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Antennal lobe projection destinations of *Helicoverpa zea* male olfactory receptor neurons responsive to heliothine sex pheromone components

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Abstract We used single sensillum recordings to define male *Helicoverpa zea* olfactory receptor neuron physiology followed by cobalt staining to trace the axons to destination glomeruli of the antennal lobe. Receptor neurons in type A sensilla that respond to the major pheromone component, (*Z*)-11-hexadecenal, projected axons to the cumulus of the macroglomerular complex (MGC). In approximately 40% of these sensilla a second receptor neuron was stained that projected consistently to a specific glomerulus residing in a previously unrecognized glomerular complex with six other glomeruli stationed immediately posterior to the MGC. Cobalt staining corroborated by calcium imaging showed that receptor neurons in type C sensilla sensitive to (*Z*)-9-hexadecenal projected to the dorsomedial posterior glomerulus of the MGC, whereas the co-compartmentalized antagonist-sensitive neurons projected to the dorsomedial anterior glomerulus. We also discovered that the olfactory receptor neurons in type B sensilla exhibit the same axonal projections as those in type C sensilla. Thus, it seems that type B sensilla are anatomically type C with regard to the projection destinations of the two receptor neurons, but physiologically one of the receptor neurons is now unresponsive to everything except (*Z*)-9-tetradecenal, and the other responds to none of the pheromone-related odorants tested.

Keywords *Helicoverpa zea* · Single sensillum recording · Cobalt staining · Calcium imaging · Sex pheromone

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Abbreviations AL: Antennal lobe · DMA: Dorsomedial anterior · DMP: Dorsomedial posterior · MGC: Macroglomerular complex · *ORN*: Olfactory receptor neuron · PCx: Posterior complex · Z9-14:Ald: (*Z*)-9-tetradecenal · Z9-16:Ald: (*Z*)-9-hexadecenal · Z11-16:Ac: (*Z*)-11-hexadecenyl acetate · Z11-16:Ald: (*Z*)-11-hexadecenal · Z11-16:OH: (*Z*) -11-hexadecenol

Introduction

The larvae of *Helicoverpa zea* are serious crop pests having a wide host range including corn, soybean, cotton, sorghum, peanuts, and lettuce in North America (Neunzig 1963; Martin et al. 1976). Adult moths use (*Z*)-11-hexadecenal (Z11-16:Ald) as a major sex pheromone component (Roelofs et al. 1974; Klun et al. 1979). The female also produces (*Z*)-9-hexadecenal (Z9-16:Ald) as an essential secondary component for male attraction in a proportion of ~2% relative to the Z11-16:Ald. Two other compounds, (*Z*)-7-hexadecenal (Z7-16:Ald), and hexadecanal (16:Ald), are found in the pheromone gland but have no effect on upwind flight or courtship behavior of male *H. zea* when combined with the binary pheromone blend of Z11-16:Ald and Z9-16:Ald in both field and wind tunnel studies (Klun et al. 1980; Vetter and Baker 1984). Two other North American heliothine moths, *Heliothis virescens* and *Heliothis subflexa*, also use Z11-16:Ald as a major pheromone component. However, *H. zea* males are not attracted to females of other sympatric heliothine species due to the behaviorally antagonistic effects of several pheromone components released by females of these species. Addition of (*Z*)-9-tetradecenal (Z9-14:Ald), an essential secondary pheromone component of *H. virescens* (Vetter and Baker 1983; Vickers et al. 1991), to the *H. zea* blend significantly decreases *H. zea* male attraction (Shaver et al. 1982). Interestingly, this compound, blended with

Z11-16:Ald, was capable of substituting for Z9-16:Ald, the essential secondary component (Vickers et al. 1991). Two pheromone components of *H. subflexa*, (*Z*)-11-hexadecenyl acetate (Z11-16:Ac) and (*Z*)-11-hexadecen-1-ol (Z11-16:OH), also are behavioral antagonists to male *H. zea* attraction (Shaver et al. 1982; Fadamiro and Baker 1997; Quero and Baker 1999; Quero et al. 2001).

The behavioral effects of these inter- and intraspecific pheromonal compounds have been linked with neurophysiological activities of olfactory receptor neurons (ORNs) housed within three different physiological types of trichoid sensilla on the antennae of male *H. zea* (Cossé et al. 1998). The majority (71%) of examined sensilla have an ORN that responds exclusively to Z11-16:Ald (type A sensilla). Approximately 10% of the sensilla have an ORN that responds to Z9-14:Ald only (type B sensilla). Type C sensilla (19%) contain one ORN exhibiting larger spikes that responds equally well to both Z9-16:Ald and Z9-14:Ald as well as a second ORN that has small spikes and responds to the behavioral antagonists Z11-16:Ac and Z11-16:OH. The *H. virescens* secondary pheromone component Z9-14:Ald also stimulates this ORN, but at a higher concentration. The presence of antagonistic ORNs responding to the interspecific pheromone components helps explain how male *H. zea* discriminates conspecific pheromone blends from others (Baker et al. 1998; Cossé et al. 1998). The dose-dependent agonistic and antagonistic effects of Z9-14:Ald on *H. zea* male behavior (Vickers et al. 1991) correlate with the physiological responses to Z9-14:Ald of the two different co-compartmentalized ORNs in the type C sensillum. However, whether the Z9-14:Ald-selective ORNs in the type-B sensilla contribute to agonistic or antagonistic behavioral effects has remained unclear.

Histophysiological studies of projection neurons (PNs) in the *H. zea* macroglomerular complex (MGC) of the antennal lobe (AL) have helped us begin to understand how information about the presence of pheromone-related compounds in blends is relayed to higher centers (Christensen et al. 1991; Vickers et al. 1998). Because the glomeruli are known to be structural and functional units of olfactory information processing in the AL, staining and tracking physiologically identified PNs has elucidated the output characteristics of such neurons exiting each target glomerulus. However, the glomeruli-specific location and output activities of noctuid moth PNs have occasionally been found not to correspond to the activities and glomerular arborization locations of ORNs (Anton and Hansson 1999), despite there being a good correspondence in the majority of cases (Berg et al. 1998; Galizia et al. 2000). In the present article, we have examined the arborization destinations of *H. zea* ORNs in the type A, B, and C sensilla through both cobalt staining (Hansson et al. 1992) and calcium imaging (Joerges et al. 1997; Galizia et al. 2000; Berg et al. 2002; Carlsson et al. 2002). Our goal was to relate these ORN input destinations with PN outputs found in previous studies (Vickers et al. 1998).

Materials and methods

Insects

The *H. zea* colony was maintained on a 16:8-h (light:dark) photoperiod at 25°C, 40–50% RH. Larvae were reared on a modified pinto bean diet (Shorey and Hale 1965). Males and females were separated in the pupal stage and housed in separate growth chambers in different rooms. Male pupae were sent to Sweden for the calcium-imaging experiments through express mail.

Chemical stimuli

The synthetic pheromone-related compounds Z11-16:Ald, Z9-16:Ald, Z9-14:Ald, Z11-16:Ac, and Z11-16:OH (Bedoukian Research Inc., Danbury, CT, USA; purities >98%, verified by gas chromatography) were obtained from our laboratory stock. Serial dilutions of these compounds were made in HPLC-grade hexane and stored at −20°C. To prepare stimulus cartridges for each compound, 10 µl of each diluted solution was loaded onto a 0.7×2.5-cm^2 filter-paper strip, the solvent was allowed to evaporate, and then the paper strip was placed in a glass Pasteur pipette. For the mixture of Z11-16:Ald and (±)-linalool, 10 µg (±)-linalool in 10 µl hexane was added to a filter paper onto which 10 µg Z11-16:Ald had already been loaded, and the solvent was allowed to evaporate. New cartridges were prepared every 5 days and stored at −20°C when not in use after being sealed with an aluminum foil.

