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Projections of Male-Specific Receptor Neurons in the Antennal Lobe of the Oriental Tobacco Budworm Moth, *Helicoverpa assulta:* A Unique Glomerular Organization among Related Species

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ABSTRACT

The macroglomerular complex in the primary olfactory center of male moths receives information from numerous pheromone-detecting receptor neurons housed in specific sensilla located on the antennae. We investigated the functional organization of the three glomeruli constituting this complex in *Helicoverpa assulta*, a unique species among heliothine moths as concerns the composition of the pheromone blend. By tip recordings from the male-specific receptor neurons combined with cobalt-lysine stainings, the axon terminals in the brain were traced and subsequently reconstructed by camera lucida drawings. Some were also reconstructed in a digital form. The results showed that the sensilla could be classified into two functional types. A major category housed two colocalized receptor neurons, one responding to the primary pheromone component *cis*-9-hexadecenal and the other to the behavioral antagonists *cis*-9-tetradecenal and *cis*-9-hexadecenol. Cobalt-lysine applied to this sensillum type consistently resulted in two stained axons, each terminating in one of the two large subunits of the macroglomerular complex: the cumulus or the dorsomedial glomerulus. The second, less frequently appearing sensillum type contained a receptor neuron responding to the second pheromone component, *cis*-11-hexadecenal. Dye applied to this type resulted in stained axon projections in the ventral glomerulus. In an evolutionary context it is particularly interesting that differences of related heliothine species are reflected in the functional organization of the MGC compartments. J. Comp. Neurol. 486:209–220, 2005.

Indexing terms: pheromones; sensory neurons; macroglomerular complex; electrophysiology; anatomical reconstruction; heliothine moths

Among the heliothine species studied so far, the Oriental tobacco budworm moth, *Helicoverpa assulta*, is unique as concerns the composition of the pheromone blend. Whereas most of the species rely on *cis*-11-hexadecenal (Z11-16:AL) as the major pheromone component, *H. assulta* utilizes this substance as the second component, and *cis*-9-hexadecenal (Z9-16:AL) as the major one (Sugie et al., 1991; Cork et al., 1992; Park et al., 1994). The optimal ratio between the major and the second principal pheromone component is 20:1 in the Korean population of *H. assulta* (Cork et al., 1992).

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In a previous electrophysiological investigation, the receptor neurons on the male antennae of *H. assulta* were tested for sensitivity to all known components produced by the various heliothine species. In these single cell recordings, using tungsten microelectrodes, three neuron types were identified; one type responding to the primary pheromone component Z9-16:AL, a second type to Z9-14:AL, and Z9-16:OH—neither of which are produced by the conspecific female, and a third type to the second pheromone component Z11-16:AL (Berg and Mustaparta, 1995). The two former and most abundant neuron types (90%) consistently appeared in the same recordings. The behavioral significance of Z9-14:AL as well as Z9-16:OH have been tested in field studies, showing that increased amounts of each substance added to the binary pheromone blend reduce the attraction of *H. assulta* males (Cork et al., 1992; Boo et al., 1995). Thus, it is likely that both compounds act as interspecific signals contributing to the isolation between *H. assulta* and sympatric species, e.g., *Heliothis peltigera* which utilizes Z9-14:AL as the second pheromone constituent (Dunkelblum and Kehat, 1989). The anatomical arrangement of the antennal lobe glomeruli which receive the olfactory information from the receptor neurons was recently described in the *H. assulta* male by antibody staining combined with confocal microscopy reconstructions (Berg et al., 2002). The digital atlas comprised three MGC subunits that had been reported previously (Berg and Mustaparta, 1999).

The subfamily *Heliothinae* includes several genera, among them *Helicoverpa* and *Heliothis*. Studies of the phylogeny within species of *Helicoverpa*, using classical morphological characters (Mitter et al., 1993) as well as genetic markers (Fang et al., 1997; Cho et al., 1995), have shown that the oligophagous *H. assulta* is relatively closely related to the polyphagous *Helicoverpa armigera* and *H. zea*. From a comparative perspective, it is interesting to study the structure and function of the sexually dimorphic glomeruli that constitute the macroglomerular complex (MGC) across various heliothine species. The general spatial arrangement of three or four spherical units including a large centrally located glomerulus, the cumulus, has been pointed out. In species of *Helicoverpa* the number is three (Christensen et al., 1991; Vickers et al., 1998; Berg et al., 2002; H. Skiri, pers. commun.) and in species of *Heliothis* four (Berg et al., 1998, 2002; Vickers et al., 1998; Vickers and Christensen, 2003). Whereas the closely related *H. zea* and *H. armigera* have a similar glomerular organization of the MGC, comprising two dorsomedially located glomeruli in addition to the cumulus, *H. assulta* has a somewhat different organization comprising one relatively large dorsomedial unit and one ventral in addition to the cumulus. Compared to the MGC of *Heliothis virescens* and *Heliothis subflexa*, having a four-unit MGC comprising one large dorsomedial unit and two smaller ventral units in addition to the cumulus, the spatial arrangement of the MGC-units of *H. assulta* also resembles that of the *Heliothis* genus.

The projections of the male-specific receptor neurons in the MGC have been studied in several species of moths (Hansson et al., 1992, 1995; Ochieng et al., 1995; Christensen et al., 1995b; Todd et al., 1995; Berg et al., 1998). In *H. virescens*, each of the four MGC-units receives information mediated by one of four physiologically characterized receptor neuron types. The cumulus and the dorsomedial unit receive input from the neurons tuned to the primary and the secondary pheromone component, respectively, whereas the two smaller ventral units receive input from two other neuron types, one of which is involved in interspecific communication (Hansson et al., 1995; Berg et al., 1998). The functional significance of the MGC has also been thoroughly studied by staining physiologically characterized antennal lobe neurons in *H. virescens*, as well as in *H. zea* and *H. subflexa* (Christensen et al., 1991, 1995; Berg et al., 1998; Vickers et al., 1998; Vickers and Christensen, 2003).

The identification of individual glomeruli across preparations within one species and between the different species makes it possible to describe the functional significance of particular glomeruli and to study the data in a comparative context. The aim of the present investigation was to determine the male-specific sensillum types and to elucidate the functional relationships between the previously characterized receptor neurons and the MGC subunits of *H. assulta* by tracing the axonal projections in the antennal lobe. The specific combination of the pheromone blend of *H. assulta* compared to those of related heliothine moths makes this particular species interesting, not only to gain insight into how the olfactory system encodes the signal information, but also how the peripheral and central pathways have evolved similarities and differences across the related species.

