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Spatial Organization of Antennal Olfactory Sensory Neurons in the Female *Spodoptera littoralis* Moth: Differences in Sensitivity and Temporal Characteristics

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Abstract

Single-cell recordings from olfactory sensory neurons (OSNs), housed in sensilla located at the base and at the tip of the antenna, showed selective responses to plant odors and female sex pheromone in this polyphagous moth. A spatial variation existed in sensitivity: OSNs present on the more proximal segment (P) were more sensitive than those on the more distal segment (D). OSNs of the 2 locations also differed in temporal characteristics: OSNs on P had shorter latency and displayed more phasic responses, whereas those on D had more tonic responses, especially at low stimulus concentrations. The 196 OSNs responding to our 35 monomolecular stimuli in the screening were housed in 32 functional sensillum types: 27 in basiconic, 3 in long-trichoid, 2 in coeloconic, and 3 in auricillic sensilla. The OSNs in basiconic, coeloconic, and auricillic sensilla responded to plant-associated odorants, whereas OSNs in long-trichoid sensilla responded to female-produced sex pheromone components. Short-trichoid sensilla showed spontaneous activity, but no responses to any odorant tested. OSN specificity to plant stimuli ranged from highly specific to broadly tuned, but it did not differ clearly from females in more specialized moths. OSN response diversity is discussed in terms of olfactory coding, behavior, and ecological specialization.

Key words: antennal architecture, Lepidoptera, plant volatiles, single sensillum recordings, spatial variation, tuning

Introduction

Selection of host plants is a major task in the life of phytophagous insects and requires sophisticated sensory systems (Bernays 2001; Bruce et al. 2005; Bruce and Pickett 2011). Many insects rely primarily on olfactory cues to locate and discriminate host plants from non-hosts for feeding and oviposition (Ramaswamy 1988; Renwick 1989; Gregg et al. 2010; Jactel et al. 2011). In moths, these volatile cues are detected by olfactory sensory neurons (OSNs) enclosed in sensilla distributed across the antennal surface (Hallberg et al. 1994; Shields and Hildebrand 1999a, 1999b). The OSN membranes carry the olfactory receptor (OR) proteins, the *Or* genes of which have been characterized in several insects by genomic or transcriptomic approaches (Fabrice et al. 2011; Grosse-Wilde et al. 2011; Sachse and Krieger 2011). However, detailed *Or* specificities have been established in only a few insects, primarily in *Drosophila* (Hallem et al. 2004; Galizia and Rössler 2010).

Functional characterization of OSNs in different insect species has revealed basic concepts of odor coding (Dobritsa et al. 2003; Hallem et al. 2004; Bruce et al. 2005; Ignell and Hansson 2005; de Bruyne and Baker 2008) and provides valuable insight into how input from OSNs is represented in the central nervous system (CNS) (Anton and Hansson 1994, 1995; Mustaparta 2002; Carlsson and Hansson

2003; Schlief and Wilson 2007; Galizia and Rössler 2010) and results in behavioral output (Schlief and Wilson 2007; Riffell et al. 2009). There is a general understanding of male moth physiological and behavioral response toward female-produced sex pheromone (Hansson 1995; Touhara and Vosshall 2009) and of the behavioral role of plant-produced volatiles in female moths (Tasin et al. 2006; Riffell et al. 2009; Gregg et al. 2010; Bruce and Pickett 2011). However, our understanding of neural mechanisms underlying host-plant selection in female moths is limited. Available neurophysiological data on the sensory ecology of female moths in specialist herbivore species suggest that their ability to detect and discriminate odors emitted by plants is determined by OSNs specifically tuned to host-plant volatiles (Shields and Hildebrand 2001; Stranden et al. 2003; Røstelien et al. 2005). One would expect more polyphagous insects, like the Egyptian cotton leaf worm, *Spodoptera littoralis*, to differ from specialist feeders by having a more diverse (Spellerberg and Fedor 2003) array of OSNs, but to date, only a few polyphagous insects have been studied (de Bruyne and Baker 2008).

Spodoptera littoralis (Boisd.) (Lepidoptera: Noctuidae) uses a broad range of species from many different families as host plants (Brown and Dewhurst 1975). However, in spite of this polyphagy, there is evidence of host selection; in behavioral experiments, female *S. littoralis* discriminate between food sources and oviposition sites and host plants of different quality (Anderson et al. 1993, 2011; Anderson and Alborn 1999; Saveer et al. 2012; Thöming G, Larsson MC, Hansson BS, Anderson P, unpublished data). Previous studies have shown that female *S. littoralis* have olfactory sensilla that respond to a wide variety of plant odors (Anderson et al. 1995; Jönsson and Anderson 1999). However, a complete morphological description and functional–morphological map of female antennal olfactory sensilla are lacking. Spatial variation of sensilla or OSNs of different functional types along filiform antenna is partly known (Ignell and Hansson 2005). However, to our knowledge, spatial variation of sensillum function from proximal and distal parts of the antenna has so far not been studied in female moths.

In this study, we mapped the morphological and functional characteristics of antennal olfactory sensilla in female *S. littoralis*, using scanning electron microscopy and single sensillum recordings (SSRs), respectively. Additionally, we studied the distribution of sensillum types and differences in sensitivity and temporal response between proximally and distally located OSNs.

Materials and methods

Insects

Spodoptera littoralis, originating from and annually supplemented with field-collected moths from Egypt, were reared on an artificial diet (Hinks and Byers 1976), using potatoes instead of beans. All developmental stages of the insects were kept at 25 °C, 60–70% relative humidity and at a 16:8 h light:-dark photoperiod. Pupae were collected from the food, sexed, and transferred to 30 × 30 × 30 cm Plexiglas cages in sex-specific rearing chambers. Adult virgin females, 2–4 days of age, were used for the experiments.

Scanning electron microscopy

Heads with intact antennae were fixed in 70% ethanol overnight at 4 °C and then dehydrated sequentially in 80%, 90% and 100% ethanol. Critical point dried (BAL-TEC CPD 030 Critical point Dryer) antennae were glued on metal sockets and coated with gold/palladium (Polaron SC7640 sputter coater) and analyzed under a scanning electron microscope (SEM; JEOL JSM-5600LV). The numbers of each morphological sensillum type were counted from SEM pictures of the 15th segment from the tip (D) and the 15th segment from the base (P) of the antenna ($n = 3$).

Electrophysiology

Chemical stimuli, preparation, and stimulation

The stimuli tested included a wide range of compounds that have been used in earlier studies on *S. littoralis*: green leaf volatiles (GLVs), floral volatiles (FLVs) (Anderson et al. 1995; Galizia et al. 2002), oviposition deterrents (OVD) (Anderson et al. 1993), and herbivore-induced plant odors (Jönsson and Anderson 1999). In addition, 5 female-produced sex pheromone (Sex Ph) components (Campion et al. 1980) were included in the screening (Table 1). An additional panel of volatiles (Table 2) was tested only on short-trichoid sensilla, due to the lack of responses to the first panel tested.

Stimuli were applied as 10 μL aliquots on a 7 × 15 mm piece of filter paper (Whatman No. 1) that was inserted into a Pasteur pipette (15 cm long, soda lime glass; VWR International). For screening, we used pipettes loaded with 10 μg for the compounds dissolved in paraffin oil and with 100 ng for the compounds dissolved in hexane, ethyl acetate, or water. In the dose–response trials, doses of 10 ng–1 mg for the compounds dissolved in paraffin oil, and 100 pg–1 mg for the compounds dissolved in hexane were tested, starting with the lowest dose. Pipettes with filter papers containing 10 μL of solvent and a blank pipette were used as controls. For screening, loaded pipettes were used at most 4 times for stimulation (Andersson et al. 2012). For dose–response trials, the pipettes were used only once. The loaded pipettes were kept in a freezer at –18 °C when not in use.