For attempts to stimulate co-compartmentalized "silent" ORNs in sensilla, we used 28 different volatile organic compounds at a loading of 100 µg on a filter paper in Pasteur pipettes. These compounds were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), Fluka Chemika (Buchs, Switzerland), and Bedoukian Research Inc., and were >97% free of other volatile impurities. The compounds were (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, 1-hexanol, phenylacetaldehyde, ocimene, α-humulene, *cis*-nerolidol, α-pinene, 2-phenylethanol, benzaldehyde, geraniol, (±)-linalool, indole, terpinyl acetate, 3-carene, α-copaene, (−)-(*E*)-caryophyllene, ethyl caproate, citronellal, allyl isothiocyanate, methyl jasmonate, methyl salicylate, dimethyltrisulfide, isovaleric acid, 1-octen-3-ol, (*E*)-β-farnesene, and 6-methyl-5-hepten-2-one.

Single sensillum recordings

The cut-sensillum technique (Kaissling 1974; Van der Pers and Den Otter 1978) was used to record the activities of ORNs housed within individual antennal sensilla. A male *H. zea* was restrained inside a disposable pipette tip; the narrow end of the pipette tip was cut to allow the male's head to pass through. The head and

antennae were immobilized in the proper orientation using dental wax. A Ag/AgCl wire was inserted into the abdomen to serve as a reference electrode and was then secured with an alligator clip attached in turn to the end of one of the micromanipulators. By maneuvering this micromanipulator, the tip of one of the many sexually dimorphic long trichoid sensilla was placed on the edge of a vertically positioned metal knife fashioned by sharpening an insect pin. The sensillum tip was cut off using a horizontally positioned glass knife held in place by a second micromanipulator. The cut end of the sensillum was then contacted by a saline-filled glass micropipette, and an electrical connection was established. The AC signals from the ORN were amplified using a high-impedance amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA), and the action potentials were digitally recorded on VHS tapes or directly onto a personal computer by means of data processing software (Syntech Autospike 3.2; Syntech, Hilversum, The Netherlands).

Olfactory stimuli (50-ms pulses at 40 ml/s flow rate) were delivered into a continuous charcoal-filtered humidified airstream (10 ml/s) by means of a stimulus flow-controller device (SFC-2; Syntech). The odor-laden air pulse passed through a 14-cm-long glass tube (8 mm ID) whose outlet was positioned 2 cm from the antenna. Linear flow through this continuous airstream was ~0.3 m/s. At least 30 s was allowed to elapse between consecutive stimulations in order to minimize adaptation.

Staining of ORNs with cobalt lysine

To try to find the axonal target arborization destination in particular glomeruli of the AL for each electrophysiologically recorded ORN, we used a cobalt lysine staining technique, with a minor modification, following the protocol of Hansson et al. (1995), Ochieng et al. (1995), and Todd et al. (1995). The recording electrode was replaced by a new electrode filled with 0.5 M cobalt lysine solution after the ORNs residing in the sensillum had been identified with regard to their response specificity. The cobalt lysine electrode was then used to contact the sensillum for 10 min, during which time one of the pheromone-related compounds, to which one of the ORNs was tuned, was puffed at a frequency of 0.7 Hz with a 50-ms pulse duration at 40 ml/s flow rate. Afterwards, the moth was kept at 4°C for 2 days. The brain was then dissected in saline (150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 10 mM TES, and pH 7.0) and was then treated with ammonium sulfide solution for the cobalt precipitation. Brains were washed four times for 5 min each with saline with 2% sucrose. After fixation, the stained neurons were intensified with silver (Bacon and Altman 1977). Brains were dehydrated through a graded series of ethanol, cleared with methyl salicylate, and viewed as whole mounts under a light microscopy. Brains were preliminarily counterstained for differentially enhancing cell bodies and glomeruli using modified Lee's methylene blue-basic Fuchsin solution (Bennett et al. 1976). Our modified solution used 0.5% methylene blue + 0.5% azure II in 1% borate:0.5% basic fuchsin in 95% ethanol:100% ethanol = 1:2:1, or 0.15% methylene blue, 0.15% azure II, and 0.3% basic fuchsin in 70% alcohol for 10 min. The stained whole brains were washed with 70% alcohol and dehydrated sequentially with 95%, pure ethanol (twice), and twice with propylene oxide. The infiltration procedure was performed using a mixture of propylene oxide and Durcupan resin (Sigma-Aldrich, St. Louis, MO, USA). The propylene oxide was allowed to evaporate at room temperature overnight, and then the brains were embedded in pure Durcupan resin. Most brains were oriented for frontal sections, but a few were prepared for horizontal sections. After curing at 60°C for 2 days, brains were sectioned at 10-µm thickness and digitally captured. The levels of digitally captured images were modified using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA) for better contrast.

Calcium imaging

Preparation of animals and optical recordings were performed as described previously (Carlsson et al. 2002; Carlsson and Hansson 2003). Briefly, the animals were restrained in plastic tubes and firmly fixed with dental wax. A window was cut in the head between the compound eyes, and then glands and muscles and tracheae removed to allow AL observations. A calcium-sensitive dye (CaGR-2-AM; Molecular Probes, Eugene, OR, USA) was bath-applied to the uncovered brain. The dye was dissolved in 20% Pluronic F-127 in dimethyl sulfoxide (Molecular Probes) and diluted in saline (Christensen and Hildebrand 1987) to a final concentration of ~30 µM. After incubation (~60 min in 10–12°C) and rinsing in saline, recordings were performed in vivo.

We used an air-cooled imaging system (TILL Photonics, Gräfelfing, Germany) with a 12 bit slow-scan charge-coupled device camera. Filter settings were dichroic, 500 nm, and emission LP, 515 nm, and the preparation was excited at 475 nm. Sequences of 40 frames and a sampling rate of 4 Hz (200-ms exposure time) were recorded through an upright microscope (Olympus, Tokyo, Japan) with a ×20 (numerical aperture 0.50; Olympus) air objective. Stimulation started at frame 12 and lasted either 100 ms or 1 s. On-chip binning (2×2) was performed, which resulted in a final image size of 320×240 pixels. Execution of protocols and initial analyses of data were made using the software Till-vision (TILL Photonics).

A moistened and charcoal-filtered continuous airstream (30 ml/s) was used to flush away odors from the antenna ipsilateral to the recorded AL through a glass tube (7 mm ID). The glass tube ended at ~10 mm from the antenna. An empty Pasteur pipette was inserted through a small hole in the glass tube, blowing an

airstream of ~5 ml/s. Air was blown (~5 ml/s) through the odor-loaded pipette by a manually triggered puffer device (Syntech) into the continuous airstream. During stimulation, the airstream was switched from the empty pipette to the odor-loaded pipette, thereby minimizing mechanical influences. Odorants (conspecific pheromones or behavioral antagonists) were diluted in hexane and applied on the filter papers (5×15 mm^2). Filter papers were inserted in Pasteur pipettes that were then sealed with Parafilm and stored in a freezer (−20°C) until experiment started. A syringe with a filter paper loaded with solvent served as a control. Every fifth stimulation was done with a control.

Background fluorescence (F) was defined as an average of frames 2–11, i.e., before onset of stimulation. F was subtracted from all frames to yield dF, and signals were expressed as dF/F, i.e., a relative change in fluorescence over background fluorescence. For image presentation, an average of frames 14–18 (peak of activity) of a bleaching-corrected sequence was calculated, and an average of frames 2–11 (prestimulation) was subtracted. The resulting image was subsequently filtered with a spatial average low-pass filter (13×13 pixels) and false-color coded to its entire intensity range.

