups fitted with cardboard lids (WLM Inc., Newark, NJ). Once the larvae had pupated they were removed from the cups and pupae were segregated by sex. Male pupae were placed in an environmental chamber (Percival Scientific, Boone, IA) at 25°C, 60% RH, 14:10 L:D reversed light cycle and allowed to eclose. Males were aged daily and cohorts of aged males were kept in plastic containers. Males between the ages of 3 and 10 days were utilized in neurophysiological experiments.

Stimulation

Details of the preparation of animals for recording and the experimental set-up have been described in detail previously (Christensen *et al*., 1995; Vickers *et al*., 1998). All olfactory stimuli were delivered as a series of five 40 ms pulses (interpulse interval 300 ms) from an odor cartridge directed at one antenna. The rapid presentation of brief odor pulses allows an assessment of the ability of each neuron to follow the temporal pattern of the stimulus. The odorants included the following single compounds that have been identified as pheromone components in *H. subflexa* or other closely related heliothine moths, including *H. virescens* and *H. zea*: (*Z*)-11-hexadecenal (Z11–16:Ald), (*Z*)-9-tetradecenal (Z9–14:Ald), (*Z*)-9-hexadecenal (Z9–16:Ald), (*Z*)-11-hexadecenyl acetate (Z11–16:Ac), (*Z*)-11-hexadecenol (Z11–16:OH). In addition, several synthetic mixtures were tested: *H. subflexa* 4-mix, *H. subflexa* 3-mix; *H. virescens* 1:0.5 blend, *H. virescens* 2-mix; and *H. virescens* 6-mix (ratios detailed in Table 1). As eight positions on the odor cartridge-holder assembly were available only two blends (along with the five single odorants and a blank control) were tested against any given neuron. Typically, one *H. subflexa* and one *H. virescens* mixture were used. In earlier experiments, an *H. subflexa* female pheromone gland extract was used as a stimulus. Gland extracts were prepared by removing the female pheromone gland during the fourth to sixth hour of scotophase and placing it in 50 µl hexane for 1 min. Single odorants were loaded onto filter paper strips (0.7 cm × 3.5 cm, Whatman No. 1, Maidstone, UK) as 10 µl aliquots to give a final dosage of 10 ng. For blends, individual compounds were loaded in the stated ratio with respect to 10 ng of Z11–16:Ald. Thus, a 1:0.05 mixture of Z11–16:Ald:Z9–14:Ald had a dosage of 10 ng Z11–16:Ald and 0.5 ng Z9–14:Ald loaded onto the filter paper substrate. A blank cartridge containing only a filter paper strip was used as a control.

Morphology

Intracellular neurophysiological recordings were obtained using borosilicate glass microelectrodes filled at the tip with Lucifer yellow CH (LY, 4–6% in 0.2 M LiCl) and backfilled with 2 M LiCl. Resistance of the microelectrodes varied between 150 and 400 MΩ. Following physiological characterization, LY was injected into the neuron by passing

negative direct current (up to 1 nA for 10 min). Details of histological procedures to prepare brains for microscopic examination have been provided in detail previously (Vickers *et al*., 1998). Wholemount and sectioned material were examined on either a Bio-Rad MRC 600 (equipped with: Nikon Optiphot-2 microscope, 100 mW Argon laser light source, filter cube: 457 nm excitation) or Zeiss LSM 510 (25 mW Argon laser light source, excitation: 458/514 nm dichroic, emission: LP 475 nm) laser scanning confocal microscopes (LSCM). Serial optical images were collected at intervals of either 1 or 2 μm.

Neurophysiological analysis

Intracellular responses were monitored continuously by oscilloscope and recorded on either FM tape (Vetter model D instrumentation tape recorder) or VHS tape (Vetter PCM recorder). Analysis of action potential trains was performed off-line using either Axon Instruments' Axoscope or Run Technologies' Datapac2000. Acquisition was triggered on the rising phase of the first stimulus pulse and 2 s of pre-trigger and 4 s of post-trigger data were recorded. Acquired records were then analyzed for neuronal spiking activity by setting user-defined thresholds that varied according to the quality of the recording. Spike data were then converted into instantaneous frequency–time histograms (IFTs) so that responses could be compared across stimuli within the same neuron or across different neurons.

Results

Morphology of the MGC in *H. subflexa* males

Detailed morphological examination of the AL in male *H. subflexa* moths revealed that the MGC is dominated by a large glomerulus situated near the entrance of the antennal nerve into the AL (Figure 1B) as demonstrated in other noctuid species (Christensen *et al*., 1991, 1995; Hansson *et al*., 1994; Anton and Hansson, 1995; Berg *et al*., 1998, 2002; Vickers *et al*., 1998). In the sphingid moth, *Manduca sexta* as well as two other heliothine moths, this glomerulus was named the 'cumulus' because of its complex, multi-lobed structure (Hansson *et al*., 1992; Vickers *et al*., 1998; Berg *et al*., 2002). We therefore use the same term for this large glomerulus in *H. subflexa*. Three additional satellite glomeruli are positioned near the cumulus in a precise spatial arrangement that is indistinguishable from the *H. virescens* MGC (Figure 1A). We previously devised a scheme for naming each satellite glomerulus according to its spatial position relative to the cumulus. In *H. virescens* these are the AM (antero-medial), VM (ventro-medial) and DM (dorso-

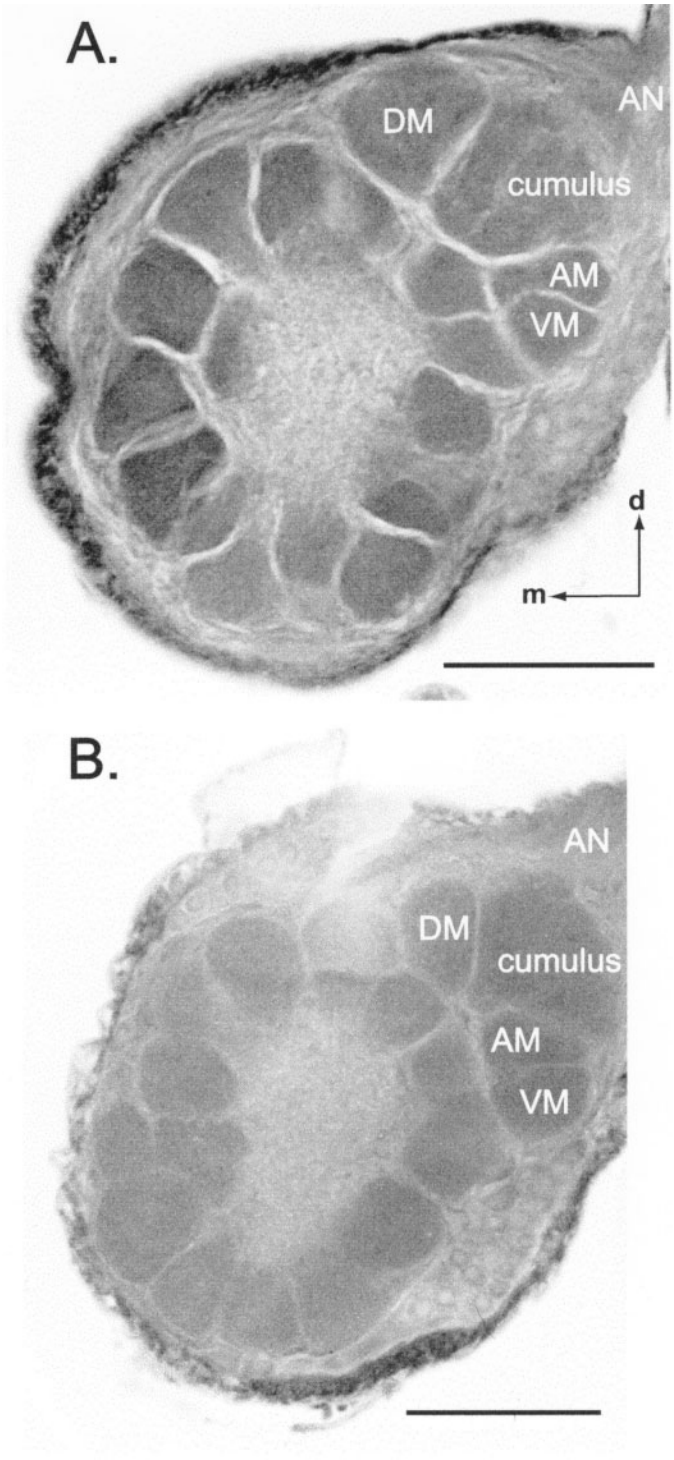

Figure 1 Comparison of the macroglomerular complexes (MGCs) in *H. virescens* **(A)** and *H. subflexa* **(B)** male moths. (A) Confocal photomicrograph showing a frontal view of a slice through the antennal lobe (AL) of a male *H. virescens*. At this depth (~60 μm from the AL surface), the four glomeruli of the MGC were clearly visible. (B) Confocal photomicrograph at approximately the same depth below the AL surface in *H. subflexa* revealed an MGC comprised of four glomeruli in a spatial configuration that was virtually indistinguishable from that of *H. virescens*. In both species a large, multi-lobed glomerulus (the cumulus) occupied the area closest to the entrance of the antennal nerve (AN). The three remaining 'satellite' glomeruli were arranged around the cumulus. Because of their anatomical similarity the same nomenclature was used to describe the satellite glomeruli in both species: AM = antero-medial; DM = dorso-medial; VM = ventro-medial. Scale bar = 100 μm. Dorsal, d; medial, m.