Abbreviations

H. assulta	Helicoverpa assulta
H. armigera	Helicoverpa armigera
H. zea	Helicoverpa zea
H. virescens	Heliothis virescens
H. peltigera	Heliothis peltigera
H. subflexa	Heliothis subflexa
M. sexta	Manduca sexta
S. littoralis	Spodoptera littoralis
T. ni	Trichoplusia ni
MGC	macroglomerular complex
Z9-16:AL	*cis*-9-hexadecenal
Z11-16:AL	*cis*-11-hexadecenal
Z9-14:AL	*cis*-9-tetradecenal
Z9-16:OH	*cis*-9-hexadecenol
Z11-16:OH	*cis*-11-hexadecenol
Z9-16:AC	*cis*-11-hexadecenyl acetate
Z9-16:AC	*cis*-9-hexadecenyl acetate

MATERIALS AND METHODS

Insects and preparation

Helicoverpa assulta (Lepidoptera: Noctuidae) pupae, originating from a lab culture, were kindly provided by Dr. K.S. Boo (University of Seoul, Korea). Male and female pupae were separated and kept in an incubator on a phase-shifted LD 14:10-hour photoperiod at 23°C. Each day, moths that had eclosed were placed in a container marked with the date. The males were allowed to feed on honey-water. Most animals were studied 1–3 days after eclosion.

The insect was prepared as described by Hansson et al. (1995). In principle, the moth was restrained inside a plastic jacket (a 1-ml pipette tip cut at the tapered end) by pushing it into the jacket so that the head and the antennae were exposed. The antennae were fastened in the desired position by covering the head and proximal seg-

ments with utility wax (Kerr). The hair tips of several *sensilla trichodea* type 1 (Koh et al., 1995) were cut by glass knives as described by Van der Pers and Den Otter (1978).

Electrophysiology and morphology

To categorize the sensillum types, the cut-sensillum technique was used (Kaissling, 1974). By placing a glass capillary electrode (10 μm tip diameter) filled with Ringer's solution over the cut end of one male-specific sensillum, the activity of receptor neurons within the individual sensillum was recorded. A chloridized silver wire inserted into the abdomen of the insect served as the indifferent electrode. Since the male-specific receptor neurons have been functionally characterized previously (Berg and Mustaparta, 1995), the aim of the electrophysiological experiments was to determine the categories of sensilla by identifying which of the neuron types were present in each sensillum. Neuronal spiking activity was monitored continuously by oscilloscope and loudspeaker and stored on an instrumentation tape recorder. Responses were measured by counting the number of nerve impulses during the 500-ms stimulation period; a frequency higher than that displayed during spontaneous activity was determined as a response. The spontaneous activity was measured over a period of 10 seconds prior to the first stimulation. To discriminate the different spike amplitudes of the colocalized responding neuron types the spike sorting tool of the program Spike2 was used. After identifying the functional types of the receptor neurons, i.e., determining the compounds to which they responded best, the Ringer electrode was replaced with another electrode filled with 0.5 mol^{-1} cobalt-lysine solution (pH 7.4) for marking the neurons (Lázár, 1978). The Ringer electrode was used to prevent multiple stainings, which may occur when subsequent recordings are performed from several sensilla with a cobalt-filled electrode. During the 1-hour staining period the neurons were stimulated with the key compound (usually 10 μg) applied as 50-ms pulses at a frequency of 0.5 Hz (Hansson et al., 1995). For mass stainings of male-specific receptor neurons, a cobalt-filled electrode was placed over several cut *sensilla trichodea* type 1 of one antennal segment. After the cobalt application, the preparation was placed in a refrigerator at 4°C for 2 days, allowing anterograde transportation of the dye in the axons. The brain was then dissected from the head capsule and submerged in 1 ml Ringer's solution with three drops of concentrated ammonium sulfide (38%). The following fixation-intensification procedure was the same as used by Hansson et al. (1995). After 10 minutes the brain was rinsed four times in physiological saline and fixed in alcohol-acetic acid-formaldehyde (8:1:1) for 24 hours at 4°C. The fixation was followed by rehydration and silver intensification according to Timm (1958). Then the brain was dehydrated, cleared in methyl salicylate, and viewed as a whole mount in light microscope. Stained specimens were infiltrated with propylene oxide and embedded in epoxy resin (Durcupan, Fluka, Buchs, Switzerland). Each brain was sliced in 10-μm sections, mounted, and counterstained with 1% methylene blue buffered in 1% sodium borate. Reconstructions of the antennal lobes with the stained neurons were made from drawings of the sections, using a light microscope equipped with a camera lucida attachment. Some of the brain section series were also subjected to computerized 3D reconstruction.

Stimulation

The odors were delivered via an air puff into a filtered and moistened air stream (25 ml s^{-1}, 0.5 m s^{-1}) blowing continuously over the insect antenna (Hansson et al., 1995; Berg et al., 1998). The air puff (3 ml s^{-1}, 500 ms) was led through a Pasteur pipette containing a filter paper with the compound. Both air flows were regulated by flow meters. To avoid contamination of odorants, the air around the preparation was continuously removed by a sucking device.

As test stimuli the two pheromone components Z9-16:AL and Z11-16:AL and the interspecific signal Z9-14:AL were used. Each compound was diluted in hexane before the desired amounts, 1, 10, and 20 μg, were applied to filter paper. In addition, a mixture of Z9-16:AL and Z9-14:AL in a 1:1 ratio (i.e., 1 μg + 1 μg, 10 μg + 10 μg) was tested. The neurons were screened for sensitivities to the three components for determining their specificities. To confirm that the results were in accordance with previous findings (Berg and Mustaparta, 1995), four additional substances produced by heliothine moths, *cis*-9-hexadecenyl acetate (Z9-16:AC), *cis*-11-hexadecenyl acetate (Z11-16:AC), *cis*-9-hexadecenol (Z9-16:OH), and *cis*-11-hexadecenol (Z11-16:OH), were tested in 11 recordings, seven from the two colocalized neuron types, tuned to Z9-16:AL and Z9-14:AL/Z9-16:OH, and four from the remaining neuron type, tuned to Z11-16:AL. The odor sources were renewed between the experiments. The compounds, kindly provided by Dr. J.G. Tumlinson (USDA, Gainesville, FL), were purified by HPLC on silica, reverse phase, or $AgNO_3$-treated silica columns and analyzed for purity and conformation of structure by capillary gas chromatography and mass spectrometry (Heath and Tumlinson 1984). The compounds had a purity greater than 99%. A blank cartridge containing pure filter paper was used as control.

Digital 3D reconstructions

In seven cases the section drawings were digitized using a PC equipped with a modified version of the program MicroTrace (Leergaard and Bjaalie, 1995). The distribution of stained axon terminals was coded as points in x,y space. Outlines of the glomeruli and the antennal lobe were coded as closed contour lines. Each section, and all coordinates, were assigned a common z-coordinate, which was defined by the section thickness and serial number. The stacks of digitized drawings were transferred to a Silicon Graphics (Mountain View, CA) workstation for 3D reconstruction and visualization, using program Micro3D (Neural Systems and Graphics Computing Laboratory, University of Oslo, Oslo, Norway, www.nesys.uio.no; see also Leergaard and Bjaalie, 1995a,b; Berg et al., 1998; Leergaard and Bjaalie, 2002; Bjaalie, 2002). The surfaces of the glomeruli and antennal lobes were reconstructed using a standard triangulation method included in Micro3D (for further details, see procedures described in Berg et al., 1998). The partly incomplete surfaces at the anterior and posterior parts of each digitally reconstructed glomerulus are due to the data program.