Results

Our histological examinations of the *H. zea* AL (Fig. 1) were consistent with other reports (Vickers et al. 1998) showing an MGC comprised of three glomeruli called the cumulus, the dorsomedial posterior (DMP), and the dorsomedial anterior (DMA) (Fig. 1a), along with numerous ordinary glomeruli clearly set apart from these three MGC glomeruli (Fig. 1b, c). However, we also identified a previously undescribed complex of glomeruli residing just posterior to the MGC in every male that we examined, and we have named this the posterior complex (PCx) (Figs. 1c, 2). The PCx is comprised of seven glomeruli in which the MGC is nestled, with many of the glomeruli in the complex representing the most posterior area of this most dorsal portion of the AL (Fig. 1). The PCx is also characterized by a confluent bundle of fibers whose main trunk is oriented toward the center of the AL and that also eventually merges with the fiber bundle from the MGC (Figs. 1b, 2c). The numbers we assigned to the PCx glomeruli were determined first according to the order of their dorsal-to-ventral positions, after examining serial sections from

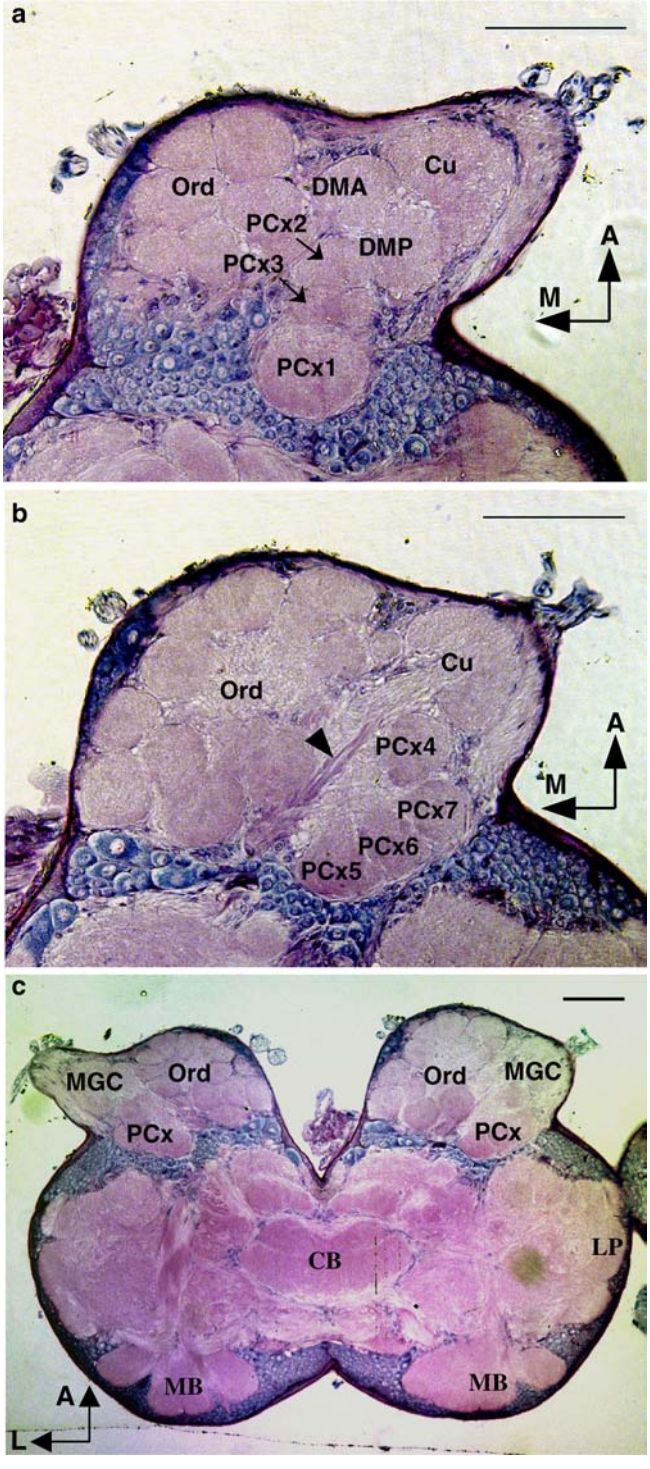

Fig. 1 Dorsal view of the antennal lobe (*AL*) of *Helicoverpa zea* brain (*horizontal sections*) showing position of the PCx relative to macroglomerular complex (*MGC*) and ordinary glomeruli (*Ord*). **a** Section at ~70 µm depth from the most dorsal surface. Three MGC compartments (Cu, DMP, and DMA) and three glomeruli of the posterior complex, PCx (*PCx1, 2, 3*) are visible. **b** Depth of 130 µm showing the cumulus (*Cu*) of the MGC and four more glomeruli of the PCx (*PCx4, 5, 6, 7*). A confluent bundle of the fibers from interneurons from the MGC and the PCx is indicated (*arrowhead*). **c** Low-magnification overview of the brain, showing the positions of MGC, Ord, and PCx relative to the protocerebrum. Mushroom bodies (*MB*) of the protocerebrum are indicated at the posterior part of the brain, and the central body (*CB*) and lateral protocerebrum (*LP*) also are shown. *Cu* cumulus, *DMA* dorsomedial anterior, *DMP* dorsomedial posterior, *Ord* ordinary glomeruli, *MB* mushroom body, *CB* central body, *LP* lateral protocerebrum, *A* anterior, *M* medial, *L* lateral. *Scale bar* 100 µm

six males (8 ALs), followed by anterior–posterior and medial–lateral number assignments.

Single sensillum recording and staining

Most of the examined sensilla were selected between the 10th and 20th flagellomeres, counted from the base of the antenna. The average number of flagella comprising a male antenna was 75 ($n = 6$) with a range of 72–79. This result is slightly different from the reports of Callahan (1969) and Grant et al. (1989) who counted an average of 82 flagellomeres on *H. zea* antennae.

Type A sensilla

We physiologically identified 134 type A sensilla. In these, action potentials from a large-spiking ORN were evoked after stimulation by Z11-16:Ald only, and not by any of the other heliothine pheromone components (Fig. 3a). A second ORN having a spike amplitude