Single sensillum recordings

Two antennal segments (P and D, corresponding to the 15th flagellomeres from the proximal and distal ends of the antenna, respectively) were selected as representatives of the base and the tip of the antenna, and SSRs were made from

Table 1 Synthetic compounds used in screening of functional classes of OSNs

Number	Stimulus compound	Solvent	Dose	Purity (%)	CAS number	Source
1[a]	(*Z*)9,(*E*)11-14:OAc	H	100 ng	95	50767-79-8	Pherobank
2[a,b]	(*Z*)9,(*E*)12-14:OAc	H	100 ng	96	30507-70-1	Pherobank
3[a,b]	(*Z*)9-14:OAc	H	100 ng	96	16725-53-4	Pherobank
4[a]	(*Z*)7-12:OAc	H	100 ng	97	14959-86-5	Pherobank
5[a]	(*Z*)9-14:OH	H	100 ng	98	35153-15-2	Pherobank
6	β-caryophyllene	H	100 ng	98.5	87-44-5	Aldrich
7	α-humulene	H	100 ng	98	6753-98-6	Aldrich
8[b]	α-pinene	H	100 ng	98	7785-26-4	Aldrich
9[b]	(*E*,*E*)-α-farnesene	H	100 ng	99	502-61-4	Bedoukian
10[b]	α-copaene	H	100 ng	98	3856-25-5	inc.
11[b]	Thymol	H	100 ng	99.5	89-83-8	Fluka
12[b]	Geraniol	H	100 ng	98	106-24-1	Gift from Prof. Monika Hilker, Berlin, Germany
13[b]	Eugenol	H	100 ng	98	97-53-0	Aldrich
14[b]	Nonanal	H	100 ng	95	124-19-6	Aldrich
15[b]	Decanal	H	100 ng	99	112-31-2	Aldrich
16[b]	(±)-phytol	H	100 ng	99	7541-49-3	Aldrich
17	Indole	H	100 ng	99	120-72-9	Aldrich
18	Carvacrol	H	100 ng	98	499-75-2	Aldrich
19	(±)-nerolidol	H	100 ng	98	7212-44-4	Aldrich
20	(±)-linalool	H	100 ng	97	78-70-6	Aldrich
21	*(E,E)*-farnesol	H	100 ng	95	106-28-5	Aldrich
22	1-methoxy-4-(2-propenyl)-benzene = estragol	H	100 ng	96	77525-18-9	SIGMA
23	4,8,12-trimethyl-1,(*E*)3,(*E*)7,11-tridecatetraene	H	100 ng	98	62235-06-7	Aldrich
24	4,8-dimethyl-1,(*E*)3,7-nonatriene	H	100 ng	99	51911-82-1	Gift from Prof. Wittko Francke, Hamburg, Germany
25	*Z*-jasmone	H	100 ng	98	6261-18-3	Gift from Prof. Wittko Francke, Hamburg, Germany
26	(*E*)2-hexenol	P.o	10 µg	96	928-97-2	Aldrich
27	(*Z*)3-hexenol	P.o	10 µg	98	928-96-1	Aldrich
28	1-hexanol	P.o	10 µg	98	111-27-3	Aldrich
29	1-heptanol	P.o	10 µg	99	111-70-6	Aldrich
30	1-nonanol	P.o	10 µg	99.5	143-08-8	Aldrich
31	1-octanol	P.o	10 µg	99.5	111-87-5	Aldrich
32	(*E*)2-hexenal	P.o	10 µg	98	6728-26-3	Aldrich
33	Benzaldehyde	P.o	10 µg	99.5	100-52-7	Aldrich
34	2-phenylacetaldehyde	P.o	10 µg	98	122-78-1	Aldrich
35	(*Z*)3-hexenyl acetate	P.o	10 µg	98	3681-71-8	Aldrich

CAS stands for Chemical Abstracts Service, H for hexane, and P.o for Paraffin oil.
[a]Ten micrograms dose was used for screening of Ph-OSN housed in LT sensilla.
[b]Did not elicit response in any OSN.

Table 2 Synthetic compounds used in screening of functional classes of OSNs, tested only on ST sensilla in addition to the compounds listed in "Table 1"

Number	Compound name	Number	Compound name
1	2-hydroxypropanoic acid	38	Hexyl hexanoate
2	Propanoic acid	39	(*E*)2-hexenyl acetate
3	Butanoic acid	40	(*Z*)3-hexenyl (*E*)-2-methyl-2-butenoate
4	Pentanoic acid	41	(*Z*)3-hexenyl butyrate
5	Hexanoic acid	42	(*Z*)3-hexenyl isobutanoate
6	Heptanoic acid	43	(*Z*)3-hexenyl benzoate
7	2-ketobutanoic acid	44	(*E*)3-hexenyl butanoate
8	2-ketopentanoic acid	45	(*Z*)3-hexenyl 2-methyl-butanoate
9	Methyl eugenol	46	Isopropyl acetate
10	Benzyl alcohol	47	3-methylbutyl acetate
11	Phenol	48	3-methylbutyl butanoate
12	4-ethylphenol	49	3-methylpropyl acetate
13	(*E*)3-hexenyl acetate	50	3-methylpropyl 2-methylpropanoate
14	(*E*)3-hexenol	51	3-methylbutyl 2-methylpropanoate
15	3-octanol	52	Methyl anthranilate
16	1-octen-3-ol	53	Methyl cinnamate
17	2-phenyl ethanol	54	Geranyl acetate
18	(*E*)-anethol	55	Methyl salicylate
19	2,3-butandiol	56	2-phenylethyl propanoate
20	β-citronellol	57	Methyl jasmonate
21	Hexanal	58	Acetophenone
22	Cinnamic aldehyde	59	γ-hexalactone
23	Methyl benzoate	60	± δ-decalactone
24	Methyl propanoate	61	γ-octalactone
25	Methyl butanoate	62	γ-undecalactone
26	Methyl hexanoate	63	1,4-benzoquinone
27	Methyl octanoate	64	2-methyl-1,4-benzoquinone
28	Ethyl propionate	65	1-indanone
29	Ethyl butanoate	66	3-hydroxybutanone
30	Ethyl-3-hydroxy-butyrate	67	2-phenylacetonitril
31	Ethyl hexanoate	68	1,4-diaminobutane
32	Ethyl (*E,Z*)-2,4-decadienoate	69	Benzyl methyl ether
33	Propyl butyrate	70	(*E*)-ocimene
34	Butyl butanoate	71	Ammonia
35	Butyl isobutanoate	72	3-carene
36	Hexyl acetate	73	Carbon dioxide (CO_2)
37	Hexyl butanoate		

the 6 morphological types of sensilla identified in the SEM study using standard equipment and well-established experimental procedures (Andersson et al. 2009). A female moth was restrained in a plastic pipette tip with only the head protruding, and a tungsten wire, serving as a reference electrode, was inserted into the abdomen. The head was immobilized with dental wax (Surgident periphery wax; Heraeus Kulzer GmbH), and one of the exposed antennae was fixed on a microscope glass slide (76 × 26 mm, Menzel-Glaser) using double-sided sticky tape. SSRs were performed under a light microscope (Nikon FN-S2N) with ×750 magnification, using electrolytically sharpened tungsten electrodes (Clark Instruments Ltd). The recording electrode was attached to an olfactory probe (INR-02; Syntech), and by using a micromanipulator (Syntech), its tip was inserted into the base of a sensillum in the case of trichoid and basiconic sensilla or into the sensillum cavity of the other sensilla types until electrical contact with the OSNs was established. The spike activity was monitored on a computer screen, and the data were recorded using Autospike software (Syntech).

A stream of charcoal filtered humidified air was continuously flushed over the antenna (1 L min^{-1}), through a 14-cm long glass tube (7 mm inner diameter), which terminated 1.5 cm from the antenna. In some cases, where the same preparation (insect antenna) was used to record responses from both distal and proximal segments, the preparation was moved to bring the corresponding antennal segment under the microscope (maximum 2 antennal segments at the time could be observed at the magnification used) to record from it while the ventrolateral surface of the antenna was facing the airflow. However, the position of the glass tube carrying the airstream was not changed, giving the identical antennal orientation in relation to the airstream and avoiding any differences in orientation of the airstream in relation to the different antennal parts. During odor stimulation, a 0.5-s air pulse (0.5 L/min), controlled by a stimulus controller (CS-55; Syntech), was passed through the stimulus pipette, which was inserted into a hole in the glass tube. Compounds were tested randomly, either with an interval of at least 15 s or in cases where the response was strong, when the spiking activity of the neurons had returned to normal spontaneous activity. The topographical positions of responding sensilla were mapped to estimate the spatial organization of functional classes of OSNs.

Data analysis

The change in neural activity after stimulation was determined by counting the number of spikes during the first 0.5 s of the stimulus response and subtracting the spontaneous spike activity during the 0.5 s prestimulation period just before the onset of response. If the solvent or control elicited any response, the odor responses for the compounds eliciting responses in that functional type were calculated after subtracting the response to the control. An excitation of less than 15 Hz above spontaneous activity was considered as ''no response''. The OSNs were classified into different functional classes based on the morphology of the sensillum they were housed in, the amplitude of the responding neuron, and their activating odorants.