Data analyses

The final figures were edited with Adobe PageMaker 7.0 (Mountain View, CA), and some were adjusted for brightness and contrast with Adobe PhotoShop 7.0.

TABLE 1. Number of MGC Units with Stained Projections Obtained by Cobalt Applied to the Two Physiological Categories of Male-Specific Sensilla

Sensillum type (Receptor neuron type)	Number of sensilla contacted	Number of successful stainings	Number of MGC-units with stained axons:					
			Cumulus + DM unit	Cumulus alone	DM unit alone	Ventral unit alone	Ventral unit + cumulus/DM	Ventral unit + ordinary glom
C (Z9-16:AL/Z9-14:AL)	142	32	29[1]	1	2			
A (Z11-16:AL)	13	6				2	2[2]	2

[1]One preparation showed two additional axon terminals in two ordinary glomeruli.
[2]One of the two preparations showed one additional projection in the DM unit and the other two additional projections: one in the cumulus and one in the DM unit.
DM, dorsomedial.

RESULTS

Recordings were obtained from 155 male-specific *sensilla trichodea* type 1, located on the flagellar segments numbers 4–18. According to the neuronal responses, the sensilla could be classified into two different categories. The most abundant type (about 95%) contained two colocalized receptor neurons: one with large-spiking amplitudes responding to the primary pheromone component Z9-16:AL, and the other, with smaller amplitudes, responding to the interspecific signals Z9-14:AL and Z9-16:OH. The less frequently occurring sensillum type (about 5%) contained a receptor neuron responding to the secondary pheromone component Z11-16:AL. Out of 67 staining attempts, 38 were successful (Table 1).

Cobalt-lysine applied to many cut hairs of the *sensilla trichodea* type 1 showed the general distribution of the male-specific receptor neuron terminals in the antennal lobe with the major portion of labeled axons targeting the area close to the entrance of the antennal nerve (Fig. 1B), i.e., the area of the MGC as shown in Figure 1A,C–E.

Sensilla containing two colocalized receptor neurons responding to the primary pheromone component, Z9-16:AL, and the behavioral antagonists, Z9-14:AL and Z9-16:OH

The major group, comprising 142 sensilla (about 95%), contained two colocalized receptor neurons in each sensillum: one large-amplitude spiking neuron responding specifically to the major pheromone component Z9-16:AL (n = 142), and a second, distinctly smaller-amplitude spiking neuron responding to the interspecific signals Z9-14:AL (n = 142) and Z9-16:OH (n = 7) (Fig. 2A,B). The consistent and evident difference in amplitudes made it easy to distinguish the spiking activities of the two neurons (Fig. 2C). The identification of the neuron types was confirmed by the concordance between the data presented here and those previously reported by Berg and Mustaparta (1995). The large-amplitude Z9-16:AL neurons responded, in addition to the key compound, weakly to the highest concentration of Z9-14:AL (n = 142) (Fig. 2A,B,D). The small-amplitude Z9-14:AL/Z9-16:OH neurons responded relatively strongly to two substances: best to Z9-14:AL (n = 142) and second best to Z9-16:OH (n = 7) (Fig. 2A,B,E). These neurons showed no response to Z9-16:AL (n = 142). None of the two colocalized receptor neuron types responded to Z11-16:AL, Z9-16:AC, Z11-16:AC, or Z11-16:OH (n = 7) (Fig. 2A,B,D,E). Both neurons responded simultaneously to the mixture of Z9-16:AL and Z9-14:AL (Fig. 2A). According to previous labeling schemes this category of sensilla is named type C (Baker et al., 2004).

Cobalt-lysine was applied to 59 of the sensilla and resulted in 32 successfully stained preparations. Most of them (28) showed two stained axons, one terminating in the cumulus and the other in the dorsomedial glomerulus (Fig. 3). Stimulation during the initial staining period was performed with either Z9-16:AL, Z9-14:AL, or a mixture of the two compounds. Three other stained preparations showed one labeled axon, two with the axon projecting in the dorsomedial compartment, and one with the axon in the cumulus. For all three, Z9-14:AL was used as stimulus during the initial staining period. One preparation showed four labeled axons targeting four compartments: the cumulus, the dorsomedial unit, and two ordinary glomeruli.

Sensilla containing a receptor neuron responding to the second pheromone component, Z11-16:AL

Recordings from sensilla containing a receptor neuron responding to the second pheromone component, Z11-16:AL, were obtained in 13 experiments (about 5%). Again, the identification of the neuron type was confirmed by the consistency between the present data and those previously reported (Berg and Mustaparta, 1995). Of the substances tested, only the key compound elicited a response (Fig. 4A–C). In some of the recordings activity of one small-amplitude, nonresponding neuron was recorded simultaneously with the Z11-16:AL-specific neuron (asterisk, Fig. 4A). According to the scheme of Baker et al. (2004), these sensilla are named type A.

Six of eight staining attempts were successful, all showing axon terminals in the ventral MGC glomerulus (Fig. 5). Whereas two of the six specimens had a single stained neuron (Fig. 5F), four showed additional axons projecting in other glomeruli: two with an axon targeting an ordinary glomerulus (Fig. 5A–D), one with an axon targeting the dorsomedial MGC unit, and one with two axons targeting the cumulus and the dorsomedial unit (Table 1). In all experiments, stimulation was performed with Z11-16:AL during the staining period.

DISCUSSION

The results presented here indicate that the male-specific receptor neurons with similar physiological response characteristics have a consistent projection pattern within the MGC across different individuals. The numerous colocalized receptor neurons responding to the primary pheromone component, Z9-16:AL, and the behavioral antagonists, Z9-14:AL and Z9-16:OH, target the cumulus and the dorsomedial glomerulus, whereas the infrequently occurring receptor neurons responding to the

