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Detection of Sex Pheromone Components in *Manduca sexta* (L.)

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Abstract

The ability of olfactory receptor neurons to detect female-produced sex pheromone components and a limited sample of potential host plant odours was studied by single-sensillum recordings from olfactory sensilla present on male and female antennae in *Manduca sexta*. The majority of pheromone-sensitive receptor neurons examined in males was specialized for detection of the two major pheromone components, *E*10,*Z*12-hexadecadienal and *E*10,*E*12,*Z*14-hexadecatrienal or *E*10, *E*12,*E*14-hexadecatrienal. New olfactory receptor neurons tuned to the minor components *E*10,*E*12-hexadecadienal and *Z*11-hexadecenal were found. In females, olfactory receptor neurons specific to Z11-hexadecanal were discovered. Pheromone components and host volatiles were detected by separate sets of receptor neurons.

Introduction

The tobacco hawk moth, *Manduca sexta* (Linnaeus) (Lepidoptera, Sphingidae), is one of the most thoroughly studied insect models used in olfactory research. The main interest has been directed towards the central nervous system, especially to processing of the two main sex pheromone components in males [for review see (Hildebrand, 1995, 1996; Hildebrand and Shepherd, 1997)]. In comparison, the peripheral aspects of pheromone detection have not been studied so intensely (Kaissling *et al*., 1989).

Twelve pheromone-like compounds have been identified in solvent rinses of the female sex pheromone gland (Tumlinson *et al*., 1989). Behavioural observations in the wind tunnel and in the field revealed that eight of them, namely hexadecanal (16:Ald), three isomeric hexadecenals [*Z*9–16:Ald, *E*11–16:Ald and *Z*11–16:Ald (Z9, E11 and Z11, respectively)], two isomeric hexadecadienals [*E*10,*E*12–16: Ald (EE) and *E*10,*Z*12–16:Ald (EZ; bombykal)] and two isomeric hexadecatrienals [*E*10,*E*12,*E*14–16:Ald (EEE) and *E*10,*E*12,*Z*14–16:Ald (EEZ)], play a role in the attraction of males to conspecific females (Starrat *et al*., 1979; Tumlinson *et al*., 1989, 1994).

Males detect pheromone by olfactory receptor neurons (ORNs) within male-specific sensilla trichodea (Sanes and Hildebrand, 1976; Schweitzer *et al*., 1976; Keil, 1989; Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a,b). Two morphological classes of sensilla trichodea—type I and II—have been found on the male antennal flagellum (Sanes and Hildebrand, 1976; Keil 1989; Lee and Strausfeld, 1990). Type I trichoid sensilla form typical arch-like rows on the dorsal and ventral surfaces of each annulus. Slender type II trichoid sensilla are located, together with sensilla basiconica and other sensillar types, in areas of the annulus not occupied by type I elements (Lee and Strausfeld, 1990).

Though females lack type I trichoid hairs, they possess trichoid elements too (Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a,b). Similarly as in males, slender A and stout B elements have been recognized (Shields and Hildebrand, 1999a,b). The female-specific trichoid types do not exceed 50 nm in length and are distributed over the annular surfaces among the population of much shorter sensilla basiconica. Females are considered to be pheromone anosmic (Schweitzer *et al*., 1976; Hildebrand, 1996).

Previous electrophysiological recordings from male-specific type I trichoid sensilla of *M. sexta* showed that one of the two ORN types present is specific to EZ (the most prominent component in the pheromone blend) and the second, to either EEZ or EEE (Kaissling *et al*., 1989). No other male-specific ORNs have been characterized. But it has been shown that minor pheromone components have physiological effects in the brain (Christensen *et al*., 1989b). In females, no ORNs tuned to EZ and/or *E*11,*Z*13-pentadecadienal (a mimic of EEZ) were observed when single sensillum responses were recorded from type A sensilla trichodea (Shields and Hildebrand, 2001). But expression of the 'male-specific' pheromone binding protein in female antennae has been reported (Györgyi *et al*., 1988; Vogt *et al*.,

1991). The aim of our study was to find out how males detect minor pheromone components and if there are any pheromone-sensitive ORNs on female antennae.

Material and methods

Insects and electrophysiological recordings

Manduca sexta moths were reared on an artificial diet [modified from that of Bell and Joachim (Bell and Joachim, 1976)] under a L:D 16:8 photoperiod regime (23–25°C, 40–50% relative humidity). Males and females 1–2 days old were used for experiments. Moths were restrained in a tightly fitting plastic tube. The head was encased in wax with the antennae firmly fixed at their bases.