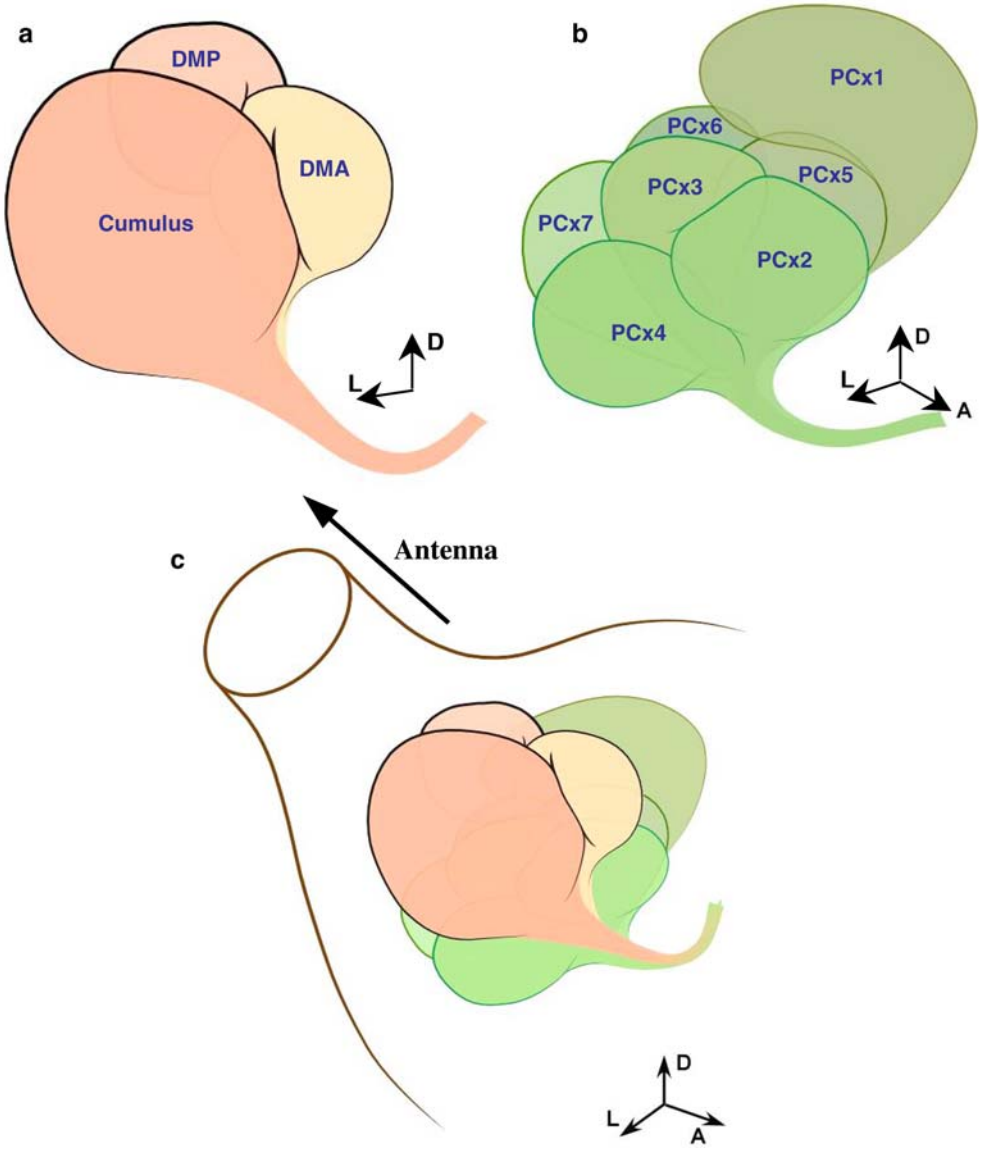

Fig. 2 Schematic frontal view diagram of the glomerular topographies of the MGC and the PCx in the male *H. zea* AL. **a** MGC is comprised of three glomeruli: Cu, dorsomedial anterior (*DMA*), and dorsomedial posterior (*DMP*). **b** The PCx is comprised of seven glomeruli: PCx1–7. The numbers following PCx are designated by the order of the glomerular arrangement in the AL: dorsal to ventral, anterior to posterior, and medial to lateral. The PCx1 is the most dorsally positioned glomerulus in this complex and is the arborization destination of the second (“silent”) olfactory receptor neuron (*ORN*) from the type A sensilla. This schematic diagram represents an average male AL reconstructed after examination of serial 10-μm sections from eight individual *H. zea* males. **c** Combined view, showing that the PCx is situated posterior to the MGC. Outgoing neurons from both the MGC and the PCx seem to form a common tract. *D* dorsal, *L* lateral, *A* anterior

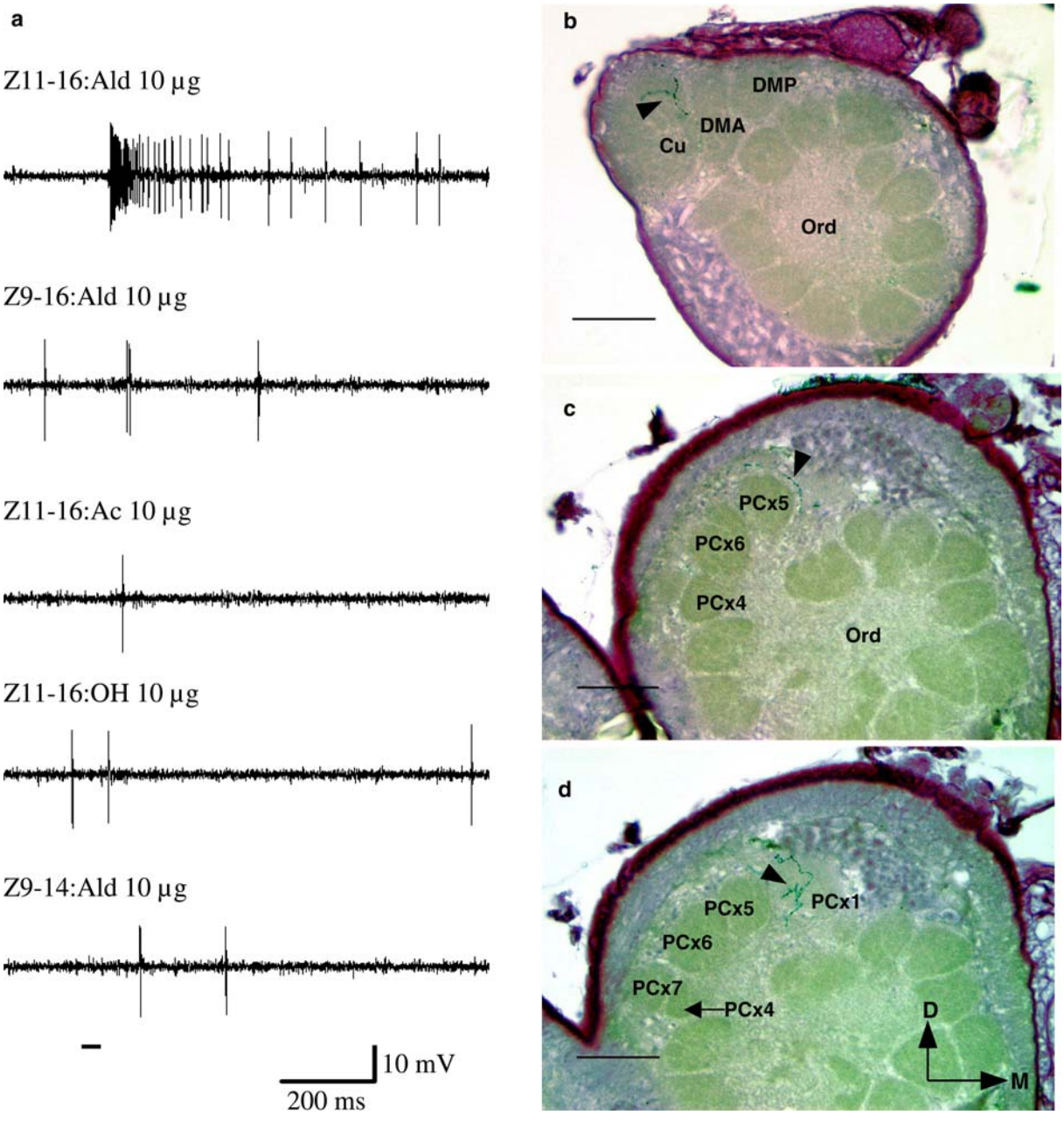

Fig. 3 **a** Spike train of an ORN from an *H. zea* type A sensillum. Action potentials were triggered only by stimulation with Z11-16:Ald. **b–d** Frontal view of a male *H. zea* AL, showing the arborization destinations of two ORNs that were stained within one type A sensillum. **b** One stained axon (*arrowhead*) arborized in the Cu. **c, d** A second ORN arborized in the dorsal-most glomerulus (PCx1) of the posterior complex. The section in (**b**) is ~80 µm posterior from the frontal surface. The section in (**c**) is 100 µm posterior to (**b**). In (**d**), the section is ~10 µm posterior to (**c**) and the axonal path of the stained second ORN (*arrowhead*) is shown traveling (in **c**) around glomerulus PCx5 of the posterior complex to arborize in glomerulus PCx1 (*arrowhead* in **d**). *Cu* cumulus, *DMA* dorsomedial anterior, *DMP* dorsomedial posterior, *Ord* ordinary glomeruli, *D* dorsal, *M* medial. *Scale bar* 50 µm

barely discernible from background noise could occasionally be seen in recordings from these sensilla (Fig. 3a, top trace). None of the 28 volatile organic compounds that we tested for 15 type A sensilla caused firing from either this small-spiking ORN or the large-spiking ORN.

Attempts to stain ORNs in these 134 sensilla resulted in 40 successful stains. Of these, 23 (~57.5%) had single stains, with one ORN projecting to the cumulus glomerulus of the MGC (Vickers et al. 1998) (Table 1). In the other 17 preparations (42.5%), two ORNs were stained. In these preparations, one ORN projected to the cumulus (Fig. 3b) and the other projected to a glomerulus, glomerulus 1 (PCx1) of the posterior complex, located on an average more than 100 µm posterior to the cumulus (Fig. 3c, d).