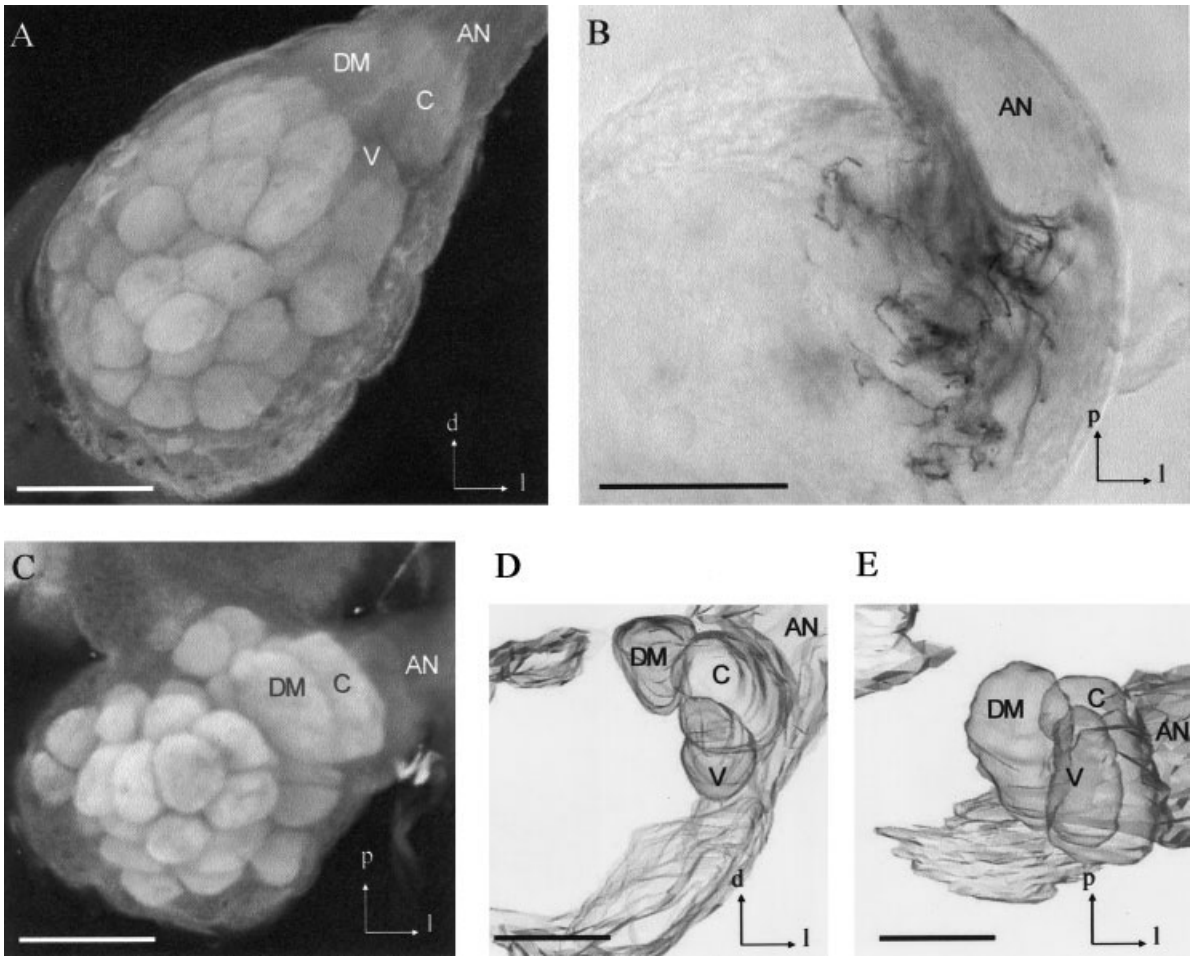

Fig. 1. The antennal lobe of *Helicoverpa assulta* male, showing the location of the macroglomerular complex (MGC) situated close to the entrance of the antennal nerve. **A:** 3D confocal reconstruction of the antennal lobe in a frontal view. The MGC units and the numerous ordinary glomeruli are visible in the immunostained preparation. **B:** Whole-mount preparation in a dorsal (slightly frontally tilted) view showing mass stainings of axon terminal projections of the male-specific *sensilla trichodea* type 1. As demonstrated, the projections target the MGC area. **C:** 3D confocal reconstruction of an immunostained antennal lobe in a dorsal view showing the appearance of only two MGC glomeruli, the cumulus and the dorsomedial unit. **D:** Reconstruction of the three MGC units, the cumulus, the dorsomedial unit, and the ventral unit, in a frontal view. **E:** Reconstruction of the three MGC units in a dorsal view, showing the ventral MGC-unit situated below the cumulus. C, cumulus; DM, dorsomedial glomerulus; V, ventral glomerulus; AN, antennal nerve. d, dorsal; l, lateral; p, posterior. Scale bars = 100 μm.

secondary pheromone component, Z11-16:AL, target the ventral glomerulus. Thus, the three-compartment MGC of *H. assulta* receives input from three physiological types of male-specific receptor neurons. The data indicate that the two enlarged glomeruli receive information about the primary pheromone component and the behavioral antagonists, while the smallest glomerulus receives information about the secondary pheromone constituent.

Functional organization of the MGC in a comparative perspective

Since there seems to be a general principle that the cumulus is involved in processing information about the primary pheromone component—Z11-16:AL in most heliothine species studied so far, i.e., *H. zea, H. armigera, H. virescens* and *H. subflexa*—the cumulus has generally been interpreted as a unit tuned to Z11-16:AL in this subfamily (Christensen et al., 1991, 1995a; Hansson et al., 1995; Berg et al., 1998; Vickers et al., 1998; Skiri, 1999; Galizia et al., 2000; Vickers and Christensen, 2003; Skiri et al., 2004). Based on the present results, we can conclude that the particular odor to which the cumulus is associated has shifted in *H. assulta* as compared to the other species. Obviously, the principle of the cumulus as a glomerulus dedicated to the primary pheromone component, Z9-16:AL in *H. assulta*, is a possible reason for the shift. The size of the cumulus also corresponds with the large number of receptor neurons tuned to Z9-16:AL. Furthermore, the restricted number of receptor neurons tuned to Z11-16:AL, which here terminate in a smaller ventrally located MGC unit, probably reflects the particular role of this substance—acting as the secondary pheromone component. Whether the small unit tuned to Z11-16:AL was displaced and diminished over evolutionary time by the enlargement of another glomerulus, e.g., one tuned to Z9-16:AL, leaves room for speculation. An exception from