The neuronal activity was recorded extracellularly by means of an electrolytically sharpened tungsten electrode penetrating the antennal cuticle at the base of a sensillum (Hubel, 1957; Boeckh, 1962). The position of each electrode contact was recorded to achieve information about the distribution of different physiological types of ORNs on antennal annuli. The recording electrode was connected to a high impedance AC amplifier (Syntech, Hilversum, The Netherlands) operating at 1000 times amplification and with a 500 Hz bandpass filter. The indifferent electrode (Ag/AgCl wire) was inserted into the moth's abdomen. An audio-monitor was used to indicate contact quality. The signals were observed on a Phillips oscilloscope and recorded on a Vetter videocassette recorder, SLF-750HF (Vetter, NJ) for later processing. Responses were then digitized (sampling rate 10 416 samples/s) and PC analysed using Syntech Auto-spike software version 3.0 and 4.0 (Syntech). Experiments were performed on 20 males and 25 females.

Chemicals

The commercially available host plant-related volatiles were used as representatives of non-pheromonal stimuli. Among the selected compounds were those affecting oviposition in *M. sexta* (Tichenor and Seigler, 1980) or compounds identified in emanations of tomato and tobacco, the preferred host plants in *M. sexta* (Andersen *et al*., 1986, 1988; Buttery *et al*., 1987a,b; Loughrin *et al*., 1990). The selected volatiles and their purities, determined by gas chromatography, are listed in Table 1.

Pheromone components were synthesized in the laboratory. Monounsaturated aldehydes were prepared from corresponding alkenols by a simple oxidation procedure with pyridinium chlorochromate (PCC) (Corey and Suggs, 1975). The starting alkenols were synthesized by an alkyne chain elongation (from ω-bromo alkanols and corresponding 1-alkynes) and subsequent reduction/hydrogenation of the triple bond.

The key intermediate for synthesis of 10,12-hexadecadienals, O-protected 1-iodo-*E*1-undecen-11-ol, was prepared from O-protected 10-undecyn-1-ol by a standard hydroalumination/iodation procedure (Tellier and Descoins, 1991). The palladium catalysed cross-coupling reaction of this iodocompound with 1-diisobutylaluminium-*E*1-pentene (Negishi *et al*., 1988) directly provided the required *E*10, *E*12-dienic system. The product of this coupling was deprotected and oxidized (PCC) to a desired *E*10,*E*12-hexadecadienal (EE). Isomeric *E*10,*Z*12-hexadecadienal (EZ; bombykal) was prepared in a similar way. The palladium catalysed cross-coupling of the key intermediate with 1-pentyne (Ratovelomanana and Linstrumelle, 1981) was followed by a hydroboration with dicyclohexylborane, which gave the corresponding O-protected *E*10,*Z*12-dienic alcohol. The last steps of the synthesis were the same as in the case of the above-mentioned *E*10,*E*12-hexadecadienal.

*E*10,*E*12,*E*14-hexadecatrienal was prepared according to the following procedure: the triphenylphosphosphonium salt prepared from 10-bromo-1-decanol was converted to the corresponding ylide which was reacted with sorbinal in the presence of LiBr and excess of base. From the obtained mixture of *Z*10,*E*12,*E*14-hexadecatrienol (major product) and the *E*10,*E*12,*E*14-isomer, the latter isomer was isolated by an urea complex inclusion procedure, then oxidized to the desired product, *E*10,*E*12,*E*14–16:Ald (EEE) by Swern oxidation.

The synthesis of *E*10,*E*12,*Z*14-hexadecatrienal started with oxidation of 1-(2-tetrahydropyranyloxy)-10-bromodecane to the corresponding O-protected decanal by

Table 1 List of host-plant related volatiles used in the study

Compound	Purity	References
1. (*Z*)3-Hexenol	99	1,3
2. (*R*)-(+)-Limonene	97	1
3. (+)-δ-4-Carene	92	1
4. Terpinolene	90	1
5. Benzaldehyde	96	1,2,3
6. (*S*)-(–)-β-Pinene	99	1
7. (*S*)-(–)-α-Pinene	97	1
8. Myrcene	92	1
9. (*E*)2-Hexenal	85	1,3
10. 2-Phenyletanol	99	1
11. Benzylalkohol	78	2,4
12. Phenylacetaldehyde	99	
13. Linalool	92	1,2,3
14. β-Caryophyllene	89	1,2,3
15. Geraniol	99	1
16. Methylsalicylate	97	2,3
17. (*E*)2-Hexenylacetate	99	
18. (*E*)-β-Ocimene	99	2,3
19. (*E*)-β-Farnesene	98	3
20. Geranylacetone	73	4
21. (*Z*)3-Hexenylacetate	97	3
22. Methyl-jasmonate	98	1
23. Humulene	79	2

References: 1, tomato leaf (Buttery *et al.*, 1987); 2, tobacco flowers (Loughrin *et al*., 1990); 3, tobacco leaf (Andersen *et al.*, 1988); 4, EAG (Tichenor and Seigler, 1980).