Table 1 Summary of the glomerular arborization destinations of olfactory receptor neurons from three different types of *Helicoverpa zea* male sensilla, as determined by cobalt back-filling of physiologically characterized neurons

Sensilla type	Stained sensilla (attempts)	Target glomeruli				
		Single target			Double targets	
		Cumulus	DMA	DMP	Cumulus and PCx1	DMA and DMP
Type A	40 (134)	23	0	0	17	0
Type B	2 (3)	0	0	0	0	2
Type C	26 (58)	0	2[a]	0	0	24[b]

DMA dorsomedial anterior, *DMP* dorsomedial posterior, *PCx1* posterior complex glomerulus 1
[a]One of these occasions stained two axons but one axon faded out in its pathway with no target glomerulus
[b]One sensillum possessed three stained axons with two projecting into DMP

During the application of cobalt stain into the cut sensillum, the antenna was exposed to repetitive stimulation (0.7 Hz) with either 10 μg of Z11-16:Ald, 10 μg of (±)-linalool, or a mixture of 10 μg Z11-16:Ald and 10 μg (±)-linalool. (±)-Linalool was used as a stimulus because of the enhanced firing that occurs in pheromone-sensitive ORNs when the antenna is stimulated with a mixture of (±)-linalool and Z11-16:Ald (Ochieng et al. 2002). We thought there might possibly be activity-dependent cobalt uptake in one of the ORNs with (±)-linalool-Z11-16:Ald mixtures. However, the uptake of cobalt stain was not influenced by which stimulus was used, with approximately one-half of all preparations resulting in one stained ORN and the other half showing two stained ORNs. Single stains always involved one ORN projecting only to the cumulus glomerulus, and those showing two stained ORNs always included one projecting to the cumulus and the other projecting to the PCx1.

Type B sensilla

In trying to find type B sensilla, 20 sensilla were sampled between the basal and the middle region of the antenna (from the base to approximately the 25th flagellomere), 133 were sampled from the middle region (between the 25th and the 50th flagellomere), and 24 were even more distally located. These sensilla, housing ORNs exhibiting action potentials only in response to Z9-14:Ald (Fig. 4a), were rarely encountered. However, of the three such type B sensilla we found (at flagellomeres 34 and 35), two produced ORNs that were successfully stained. These both exhibited two stained axons, one projecting into the DMA glomerulus and the other into the DMP glomerulus, respectively (Fig. 4b–d, Table 1), which are the same arborization locations as the ORNs from type C sensilla (see below). The stimulus used during cobalt introduction for both sensilla was Z9-14:Ald.

Type C sensilla

The physiological identification of type C sensilla was done by means of their response profiles, which were consistent with the findings of Cossé et al. (1998). Larger amplitude action potentials were evoked after stimulation with Z9-16:Ald and also Z9-14:Ald, whereas smaller action potentials were elicited after stimulation with Z11-16:Ac, Z11-16:OH, and Z9-14:Ald (Fig. 5a). Previous studies using differential adaptation had already shown that it is one large-spiking ORN that responds to both Z9-16:Ald and Z9-14:Ald (Cossé et al. 1998). Similarly, there is only one smaller-spiking ORN that responds to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald (Cossé et al. 1998).

Attempts to stain ORNs in these 58 sensilla resulted in 26 successful stains. Out of these preparations, 24 (~92%) showed two separate ORNs, each projecting to the two smaller glomeruli of the MGC, the DMA and the DMP glomeruli (Vickers et al. 1998) (Fig. 5b, c, Table 1). Although nearly all preparations with multiple stains (23/24) showed two stained neurons that targeted two glomeruli, one preparation showed three stained neurons. Two of these neurons projected axons to the DMP glomerulus and one to the DMA glomerulus. The other 2 preparations (out of 26) had a single ORN projecting to the DMA glomerulus. No apparent differences in resulting stains were found when either Z11-16:Ac, Z9-16:Ald, or a mixture of the two were used to stimulate the ORNs during cobalt uptake; two stained axons projecting to the same two glomeruli were obtained, except in two cases when only one stained ORN was obtained (Fig. 5b, c, Table 1).

Calcium imaging

With the use of the calcium imaging technique, increases in intracellular calcium concentrations in glomeruli in response to antennal stimulation with pheromone-related compounds were observed in eight male moths (Table 2). Two different doses (1 and 10 μg) of pheromone components were applied. Stimulation with the major component, Z11-16:Ald, increased calcium concentration prominently in the cumulus (Fig 6a). Exposure of the antenna to the behavioral antagonist Z11-16:Ac showed maximum activity in the DMA glomerulus (Fig. 6b), whereas