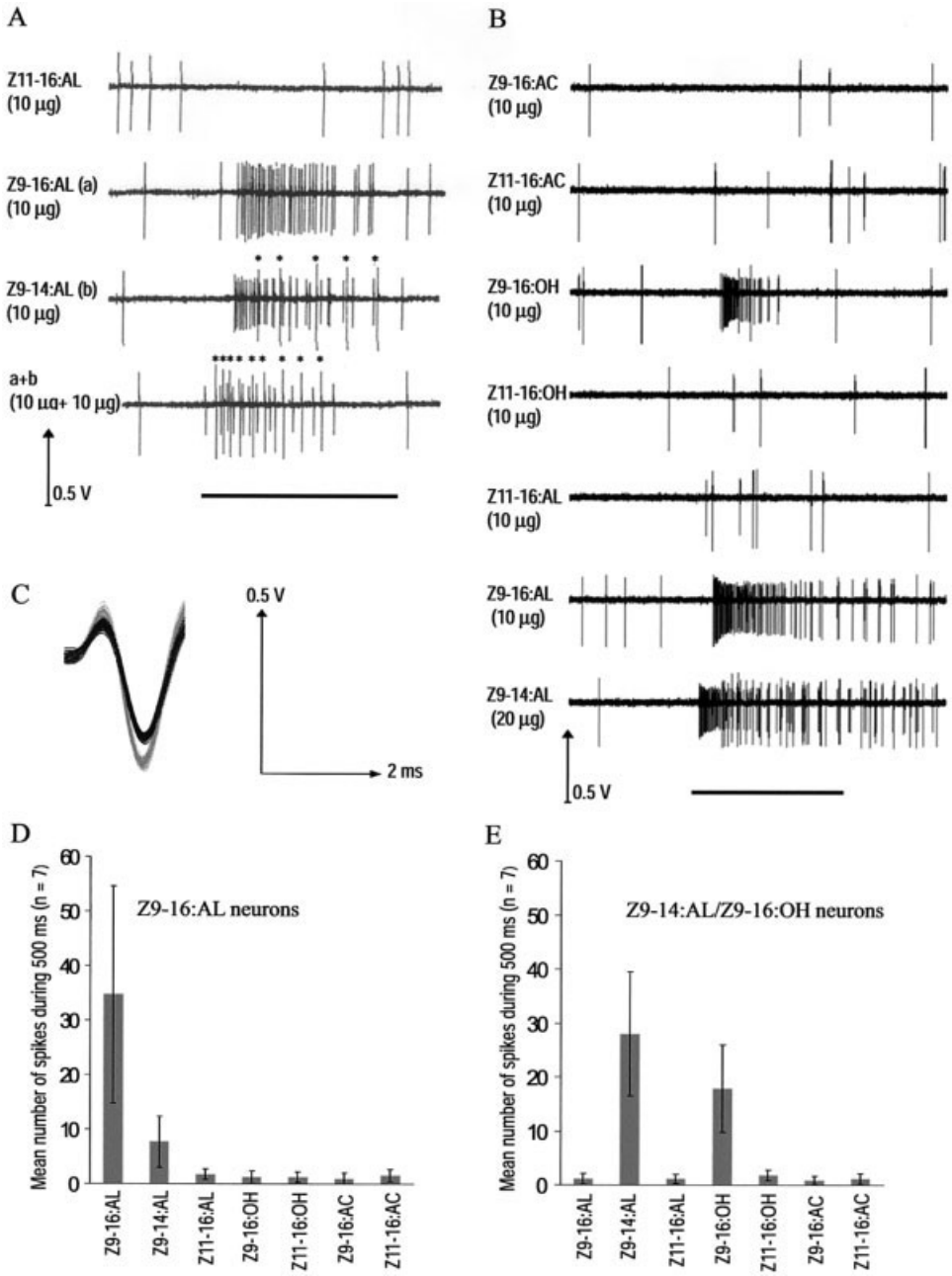

Fig. 2. Cut-sensillum recordings from the two colocalized receptor neurons responding to the primary pheromone component Z9-16:AL and the behavioral antagonists Z9-14:AL/Z9-16:OH, respectively, and histograms showing spike frequencies (mean ± SD) of the same two neuron types during stimulation with the single substances. **A:** Recording showing a large-spiking neuron responding to Z9-16:AL (a) and a small-spiking neuron responding to Z9-14:AL (b). None of the neurons responded to the secondary pheromone component Z11-16:AL. Whereas the small-amplitude neuron did not respond to the major pheromone substance Z9-16:AL, the large-amplitude neuron responded weakly to the highest concentration of Z9-14:AL (b). The mixture of the two compounds (a+b) elicited simultaneous but weaker responses from the two neurons. The large amplitudes are indicated by asterisks during the responses to Z9-14:AL and the mixture. **B:** Recording from two similar, colocalized neurons showing a strong excitation by the large-spiking neuron to Z9-16:AL and the small-spiking neuron to Z9-14:AL. In addition, excitation of the small-spiking neuron to Z9-16:OH is demonstrated. No responses were obtained to stimulation with Z9-16:AC, Z11-16:AC, Z11-16:OH, or Z11-16:AL. The stimuli were applied in the same order as listed. Because of electric noise the response to 20 µg Z9-14:AL, instead of 10 µg, is shown. The stimulation period (500 ms) is indicated below. **C:** The wave-forms of spikes sampled during 15 seconds of spontaneous activity (same neurons as in B) indicating the presence of two neuron types. The signal was amplified about 2,000 times. **D:** Histograms showing the mean number of spikes in seven Z9-16:AL neurons during the 500 ms stimulation period. Seven substances were tested at 10 µg. **E:** Histograms showing the mean number of spikes in seven Z9-14:AL neurons during 500 ms stimulation period. The same substances were tested at 10 µg.