N-methylmorpholine N-oxide. This aldehyde was reacted with the anion of methyl 4-dimethylphosphonate-*E*2-butenoate (Wadsworth–Horner–Emmons reaction). The hydroxy function in the resulting dienoate was deprotected before the ester functionality was converted to an aldehyde by use of diisobutylaluminium hydride and manganese dioxide in two subsequent reactions. The product, 14-hydroxy-*E*2,*E*4-tetradecadienal, was converted to the desired *E*10,*E*12,*Z*14-hexadecatrienol by a reaction with a corresponding ylide. The trienol was oxidized (Swern oxidation) to 10*E*,12*E*,14*Z*-16:Ald (EEZ) in the last step of the synthesis.

The purity of all synthetic pheromone components used was between 95 and 99% (as determined by GC–MS and HPLC–MS).

Stocks of test compounds were prepared by diluting the neat compound in hexane in decadic steps. From each stock concentration, 10 µl were pipetted onto a strip of filter paper (~10 × 15 mm) placed in a Pasteur pipette, where the solvent was allowed to evaporate. The amount of substance in pipettes ranged from 100 pg to 1 µg in decadic steps for the dose–response trials. For screening, 100 ng were used in each pipette. Blank stimulations were performed with a cartridge containing a filter paper onto which only solvent had been applied. The test cartridges were kept at –20°C when they were not used to prevent degradation of the compounds. New pheromone cartridges were prepared every second day, cartridges loaded with plant volatiles were prepared prior to every experiment.

Odour delivery system

The antenna was continuously ventilated with a stream of purified, humidified air (0.5 m/s) that passed through a glass tube (8 mm i.d.) with the outlet (3 mm i.d.) positioned 0.5 cm from the antenna. Neurons were stimulated with 0.5 s puffs of each component by injecting 1 ml of air from the odour cartridge into a continuous air-stream through a hole (i.d. 0.4 cm) in the glass tube located 15 cm from the outlet. Odour stimulations were controlled by a Syntech stimulus controller operated by a foot switch. The time of closed switch was indicated on the computer screen as a stimulus bar. In selective blocking experiments, two stimulation channels were synchronized to deliver the blocking and test stimuli (duration 0.3 s) with a 0.1 s interval.

Each time a contact with a sensillum was established, the spontaneous activity of associated ORNs was recorded for 30 s and the number of neurons within a sensillum was determined. Then, pheromone and host plant-related compounds at the screening dose, and a blank, were used to test whether any ORN of the contacted sensillum gave a response stronger than the blank. If an ORN responded to any of the test substances, dose–response trials were performed. The test substances were presented to the antenna at all dose levels, starting with the lowest doses. At lower doses (<100 ng) the stimuli were presented with an interval of 60 s, at higher doses the ORNs were allowed to recover for longer periods up to 5 min. Spikes were counted during the period of stimulation. When a dose–response curve for a key compound was established, the lowest dose that gave responses significantly higher than the spontaneous activity was determined by a Wilcoxon rank test (one-sided, $P < 0.05$). When all tests were done, the antenna was fixed in a new position that made it possible to contact previously un-stimulated sensilla.

Selective blocking technique

In sensilla where spike amplitudes of individual ORNs could not be discriminated, separation of individual ORNs within the sensillum was performed using a technique modified from differential adaptation as described elsewhere (Payne and Dickens, 1976; Kaissling *et al*., 1989). Initially, the sensillum was exposed to 0.3 s stimulation with one compound active in the screening procedure (blocking compound, 500 ng) and then, within 0.1 s interval, with another compound active in the screening procedure (test compound, 100 ng). If blocking and test compounds were detected by the same ORN, no response or a weak response was supposed to be elicited by the test compound. If the test compound was detected by a different ORN within a sensillum, the response to the test compound was considered to be more or less unaffected by blocking.

Results

Sensillar classification

The male-specific type I sensilla trichodea (long trichoids) of the phallanxes were determined unambiguously due to their anatomical separation and length. Outside phallanxes however, morphological characteristics visible in the stereomicroscope of the recording set-up did not allow clear discrimination among other, much shorter, morphological types (e.g. shortest type I sensilla trichodea, type II sensilla trichodea and/or sensilla basiconica). All sensilla outside phalanxes were therefore assigned as short ones. Similarly, morphological types were not distinguished in females.