The variation in spiking frequency between 5 classes of OSNs, common to both segments, due to factors of OSN class, OSN location, and dose was analyses by analysis of variance (ANOVA) (using Generalized Linear Models, IBM SPSS 19). The overall (pooled doses) means of OSN responses were compared by paired sample *t*-test (IBM SPSS 19) between distally and proximally located OSNs belonging to the same functional class. The sensitivity of OSNs to different doses of individual compounds was tested by a nonparametric paired samples Wilcoxon signed-ranks test (IBM SPSS 19). Parameter estimates are given as mean ± standard error of the mean. The α-level was set at 0.05.

Temporal analysis

The dose responses to β-caryophyllene and α-humulene were analyzed to quantify the differences in temporal characteristics of responses recorded from an OSN (BC3A) found on both antennal segments. For the analysis, the raw recorded spike signals (potentials over time) were filtered using a continuous wavelet transform, akin to a Fourier transformation with both high- and low-pass filters, and a second degree Paul wavelet (Nenadic and Burdick 2004). The scale of temporal integration width was automatically selected for best fit. Filtered signals were interpolated using cubic B-spline. Using the first derivate of these interpolations, spike timing and amplitude were extracted. Spikes were classified as either noise or ''real'' spikes using kernel density estimations of subpopulation Gaussian distributions (Parzen 1962) of the spike size distribution modes. For each spike, the time averaged frequency was calculated based on 10 spikes divided by the time between the fifth last and fifth future spike.

The latency was calculated as the time between the starting point of stimulation and the onset of response. The duration of response was the time between the onset and the termination of the excitation, that is, the time point where the number of spikes/100 ms were equal to the number of spikes/100 ms registered in the spontaneous activity before stimulation. The difference in latency and duration of excitations between OSNs located proximally and distally was tested by paired sample *t*-test.

Results

Antennal morphology

The female *S. littoralis* antenna comprises an average of 66 ± 4 segments ($n = 10$). According to morphology, we identified 6 distinct types of olfactory sensilla: basiconic (BC), short trichoid (ST), long trichoid (LT), coeloconic (CC), auricillic (AC), and grooved peg (GP) on the antennal surface

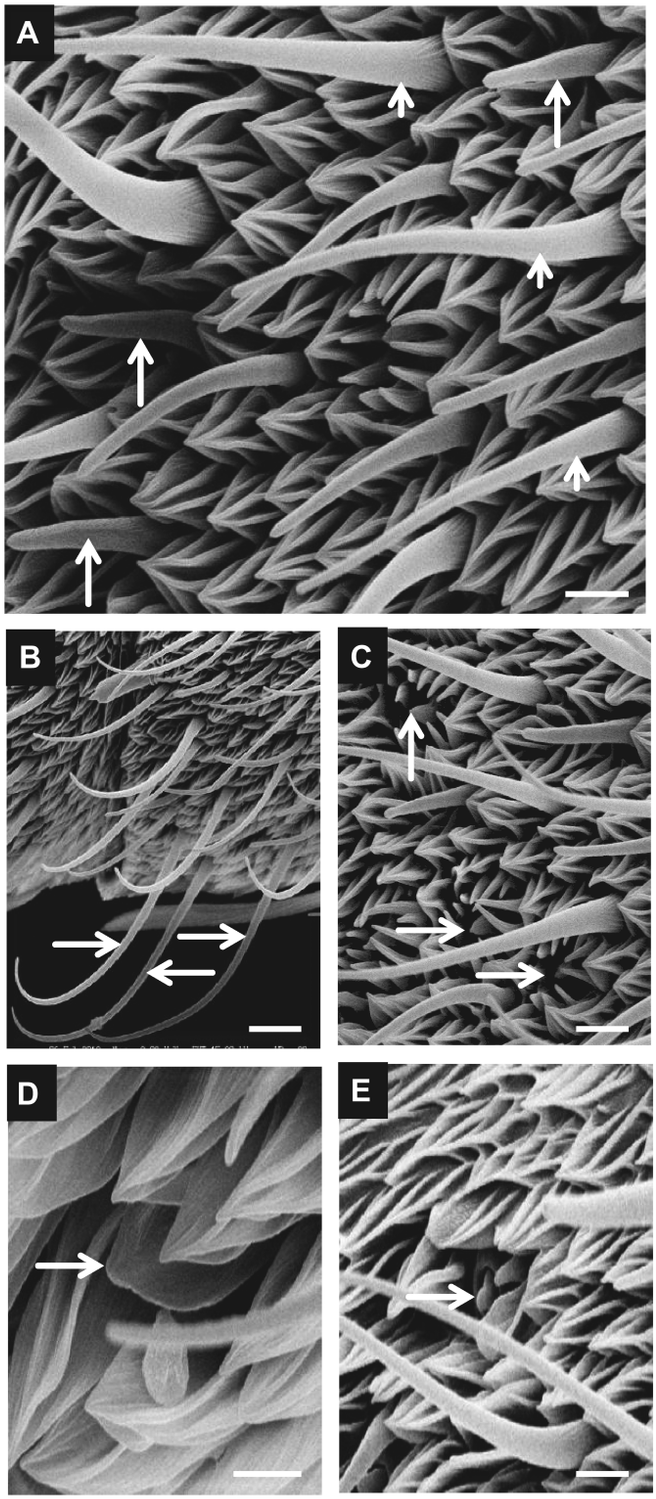

Figure 1 We found 6 morphological types of antennal olfactory sensilla in female *Spodoptera littoralis*. (**A**) Short trichoid (ST) (short arrows), a new type not earlier distinguished from the basiconic (BC) sensilla (long arrows). (**B**) Long-trichoid (LT) sensilla (arrows) are present at the lateral surfaces. (**C**) Coeloconic (CC) sensilla (arrows). (**D**) Auricilic (AC) sensilla (arrow). (**E**) Grooved peg (GP) sensilla (arrow). Bars represent a scale of 5 μm except in (D) where the bar represents a scale of 2 μm in the SEM micrographs.

(Figure 1). The ST was the dominant sensillum type (50 ± 6 per segment), averaging 35 μm in length and 2.25 μm in diameter at the base and tapering to 0.5 μm diameter at the tip (Figure 1A). The BC was the second most common sensillum type (38 ± 4 per segment), on average 12 μm long, 1.5 μm in diameter at the base, and tapering to 0.75 μm at the tip (Figure 1A). Both types were found on the ventral and lateral surfaces of the antenna. Twelve ± 2 LT sensilla were present on the lateral edges of each segment (6 ± 1 on each lateral edge) and averaged 48 μm in length, 1.5 μm in diameter at the base, and tapered to 0.56 μm (Figure 1B). Seven ± 2 CC sensilla were found on each segment, mostly located on the ventral surface of the antenna, and averaged 4 μm in length and were 1.5 μm thick at the base (Figure 1C). This sensillum type appears as a peg, recessed in a pit surrounded by 12 cuticular spines, which are pointing inward and form a circle around the peg. Four AC sensilla were found, 2 on each lateral edge close to the distal margin of each segment, situated in cavities that were partially covered by scales (5 μm long and 4 μm wide at the base) (Figure 1D). The GP sensilla (4 ± 1) were of very small size (only 2 μm long and 1.5 μm in diameter) and were mainly found on the ventrolateral surface and close to the distal margin of each segment (Figure 1E).

Functional classification of OSNs

Altogether, OSNs present in 452 sensilla on the P and D segments were screened for physiological responses to all test odorants. Out of these, 182 were BC (86 D and 96 P), 180 ST (125 D and 55 P), 40 LT (17 D and 23 P), 38 CC (24 D and 14 P), 10 AC (3 D and 7 P), and 2 GP (1 D and 1 P) sensilla. For the numbers of encountered sensilla containing activated OSNs on D and P, see Tables 3 and 4. None of the ST or GP sensilla responded to any odorants tested. Furthermore, the ST sensilla did not respond to an extended panel of 73 compounds (Table 2).