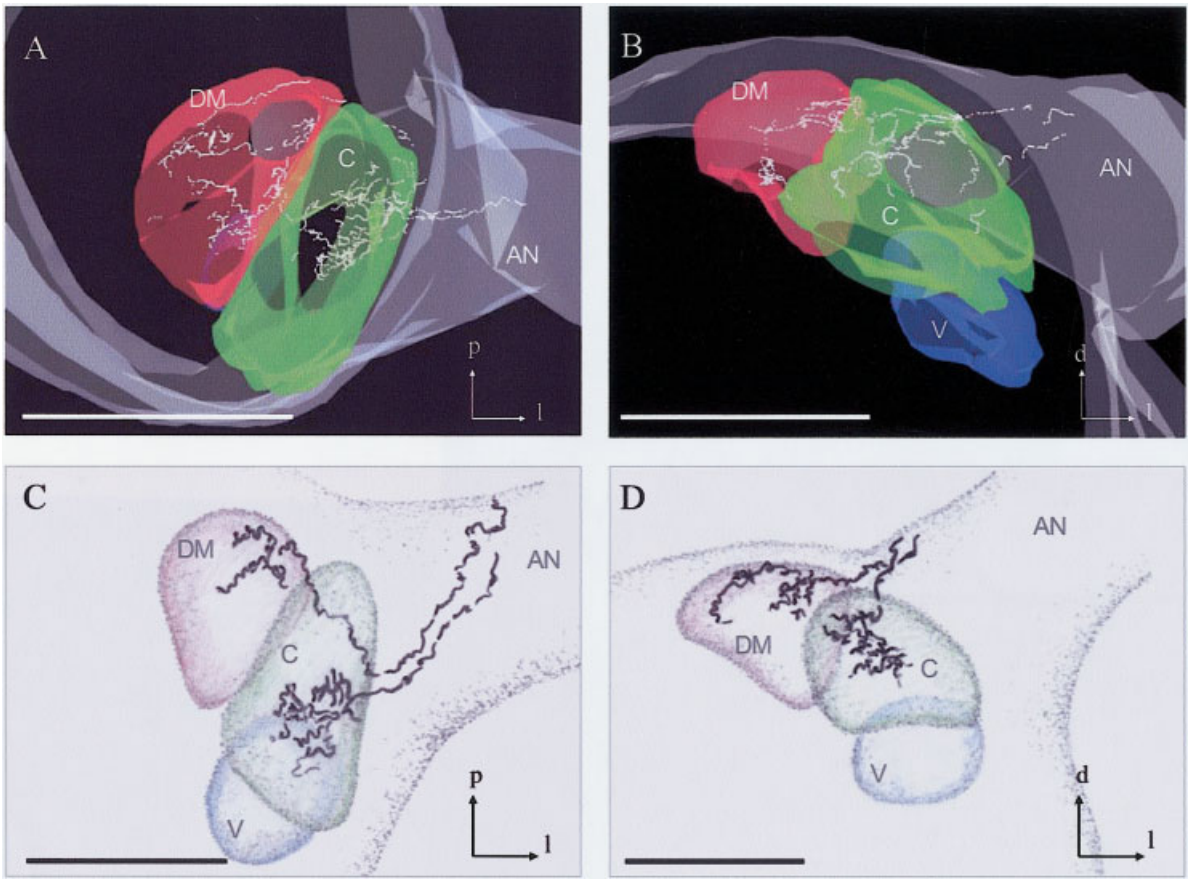

Fig. 3. Projections of the Z9-16:AL and the Z9-14:AL/Z9-16:OH specific receptor neuron types terminating in the two large units of the macroglomerular complex (MGC), the cumulus and the dorsomedial glomerulus. **A:** Digital 3D reconstruction in a dorsal view, showing two stained neurons projecting in the MGC. The axons terminated in the two large MGC glomeruli: one in the cumulus and the other in the dorsomedial subunit. **B:** Digital 3D reconstruction of two stained neurons in a frontally oriented brain, showing projections in the cumulus and the dorsomedial unit. **C:** Camera lucida reconstruction of a dorsally oriented brain showing two stained axon terminals targeting the cumulus and the dorsomedial glomerulus, respectively. **D:** Camera lucida reconstruction of a frontally oriented brain showing two stained axon terminals targeting the cumulus and the dorsomedial glomerulus, respectively. C, cumulus; DM, dorsomedial glomerulus; V, ventral glomerulus; AN, antennal nerve. Scale bars = 100 μm.

the general rule of using Z11-16:AL as a pheromone constituent at all has recently been reported in *Helicoverpa gelotopoeon,* which instead utilizes the substance as an interspecific signal interrupting the pheromone attraction (Cork and Lobos, 2003). The present results occasionally showing stainings in one or two glomeruli in addition to the ventral unit (Fig. 5, Table 1), indicate that the A-type sensilla comprises different subpopulations. Interestingly, in *H. virescens* the presence of stained terminals in one ordinary glomerulus—in addition to the consistent labelings in the cumulus—has also been reported when dye was applied to the sensilla housing the Z11-16:AL neurons (Berg et al., 1998). Altogether, the distinctive characteristics concerning the pheromonal odor tuning of individual MGC units in *H. assulta* seems to mirror the particular composition of the pheromone blend in this species. Obviously, characteristics like these are connected to the divergence of heliothine species.

The particular projection pattern including a nonpheromonal pathway targeting one of the two large MGC units is consistent with the numerous receptor neurons tuned to compounds that are not part of the conspecific pheromone blend in *H. assulta*. These findings strengthen the impression that the general function of the MGC in the subfamily *Heliothinae* is not only processing of pheromone information but also information about interspecific signals. It cannot be definitively concluded which of the two colocalized neuron types projects in the cumulus and the dorsomedial unit. As mentioned above, the role of the cumulus as the common site for processing information about the primary pheromone component has been demonstrated in several heliothine species. Also in other Lepidopterans such as *Manduca sexta, Bombyx mori, Spodoptera littoralis*, and *Trichoplusia ni*, the general role of the cumulus in processing pheromone information has been examined (Koontz and Schneider, 1987; Hansson et al., 1991, 2003; Anton and Hansson, 1995; Ochieng et al., 1995; Todd et al., 1995; Carlsson et al., 2002; Kanzaki et al., 2003). A corresponding functional organization in *H. assulta* would mean that the cumulus receives input from the receptor neurons tuned to Z9-16:AL, further

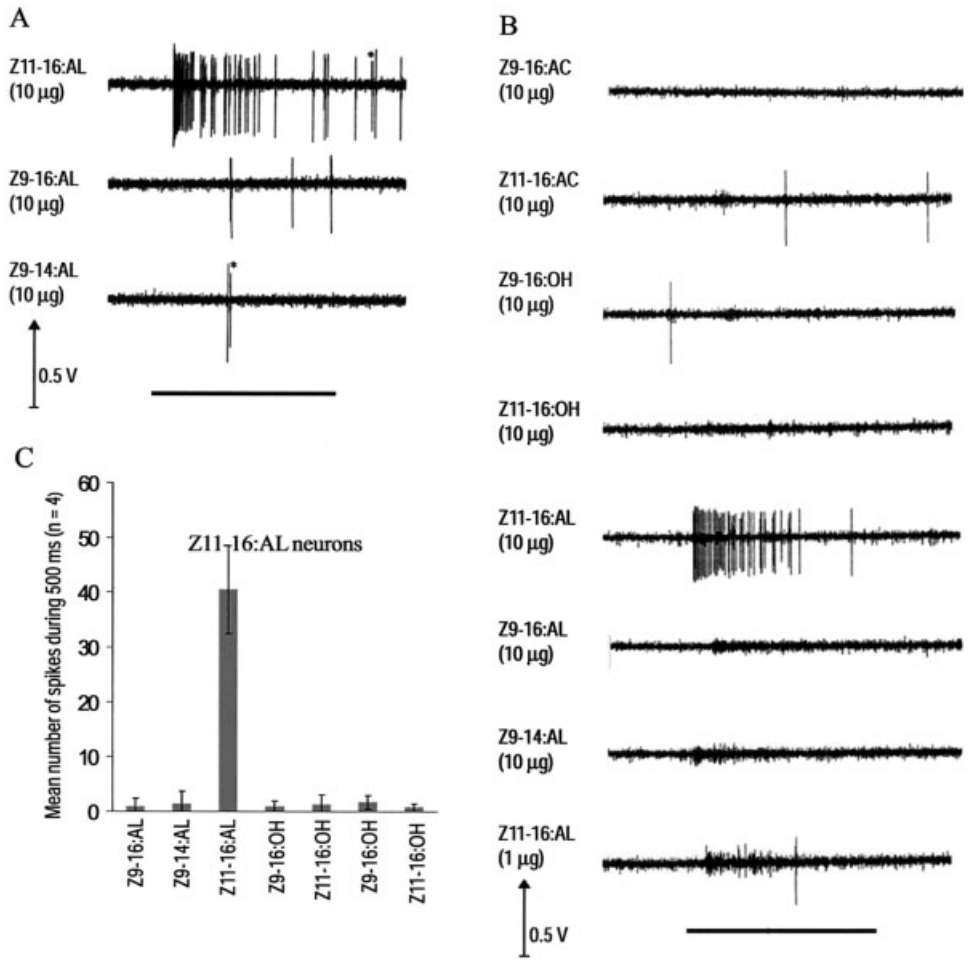

Fig. 4. Two cut-sensillum recordings showing activity of the receptor neuron type responding to the secondary pheromone component Z11-16:AL and histogram showing spike frequencies (mean ± SD) during stimulation with the seven substances. **A:** Response from the neuron to stimulation with Z11-16:AL at 10 µg. No responses to the primary pheromone component, Z9-16:AL, or to the behavioral antagonist, Z9-14:AL, appeared. Activity of a smaller-amplitude spiking neuron occasionally occurred (examples indicated by an asterisk). **B:** Recording from a similar neuron type showing selective responses to Z11-16:AL. No responses were elicited by the other substances (including Z9-16:AC, Z11-16:AC, Z9-16:OH, and Z11-16:OH) or 1 µg Z11-16:AL. The stimulation period (500 ms) is indicated below. The signal was amplified about 2,000 times. **C:** Histograms showing the mean number of spikes in four Z11-16:AL neurons during 500 ms stimulation period. Seven substances were tested at 10 µg.