Sensillar physiology

Out of 431 sensilla investigated in males, 170 sensilla were type I trichoids (long sensilla) and 261 were contacted in areas outside phallanxes (short sensilla). All long trichoids examined contained pheromone-sensitive ORNs (the representation of all ORN types found on male and female antennae is summarized in Table 2). Out of the 261 short sensilla, 86 contained pheromone-sensitive ORNs. In 81 short hairs, ORNs sensitive to one or more host plant odours were found (detailed physiological results will be reported elsewhere). In seven contacts, ORNs responded equally to pure air and to all applied stimuli. In 87 sensilla, associated ORNs did not respond to any compound tested.

In females, 200 sensilla were studied. Out of all impaled

Table 2 Representation of different physiological ORN types found on male and female antennae of *M. sexta*

	Males (n = 431)		Females
	Long (n = 170)	Short (n = 261)	Short (n = 200)
Pheromone-sensitive	170	86	8
EZ and EEZ	134	44	0
EZ and EE	16	10	0
EZ and EEE	3	1	0
EZ	4	5	0
EEZ	1	2	0
Z11	0	14	8
Unspecified	12	10	0
Plant-odour sensitive	0	81	121
Phenylethanol	0	18	26
Benzylalcohol	0	18	21
Phenylacetaldehyde	0	1	2
Linalool	0	10	22
β-Caryophyllene	0	10	13
Geraniol	0	5	8
Methylsalicylate	0	10	5
β-Farnesene	0	7	9
Geranylacetone	0	5	16
(*Z*)3-Hexenylacetate	0	5	5
Air	0	7	0
Unknown	0	94	71

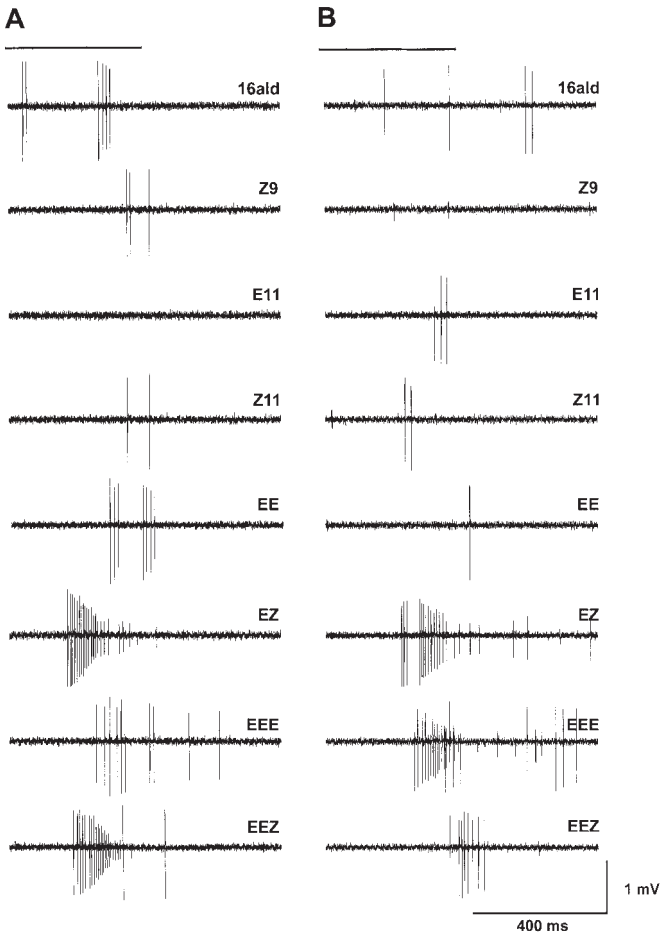

Figure 1 **A** and **B**.

sensilla, 121 contained ORNs sensitive to one or more host plant odours, 71 sensilla did not respond to any odour tested, eight ORNs were found to be specific to Z11.

In most contacts in both sexes, the spontaneous activity showed more than one class of spike amplitudes, indicating the presence of two or three ORNs.

Type I sensilla trichodea

In agreement with previously published data, male-specific sensilla trichodea type I contained two cells. In the majority of them, an EZ-specific neuron was paired with an EEZ-specific one (Figure 1A). In only four sensilla, the trienal-specific cell showed higher sensitivity to EEE than to EEZ (Figure 1B). In 16 long hairs, the EZ cell was associated with a so far unknown ORN type tuned to EE. In some cases, the EE cell responded selectively to EE (Figure 1C). In others, however, the EE cell responded also to EEE but at a somewhat lower sensitivity (Figure 1D). In few contacts, only one ORN—tuned either to EZ or EEZ—was found. Due to very high cross-reactivity and/or contact deterioration, the specificity of associated ORNs was not determined in 12 contacts.