Electrophysiological recordings from the different sensillum types consistently showed spontaneous action potentials (spikes), which in most recordings could be resolved into 2 populations based on their amplitudes (Figure 2A). Here, we refer to the neuron with the larger spike amplitude as the A-neuron and the one with the smaller spike amplitude as the B-neuron (Figure 2A). Most contacts revealed spontaneous activity from 2 OSNs, but in most cases, only one of them responded to a tested odorant. Based on the morphology of the sensillum they were housed in, the amplitude of the responding OSNs, and their activating odorants, 35 functional classes of OSNs housed in 32 functional sensillum types could be distinguished. Of these, 27 classes were found among the BC sensilla and the remaining 8 classes among the LT, CC, and AC sensilla (Tables 3 and 4). The OSN classes could be grouped into 3 categories based on their response specificity: 1) highly selective, where a single odorant elicited a response (46%), 2) less selective, where a narrow range of 2–4, often structurally similar odorants elicited a response

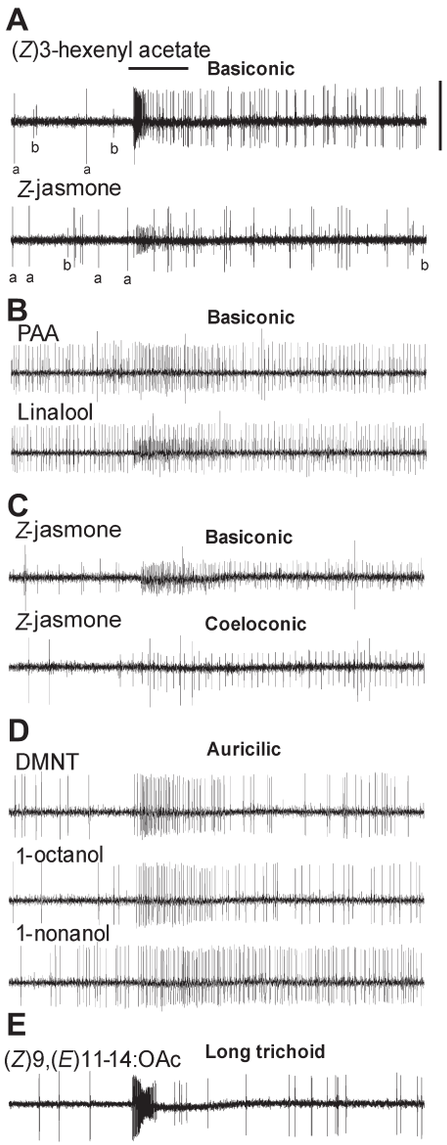

Figure 2 Temporal pattern variability in SSRs from OSNs housed in different morphological sensillum types. (**A**) Two OSN classes (BC22A and BC22B) where A and B neurons show phasic responses to (*Z*)3-hexenyl acetate and *Z*-jasmone, respectively, whereas in (**B**) OSN classes (BC19A and BC19B), A and B neurons show phasic–tonic responses to phenylacetaldehyde (PAA) and linalool, respectively. (**C**) An OSN housed in BC sensilla (BC7B) where the B-neuron shows phasic–tonic response to *Z*-jasmone, whereas in another OSN housed in CC sensilla (CC1B), the B-neuron shows weak tonic response to the same compound. (**D**) An OSN (AC1A) housed in AC sensilla that responded differently to 3 compounds, where the A-neuron shows phasic–tonic responses to 4,8-dimethyl-1,(*E*)3,7-nonatriene (DMNT) and 1-octanol, but a tonic response to 1-nonanol. (**E**) The LT1A OSN, where the A-neuron shows a strongly phasic response accompanied by strong pinching in spike amplitude to the major pheromone component (*Z*)9,(*E*)11-14:OAc. Horizontal bar represents 0.5 s and vertical represents 2 mV.

(38%), and 3) generalist, where 5–13 compounds stimulated the OSN (16%). OSN classes detecting GLV and FLV were the most common (Supplementary Figure S1). Of the 35 tested odorants, 11 did not elicit a clear response in any OSN (Table 1).

The 27 OSN classes identified in BC sensilla were housed in 24 functional sensillum types, BC1–BC24 (Tables 3 and 4). The OSNs housed in BC sensilla responded exclusively to plant-related compounds. One OSN, BC1A, responded specifically to PAA (phenylacetaldehyde), whereas the BC2A neuron responded to both PAA and (*Z*)3-hexenyl acetate. BC3A responded to 2 structurally similar sesquiterpenes, β-caryophyllene, and α-humulene, whereas BC4A responded to only α-humulene. The BC8A neuron, which was found only on the proximal segment, responded with high sensitivity to 3 GLV alcohols: 1-hexanol, (*E*)2-hexenol, and (*Z*)3-hexenol. When subjected to dose–response trials with these 3 GLVs, this OSN did not show any difference in response between the compounds (Supplementary Figure S2). BC11A responded specifically to our racemic linalool, but responses to racemic linalool were also found in 4 other OSN classes. In 3 functional sensillum types (12, 19, and 22), both the A and B neurons responded to tested odorants (Tables 3 and 4): BC12A responded to benzaldehyde, whereas BC12B responded to *Z*-jasmone (Tables 3 and 4); BC22A responded to (*Z*)3-hexenyl acetate, whereas BC22B responded to *Z*-jasmone (Figure 2A); BC19A responded to phenylacetaldehyde (PAA), whereas BC19B responded to linalool (Figure 2B). For response spectra of all encountered OSNs, see Tables 3 and 4.

In CC sensilla, we found only 2 functional classes of OSNs, housed in 2 functional sensillum types. Of these classes, the CC1B responded to *Z*-jasmone (Figure 2C), whereas CC2A responded to 3 GLV alcohols (Table 4).

The 3 OSN classes housed in 3 types of AC sensilla responded to a narrow range of 2–5 plant odors (Table 4). For example, AC1A responded to DMNT (*E*-4,8-dimethyl-1,3,7-nonatriene), 1-octanol, and 1-nonanol (Figure 2D)

OSNs housed in LT sensilla responded with high specificity and sensitivity to single pheromone components. LT1A responded to the main pheromone component (*Z*)9,(*E*)11-14:OAc (Figure 2E), whereas LT2A responded to (*Z*)9-14:OH, and LT3B responded to (*Z*)7-12:OAc (Table 4). These neurons were all housed in separate LT sensilla together with one other non-responding neuron.

Spatial organization and sensitivity of OSNs

Eighteen of the 35 classes of OSNs were found in common on both the distal (D) and proximal (P) segments. All of these classes were found at least one time on similar topographical positions on both antennal segments (Figure 3). Most of the OSN classes that were not found in common on both segments were found only once.

Interestingly, we observed that OSN sensitivity was dependent on location on the antenna. In general, OSNs located on the P segment were more sensitive to the test stimuli compared with the same functional class on the D segment. For example, the BC3A OSN, which responded to the 2 sesquiterpenes β-caryophyllene and α-humulene,

Table 3 Functional classes of OSNs

Morphological sensillum types	Basiconic (BC)																		
Functional sensillum types	1	2	3	4	5	6	7	8	9	10	11	12		13	14	15	16	17	18
OSN functional classes	1A	2A	3A	4A	5A	6A	7B	8A	9B	10A	11A	12A	12B	13A	14A	15A	16B	17B	18A
(*Z*)9,(E)11-14:OAc																			
(*Z*)7-12:OAc																			
(*Z*)9-14:OH																			
β-caryophyllene			•																
α-humulene			•	•															
Indole						•										•			
Carvacrol																	•		
± nerolidol														•			•		
± linalool											•			●			●		
TMTT					•														
DMNT																			
Z-jasmone							•						•						
(*E*)2-hexenal														•	•	•		●	
(*E*)2-hexenol								●	●					•	•				●
(*Z*)3-hexenol								●	•					•	●				●
1-hexanol								•	•					•	•				•
(*Z*)3-hexenyl acetate		●												•	•				•
1-heptanol														•	•	•			•
1-nonanol														•	•				
1-octanol														•	•				
Benzaldehyde									•	•		•		•	•				•
PAA	●	●												•	●	•			•
Estragol														•					
(*E,E*)-farnesol																			
Number of encountered sensilla at D	15	5	10	0	9	6	5	0	2	4	2	1		3	0	0	0	1	4
Number of encountered sensilla at P	11	11	7	2	6	5	4	3	0	5	3	4		8	4	3	2	2	1

The spike frequency (Hz) is coded as • 15–50 Hz, • 51–80 Hz, ● 81–110 Hz, and ● >110 Hz. Blank cells indicate that compound did not elicit response in that OSN. No inhibitory responses were observed in any OSN. Neurons (A and B) were differentiated on the basis of spike amplitude: A = large amplitude and B = small amplitude. Three functional sensillum types: 12, 19, and 22 contain 2 neurons (A and B) responding to different stimuli. BC stands for basiconic sensilla, TMTT (*E,E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene), DMNT (*E*-4,8-dimethyl-1,3,7-nonatriene), PAA (Phenylacetaldehyde), and estragol = 1-methoxy-4-(2-propenyl)-benzene. P = proximal and D = distal.

displayed a higher spike frequency when recorded from the P segment as compared with the D segment (Figure 4). Five classes of OSNs, common to both segments, were further characterized with dose–response tests over 6–8 decadic steps for the 5 OSN classes and the 2 positions, yielding in total 945 data points (Figure 5). There was an overall highly significant variation ($P < 0.001$) due to all the 3 factors: location, OSN class, and dose, as judged by the factorial ANOVA (Table 5).