implying that the second largest, the dorsomedial MGC-unit, receives input from the receptor neurons tuned to the behavioral antagonists, Z9-14:AL and Z9-16:OH. Previous findings, originating from the congeners *H. zea* and *H. armigera*, have reported that processing of interspecific signal information, including Z9-14:AL, is associated with a dorsomedially located MGC-unit in both species (Christensen et al., 1991; Vickers et al., 1998; Skiri, 1999). The alternative projection pattern in *H. assulta* implying that the interspecific signal pathway target the cumulus and the main pheromone pathway of the dorsomedial glomerulus would be fundamentally different from that demonstrated in all other species of *Heliothinae* studied so far. To definitively identify the functional specificities of the two large MGC-units, different techniques such as intracellular recordings combined with stainings need to be carried out.

Compartmentalization of the antennal receptor neurons

By studying the compartmentalization of the receptor neuron types on the antennae across different heliothine species, general principles governing the species-specific peripheral arrangements may be discovered. The two physiological sensillum types of *H. assulta* described here correspond to specific types previously identified in other species of *Heliothinae*. The most abundant category in *H. assulta*, the C-type, comprising the two colocalized receptor neurons tuned to Z9-16:AL and Z9-14:AL/Z9-16:OH, respectively, is partly similar to a sensillum type in *H. zea*, housing two colocalized neurons: one with a large-spiking amplitude responding to Z9-16:AL—which here serves as the second pheromone component—and one with a small-spiking amplitude responding to Z11-16:AC, Z11-16:OH, and Z9-14:AL, acting as behavioral antagonists (Cossé et

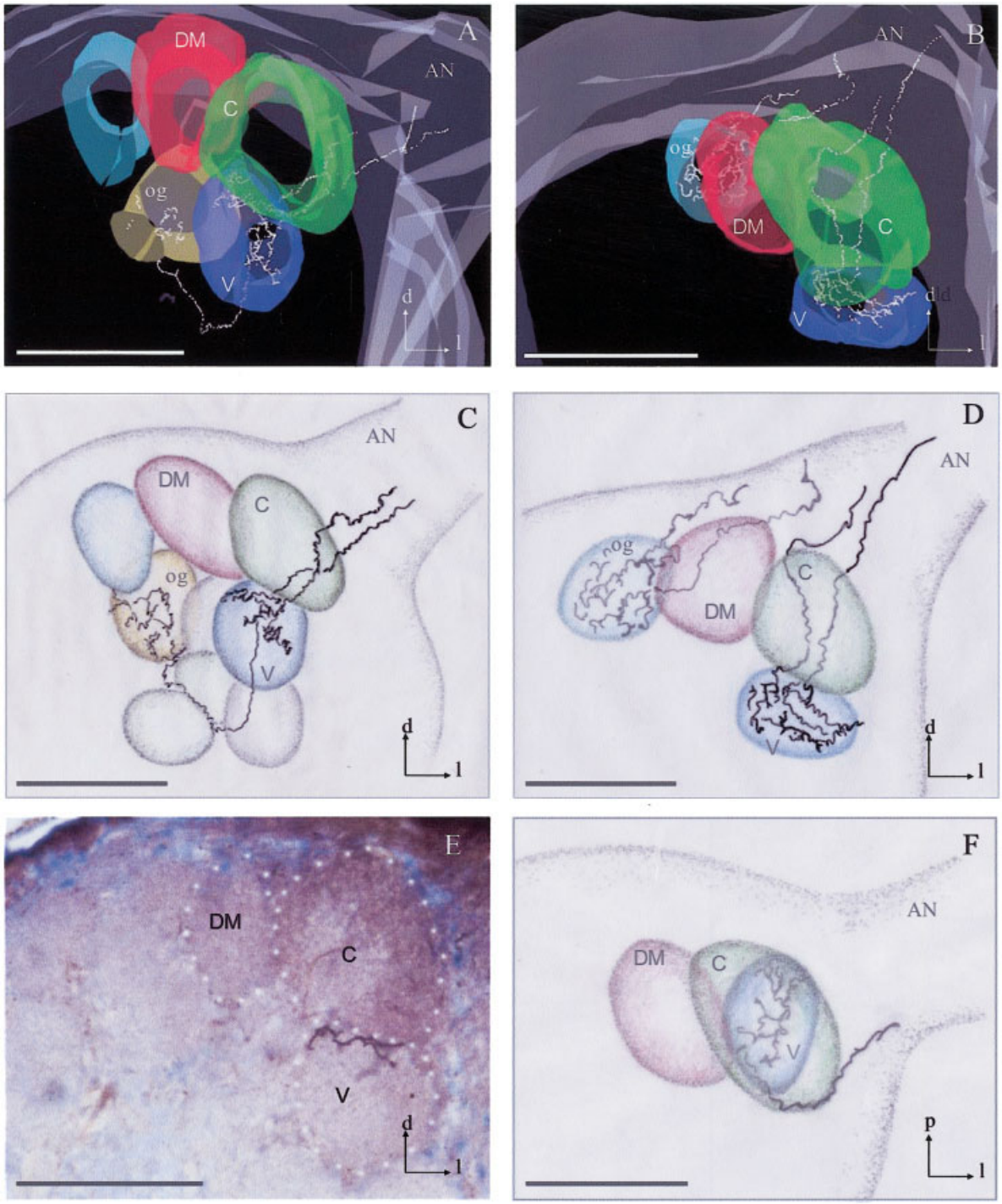

Fig. 5. Stained axon terminals originating from the sensilla housing a receptor neuron responding to the secondary pheromone component. **A:** Digital reconstruction of two axon terminals, one projecting in the ventral MGC-unit and the other in an ordinary glomerulus located posteriorly in the antennal lobe. **B:** Digital reconstruction of four axon terminals: two projecting in the ventral MGC-unit and two in one ordinary glomerulus. The latter, located adjacent to the dorsomedial glomerulus, is unit number 50 according to the atlas of Berg et al. (2002). **C:** Camera lucida reconstruction of the stained projections shown in A. **D:** Camera lucida reconstruction of the stained projections shown in B. **E:** Micrograph of one brain section at 100 μm depth showing stainings in the ventral MGC unit (same preparation as in A and C). **F:** Dorsally oriented camera lucida reconstruction of one stained axon terminating in the ventral MGC-unit, positioned below the cumulus. C, cumulus; DM, dorsomedial glomerulus; V, ventral glomerulus; og, ordinary glomerulus; AN, antennal nerve; d, dorsal; l, lateral; p, posterior. Scale bars = 100 μm.