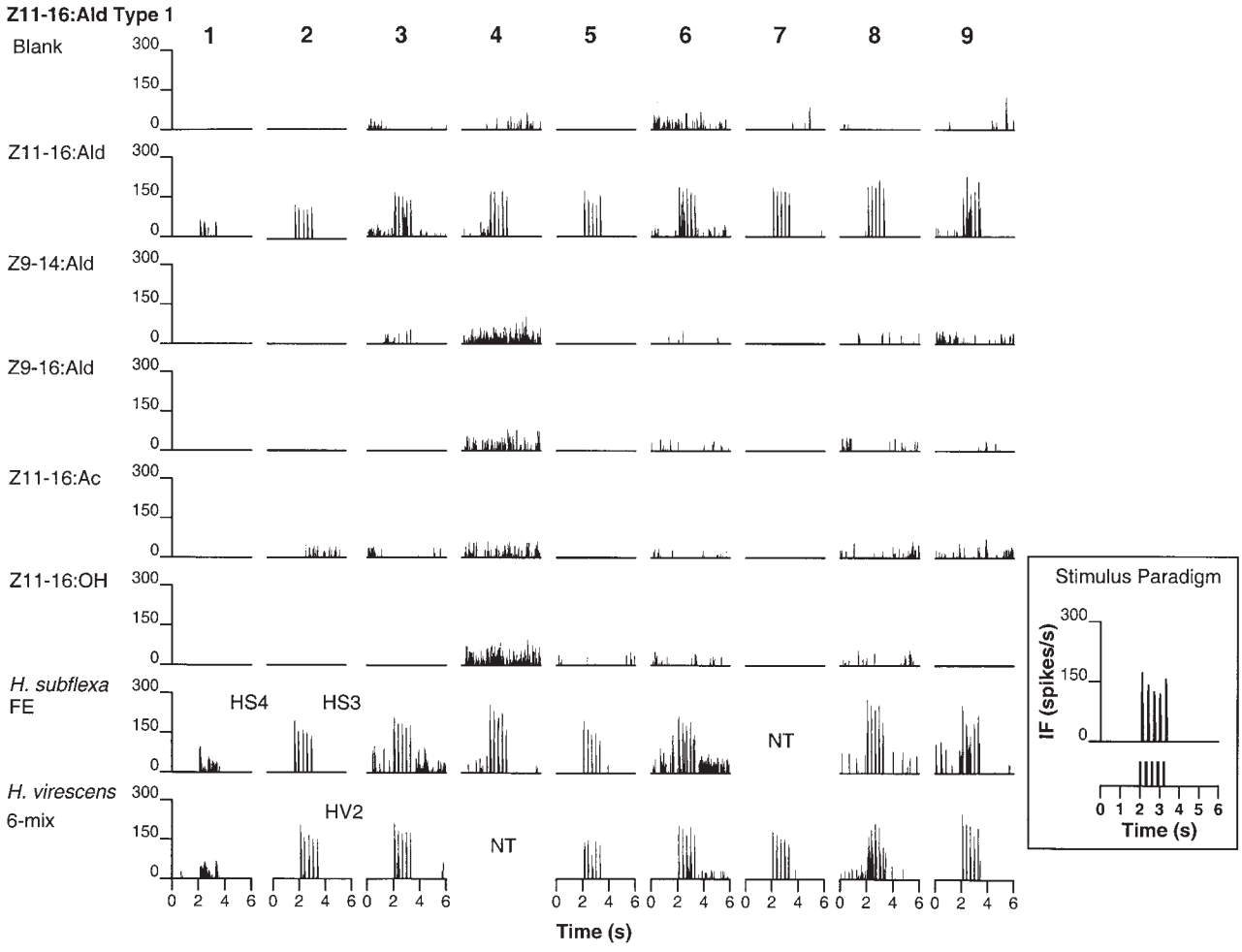

Figure 2 Instantaneous frequency (spikes/second) versus time histograms (IFTs) detailing the responses of nine Z11–16:Ald Type 1 neurons characterized in this study. Responses to antennal stimulation with Z11–16:Ald were excitatory, with each stimulus pulse eliciting a phasic burst of action potentials. Neither inhibitory nor excitatory responses were noted to other stimuli. Neurons were organized with respect to apparent threshold from highest (cell no. 1) to lowest (cell no. 9). In most cases, responses to Z11–16:Ald alone were no different than blends containing Z11–16:Ald: *H. subflexa* female equivalent (FE), 3-mix (HS3), or 4-mix (HS4), and either *H. virescens* 2-mix (HV2) or 6-mix. NT, not tested. Inset: Stimulation protocol consisted of five 40 ms pulses with an interpulse interval of 300 ms (shown below IFT histogram). Onset of the first pulse was at the 2 s mark.

Table 1 Ratios of individual compounds used to formulate synthetic mixtures to test male *H. subflexa* central olfactory projection neurons (all ratios were loaded onto a filter paper substrate with respect to a dosage of 10 ng Z11–16:Ald)

Compound	Blend				
	H. subflexa 3-mix	*H. subflexa* 4-mix	*H. virescens* 1:0.5	*H. virescens* 2-mix	*H. virescens* 6-mix
Z11–16:Ald	1	1	1	1	1
Z9–14:Ald			0.5	0.05	0.1
Z9–16:Ald	0.5	0.5			0.01
Z11–16:OH	0.1	0.1			
Z11–16:Ac		0.1			
Z7–16:Ald					0.01
14:Ald					0.05
16:Ald					0.5