An overall significantly higher sensitivity in P OSNs was found in 4 cases: β-caryophyllene and α-humulene in BC3A, TMTT in BC5A, and PAA in BC1A (Figure 5A–E). In contrast, the BC2A OSN showed a higher response in the distal location to PAA (Figure 5G). Corresponding to the overall responses, we also found significant differences at individual doses in 6 cases between OSNs located on P and D segments (Figure 5). The differences in response among OSN classes in Figure 5 correspond strictly to the 2 highly

Table 4 Functional classes of OSNs, continued

Morphological sensillum types	BC								CC		AC			LT		
Functional sensillum types	19		20	21	22		23	24	1	2	1	2	3	1	2	3
OSN classes	19A	19B	20A	21A	22A	22B	23A	24A	1B	2A	1A	2A	3A	1A	2A	3B
(*Z*)9,(*E*)11-14:OAc														●		
(*Z*)7-12:OAc																●
(*Z*)9-14:OH															●	
β-caryophyllene																
α-humulene																
Indole																
Carvacrol																
± nerolidol																
± linalool		●					●									
TMTT																
DMNT											•	●				
Z-Jasmone						•			•							
(*E*)2-hexenal			●										•			
(*E*)2-hexenol			•	●						•		●	•			
(*Z*)3-hexenol			•							•			•			
1-hexanol			•							•			•			
(*Z*)3-hexenyl acetate					●											
1-heptanol			•													
1-nonanol											•					
1-octanol											•					
Benzaldehyde			●										•			
PAA	•		●	●				●								
Estragol							•							nt	nt	nt
(*E*,*E*)-farnesol								•						nt	nt	nt
Number of encountered sensilla at D	2		1	0	0		0	0	1	0	2	0	0	5	5	1
Number of encountered sensilla at P	0		1	1	1		1	1	0	1	3	1	2	16	2	1

The spike frequency (Hz) is coded as • 15–50 Hz, • 51–80 Hz, ● 81–110 Hz, and ● >110 Hz. nt means that compound was not tested on that OSN. Blank cells indicate that compound did not elicit response in that OSN. No inhibitory responses were observed in any OSN. Neurons (A and B) were differentiated on the basis of spike amplitude: A = large amplitude and B = small amplitude. Three functional sensillum types: 12, 19, and 22 contain 2 neurons (A and B) responding to different stimuli. BC stands for basiconic; CC, coeloconic, AC, auricilic; LT, long-trichoid sensilla, TMTT (*E*,*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene), DMNT (*E*-4,8-dimethyl-1,3,7-nonatriene), PAA (Phenylacetaldehyde), and estragol = 1-methoxy-4-(2-propenyl)-benzene. P = proximal and D = distal.

significant interactions of OSN class × location and of OSN class × dose (Table 5).

Temporal characteristics of OSN responses

The temporal patterns of OSN responses varied depending on functional type, stimuli tested, and position on the antenna. For example, OSNs housed in BC sensilla showed a phasic response to *Z*-jasmone, whereas neurons responding to the same compound but housed in CC sensilla displayed a tonic response (Figure 2C).

The same OSN stimulated with different stimuli could respond with different temporal dynamic patterns. For instance, AC1A responded to 3 compounds (Figure 2D) and displayed phasic responses to both DMNT and 1-octanol but a tonic response to 1-nonanol. In contrast to all other

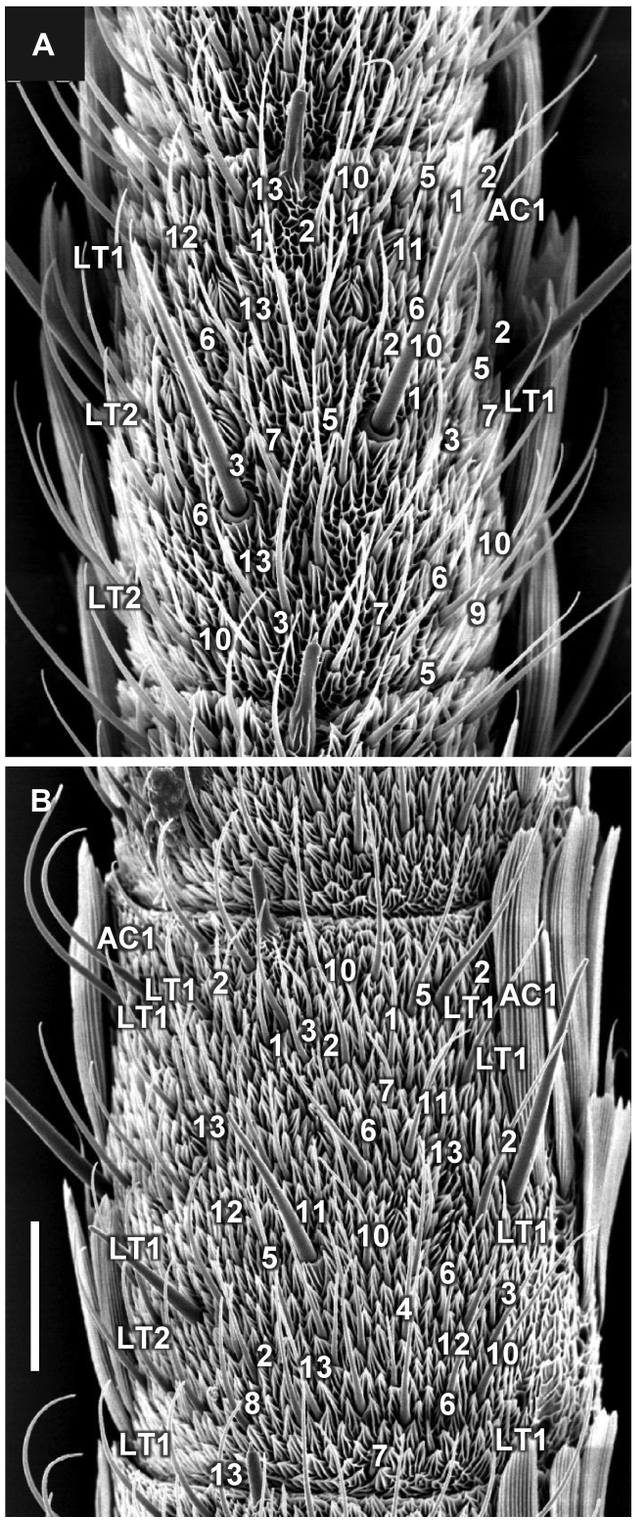

Figure 3 Positions of functional classes of OSNs were mapped on the tip and base of the antennae. (**A**) Fifteenth flagellomere from tip (distal, D) of antenna. (**B**) Fifteenth segment from the base (proximal, P) of the antenna. Numbers indicate the OSN classes belonging to BC sensilla, and numbers with LT or AC indicate OSN classes from LT sensilla and auricilic sensilla, respectively. See Tables 3 and 4 for further details of OSNs classes. Scale bar represents 50 μm for both A and B. Only those classes found in common on both segments are indicated, except OSN class 8 and 9, whose response profiles differ by only 1 compound but were present on different positions.

functional classes of OSNs, pheromone-specific OSNs housed in the LT sensilla always displayed phasic responses (Figure 2E).

An OSN housed in the same morphological or functional sensillum type but located on different antennal parts also responded differently. The distally located OSN, BC3A, displayed tonic responses at 100 pg–100 ng doses of β-caryophyllene, whereas the same type showed a more phasic response when located on the P segment (Figure 4). At the higher doses (1–100 μg), the response by the OSN changed from tonic or phasic to phasic–tonic. These temporal aspects were more or less the same for α-humulene (Figure 4).

The P OSNs that displayed phasic response modes were also characterized by higher spike frequency as compared with D OSNs, which responded in a tonic mode when stimulated with different doses of β-caryophyllene (Figure 6A,B) and α-humulene (Figure 6E,F). The OSNs from the P segment also exhibited a significantly shorter latency overall than OSNs from the D segment in response to both β-caryophyllene and α-humulene and their dose response did not overlap (Figure 6C,G). The duration of excitation increased with odor dose both at P and D locations, whereas their dose–response curves mostly overlapped (Figure 6D,H).