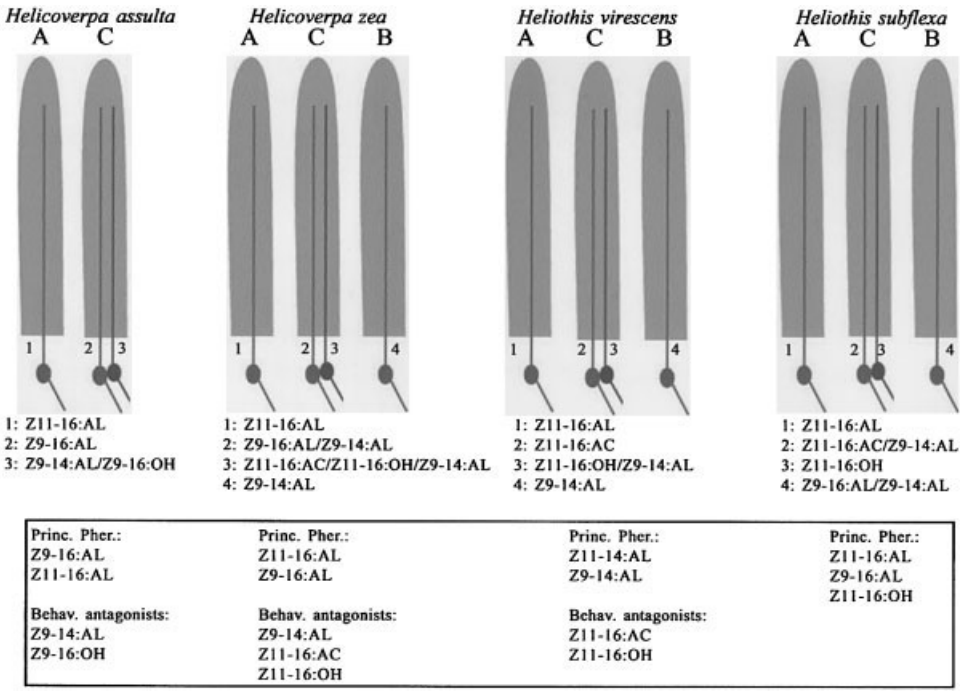

Fig. 6. Schematic drawings of the male-specific sensillum types identified in the four heliothine moths *H. assulta, H. zea, H. virescens*, and *H. subflexa* (according to Baker et al., 2004). In each species a sensillum type A, housing a neuron responding to Z11-16:AL, and a sensillum type C, containing two colocalized neurons, has been demonstrated. In both species of *Helicoverpa*, the two colocalized neurons mediate conspecific and interspecific signal information, respectively; in *H. assulta* the neuron specific for the major pheromone component (Z9-16:AL) is colocalized with the neuron specific for the behavioral antagonists (Z9-14:AL/Z9-16:OH) and in *H. zea* the neuron specific for the secondary pheromone component (Z9-16:AL) is colocalized with the neuron specific for the behavioral antagonists (Z11-16:AC/Z11-16:OH/Z9-14:AL). In *H. virescens*, the two colocalized neurons are tuned to the behavioral antagonist Z11-16:AC and to Z11-16:OH/Z9-14:AL, respectively, whereas in *H. subflexa* the neurons respond to the pheromone constituent Z11-16:OH and to Z11-16:AC/Z9-14:AL. A third sensillum type named B is identified in all species except for *H. assulta*. This type houses a single responding receptor neuron tuned to Z9-14:AL in *H. zea* and *H. virescens*, and to Z9-16:AL/Z9-14:AL in *H. subflexa*. As regards colocalization of neurons transmitting conspecific and interspecific signal information, all possible combinations occur except for the presence of two pheromone neurons in the same sensillum.

al., 1998) (Fig. 6). Compared to the numerous C-type sensilla in *H. assulta*, the corresponding type in *H. zea* were relatively few in number. In the other *Helicoverpa* species, *H. armigera*, a sensillum type housing two colocalized neurons responding to Z9-16:AL, Z9-14:AL, and Z11-16:OH has been reported (Skiri, 1999). In the species of *Heliothis, H. virescens* and *H. subflexa*, a category of sensilla with two colocalized receptor neurons, one responding to Z11-16:OH and Z9-14:AL and the other to Z11-16:AC, has been identified (Almaas and Mustaparta, 1990; Almaas et al., 1991; Hansson et al., 1995; Berg et al., 1998; Baker et al., 2004). Thus, the sensillum type housing the two colocalized receptor neurons in *H. assulta* corresponds most closely with those of the congeners, *H. zea* and *H. armigera*.

The second, infrequently occurring sensillum type of *H. assulta*, the A-type, housing the receptor neurons detecting Z11-16:AL also corresponds with a similar, but abundant type in other heliothine moths, including *H. zea, H. armigera, H. virescens*, and *H. subflexa* (Almaas and Mustaparta, 1990; 1991; Almaas et al., 1991; Hansson et al., 1995; Berg et al., 1995; Mustaparta, 1997, 1998; Cossé et al., 1998; Skiri, 1999; Baker et al., 2004). The occasional appearance of a nonresponding, small-amplitude neuron, as shown in Figure 4, is in accordance with the data from *H. virescens* (Berg et al., 1998). Together, the present and previous results suggest that the particular sensillum type is functionally similar in the heliothine species, regardless of whether Z11-16:AL serves as the primary or secondary pheromone component (Fig. 6).

Together, it seems as if several species of *Heliothinae*, including *H. assulta, H. zea,* and *H. virescens,* have conserved a pattern of compartmentalization on the antennae, by generally grouping certain types of male-specific receptor neurons (Fig. 6). Apart from a general rule implying that two pheromone neuron types never occur in the same sensillum, all possible combinations are present as concerns the colocalization of neuron types mediating interspecific and conspecific signal information; i.e., in *H. assulta* the neuron specific for the major pheromone component is colocalized with the neuron responsible for reproductive isolation, in *H. zea* the neuron specific for the secondary pheromone component is colocalized with a neuron responsible for reproductive isolation (Cossé et al., 1998), and in *H. virescens* none of the two identified pheromone neuron types, but one tuned to Z11-16:OH is colocalized with the neurons mediating interspecific signal information (Hansson et al., 1995; Berg et al., 1998; Baker, 2004). Therefore, we may conclude that the principle of separating the pheromone neuron types in differ-

ent sensilla represents a favorable organization according to detection of the conspecific pheromone blend. Apart from this particular arrangement, it seems as if the principles for compartmentalization are mainly based on the specificities of the receptor neurons, independent of the behavioral responses these signals elicit.

Together, the present results show distinctive characteristics in the male-specific olfactory pathway of *H. assulta* as compared to the other heliothine species studied so far: peripherally by a reversed ratio of the numbers of the two sensillum categories, and centrally by the alteration of odorants represented in the cumulus and the ventral MGC-unit.

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