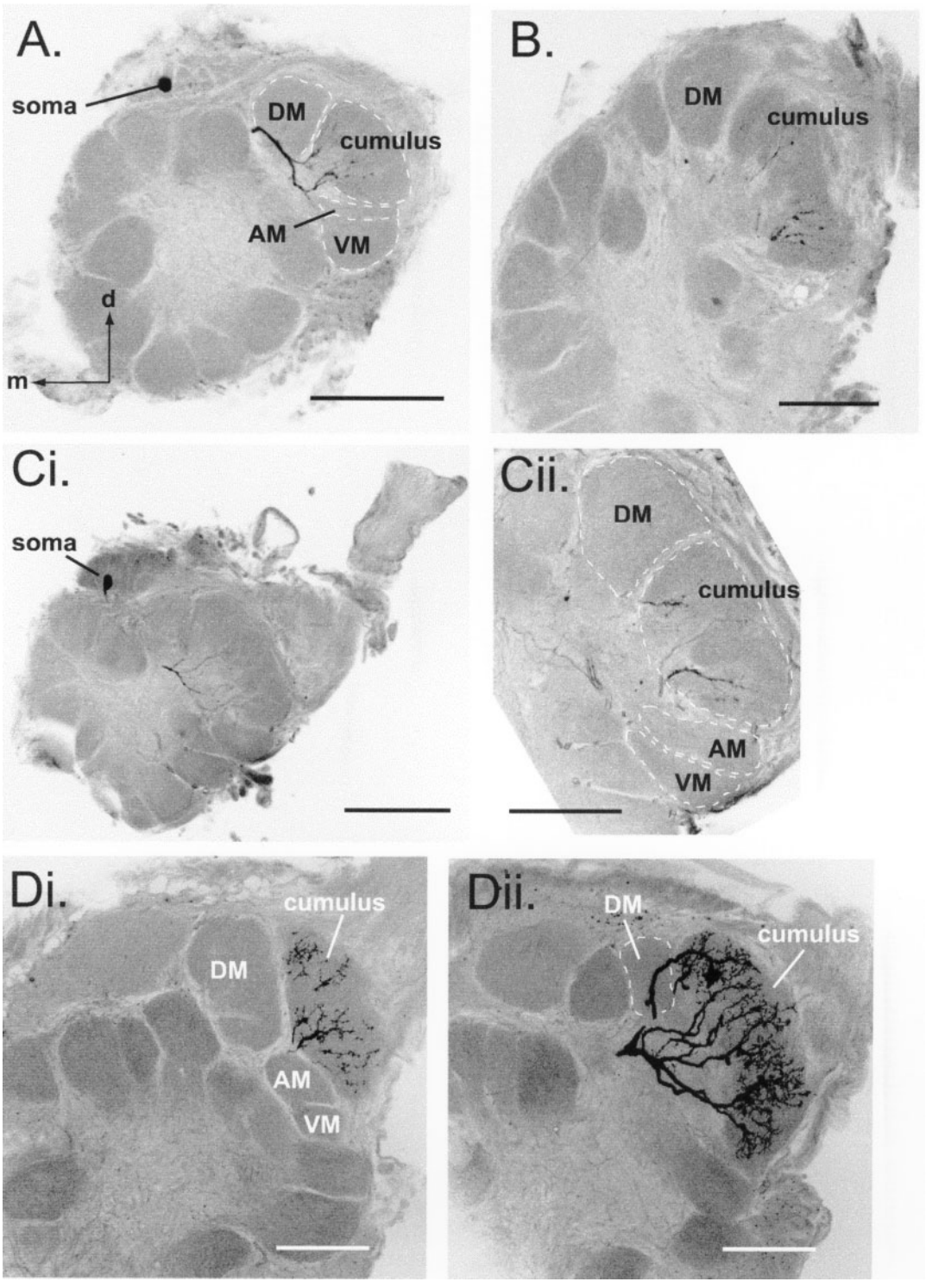

Figure 3 Morphology of four Z11–16:Ald (Type 1) PNs, based on confocal microscopy. Each neuron had a soma in the medial cell cluster and a primary neurite that gave rise to several branched dendrites that invaded the cumulus, bypassing all other glomeruli. Approximate borders of MGC glomeruli (which can become blurred when several consecutive LSCM images are combined in a projection) are outlined where necessary **(A)**. Morphology of cell 5 described in Figure 2. The primary and secondary dendritic branches of this PN clearly passed through DM en route to the cumulus without making dendritic arborizations. d, dorsal; m, medial (same orientation for B–D). **(B)** Morphology of cell 8 in Figure 2. **(C)** Morphology of cell 3 described in Figure 2. (**i**) Low power view showing the single PN with fine dendritic branches restricted to the cumulus. (**ii**) Higher magnification view of a section near the AL surface revealed that all dendritic arbors of this neuron were restricted to the cumulus. **(D)** Morphology of cell 2 described in Figure 2. (**i**) Closer to the AL surface all four MGC glomeruli were visible but dendritic arbors were restricted only to the cumulus. (**ii**) A section cut deeper through the AL illustrated how the volume of the cumulus expands posteriorly in the MGC. Notice how all dendritic arbors were confined within the borders of the cumulus even though some neurites may pass through other glomeruli (in this case DM) *en route* to the cumulus. Scale bars = 100 μm (A, Ci); 50 μm (B, Cii, Di, Dii).

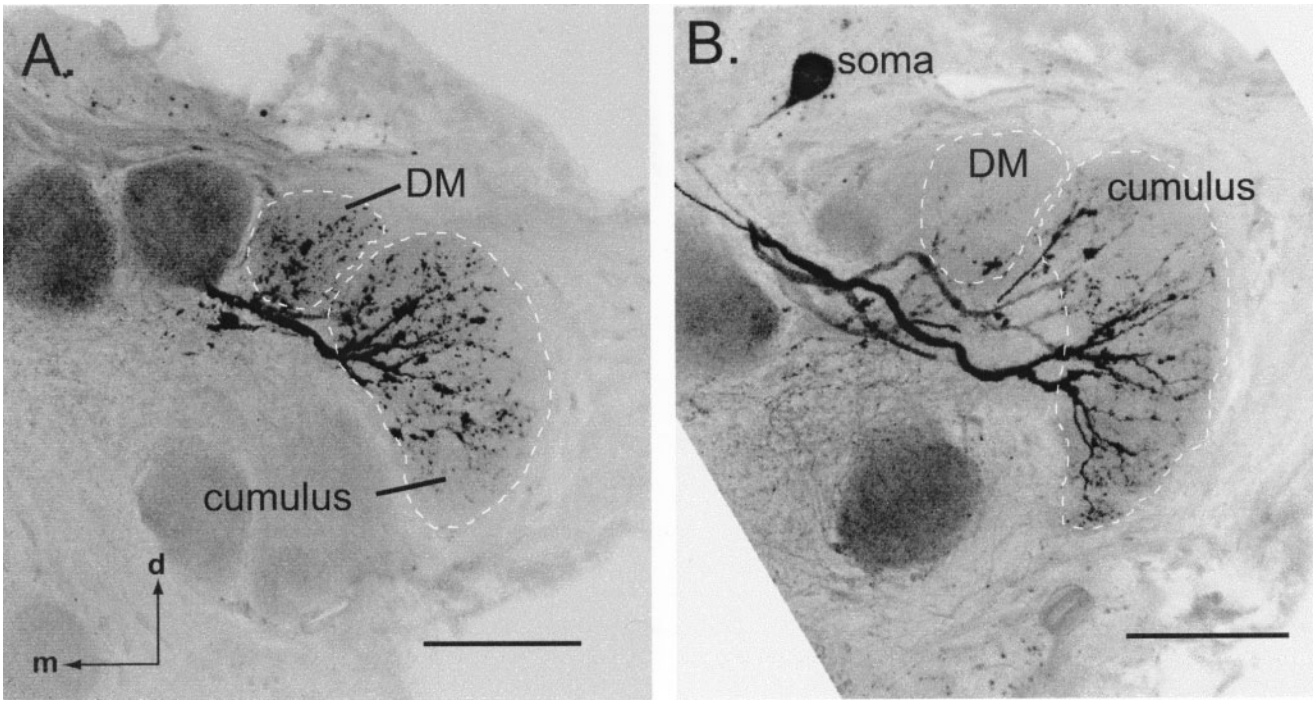

Figure 4 Confocal microscopy permitted unambiguous sorting of several PNs stained in the same preparation. In this example, one PN was more heavily stained and shows dendritic arborizations restricted to the cumulus **(A, B)**. Dorsal, d; medial, m (same orientation for B). A second, more weakly stained PN had a few branches inside DM (most noticeable in A; soma visible in B belongs to this neuron). Electrophysiological data were also obtained from two different neurons in this preparation: one was classified as Z11–16:Ald Type 1 (Figure 2, cell 4) and the other as a Z9–16:Ald Type 2 (Figure 6b, cell 4). The combined physiological and morphological data suggest that two PNs were characterized in this preparation, one that had dendrites restricted to the cumulus (and responded to Z11–16:Ald), and another that branched in DM (and responded to Z9–16:Ald). Scale bar = 50 μm.

medial) glomeruli (Vickers *et al.*, 1998). Given the close anatomical similarities between the two species, we have now adopted this nomenclature for the satellite glomeruli in *H. subflexa* (Figure 1B).

Functional characterization of antennal lobe neurons in *H. subflexa*

Successful recordings were obtained from 31 out of 48 *H. subflexa* males. In 18 of these, a total of 27 neurons responded to one or more of the test odorants or blends. Neurons were categorized according to the observed differences in their responses to the stimuli tested. *Type 1* PNs showed excitatory responses to only a single odorant and any blend containing that odorant. *Type 2* PNs also showed excitatory or inhibitory responses to one or more of the other test odorants (Christensen *et al.*, 1995; Vickers *et al.*, 1998). Included in the Type 2 category were PNs that were primarily excited by a single odorant but showed enhanced responses (either elevated peak rate of firing or improved temporal resolution in response to pulsatile stimuli) to a blend containing that odorant. Inhibitory responses were characterized by a reproducible hyperpolarization of the membrane potential with each odor pulse. Typically, these inhibitory responses were phase-locked to stimulus delivery or were observed following the offset of the last stimulus pulse. Spontaneous activity of neurons was variable but instantaneous frequencies were typically 50 Hz or less.

Z11–16:Ald-responsive PNs

About half of the odor-responsive PNs (13 out of 27 or 48%) were primarily responsive to one odorant: Z11–16:Ald (Figures 2 and 5). Nine of these PNs were exclusively responsive to Z11–16:Ald (Type 1), whereas four displayed secondary responses (either excitatory, inhibitory, or enhanced) to other pheromonal odorants (Type 2).

Type 1: Physiology

These PNs were strongly depolarized by the presence of Z11–16:Ald and showed neither excitatory nor inhibitory responses to other odorants. No quantitative or qualitative difference in response was noted between presentation of Z11–16:Ald alone, the synthetic pheromone mixtures (*H. subflexa* 3- or 4-mix and *H. virescens* 2- or 6-mix; see Table 1), or a female gland extract (FE; see Materials and methods). All nine PNs in this category were able to track the temporal dynamics of the stimulus, and responded with a discrete burst of action potentials to each of the five pulses in the stimulus train (Figure 2).