Discussion

With use of SSR technique, we present a detailed map of the peripheral olfactory system in the polyphagous moth *S. littoralis*. Beyond establishing detailed topographic and functional correlations, we have identified a novel type of variation in both sensitivity and temporal response between spatially separated OSNs of similar physiological tuning. These findings will be used as a basis for understanding host-selection mechanisms.

This is the first study where responses of the same functional cell type on different antennal parts of an insect are systematically compared. An overall higher sensitivity of OSNs recorded from the proximal segment to most stimuli tested is a new and interesting phenomenon. However, the trend of a higher sensitivity at the proximal segment was found for only 4 of the 6 compounds tested, one compound, (*Z*)3-hexenyl acetate, did not differ, whereas another, PAA in OSN BC2A, in fact had the opposite pattern. This indicates that the higher sensitivity is not a general feature due to the position of OSNs on the antenna or a recording artifact between the segments, but an actual difference in the sensitivity of the sensory neurons. This compound-specific variation with position also implies that the proximal/distal differences have

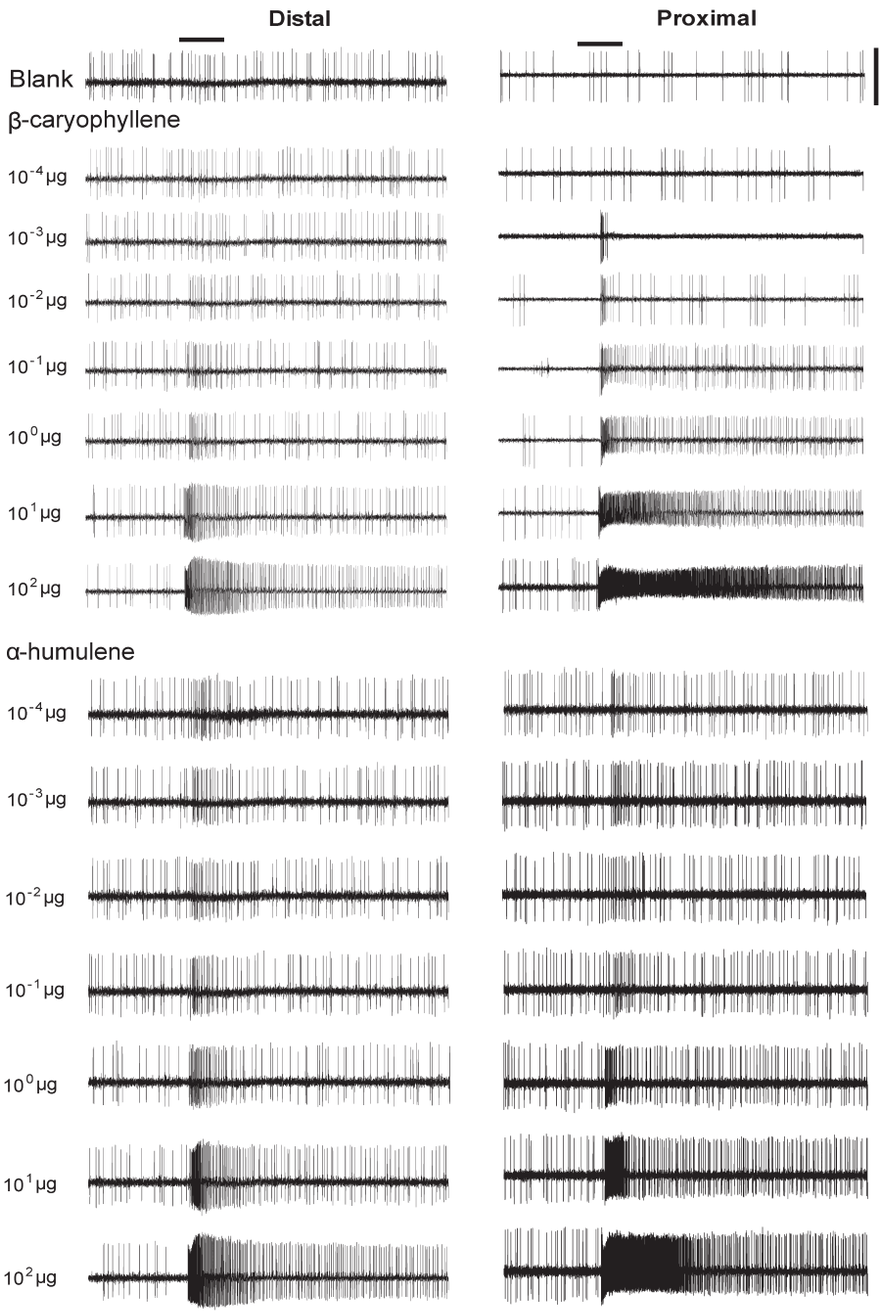

Figure 4 The sensitivity and temporal responses vary in the same OSN type between the tip and base of the antenna. Dose response of an OSN (BC3A) housed in BC sensilla on 15th antennal segments from tip and base to different doses (100 pg–100 µg on filter paper) of 2 sesquiterpenes, β-caryophyllene, and α-humulene. Horizontal scale bars indicate stimulation of 0.5 s, whereas vertical represents 5 mV.

a compound-specific relevance and thus influence the insect's perception of these host compounds. One may speculate that if the spatial variation in sensitivity is represented in the CNS, it may help in coping with signals of vastly different magnitude. Alternatively, could it be that paired lower sensitivity sensilla assemblage at antenna tips helps in close proximity, allowing orientation to point sources in clines of high concentrations?

Apart from the difference in sensitivity (firing frequency), we observed differences in temporal response characteristics (latency and duration) between distal and proximal OSNs. Temporal variations within OSNs responding to the same odorant, but housed in different sensilla, have also been found in *Heliothis virescens* (Berg et al. 1995). Spatial data in our study showed, especially at lower doses, that OSNs from the proximal segment had more phasic responses than