The ORNs present in type I sensilla trichodea displayed spikes of very similar shape and amplitude. In most of the naive (un-stimulated) sensilla, EZ spikes were slightly higher than spikes of the EEZ cell, but sensilla with both cells spiking similarly were also found. The overall spontaneous activity recorded in long trichoids was 0.93 ± 0.69 imp./s. Spontaneous spikes quite often occurred in bursts of three to five. The dose–response curves for EZ, EEZ, EE and EEE (Figure 3) show the response threshold of pheromone-sensitive ORNs at a stimulus load 1 ng (Wilcoxon rank test, one-sided, $P < 0.05$). Saturation was observed at doses ≤1 μg. At doses ≥10 ng, responses tended to be organized in an initial phasic burst of action potentials followed by a tonic rate of firing, which diminished after the end of stimulation. The frequency of spikes within a burst gradually increased (up to 200–250 Hz) with increased stimulation doses. Close to saturation (and or after repeated stimulation), the tonic phase disappeared, bursts shortened, spike amplitudes within burst rapidly declined, number of spikes decreased and action potential firing was eventually blocked (Figure 2). The increased stimulation dose reduced the latency of the neuronal response until ORN adapted (Marion-Poll and Tobin, 1992).

The ORNs associated with type I sensilla trichodea responded to non-key pheromone components only when stimulus doses were elevated substantially (>100 times). Figure 3A displays the dose–response characteristics of