Type 1: Morphology

In four individuals, a single Z11–16:Ald/Type 1 PN was stained after physiological characterization. LSCM images

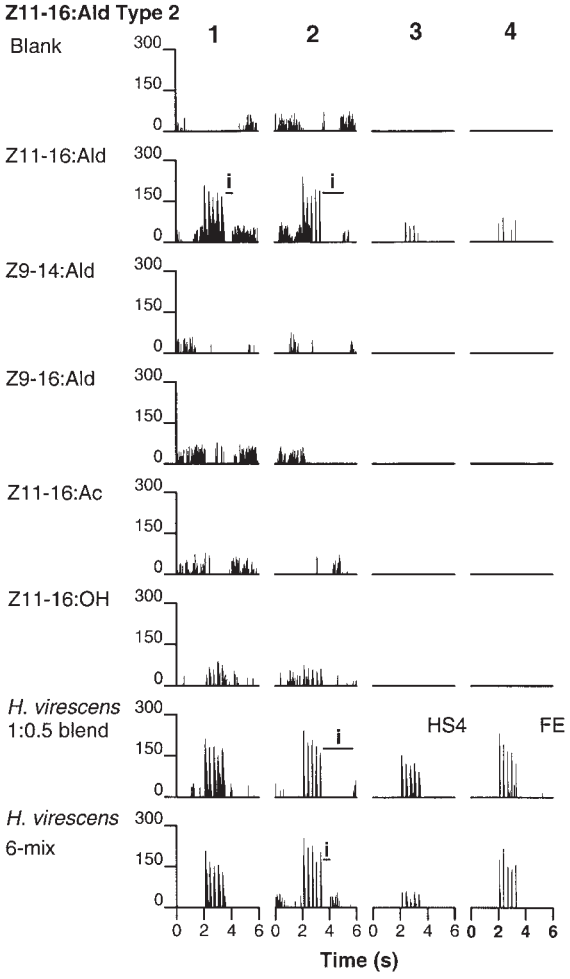

Figure 5 IFT plots of four neurons classified as Z11–16:Ald Type 2. Two neurons (cells 1 and 2) were strongly excited by Z11–16:Ald but some post-excitatory inhibition (i, duration indicated by line) was also observed. In both neurons a weak excitatory burst of impulses was noted in response to stimulation with Z11–16:OH. The populations of *H. subflexa* utilized in these experiments were derived from three different collections of field animals. The recordings that resulted in these unusual neurons were made from one population and it seems plausible that this unexpected variation in physiological profile might be due to some geographical variation in pheromone processing in different populations. Two additional neurons (cells 3 and 4) also responded to Z11–16:Ald alone but enhanced responses to the presentation of synthetic blends (*H. subflexa* 4-mix, HS4 in cell 3 and *H. virescens* 6-mix in cell 4) were observed.

revealed that in each case, the dendritic arborizations of these neurons were restricted to the largest MGC glomerulus, the cumulus (Figure 3). In a fifth individual, recordings were made separately from two neurons. One responded primarily to Z9–16:Ald (see below) while the other responded selectively to Z11–16:Ald. LSCM images confirmed staining of two PNs, each with a cell body in the medial cell cluster (Figure 4). In one PN, dendritic arborizations were restricted to DM, while in the other, branches were found exclusively in the cumulus (Figure 4). In all five preparations, the axons of stained PNs projected through the inner antenno-cerebral tract (IACT) to the protocerebrum.

Type 2: Physiology

Two PNs responded to antennal stimulation with either Z11–16:Ald or Z11–16:OH, but the responses to the two odorants were quantitatively different (Figure 5, cells 1, 2). Both PNs responded to each pulse of Z11–16:Ald with a discrete burst of action potentials. In contrast, the threshold for activation to Z11–16:OH was higher, but the responses were nonetheless phase-locked to the stimulus pulses. Two additional neurons responded to Z11–16:Ald, but the response was greatly enhanced when this odorant was presented as part of a blend (Figure 5, cells 3 and 4). None of the neurons of this type were stained successfully.

Z9–16:Ald-responsive PNs

Another third of the odor-responsive PNs (9 out of 27 or 33%) were primarily responsive to Z9–16:Ald (Figure 6). Five of these PNs were exclusively responsive to Z9–16:Ald (Type 1), whereas four displayed secondary responses (either excitatory, inhibitory, or enhanced) to other pheromonal odorants (Type 2).

Type 1: physiology

Five PNs showed low-threshold excitatory responses to antennal stimulation with Z9–16:Ald, and neither excitatory nor inhibitory responses to other test odorants. In two of these neurons (Figure 6A, cells 3–5), every odor pulse evoked a burst of spikes that was phase-locked to the stimulus train. None of the neurons in this category were stained.

Type 2: physiology

Four neurons were classified as Z9–16:Ald Type 2 (Figure 6B). Three of these had similar response profiles, showing strong depolarizing responses to Z9–16:Ald alone or to the *H. subflexa* FE-blend (Figure 6B, cells 2–4). Unlike the other PN types discussed thus far, these three neurons were also specifically inhibited by other test odorants or blends. Interestingly, even though it contained a small amount of the excitatory stimulus Z9–16:Ald, stimulation with the *H. virescens* 6-mix (Table 1) resulted in a pronounced hyperpolarization and complete inhibition of all background activity in two PNs (Figure 6B, cells 2 and 3). Therefore, the interspecific blend of six odorants had a greater effect on these neurons than did any of the individual components of the blend. This suggests that the strongly inhibitory response to the blend emerged as a result of the integration of signals converging from multiple MGC glomeruli that responded to this specific combination of odorants. The fourth Type-2 PN had a response profile that was distinctly different from others in this category. Stimulation with either Z9–16:Ald or Z9–14:Ald evoked robust excitatory responses in this neuron (Figure 6B, cell 1), while other odorants evoked neither excitatory nor inhibitory responses.

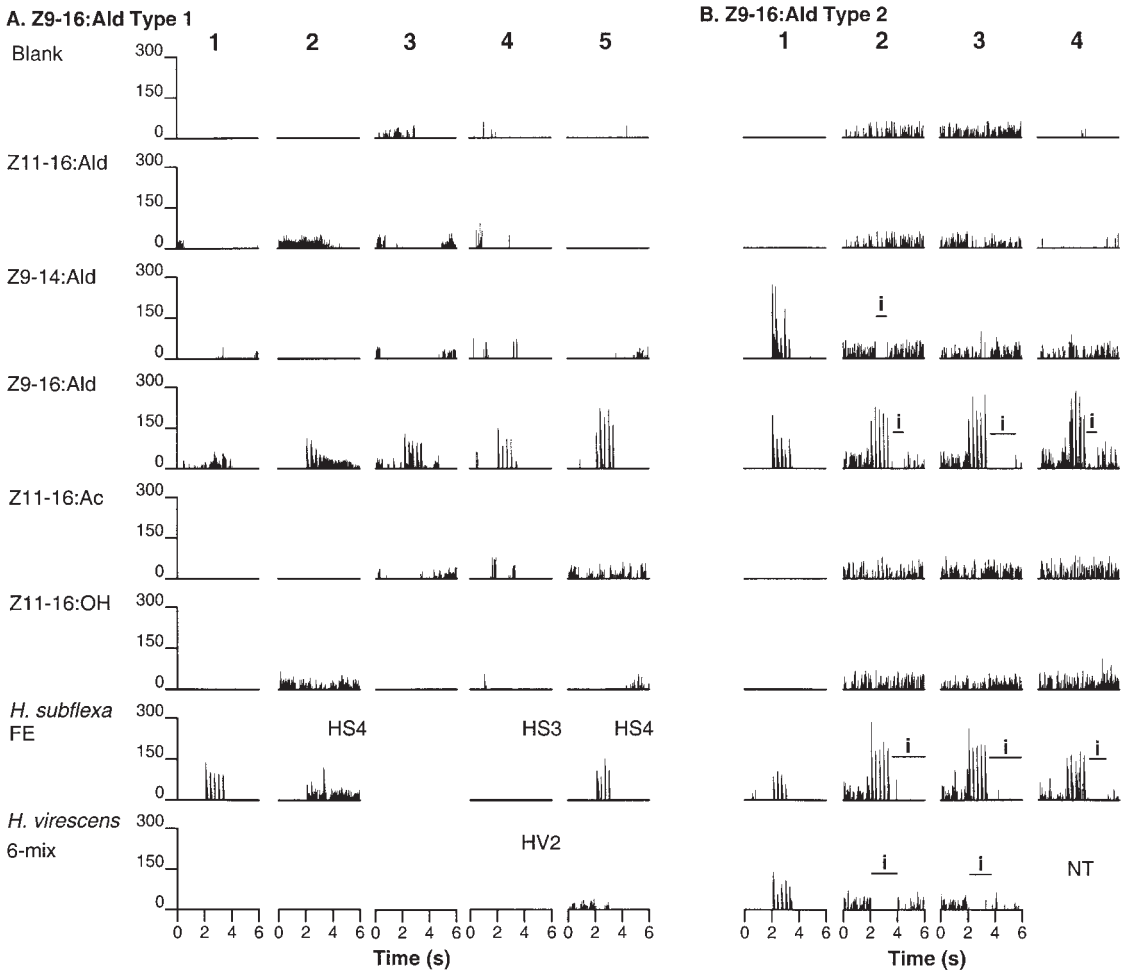

Figure 6 IFT plots of nine neurons primarily excited by stimulation with Z9–16:Ald included five Type 1 neurons and four Type 2 neurons. **(A)** Type 1 neurons were excited by Z9–16:Ald or mixtures containing this odorant. No response was noted in any neuron to the *H. virescens* 6-mix presumably because the amount of Z9–16:Ald in this mixture was below threshold. **(B)** One Type 2 neuron was also excited by Z9–14:Ald (cell 1). Three Type 2 neurons (cells 2–4) were inhibited by various compounds or blends. Other abbreviations as in Figure 2.