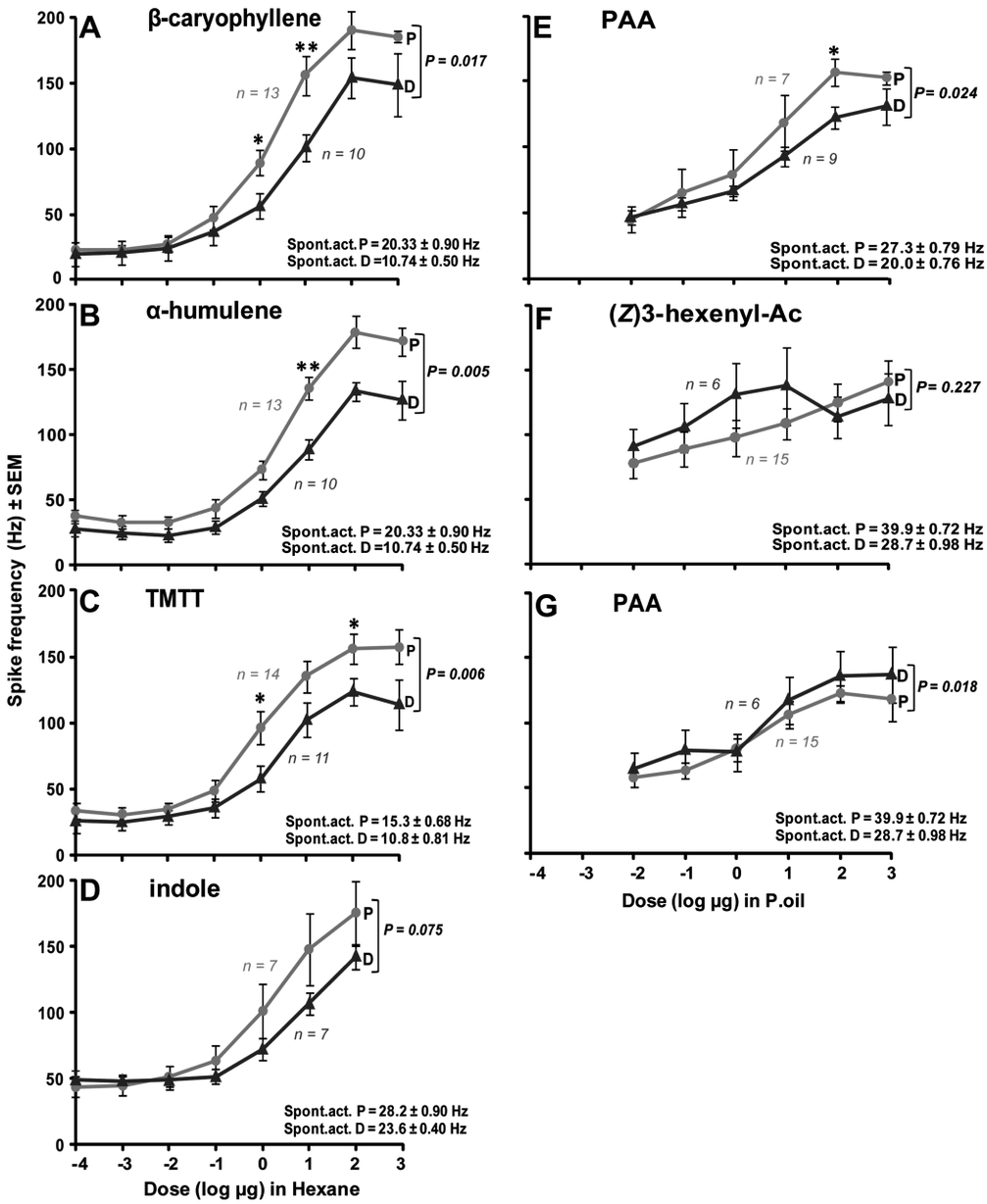

Figure 5 Sensitivity is compared among 5 functional classes of OSNs (housed in BC sensilla) from a proximal (P) and a distal segment (D) of the antenna. An OSN (BC3A) responds to both (**A**) β-caryophyllene and (**B**) α-humulene. (**C**) Responses of BC5A cell to TMTT. (**D**) The BC6A cell responses to indole. (**E**) An OSN (BC1A) responding to PAA. The BC2A cell that responds to both (**F**) (*Z*)3-hexenyl acetate and (**G**) PAA. *P* values from paired sample *t*-tests between proximal and distal OSNs (all doses pooled). The * and ** represent significant and highly significant differences at $P < 0.05$ or <0.01, respectively, at a specific dose by paired sample Wilcoxon signed-ranks test. Phenylacetaldehyde (PAA) and (TMTT) 4,8,12-trimethyl-1,(*E*)3,(*E*)7,11-tridecatetraene. The error bars represent standard error of the mean (SEM). *n* = number of replicates, and "Spont. Act." denotes spontaneous spike activity ± SEM. This figure appears in color in the online version of *Chemical Senses*.

those from distal segments. This may convey different information to the CNS regarding temporal aspects of stimulus occurrence (Almaas and Mustaparta 1991; Laurent et al. 2001; Raman et al. 2010). As flying insects encounter odor filament intermittent with pockets of clean air while flying toward the source of an odor plume (Murlis et al. 1992), the frequency of such encounters is an essential parameter in navigational strategies to keep track of an odor plume (Mafra-Neto and Carde 1994). For example, a phasic response conveys information about rapid changes in odor intensity and concentration, whereas tonic responses have been suggested to play a role in orientation behavior of an insect to keep track of an odor plume to locate the odor source by providing a short memory of a recently encountered odor stimulus (Den Otter and Van Naters 1992; de Bruyne et al. 2001).

Table 5 Analysis of OSNs spike frequency variation due to factors of OSN class, OSN location, and dose by factorial ANOVA[a]

Tests of model effects[b]				
Source	Type III		*P* value (%)	Significance level[c]
	Wald χ^2	df		
Factors				
OSN class[d]	50.1	6	<0.001	***
Location[e]	14.3	1	<0.016	***
Dose[f]	603	7	<0.001	***
Interaction of factors				
OSN class × location	41.8	6	<0.001	***
OSN class × dose	138	35	<0.001	***
Location × dose	12.0	7	10.3	NS
OSN class × location × dose	13.0	35	99.98	NS

Dependent Variable: Hz, Model: (Threshold), OSN class, location, dose, OSN class × location, OSN class × dose, location × dose, and OSN class × location × dose. df = degrees of freedom.

[a]A full range of Box–Cox transformations failed to provide the normal distribution and homogeneity of variances which are the assumptions for standard ANOVA, why we used generalized linear models (GzLM) which can handle different kinds of nonnormal distributions, with a multinomial probability distribution and a cumulative logit link function.

[b]Goodness of fit for model: Pearson χ^2/df = 1.03, omnibus test: likelihood ratio χ^2 = 997.8, df = 97, $P < 0.001$***.

[c]*** and NS represent very highly significant and nonsignificant, respectively, at $\alpha = 5\%$.

[d]OSN class and stimuli combinations, 7 levels, see Figure 5.

[e]Location at proximal or distal antennal segment (flagellomere), 2 levels.

[f]Decadic steps of amount applied to filter paper, 6–8 levels depending on solvent, see Figure 5.

It is far too early to firmly establish the behavioral and ecological relevance of the sensitivity and temporal differences found between P and D located OSNs. At the very least, however, these novel findings of spatial variation warrant a careful record of spatial sampling in future SSR mapping in insects.

The morphology of antennal olfactory sensilla described in this study resembles those previously described in moths (e.g., Hallberg 1981; Shields and Hildebrand 1999a, 1999b; Malo et al. 2004; Ansebo et al. 2005; Hill et al. 2010). The OSNs housed in the BC, CC, and AC sensilla were tuned to plant compounds and those housed in the LT sensilla to pheromone components. In spite of our extensive mapping, we found no response in the ST (the most common sensillum type) or in GP sensilla. *Spodoptera littoralis* is a polyphagous species, which uses a wide variety of plants as host that release different odors. Thus, it is possible that the ligands specific to the OSNs housed in ST and GP sensillum types were not present in our odor panels. It is also possible that these sensilla may respond to non-host volatiles (NHV) (not identified yet) emitted from plants that are unsuitable as larval food (Zhang and Schlyter 2004), as observed in the bark beetle *Ips typographus* which has close to 25% of the characterized OSNs specifically tuned to NHV (Andersson et al. 2009). To test this ad hoc hypothesis, we are presently pursuing gas chromatography (GC)-SSR studies of headspace samples from host and non-host plants. The ST sensillum type is morphologically very similar to the BC sensilla, except in size (ST are longer and wider at the base than BC). Confusingly, in the past, it was reported for female *S. littoralis* that "ST sensilla located ventrally on the antenna are tuned to plant compounds" (Anderson et al. 1995; Jönsson and Anderson 1999). It is likely that in the past that BC sensilla were classified as ST sensilla, due to a lack of today's microscope powers and of detailed information on morphology of antennal olfactory sensilla in *S. littoralis*.

The OSNs displayed different degrees of specificity when stimulated with different odors, which coincides with earlier findings in several moths and other insect species, where both specialist and generalist OSNs responding to plant volatiles have been characterized (Bruce et al. 2005; Ignell and Hansson 2005; de Bruyne and Baker 2008). The underlying reason for this has been proposed to be that insects experience a complex odor diversity, and hence, the discrimination of host plants may requires the combination of both generalist and specialist OSNs (Hansson 1995; Ignell and Hansson 2005). For OSNs housed in BC sensilla, our results are in agreement with earlier findings by Anderson et al. (1995), where specialist as well as generalist OSNs responding to GLVs, FLVs, and sesquiterpenes (β-caryophyllene and α-humulene) were very common on the antennae of female *S. littoralis*. The responses to GLVs and other plant volatiles have also been observed in many other phytophagous insect species (Bruce et al. 2005; de Bruyne and Baker 2008). More than 30% of the OSNs found in this study were tuned to phenylacetaldehyde (PAA), a compound emitted by flowers (Heath et al. 1992; Miyake et al. 1998), alone or in combination with other plant compounds, which correlates with the importance of flower nectars for a virgin female moth. For example, the BC1A neuron that specifically responds to PAA, whereas BC2A responds to (*Z*)3-hexenyl acetate in addition to PAA. PAA is a common flower produced volatile, thus representing a nectar (adult food) source, whereas (*Z*)3-hexenyl acetate is a common GLV and thus representing plant material like cotton (Röse et al. 1996), which is larval food. We also found some OSNs housed in BC and AC sensilla that were broadly tuned to GLVs, FLVs, and general plant odorants (the BC13A, BC14A, BC20A, and AC3A), which demonstrate that female *S. littoralis* moths also have generalist receptors to find plants providing both nectar source (food) and oviposition site.