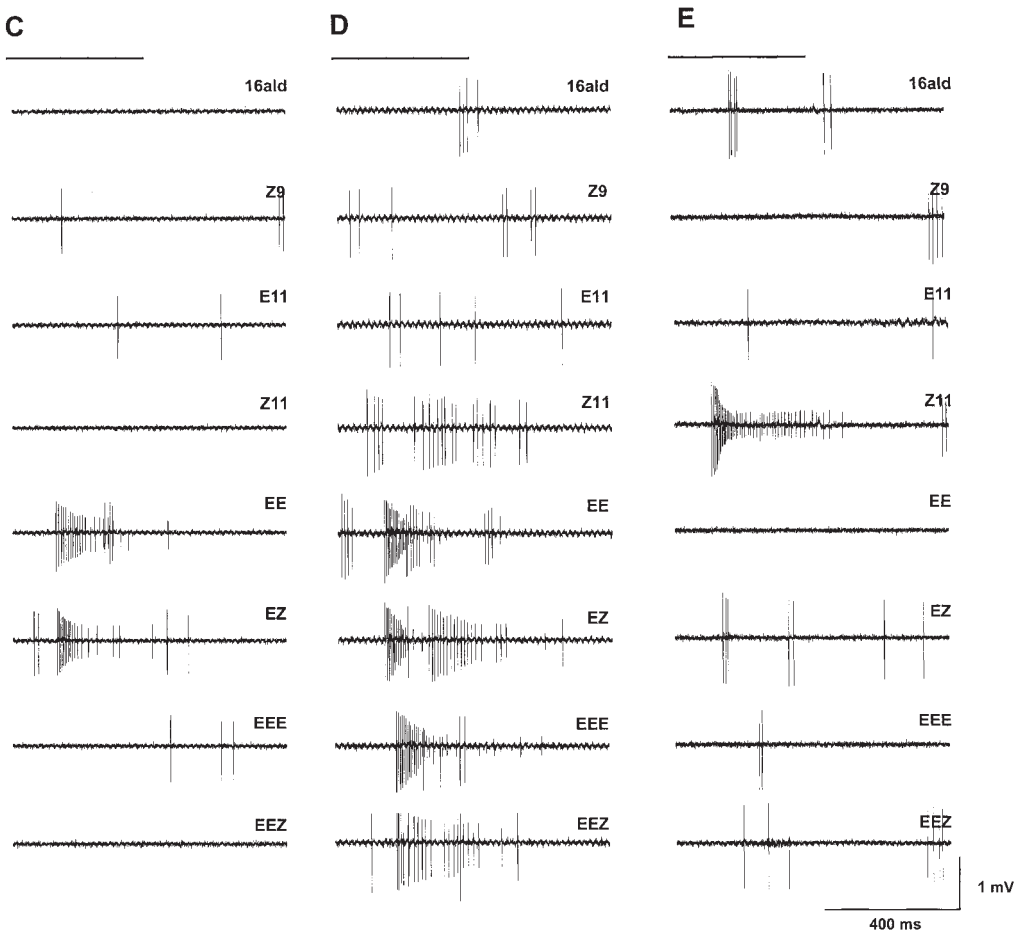

Figure 1 Physiological responses of ORNs recorded from pheromone-sensitive sensilla of male sphinx moth *M. sexta* to pheromone components. **(A)** Responses of the most abundant type I sensillum trichodeum associated with EZ- and EEZ-specific ORN. **(B)** Responses of less abundant type I sensillum trichodeum with ORNs tuned to EZ and EEE. **(C, D)** Responses of more frequent type I sensillum trichodeum containing ORNs tuned to EZ and EE (C—a sensillum where the EE cell responded selectively to EE, D—a sensillum where the EE cell was quite sensitive also to EEE, but responded with a longer latency and a lower spike frequency than to EE). **(E)** Responses of ORN tuned to Z11 associated with a morphologically unclassified sexually isomorphic sensillum. Stimulus bar = 0.5 s. The compounds were tested at 100 ng doses.

ORNs present in the most abundant type I sensilla trichodea to key and non-key pheromone components. In these sensilla, EE was the second most effective compound, followed by Z11 and E11. Compounds EEE, Z9 and 16:Ald elicited weak or no responses. Selective blocking proved that the EE was the second best stimulus for EZ-specific neurons. The EE cells responded second best to EEE.

A relatively large variation in the specificity and sensitivity of ORNs in male-specific sensilla trichoidea was observed. Some ORNs responded very specifically only to their key compound even at elevated doses (Figure 1A,B,C,E), while others were significantly sensitive also to other stimuli (Figure 1D). No ORNs within type I sensilla trichodea responded to any plant odour tested.

Selective blocking technique

The principle of how the selective blocking technique was used in our study to discriminate between neurons in a contacted sensillum is demonstrated in Figure 4. Two columns (A, B) represent typical examples of physiological responses recorded from type I sensillum trichodeum. Responses displayed in column A were obtained from the most abundant sensillum type containing EZ and EEZ neurons. The first trace displays neuronal responses to two successive EZ stimuli. The EZ-specific ORN responded to the first EZ stimulus with a strong phasic burst of spikes. By the time of the second EZ stimulus onset, the EZ cell remained blocked and EZ re-stimulation did not elicit any further spike activity (similarly, EEZ block eliminated the response of the EEZ neuron to the second EEZ stimulus—not shown). On the other hand, the EZ block did not eliminate the responses of the EEZ neuron to EEZ stimulation and vice versa (Figure 4A, the second and the third trace). A clear response to EEZ after EZ block (and the other way round) was considered as a proof that EZ and EEZ were detected by two discrete ORNs. Column B displays recordings from a

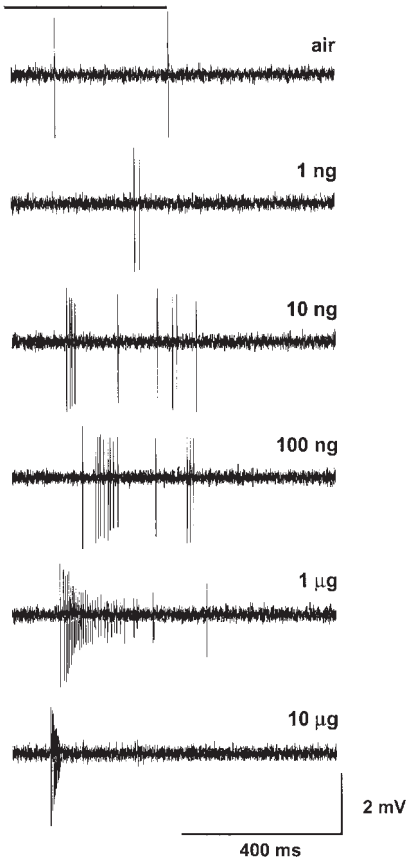

Figure 2 Physiological responses of Z11-specific ORN present in sexually isomorphic sensilla on male antennae of *M. sexta* to different dosages of Z11. The response threshold was observed at doses ≥10 ng. Above the threshold, the frequency of action potentials gradually increased with increased stimulation doses, while the latency of the response decreased. When stimulation doses increased, the pattern of spike activity were organized in an initial phasic burst of action potentials followed by a tonic rate of firing that diminished after the end of the stimulus. Close to saturation (and or after repeated stimulation), the tonic phase disappeared, bursts shortened, spike amplitudes within burst rapidly declined and action potential firing eventually blocked. The described phasic response characteristics were found in all studied pheromone-sensitive ORNs.

sensillum where an EZ-specific neuron was located together with an EE-specific one. The first and second traces show how the EZ cell responded to EZ stimulation after EE or EEE blocking (large spikes detected in the background of rapidly declining spikes of the first burst represent activity of the EZ-specific ORN). The selective blocking by EE abolished the response to EEE (Figure 4B, third trace) and vice versa (not shown). The third trace thus demonstrates that EE and EEE were detected by the same ORN not identical to the EZ one. As could be seen from averaged frequency histograms displayed in Figure 5, the selective blocking was quite efficient and reduced the spiking activity of the blocked cell considerably, while the response of other cell within the sensillum to the key stimulus remained unaffected.