Type 2: morphology

AL neurons were stained in two of the above-mentioned preparations. In the first, two different PNs were characterized (as discussed above; Figure 4). In a second preparation, recordings were obtained from two neurons with similar physiological properties (Figure 6B, cells 2 and 3) but only one neuron (a PN) was stained sufficiently after dye injection. This PN had branches that were restricted to the MGC, with particularly intense staining in DM (Figure 7). The cell body was in the lateral cluster and the axon projected through the MACT directly to the inferior lateral protocerebrum (ILP).

Z11–16:OH-responsive PNs

Five neurons out of 27 (19%) responded primarily to stimulation with Z11–16:OH (Figure 8). Two of these neurons were exclusively responsive to Z11–16:OH (Type 1), whereas another three displayed secondary responses (either excitatory, inhibitory, or enhanced) to other pheromonal odorants (Type 2).

Type 1: physiology

Two neurons exhibited rather high-threshold (but nevertheless selective) responses to Z11–16:OH (Figure 8A). For one neuron (Figure 8A, cell 1), bursts of action potentials were phase-locked to each stimulus pulse and the instantaneous frequency of each burst exceeded firing rates for any other stimulus. A second neuron (Figure 8A, cell 2) was weakly excited by Z11–16:OH when presented alone but responded much more vigorously to the FE possibly due to a greater concentration of Z11–16:OH in the gland extract. Neither neuron was stained.

Type 2: physiology

Three neurons responded to Z11–16:OH and to the *H. subflexa* FE. Responses of these neurons (Figure 8B, cells 1–3) were not quantitatively or qualitatively different

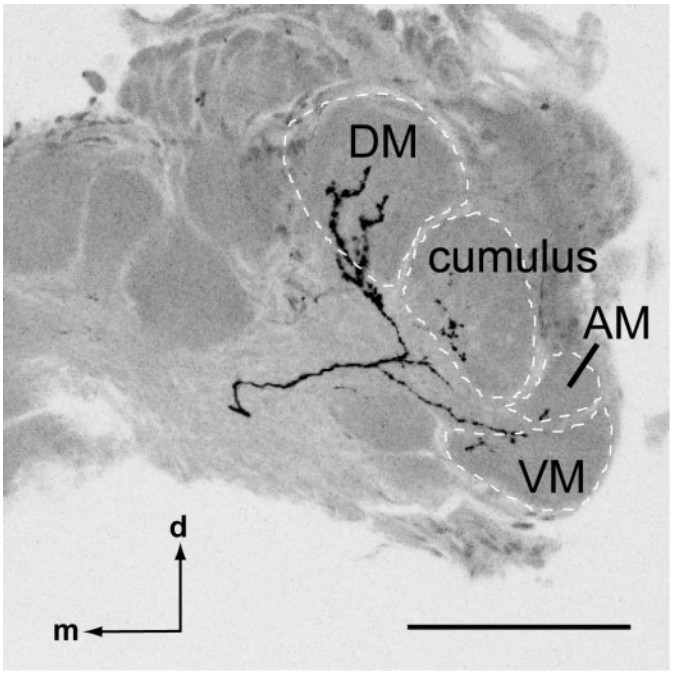

Figure 7 Staining of a multiglomerular Z9–16:Ald Type 2 PN. This neuron clearly arborized in all MGC glomeruli but had a particularly strong arborization in DM. This neuron had a soma located in the lateral cluster and, like other neurons in this class, projected out of the AL through the middle antenno-cerebral tract. Dorsal, d; medial, m. Scale bar = 50 µm.

for these two stimuli. However, secondary responses to other odorants took on different forms in these neurons. One cell (Figure 8B, cell 1) was specifically inhibited by stimulation with Z9–14:Ald. Two neurons (Figure 8B, cells 2 and 3) in this category were also excited to some extent by antennal stimulation with Z9–14:Ald.

Type 2: morphology

In one preparation LSCM images revealed a single PN with a cell body in the medial cell cluster. Arborizations were restricted to the AM glomerulus of the MGC (Figure 9).

Discussion

In *H. subflexa* a blend of three female-produced odorants (Z11–16:Ald, Z9–16:Ald, and Z11–16:OH) was necessary and sufficient to stimulate upwind flight and source location by conspecific males (Vickers, 2002). Based upon this finding, as well as previous observations from other heliothine species, we were able to make several predictions about the functional and anatomical organization of the MGC glomeruli in *H. subflexa*. First, we postulated that some olfactory PNs would show odorant-specific responses and would remain segregated in different MGC glomeruli. Second, we expected to find a functionally and anatomically distinct population of PNs that would integrate information from more than one of these input pathways (Christensen *et al*., 1991, 1995; Vickers *et al*., 1998). In the present study, we found one population of PNs that was selectively responsive to either Z11–16:Ald, Z9–16:Ald or Z11–16:OH (type 1 PNs) as well as another set of PNs with firing patterns that were modulated by more than one test odorant (type 2 PNs). As in other moths, therefore, this organization indicates that pheromone processing is not simply governed by labeled lines, but involves a complex, cross-glomerular and combinatorial code that represents the attractive pheromonal blend in *H. subflexa*.

Evolution of MGC structure and function

Processing of olfactory information in the AL glomeruli has been studied in a number of Lepidopteran species, including representatives of the families Bombycidae (*Bombyx mori*), Pyralidae (*Ostrinia nubilalis*), Saturniidae (*Antheraea polyphemus*), Sphingidae (*Manduca sexta*), and other Noctuidae (*Agrotis segetum*, *Spodoptera littoralis*, *Trichoplusia ni*) (Koontz and Schneider, 1987; Hansson *et al*., 1992, 1994; Anton and Hansson, 1995; Ochieng *et al*., 1995; Todd *et al*., 1995; Anton *et al*., 1997; Christensen, 1997; Hansson, 1997; Kanzaki *et al*., 2003). These species all utilize pheromonal blends that differ in their chemical composition, either in the specific components present, or in the relative amount of each odorant present in the blend. The number and size of glomeruli in the male MGC also vary substantially for these different species, perhaps reflecting the need to process and discriminate between different conspecific and interspecific pheromone blends (Christensen, 1997; Hansson, 1997; Anton and Homberg, 1999). These data show that the spatial arrangement of glomeruli in the MGC is evolutionarily labile, but they do not explain how the evolution of pheromone-receptor specificity, or changes in the structure and function of central olfactory pathways in the MGC, might come about. What changes in olfactory processing circuitry have occurred in *H. virescens* and *H. subflexa* to accompany their semiochemical divergence and the males' behavioral requirement for distinct pheromone blends? Morphologically, the *H. subflexa* MGC is essentially indistinguishable from that of *H. virescens* (Figure 1) but the odorant-response profiles of central olfactory PNs (and ORCs; T.C. Baker *et al*., submitted for publication) have clearly changed over the course of evolution (Figure 10). The data presented here show that as *H. virescens* and *H. subflexa* have diverged, satellite MGC glomeruli (those adjacent to the cumulus) have undergone changes in their odorant tuning. In some cases, these shifts have been accompanied by a divergence in the specific association with attractive or antagonistic odorants. Below we outline some of the basic principles of organization that are emerging from studies across several related species.