In contrast to plant volatiles, the coding of the pheromone signals in the male moth seems to rely only on highly specialist OSNs (de Bruyne and Baker 2008). Interestingly, female *S. littoralis* has a specialized type of sensilla (LT) for

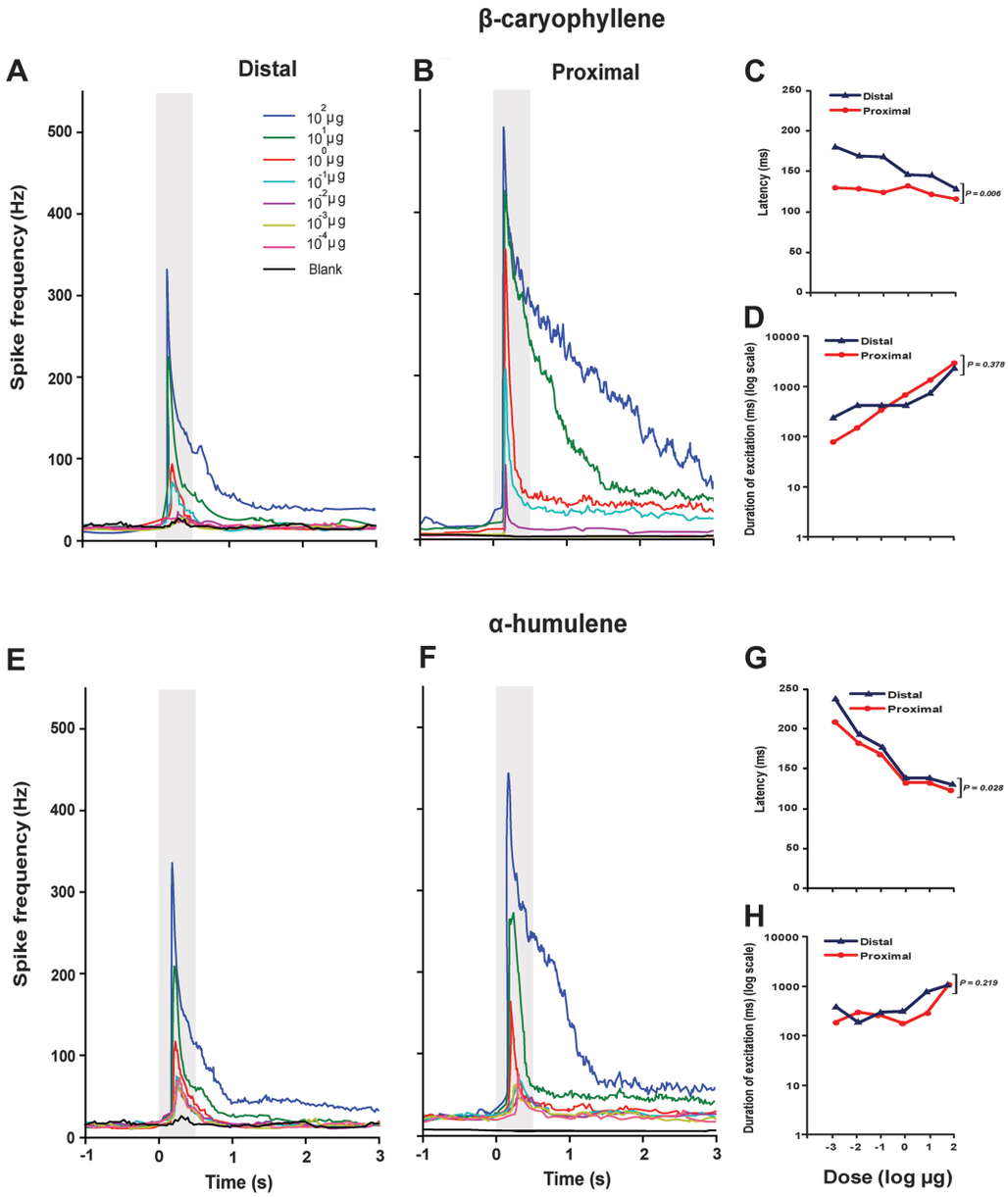

Figure 6 Spatial variation affects frequency, duration, and latency of spiking in the BC3A OSN. The temporal analysis is based on the data shown in Figure 4. Spike frequency before, during, and after stimulation to a wide dose range (100 pg–100 µg) of β-caryophyllene, from distal (**A**) and proximal (**B**) segments of the antenna, and to α-humulene from distal (**E**) and proximal (**F**) segments. For each spike, the spike frequency is determined as a moving average based on 10 spikes divided by the time between the 5th last and 5th future spike (see "Temporal analysis" in Materials and methods). The zero on horizontal scale indicates the starting time of odor stimulation, and areas highlighted in gray represent the stimulation period of 0.5 s. In each graph, the *x* axis represents a recording time of 4 s (1-s prestimulus and 3-s poststimulus periods). Latency in responses to β-caryophyllene (**C**) and α-humulene (**G**). Proximal OSNs responded with significantly shorter latencies than distal OSNs. Duration of excitation to different doses of β-caryophyllene (**D**) and α-humulene (**H**). The distal OSNs spike longer than proximal ones at lower doses of (1–100 ng) but with shorter duration at higher doses of β-caryophyllene. The distal OSNs spike longer than proximal ones at all doses of α-humulene, except for the 10 ng dose. *P* values are from paired sample *t*-tests between distal and proximal OSNs in latency and duration of excitation.

pheromone detection in which all 3 highly specific functional pheromone OSN classes were found both in our study and by Ljungberg et al. (1993). Pheromone autodetection in female moths has been observed both behaviorally (Mitchell et al. 1972) and physiologically (Nesbitt et al. 1973; Anderson et al. 1995), but the ecological significance of pheromone perception in female *S. littoralis* is not yet clear.

Does the response diversity of the OSNs of an (female) insect reflects the degree of specialization? One could hypothesize that a specialist insect would have fewer selective

types of receptors of special relevance to host odors, but of higher number, somewhat like the highly specialized male system detecting the narrow signal of its species-specific pheromone blend. The review by de Bruyne and Baker (2008) concludes that there is a small, if any, such effect of specialization in phytophagous insects. For *Spodoptera,* the closest examples are 3 other Noctuids, in which the female moths of the oligophagous *Helicoverpa assulta* and 2 polyphagous species, *Heliothis virescens* and *Helicoverpa armigera*, have at least some functionally similar types of OSNs for plant-odor detection (Stranden et al. 2003). *Spodoptera littoralis* also has some OSNs of similar response patterns as those reported by Stranden et al. (2003). Clearly, we need data from functional maps of additional generalist as well as specialist species to allow for a more in-depth comparison of the number of OSNs targeting plant volatiles. Several other studies on moths and other phytophagous insects, reviewed by Bruce et al. (2005) and de Bruyne and Baker (2008), have also reported that OSN responses to many odorants are shared across species, irrespective of oligophagy and/or polyphagy, suggesting that the fine tuning or discrimination between odorants may take place at higher levels in the olfactory system. One may also speculate that the presence of functionally similar OSNs or ORs in different species of insects are due to their common adult feeding ecology, as in most species adults feed on floral nectars, irrespective of the evolution of female preferences and larval ecology for reproduction. However, in the extremely specialized *Drosophila sechellia*, the sensory array has indeed evolved to a simpler state by an increased frequency of sensilla tuned to the volatiles from its host *Morinda citrifolia* (fruit), as compared with the other sibling species of the *D. melanogaster* species complex, for which this fruit is toxic (Dekker et al. 2006).

The evolution at the antennal level is more clearly seen when comparing insects with more fundamental differences in the feeding ecology than diet breadth in plant feeders. For example, most phytophagous species have a majority of OSNs that respond to plant compounds like GLV alcohols and terpenoids (Bruce et al. 2005), which does not seems to be the case in *Drosophila*, where most of the OSNs respond to fruit esters (de Bruyne et al. 2001; de Bruyne and Baker 2008), produced during the fermentation process of decaying fruits (food for *Drosophila* larvae). Likewise, mosquitoes have OSNs that respond to a variety of compounds derived from humans and birds, in addition to plant compounds (Ghaninia et al. 2008; Hill et al. 2009).

Our results support the hypothesis that host-plant selection may require both generalist as well as specialist receptors (Hansson 1995; Ignell and Hansson 2005). The differences in sensitivity and temporal response between OSNs due to different positions on the antenna are an intriguing observation, warranting further studies on other insects, especially those with filiform antennae.

Our work also provides a basis for studies of the olfactory molecular biology (identification and functional characterization of putative odorant receptors, i.e., deorphanization of olfactory genes) of female *S. littoralis*, ongoing in our laboratory. A large class of ST sensilla has not yet been functionally characterized. Thus, the olfactory space of the female cotton worm is likely to be much more extensive than presently known. A strategy in our future studies will be to identify novel ligands from airborne volatile collections from leaves and flowers of host and non-host plants by gas chromatography coupled-electro antennographic detection (GC-EAD) and GC-SSR.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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