Short sensilla

Extracellular recordings from short sensilla on male antennae revealed activity of one, two or three ORNs. Based on the specificity of ORNs present within the contacted sensillum, three discrete physiological subtypes of short sensilla were identified: (i) sensilla with two ORNs sensitive to the major pheromone components and/or to their isomers, the physiology of which (spontaneous activity, spike patterns, amplitudes, sensitivity and specificity) was very similar to that found in trichoid sensilla within phallanxes, (ii) sensilla with an ORN sensitive to Z11, and (iii) sensilla with one up to three ORNs sensitive to plant volatiles. The respective physiological types were found in different areas on antennal anulli (Figure 6D). The ORNs specific to major pheromone components were occasionally found on the leading edge of the annuli, in areas along the phallanxes and more frequently in the U-shaped pocket of long trichoids at the trailing edge. On the other hand, 14 ORNs tuned to Z11 were interspersed among the host odour-sensitive sensilla on the free surface of the annuli.

The spontaneous activity recorded from sensilla with the Z11-specific ORN sometimes indicated the presence of more than one ORN. The associated cell(s), however, did not respond to any stimulus tested. A typical example of physiological responses recorded from sensillum with the Z11-specific ORN is displayed in Figures 1E and 2. The responses of Z11-specific ORNs were dose-dependent at doses above 10 ng. Saturation was observed at 10 µg (Figures 2 and 3D).

Pheromone-sensitive ORNs in females

In females, eight ORNs tuned to Z11 were found. The sensilla associated with Z11 ORNs were distributed over much of the annular surface, among the population of host-odour sensitive sensilla. An accumulation in any specific area of the antennal annulus was not observed. The physiology of Z11 ORNs was similar in males and females (Figures 1E, 3D and 7).

Discussion

Our study provides evidence about the presence of two new, previously unknown, pheromone-sensitive ORN types on male antennae. The first one, EE-specific ORNs, was found in sensilla within phalanxes and between short sensilla medially along phallanxes and among inner hairs of U-shaped cul-de-sac formed by phallanxes at the trailing edge of each annulus. This distribution corresponds with the distribution of sensilla trichodea type I (Lee and Strausfeld, 1990). Previous electrophysiological study of type I sensilla trichodea reported the presence of three types of ORNs: cells tuned to EZ (ORN type A), EEZ (type B) and EEE (type C) (Kaissling *et al.* 1989). In addition to EE-specific ORNs, we found all already known receptor types. However, the EEE-specific ORNs were found in relatively lower abundance