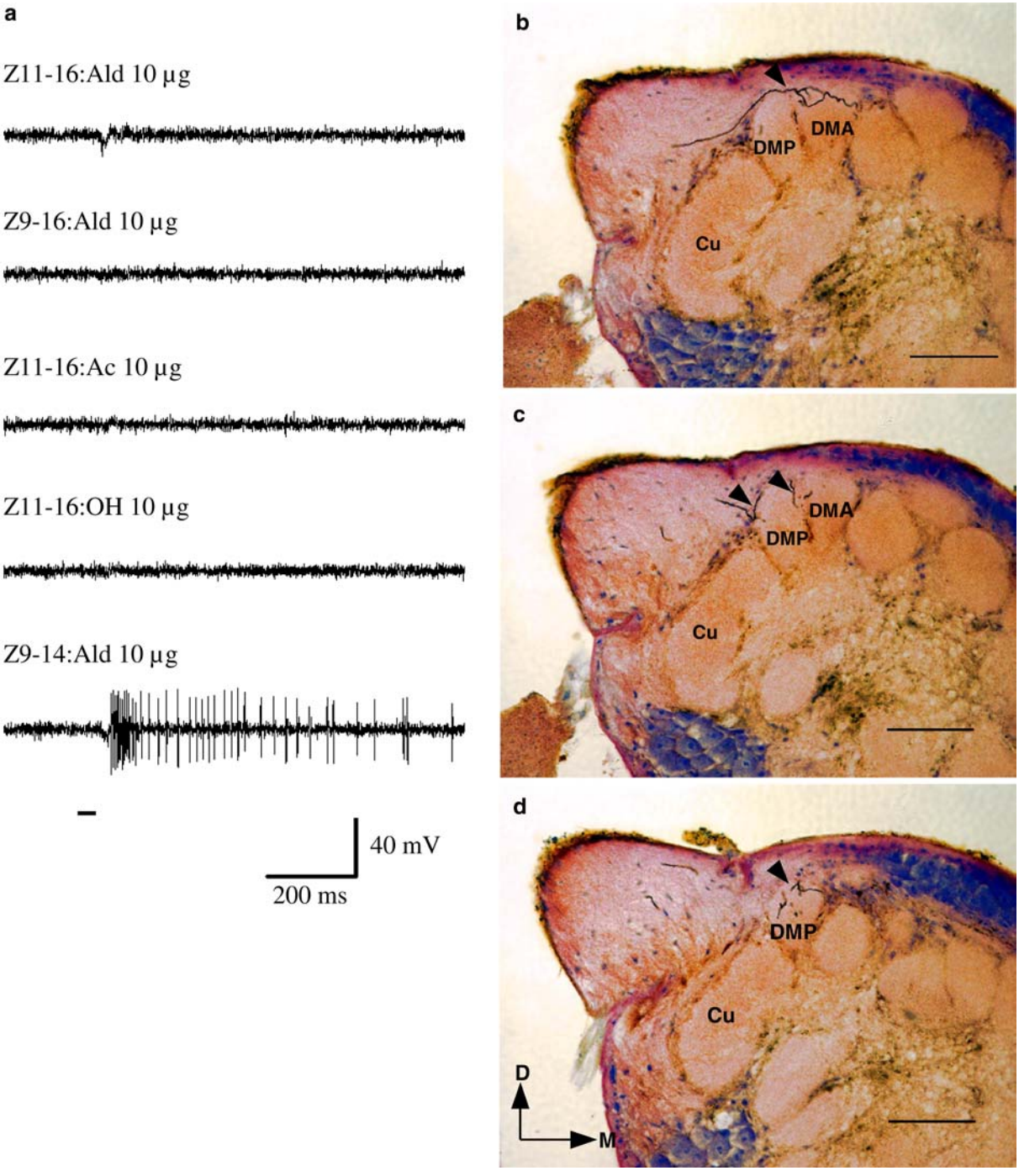

Fig. 4 **a** Responses of ORNs from a type B sensillum. Only Z9-14:Ald evokes a spike train. **b–d** Frontal view of AL showing glomerular arborization destinations of two ORNs housed within one type B sensillum. **b** One axon (*arrowhead*) arborizes in the DMA glomerulus. **c** The next section, 10 µm posterior to (**b**) shows a second axon starting to arborize (*arrowheads*) in the DMP glomerulus. **d** About 20 µm posterior to (**c**), showing more arborization of this ORN (*arrowhead*) in the DMP glomerulus. The DMA glomerulus is no longer visible in this section. *Cu* cumulus, *DMA* dorsomedial anterior, *DMP* dorsomedial posterior, *D* dorsal, *M* medial. *Scale bar* 50 µm

stimulation with the minor pheromone component Z9-16:Ald showed maximum activity in the DMP glomerulus in all attempts (Fig. 6c). These three volatiles elicited consistent results at any dosage (Table 2). In cases in which Z9-14:Ald was used for stimulation, however, the location of calcium activity in the AL was dependent on the dosage. For the two animals in which 1 µg of Z9-14:Ald was used, this dose activated the DMP glomerulus. In contrast, animals exposed to 10 µg of Z9-14:Ald displayed predominant activity in the DMA glomerulus, with some activity also occurring in the DMP glomerulus (Fig. 6d, Table 2).

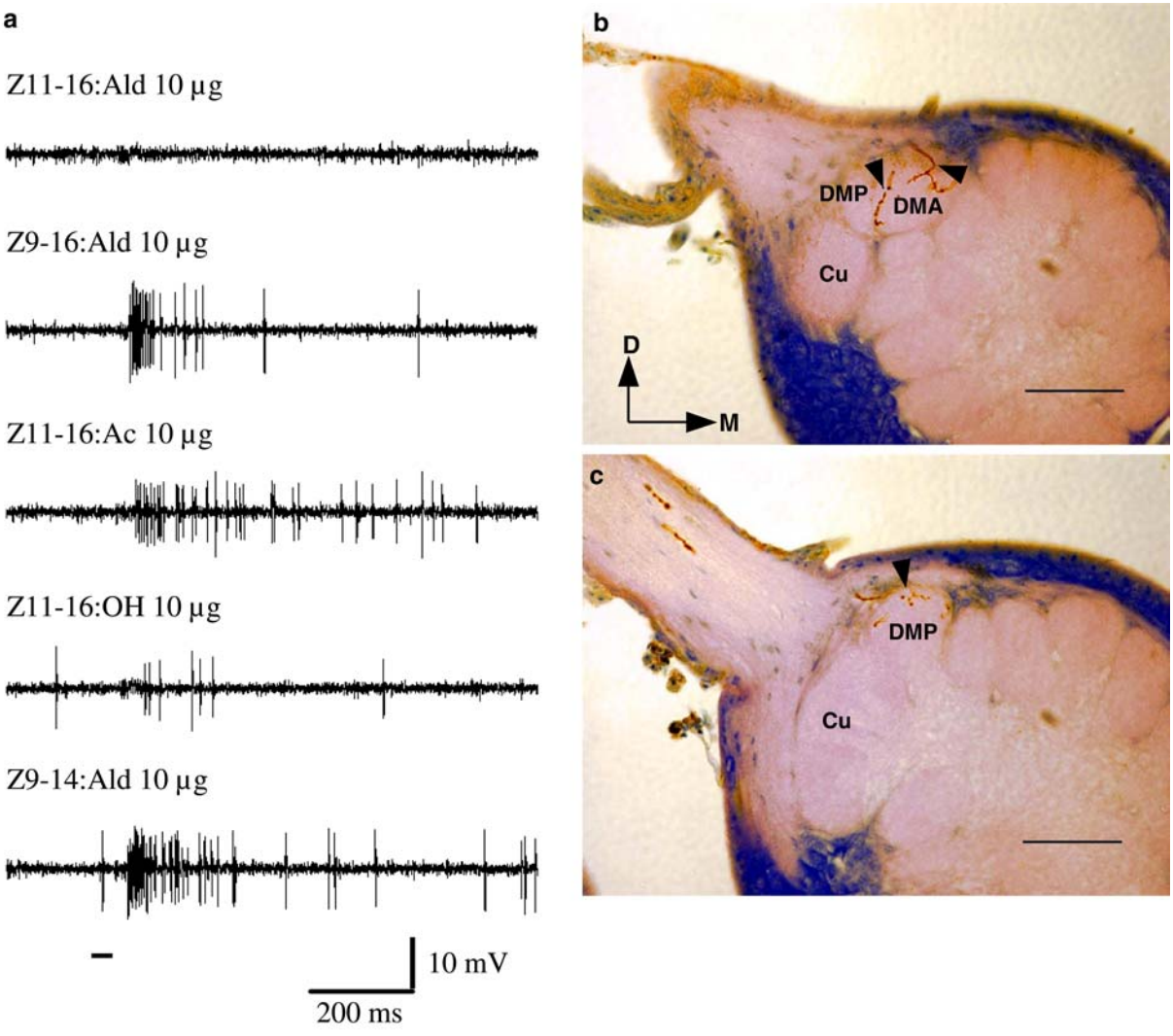

Fig. 5 **a** In type C sensilla, tracings of ORN activity show a larger amplitude spiking ORN that responds to both Z9-16:Ald and Z9-14:Ald and a smaller amplitude spiking ORN that responds to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald. **b, c** Neuronal projection patterns of the two ORNs housed within a type C sensillum (*frontal view*). **b** One axon arborized in the DMA glomerulus (*arrowheads*). **c** The second axon arborized in the DMP glomerulus (*arrowhead*). The DMA glomerulus is no longer visible in this section, which is ~50 µm posterior to (**b**). *Cu* cumulus, *DMA* dorsomedial anterior, *DMP* dorsomedial posterior, *D* dorsal, *M* medial. *Scale bar* 50 µm

Discussion

The results presented here, combined with those from previous studies (Christensen et al. 1991; Cossé et al. 1998; Vickers et al. 1998) show that in *H. zea*, as in other heliothine moths (Hansson et al. 1995; Berg. et al. 1998; Vickers and Christensen 2003), there is a correspondence between pheromone odorant-specific input to, and output from, the different MGC compartments. These relationships for *H. zea* are depicted in Fig. 7.

Without exception, cobalt stains showed that at least one axon of the examined type A sensilla (40/40) projected into the cumulus (Table 1), which is also the arborization location of Z11-16:Ald-responsive PNs (Christensen et al. 1991; Vickers et al. 1998). Our calcium-imaging experiments show that excitation from the entire population of ORNs across the antenna that are responsive to Z11-16:Ald converges onto the cumulus (Table 1, Fig. 3a). Therefore, in *H. zea*, as shown in two other North American heliothine moths (Hansson et al. 1995; Berg et al. 1998; Vickers et al. 1998; Vickers and Christensen 2003), the cumulus receives input from ORNs about the presence of the major pheromone component, Z11-16:Ald.