Principle 1. The cumulus is the site for processing the main pheromone component, Z11–16:Ald

Many heliothine species utilize Z11–16:Ald as the most abundant component of their respective pheromone blends (Arn *et al*., 1992). In both *H. virescens* and *H. zea*, PNs that

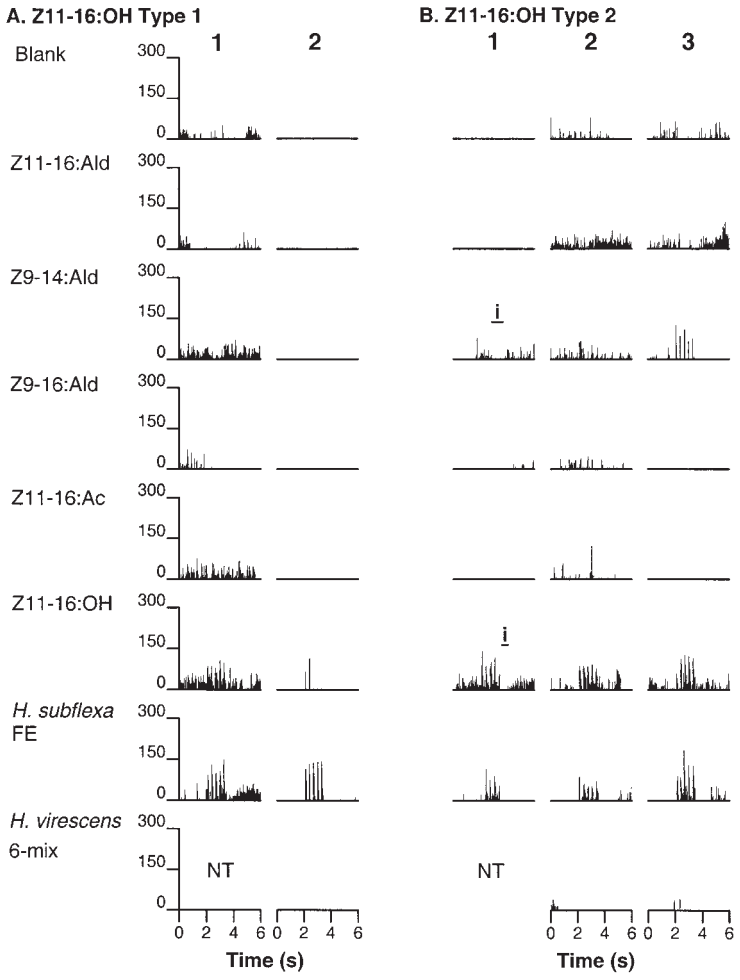

Figure 8 IFTs of five neurons classified as having a primary response to Z11–16:OH. **(A)** Two neurons responded only to the presence of Z11–16:OH (Type 1). No inhibitory or excitatory responses to other stimuli were observed. The response of cell 2 was noticeably stronger to the *H. subflexa* FE stimulus possibly because of a higher concentration of Z11–16:OH in the gland extract. **(B)** Three neurons exhibited excitatory or inhibitory responses to other compounds. Two of these neurons (cells 2 and 3) were excited by Z9–14:Ald when presented alone although the response of cell no. 2 was only barely above background. The *H. virescens* 6-mix stimulus failed to elicit any response possibly because either the amount of Z9–14:Ald in this mixture is sub-threshold or inhibitory responses to other compounds (such as Z11–16:Ald, cell 3) prevented an excitatory response.

responded to Z11–16:Ald were the most frequently encountered type of MGC neuron (58% and 56%, respectively) and Z11–16:Ald PNs that were stained always had dendritic arborizations within the cumulus (Christensen *et al.*, 1991, 1995; Vickers *et al.*, 1998). Similarly, in *H. subflexa*, Z11–16:Ald PNs were the most frequently encountered (48%), and all PNs of this type that were stained had dendritic arborizations confined to the cumulus (Figures 3 and 4). In both *H. virescens* and *H. zea*, multiglomerular PNs were also identified (Christensen *et al.*, 1991; Vickers *et al.*, 1998). These neurons always had a primary response to Z11–16:Ald, or were specifically activated by any blend containing Z11–16:Ald. Multiglomerular neurons with a primary sensitivity to Z11–16:Ald were not stained in the present study, but they also likely exist in *H. subflexa*. Nevertheless, the results obtained from stained uniglomerular PNs provide compelling evidence that the cumulus is the common site for processing the odorant Z11–16:Ald, the most abundant component of the pheromone blend in *H. subflexa* and almost all other heliothine moths studied to date.

Principle 2. Other MGC glomeruli are specific for secondary pheromone components

Additional pheromone components, specifically Z9–16:Ald and Z11–16:OH, are also required to elicit significant levels of upwind flight and source location by *H. subflexa* males (Vickers, 2002), and neurons specifically tuned to each of these odorants have been documented in this study (Figures 6 and 8). While more PNs must be stained, we now have evidence that at least a subset of these narrowly tuned PNs also have dendritic arborizations restricted to particular MGC glomeruli other than the cumulus. For example, a single PN sensitive to Z9–16:Ald was found to arborize in

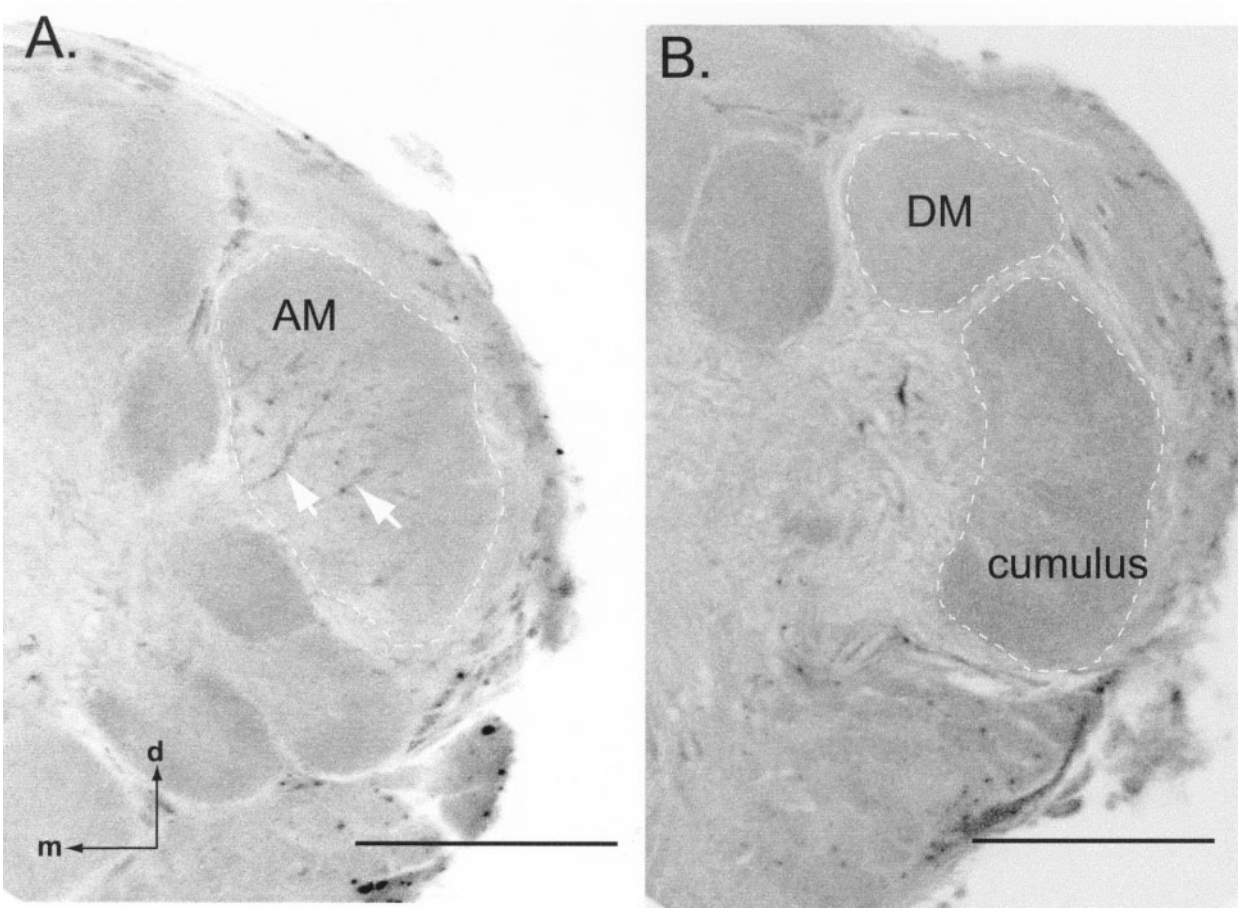

Figure 9 Confocal micrographs of a stained neuron classified as Z11–16:OH Type 2. This neuron was one of two PNs that responded to Z9–14:Ald as well as to Z11–16:OH (Figure 8B, cells 2 and 3). **(A)** Staining was weak but dendritic arborizations (the more prominent of which are indicated by arrows) were restricted to the AM glomerulus of the male MGC. The cell body of this neuron was located in the medial cluster. **(B)** No staining was visible in other MGC glomeruli including the cumulus and DM. Dorsal, d; medial, m (orientation is the same for A and B). Scale bar = 50 μm.

DM (Figure 4A). In addition, a multiglomerular PN arborizing in all MGC glomeruli, but with noticeably more branching in DM, also responded to Z9–16:Ald (Figure 7). This PN had a soma in the lateral cell cluster, and its axon projected from the AL directly to the ILP. In *H. virescens* and *H. zea*, PNs showing selectivity for a secondary component or antagonist were always uniglomerular (Vickers *et al*., 1998), which makes this type of PN in *H. subflexa* unusual. This finding may, however, be indicative of a general undersampling of PNs with somata in the lateral cell cluster. The existence of similar multiglomerular neurons was also recently reported in *B. mori*, but their function in olfactory processing remains to be clarified in this species (Kanzaki *et al*., 2003).