The unresponsive, silent ORNs co-residing with the Z11-16:Ald-sensitive ORNs in the type A sensilla project with 100% fidelity (17 of 17 cobalt stains) to the most dorsal glomerulus, PCx1, of the newly described posterior complex (Figs. 1, 2, 3d, 7, Table 1). We did not find any ORNs from any sensilla other than from the

Table 2 Summary of the focal points of maximum calcium release in the macroglomerular complex (MGC) glomeruli of *H. zea* males, as indicated by highest levels of calcium-sensitive dye activity after antennal stimulation with the indicated pheromone-related compounds

Stimulants	Dosage (μg)	Number of moths[a]	Active glomerulus		
			Cumulus	DMP	DMA
Z11-16:Ald	1	6	6		
	10	2	2		
Z9-16:Ald	1	4		4	
	10	3		3	
Z11-16:Ac	1	6			6
	10	2			2
Z9-14:Ald	1	2		2	
	10	3			3

DMA dorsomedial anterior, *DMP* dorsomedial posterior
[a]The number of individual moths in which successful recordings were made

type A that projected to the PCx1. This leads us to conclude that the secondary ORNs in the type A sensilla are not arbitrarily co-compartmentalized with the Z11-16:Ald-responsive neurons and are biochemically similar due to their axonal projection addresses into this specific PCx1 glomerulus. In *H. subflexa*, we have found a similar arrangement involving silent ORNs that are co-compartmentalized within type A sensilla with Z11-16:Ald-sensitive ORNs. The axons of these *H. subflexa* silent ORNs always project to a dorsal-most glomerulus similar in position to the PCx1 in *H. zea*, which is also located in a posterior glomerular complex (S.-G. Lee et al., unpublished data).

Spontaneous activity is often encountered from a secondary ORN co-compartmentalized with the Z11-16:Ald-responsive ORN on the male antennae of *H. virescens* and *Helicoverpa assulta* as well, but as in *H. zea* and *H. subflexa*, none of the tested compounds have been found to elicit activity from this ORN (Hansson et al. 1995; Berg et al. 1998, 2005; Baker et al. 2004). In *H. virescens*, the target glomerulus of this secondary ORN seems to correspond to the position of PCx1 in *H. zea*, with respect to the dorso-lateral position, relative to the cumulus, of what was described as an "ordinary" glomerulus in the figures of Berg et al. (1998). Similarly, the staining of secondary ORNs in *H. assulta* that are co-compartmentalized with Z11-16:Ald-sensitive ORNs resulted in arborizations that appear to occur in a PCx glomerulus (Berg et al. 2005).

For *H. zea*, we cannot say with certainty that there is no ligand that this silent ORN in the type A sensillum is tuned to, but it responded only occasionally and inconsistently to some of the compounds in the array of 28 compounds that we challenged it with in this study, plus nearly 60 others in a separate exhaustive study (S.-G. Lee

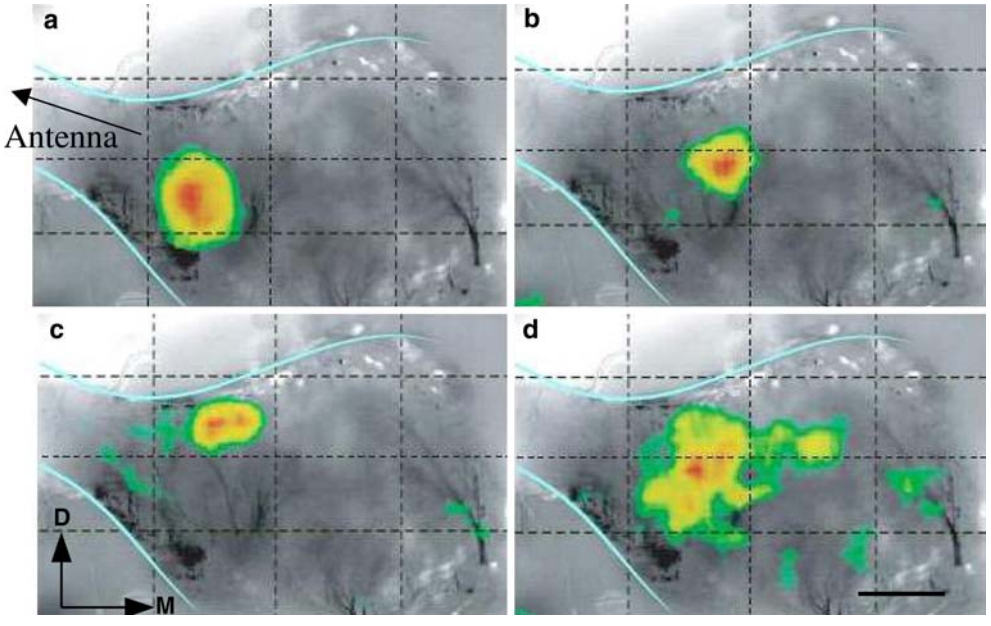

Fig. 6 Frontal view of the calcium-imaging activity from the left AL during stimulation of antennal ORNs with pheromone-related compounds. The images were left–right reversed from the originals in order to better conform to the orientations of Figs. 2, 3, 4, and 5 of this paper. The activity maps were cropped to 50% of maximal activity and superimposed on *gray-scale* images from the same recordings. **a** Stimulation from a cartridge loaded with 1 μg of Z11-16:Ald. **b** Stimulation from a cartridge loaded with 1 μg of Z11-16:Ac. **c** Stimulation from a cartridge loaded with 1 μg of Z9-16:Ald. **d** Stimulation from a cartridge loaded with 10 μg of Z9-14:Ald. The focal activity of glomeruli is distributed between both the DMA and the DMP glomeruli at the higher emission rate of Z9-14:Ald used with this animal, consistent with the physiological responses of both the large- and small-spiking ORN in type C sensilla to Z9-14:Ald (Cossé et al. 1998; this study, Fig. 4a). There are also some weaker signals from a few other glomeruli, which is probably due to the aldehyde group that could activate to a lesser degree some other types of general-odorant-tuned ORNs projecting to ordinary glomeruli. *DMA* dorsomedial anterior, *D* dorsal, *M* medial. *Scale bar* 100 μm

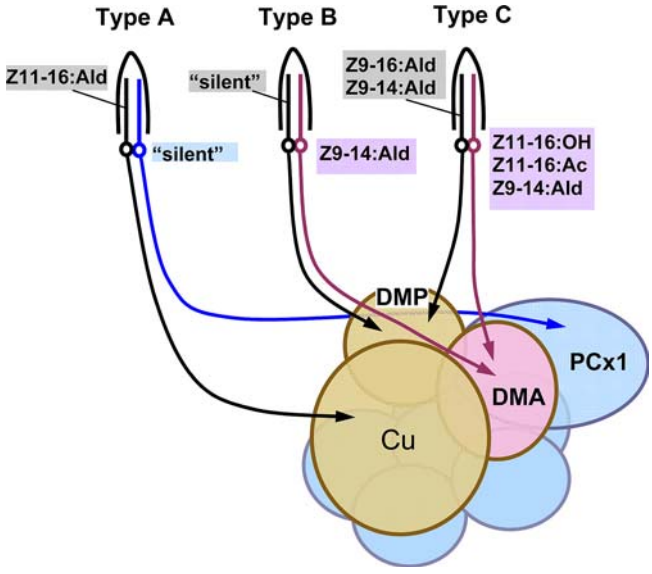

Fig. 7 Schematic diagram showing the arborization destinations of ORNs from the three classes of long trichoid sensilla on *H. zea* male antenna, constructed according to the combined results from single ORN physiological recordings (Cossé et al. 1998), cobalt stainings, and calcium imaging (this study). Axonal projections from the ORNs responsive to Z11-16:Ald in type A sensilla arborize in the cumulus glomerulus (Cu), and the "silent" ORNs from type A sensilla arborize in the PCx1 glomerulus. Type B sensilla house two ORNs; those responsive to Z9-14:Ald arborize in the DMA glomerulus and are associated with behavioral antagonism just as the small-spiking ORNs from type C sensilla. The 'silent' ORNs from type B sensilla arborize in the DMP glomerulus just as the Z9-16:Ald/Z9-14:Ald-responsive ORNs from type C sensilla. The large-spiking ORNs from type C sensilla responsive to both Z9-16:Ald and Z9-14:Ald project to the DMP glomerulus and are associated with attraction as part of the *H. zea* pheromone blend. The small-spiking ORNs in type C sensilla that are responsive to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald project to the DMA glomerulus and their activities are associated with cessation of upwind flight. *Cu* cumulus, *DMA* dorsomedial anterior, *DMP* dorsomedial posterior, *PCx1* posterior complex glomerulus 1

et al., unpublished data). In the latter study we also tried stimulation with the C16 acids corresponding to the enzymatic degradation products of heliothine aldehyde pheromone components (Prestwich et al. 1989; Tasayco and Prestwich 1990) because in other noctuid species such as *Trichoplusia ni* and *Agrotis segetum*, the ORNs that are co-compartmentalized with major component-sensitive ORNs (Hansson et al. 1990; Todd et al. 1992, 1995) are tuned to the corresponding degradation products (alcohols) resulting from esterases acting on the acetate major components (Prestwich et al., 1989). In heliothines, the enzymatic degradation product of Z11-16:Ald is (*Z*)-11-hexadecanoic acid (Z11-16:COOH), which results from the activity of aldehyde oxidase and dehydrogenase operating in the sensillar lymph (Prestwich et al. 1989). However, attempts thus far to stimulate this secondary ORN with C16 acids have not produced consistent results (S.-G. Lee et al., unpublished).

In *T. ni* and *A. segetum*, stimulation of co-compartmentalized ORNs tuned to the corresponding alcohols of the acetate pheromone components results in an antagonism of upwind flight to pheromone. These ORNs project to an MGC glomerulus and not into a separate complex such as the PCx in *H. zea*. If the silent ORNs in *H. zea* were indeed found to be sensitive to Z11-16:COOH, we would predict that this acid should be behaviorally antagonistic to *H. zea* upwind flight. However, why these ORNs should project to a glomerulus in a separate kind of complex close to the MGC and not to the MGC itself as in other noctuids is intriguing.

This question relates not just to *H. zea* but to other heliothines as well. In recordings of ORNs residing in the type A sensilla on the male antennae of the three North American heliothines that have been extensively researched (*H. zea*, *H. virescens*, and *H. subflexa*), spontaneous activity from an ORN co-compartmentalized with the Z11-16:Ald-responsive ORN has often been reported, but none of the pheromone-related test compounds have been found to elicit activity from these ORNs (Grant et al. 1989; Hansson et al. 1995; Berg et al. 1998, 2005; Baker et al. 2004).

Although not described before now, structures similar to the posterior complex can be seen in the ALs of other male heliothine moths, including *H. assulta* (Berg et al. 2002) and *H. subflexa* (S.-G. Lee et al., unpublished data). For example, in the confocal micrograph of male *H. assulta* AL (Berg et al. 2002), a cluster of seven glomeruli (G61, 50, 46, 47, 48, 49, and 60) is located posterior to the MGC that seems to have a structural conformity with the PCx of *H. zea*. In particular, the glomeruli G61 of *H. assulta* (Berg et al. 2002) and G54 of *H. virescens* (Berg et al. 2002) seem to coincide with the PCx1 that receives input from the silent ORNs from type A sensilla on *H. zea* (this study) and *H. subflexa* antennae (S.-G. Lee et al., unpublished data).

The combination of cobalt staining and calcium imaging has now also clarified the relationship of the arborization destinations of the two ORNs housed in the type C sensilla compared with the arborization locations of the PNs related to attraction and behavioral antagonism (Cossé et al. 1998; Vickers et al. 1991, 1998). The minor pheromone component Z9-16:Ald and the behavioral antagonist Z11-16:Ac, respectively, stimulate two different ORNs that are co-compartmentalized within type C sensilla (Cossé et al. 1998). Similarly, two different types of *H. zea* PNs are responsive to either Z9-16:Ald or to Z11-16:Ac, and their arborization in the DMP and DMA glomeruli, respectively, had strongly suggested that similarly tuned ORNs also should project to the DMP and DMA glomeruli (Vickers et al. 1998). In our cobalt staining studies with type C sensilla, two stained axons from these sensilla consistently arborized within the DMP and DMA glomeruli, and nowhere else (Table 1), thereby confirming the linkage between the ORNs in type C sensilla and these two target glomeruli. Subsequently, our calcium-imaging experiments were definitive, showing that Z9-16:Ald stimulation produced an increase in calcium concentration only in the DMP glomerulus (Fig. 6; Table 2). Conversely, antennal

stimulation with the behavioral antagonist Z11-16:Ac consistently increased calcium concentrations only in the DMA glomerulus (Fig. 6; Table 2). The combined results from cobalt staining and calcium imaging are consistent with the conclusion that the large-spiking ORNs in type C sensilla that are related to attraction and are sensitive to both Z9-16:Ald and Z9-14:Ald (Cossé et al. 1998) transmit their action potentials to the DMP glomerulus. PNs responsive to Z9-16:Ald also arborize in the DMP glomerulus (Vickers et al. 1998); therefore, there is a linear arrangement of Z9-16:Ald-driven input from ORNs through this glomerulus followed by Z9-16:Ald PN output.

Our cobalt stains plus the calcium-imaging experiments together clarify that the small-spiking ORN in type C sensilla related to behavioral antagonism and responsive only to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald (Cossé et al. 1998) projects to the DMA glomerulus. PNs responsive to these compounds also arborize here (Vickers et al. 1998), and now we can say that they relay information about these compounds along this line to higher order central nervous system interneurons.

Almaas et al. (1991) concluded that an ORN that they found responding both to Z9-14:Ald and Z9-16:Ald in *H. zea* was far more responsive to Z9-14:Ald, and therefore its activity was related to behavioral antagonism. They did not take into account, however, the great difference in volatility of these two compounds and they did not measure the actual amounts emitted from, not loaded into, the odor cartridges as did Cossé et al. (1998). Moreover, Almaas et al. (1991) showed no awareness that they were recording the spike activities from two ORNs (the large- and the small-spiking ORNs of Cossé et al. 1998), not just one, in the four sensilla they recorded from when stimulating with Z9-14:Ald. Because Almaas et al. (1991) lumped the separate activities of these two ORNs that are both equally responsive to Z9-14:Ald as having come from one ORN, they will have overestimated the apparent sensitivity of this 'neuron' by two-fold in response to Z9-14:Ald compared to what occurs in only the large-spiking ORN in response to Z9-16:Ald (Cossé et al. 1998). The difference in emitted versus loaded amounts of Z9-14:Ald will have added an additional ten-fold overestimation of sensitivity to Z9-14:Ald.

The results of our successful cobalt stainings of type B sensilla suggest that type B sensilla are anatomically type C sensilla, but the large-spiking ORN is now silent, being unresponsive to Z9-14:Ald and Z9-16:Ald, and the small-spiking ORN that responds to Z9-14:Ald is no longer responsive to Z11-16:Ac and Z11-16:OH (Fig. 7). Recent studies of *Drosophila melanogaster* olfactory receptor genes have shown that receptors can be expressed or repressed without the ORN changing its glomerular arborization destination (Dobritsa et al. 2003). That the fidelity of targeting a particular glomerulus does not depend on which receptors are expressed on an ORN would explain how the type B sensillar ORNs can continue to target the same glomeruli as type C ORNs, even though their responsiveness to pheromone components has apparently been repressed. Maintaining a constant glomerular destination by an ORN that has shifted its tuning spectrum due to pheromone receptor gene enhancement or repression of expression would potentially provide rich opportunities for evolutionary shifts in pheromone blends (T.C. Baker et al., submitted). There are obviously great differences in the sensitivities of what seem to be homologous ORNs in response to different subsets of pheromone components across these heliothine species. There are also striking similarities in the occurrence of co-compartmentalized silent ORNs, such as in type A sensilla across these species. A lot may be learned from studying these heliothine ORNs with regard to the expression and repression of pheromone component receptor genes.

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