Principle 3. Divergence of the odorant–glomerulus association

The odorants processed in the DM glomerulus of *H. virescens* and *H. subflexa* are different (Z9–14:Ald and Z9–16:Ald, respectively, Figure 10), but they carry a similar importance as a necessary component of the pheromone blends in these two species. For this central shift in chemical representation in the DM glomerulus of these two species to have appeared, a similar change in the specificity of the sensory input must also have occurred. Electrophysiological studies have in fact revealed evidence that some of the ORC pathways in these moths are considerably more broadly tuned than others. Sensillum recordings from the antennae of *H. subflexa* males, for example, indicate that some ORCs respond not only to Z9–16:Ald as expected, but also to Z9–14:Ald. These ORCs however, are about two orders of magnitude more sensitive to Z9–16:Ald than to Z9–14:Ald (T.C. Baker *et al*., submitted for publication). In our data set we found one Z9–16:Ald Type 2 PN (Figure 6B, cell 1) that also responded to Z9–14:Ald. A similar situation in which an olfactory pathway was found to be more broadly tuned to these same two odorants was reported in *H. zea* (Christensen *et al*., 1991; Cossé *et al*., 1998; Vickers *et al*., 1998). This pathway may explain why *H. zea* males were attracted to pheromone blends in which Z9–16:Ald was replaced with a small amount of Z9–14:Ald (Vickers *et al*., 1991). This substitution test, however, failed to attract

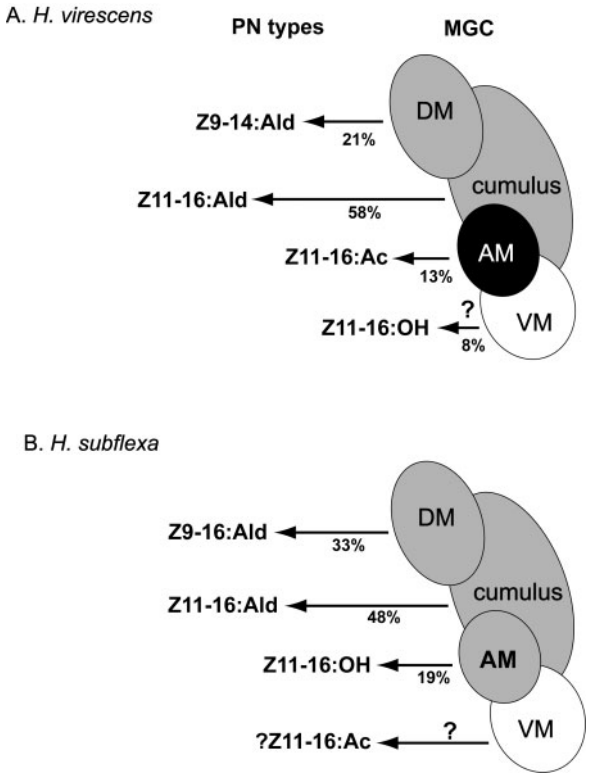

Figure 10 Comparison of projection neuron types in the MGCs of *H. virescens* and *H. subflexa*. Data for *H. virescens* were from a previous study (Vickers *et al*., 1998) but also include two neurons that responded to Z11–16:OH (Vickers, unpublished observations) (n = 24). Data for *H. subflexa* were from the current study (n = 27). The length of the arrow associated with each glomerulus corresponds to the percentage of neurons identified for each type (Type 1 and 2 neurons were combined in this representation). **(A)** In *H. virescens* a combination of activity in the cumulus and DM (encoding the presence of Z11–16:Ald and Z9–14:Ald, respectively) was required to elicit attraction. Activity in AM encodes the presence of Z11–16:Ac, a potent antagonist in this species. PNs tuned to Z11–16:OH exist and are thought to arborize in VM. **(B)** Similarly, in *H. subflexa* both the cumulus (Z11–16:Ald) and DM (Z9–16:Ald) are involved in signaling the presence of an attractive blend. Evidence suggests that the presence of Z11–16:OH, the third required component of the pheromone blend in this species, is signaled by activity in AM, indicating that the functional significance of this glomerular location has likely diverged in these two species. PNs tuned to Z11–16:Ac have not yet been encountered in *H. subflexa* but, based upon studies of olfactory receptor cells (T.C. Baker *et al*., submitted for publication), these neurons likely exist and probably have dendritic arbors restricted to VM.

H. subflexa (Vickers, 2002), reflecting perhaps the much lower affinity of Z9–16:Ald-sensitive ORCs in this species for Z9–14:Ald, as compared with *H. zea* (T.C. Baker *et al*., submitted for publication).

The capacity of some ORCs and PNs to respond to Z9–14:Ald in addition to other odorants is an increasingly common theme amongst heliothine moths (Cossé *et al*., 1998; Vickers *et al*., 1998) and could be a major factor in speciation. In the current study, one Z9–16:Ald Type 2 PN (Figure 6B, cell 1) and two Z11–16:OH Type 2 PNs (Figure 8B, cells 2, 3) also responded to Z9–14:Ald. The occurrence of these more broadly tuned sensory and central neurons suggests that the divergence of a single olfactory pathway into different sub-pathways with differing degrees of odorant selectivity may eventually lead to functional shifts in the odorant specificity of a given glomerulus.

Principle 4. Divergence of functional significance in MGC glomeruli

Finally, our results also indicate that the functional significance of at least one of the satellite MGC glomeruli can shift through evolution. For example, in *H. virescens*, it was discovered that PNs responsive to Z11–16:Ac (a potent behavioral antagonist) innervated only the AM glomerulus (Vickers *et al*., 1998). In contrast, studies of ORCs (Berg *et al*., 1998), synaptic activity associated with ORCs (Galizia *et al*., 2000), and PNs (Vickers, unpublished observations) in *H. virescens* indicated that Z11–16:OH/Z9–14:Ald responses were mediated through the VM glomerulus. Surprisingly, in the current study of *H. subflexa*, the only stained PN that was activated by both of these attractive odorants was localized not to VM, but to the AM glomerulus, which serves the antagonist function in *H. virescens* (Figure 9A). We speculate that Z11–16:OH-specific PNs (Figure 8) also arborize in this glomerulus and that activity in AM would be required in combination with that of the cumulus and DM for *H. subflexa* males to fly upwind (Figure 10). If this result can be confirmed by additional PN recording and staining, it would suggest that the odorants associated with the AM glomerulus, as well as the specific behavioral significance of activating this particular glomerulus, have diverged in these two heliothine species.

Acknowledgements

Supported by the National Science Foundation, IBN-9905683 to NJV. Thanks to Dr John Hildebrand for helpful discussion and encouragement during the early stages of this project. Many thanks to Dr Fred Gould, Dr Amy Sheck, Mark Sisterson, and Sara Oppenheim of North Carolina State University for their help in establishing a series of *H. subflexa* colonies and for their suggestions for colony maintenance. Thanks to the following for help with rearing and maintenance of moth colonies and data analysis: Keri Swearingen, Dave Kelly, Matt Pond.

References

Almaas, T.J. and **Mustaparta, H.** (1990) *Pheromone reception in tobacco budworm moth,* Heliothis virescens*.* J. Chem. Ecol., 16, 1331–1347.

Almaas, T.J. and **Mustaparta, H.** (1991) Heliothis virescens*: response characteristics of the receptor neurons in sensilla trichodea type 1 and type 2.* J. Chem. Ecol., 17, 953–972.

Almaas, T.J., Christensen, T.A. and **Mustaparta, H.** (1991) *Chemical communication in heliothine moths: 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.

Anton, S. and **Hansson, B.S.** (1995) *Sex pheromone and plant-associated odour processing in antennal lobe interneurons of male* Spodoptera littoralis *(Lepidoptera: noctuidae).* J. Comp. Neurol., 176, 773–789.

Anton, S. and **Homberg, U.** (1999) Antennal lobe structure. In Hansson, BS (ed.), Insect Olfaction. Springer-Verlag, Berlin. pp. 97–124.

Anton, S., Löfstedt, C. and **Hansson, B.S.** (1997) *Central nervous processing of sex pheromones in two strains of the European corn borer* Ostrinia nubilalis *(Lepidoptera: pyralidae).* J. Exp. Biol., 200, 1073–1087.

Arn, H., Tóth M. and **Preisner, E.** (1992) List of Sex Pheromones of Lepidoptera and Related Attractants, 2nd edn. International Organization for Biological Control, Montfavet, France.

Berg, B.G., Almaas, T.J., Bjaalie, J.G. and **Mustaparta, H.** (1998) *The macroglomerular complex of the antennal lobe in the tobacco budworm moth* Heliothis virescens*: specified subdivision in four compartments according to information about biologically significant compounds.* J. Comp. Physiol. A, 183, 669–682.

Berg, B.G., Galizia, C.G., Brandt, R. and **Mustaparta, H.** (2002) *Digital atlases of the antennal lobe in two species of tobacco budworm moths, the oriental* Helicoverpa assulta *(male) and the American* Heliothis virescens *(male and female).* J. Comp. Neurol., 446, 123–134.

Cho, S., Mitchell, A., Regier, J.C., Mitter, C., Poole, R.W., Friedlander, T.P. and **Zhao, S.** (1995) *A highly conserved nuclear gene for low-level phylogenetics: elongation factor-1 alpha recovers morphology-based tree for heliothine moths.* Mol. Biol. Evol., 12, 650–656.

Christensen, T.A. (1997) Anatomical and physiological diversity in the central processing of sex-pheromonal information in different moth species. In Cardé, RT and Minks, AK (eds) Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 184–193.

Christensen, T.A. and **White, J.** (2000) Representation of olfactory information in the brain. In Finger, TE, Silver, WL and Restrepo, D (eds), The Neurobiology of Taste and Smell, vol. II. John Wiley & Sons, New York, pp 201–232.

Christensen, T.A., Mustaparta, H. and **Hildebrand, J.G.** (1991) *Chemical communication in heliothine moths: central processing of intra- and interspecific olfactory messages in the male corn earworm moth* Helicoverpa zea*.* J. Comp. Physiol. A, 169, 259–274.

Christensen, T.A., Mustaparta, H. and **Hildebrand, J.G.** (1995) *Chemical communication in heliothine moths: parallel pathways for information processing in the macroglomerular complex of the male tobacco budworm moth* Heliothis virescens*.* J. Comp. Physiol. A, 177, 545–557.

Cossé, A.A., Todd, J.L. and **Baker, T.C.** (1998) *Neurons discovered in male* Helicoverpa zea *antennae that correlate with pheromone-mediated attraction and interspecific antagonism.* J. Comp. Physiol. A, 182, 585–594.

David, C.T. and **Birch, M.C.** (1989) Pheromones and insect behavior. In Jutsum, AR and Gordon, RFS (eds), Insect Pheromones in Plant Protection. New York, John Wiley, pp. 17–35.

Fang, Q.Q., Cho, S., Regier, J.C., Mitter, C., Matthews, M., Poole, R.W., Friedlander, T.P. and **Zhao, S.** (1997) *A new nuclear gene for insect phylogenetics: DOPA decarboxylase is informative of relationships within Heliothinae (Lepidoptera: Noctuidae).* Syst. Biol., 46, 269–283.

Galizia, C.G., Sachse, S. and **Mustaparta, H.** (2000) *Calcium responses to pheromone and plant odours in the antennal lobe of the male and female moth* Heliothis virescens. J. Comp. Physiol. A, 186, 1049–1063.

Hansson, B.S. (1997) *Antennal lobe projection patterns of pheromone-specific olfactory receptor neurons in moths.* In Cardé, RT and Minks, AK (eds), Insect Pheromone Research: New Directions. Chapman and Hall, New York, pp. 164–183.

Hansson, B.S., Ljungberg, H., Hallberg, E. and **Löfstedt, C.** (1992) *Functional specialization of olfactory glomeruli in a moth.* Science, 256, 1313–1315.

Hansson, B.S., Anton, S. and **Christensen, T.A.** (1994) *Structure and function of antennal lobe neurons in the male turnip moth,* Agrotis segetum *(Lepidoptera: Noctuidae).* J. Comp. Physiol. A, 175, 547–562.

Hansson, B.S., Almaas, T.J. and **Anton, S.** (1995) *Chemical communication in heliothine moths: antennal lobe projection patterns of pheromone-detecting olfactory receptor neurons in the male* Heliothis virescens *(Lepidoptera: Noctuidae).* J. Comp. Physiol. A, 177, 535–543.

Heath, R.R., McLaughlin, J.R., Proshold, R. and **Teal, P.E.A.** (1991) *Periodicity of female sex pheromone titer and release in* Heliothis subflexa *and* H. virescens *(Lepidoptera: Noctuidae).* Ann. Entomol. Soc. Am., 84, 182–189.

Klun, J.A., Bierl-Leonhardt. B.A., Plimmer. J.R., Sparks, A.N., Primiani, M., Chapman, O.L., Lepone, G. and **Lee, G.H.** (1980) *Sex pheromone chemistry of the female tobacco budworm moth,* Heliothis virescens*.* J. Chem. Ecol., 6, 177–183.

Klun, J.A., Leonhardt, B.A., Lopez, J.D., Jr. and **Lachance, L.E.** (1982) *Female* Heliothis subflexa *(Lepidoptera: Noctuidae) sex pheromone: chemistry and congeneric comparisons.* Environ. Entomol., 11, 1084–1090.

Kanzaki, R., Soo, K., Seki, Y. and **Wada, S.** (2003) *Projections to higher olfactory centers from subdivisions of the antennal lobe macroglomerular complex of the male silkmoth.* Chem. Senses, 28, 113–130.

Koontz, M.A. and **Schneider, D.** (1987) *Sexual dimorphism in neuronal projections from the antennae of silk moths (*Bombyx mori*,* Antheraea polyphemus*) and the gypsy moth (*Lymantria dispar*).* Cell. Tiss. Res., 249, 39–50.

Mitter, C., Poole, R.W. and **Matthews, M.** (1993) *Biosystematics of the Heliothinae (Lepidoptera: Noctuidae).* Annu. Rev. Entomol., 38, 207–225.

Ochieng, S.A., Anderson, P. and **Hansson, B.S.** (1995) *Antennal lobe projection patterns of olfactory receptor neurons involved in sex pheromone detection in* Spodoptera littoralis *(Lepidoptera: Noctuidae).* Tiss. Cell., 27, 221–232.

Roelofs, W.L., Hill, A.S., Cardé, R.T. and **Baker, T.C.** (1974) *Two sex pheromone components of the tobacco budworm moth,* Heliothis virescens*.* Life Sci., 14, 1555–1562.

Shorey, H.H. and **Hale, R.L.** (1965) *Mass-rearing of the larvae of nine noctuid species on a simple artificial medium.* J. Econ. Entomol., 58, 522–524.

Teal, P.E.A. and **Tumlinson, J.H.** (1997) *Effects of interspecific hybridization between* Heliothis virescens *and* H. subflexa *(Lepidoptera: Noctuidae) on the sex pheromone communication system.* In Cardé, R.T. and Minks, A.K. (eds), Insect Pheromone Research: New Directions. Chapman & Hall, New York, pp. 535–547.

Teal, P.E.A., Heath, R.R., Tumlinson, J.H. and **McLaughlin, J.R.** (1981) *Identification of a sex pheromone of* Heliothis subflexa *(GN.) (Lepidoptera: Noctuidae) and field trapping studies using different blends of components.* J. Chem. Ecol., 7, 1011–1022.

Teal, P.E.A., Tumlinson, J.H. and **Heath, R.R.** (1986) *Chemical and behavioral analyses of volatile sex pheromone components released by calling* Heliothis virescens *(F.) females (Lepidoptera: Noctuidae).* J. Chem. Ecol., 12, 107–126.

Todd, J.L., Anton, S., Hansson, B.S. and **Baker, T.C.** (1995) *Functional organization of the macroglomerular complex related to behaviourally*

expressed olfactory redundancy in male cabbage looper moths. Physiol. Entomol., 20, 349–361.

Tumlinson, J.H., Hendricks, D.E., Mitchell, E.R., Doolittle, R.E. and **Brennan, M.M.** (1975) *Isolation, identification, and synthesis of the sex pheromone of the tobacco budworm.* J. Chem. Ecol., 1, 203–214.

Vetter, R.S. and **Baker, T.C.** (1983) *Behavioral responses of male* Heliothis virescens *in a sustained-flight tunnel to combinations of seven compounds identified from female sex pheromone glands.* J. Chem. Ecol., 9, 747–759.

Vickers, N.J. (2002) *Defining a synthetic pheromone blend attractive to male* Heliothis subflexa *under wind tunnel conditions.* J. Chem. Ecol., 28, 1267–1279.

Vickers, N.J. and **Baker, T.C.** (1997) *Chemical communication in heliothine moths: correlation between diminished responses to point-source plumes and single filaments similarly tainted with a behavioral antagonist.* J. Comp. Physiol. A, 180, 523–536.

Vickers, N.J., Christensen, T.A., Mustaparta, H. and **Baker, T.C.** (1991) *Chemical communication in heliothine moths: flight behavior of male Helicoverpa zea and* Heliothis virescens *in response to varying ratios of intra- and interspecific sex pheromone components*. J. Comp. Physiol. A, 169, 275–280.

Vickers, N.J., Christensen, T.A. and **Hildebrand, J.G.** (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.

Accepted April 2, 2003