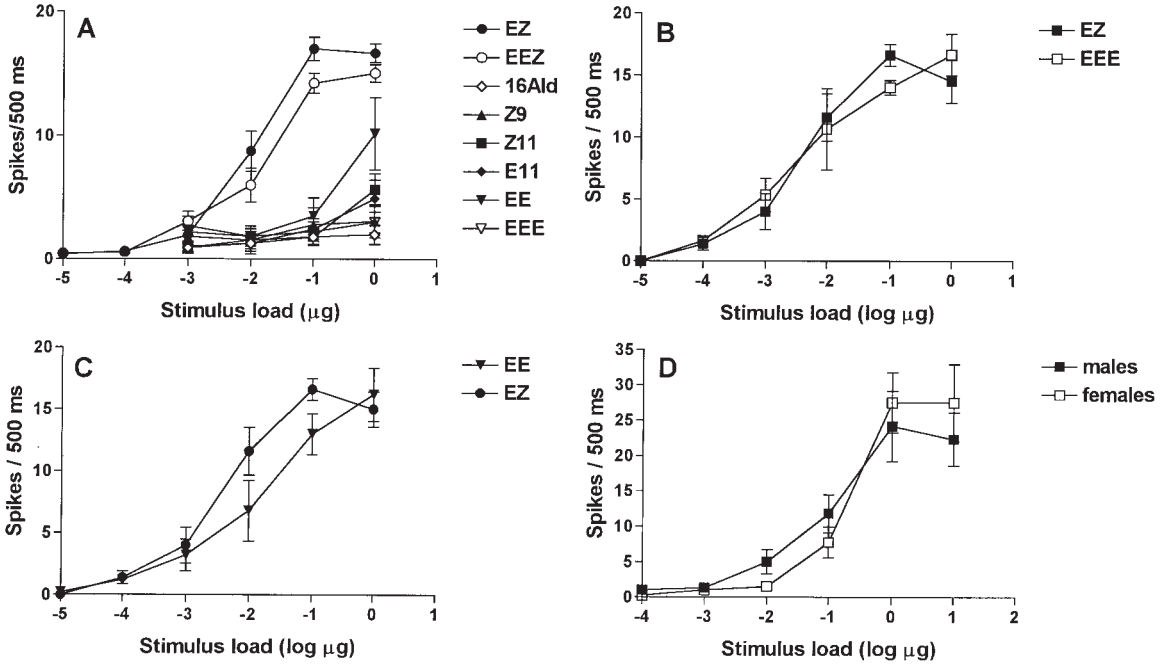

Figure 3 Dose–response curves obtained from pheromone-sensitive sensilla of male and female antennae. **(A)** Dose–response curves of EZ- and EEZ-specific ORNs to EZ, EEZ and to minor pheromone components—Z11, E11, Z9 and 16:Ald. Each point represents mean of 10 values ($n = 10$) obtained from 10 different sensilla of the respective type. **(B)** Dose–response relationship of sensilla associated with EZ and EEE ORN types ($n = 2$). **(C)** Dose–response curves of sensilla with EZ and EE ORNs ($n = 10$). **(D)** Dose–response curves of Z11-specific ORNs found on male (■, $n = 3$) and female (□, $n = 3$) antennae. The *y*-axis represents the number of spikes elicited during 500 ms stimulation by a given dose, the *x*-axis delineates the stimulation intensity given by an amount of the stimulus compound loaded onto a filter paper disc in the stimulation pipette. Vertical lines indicate the standard error of the mean.

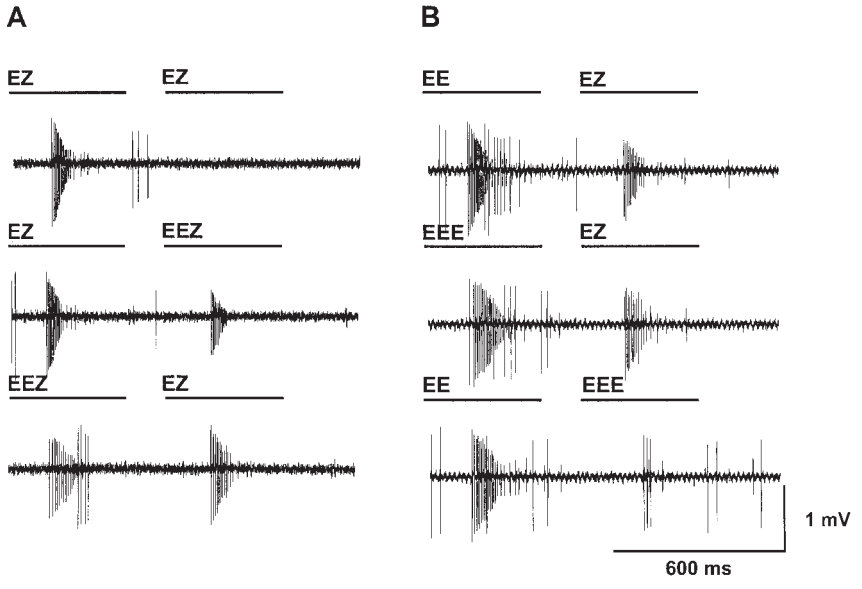

Figure 4 Physiological responses of two different type I sensilla trichodea during selective blocking. Two stimuli (500 and 100 ng, respectively) lasting 300 ms were presented with an 100 ms interval (as indicated by the markers above every recording). **(A)** Recordings from the most abundant type I sensillum trichodeum with EZ and EEZ ORNs. Double stimulation (the upper trace) with EZ elicits a response of the EZ-specific neuron to the first, but not to the second stimulus. The second trace, recorded from the same sensillum 3 min later, shows the unaffected response to EEZ after EZ block and the other way round (the third trace, the same sensillum 3 min later), indicating that EZ and EEZ were detected by two discrete ORNs. In this particular sensillum, the ORNs responded with different spike amplitudes. **(B)** Type I sensillum trichodeum with EZ and EE neurons. Again, three different traces represent three different combinations of two sequential stimuli on the same sensillum. The EZ neuron responded to EZ stimulation after EE block (first trace). In this particular sensillum, the EEE elicited significant response also but, similar to EE, did not block EZ neuron (second trace). However, EE block abolished the response to EEE, indicating that EE and EEE were detected by the same ORN. The time scale of recordings and magnitude of recorded potentials are indicated at the bottom-right corner of the figure.

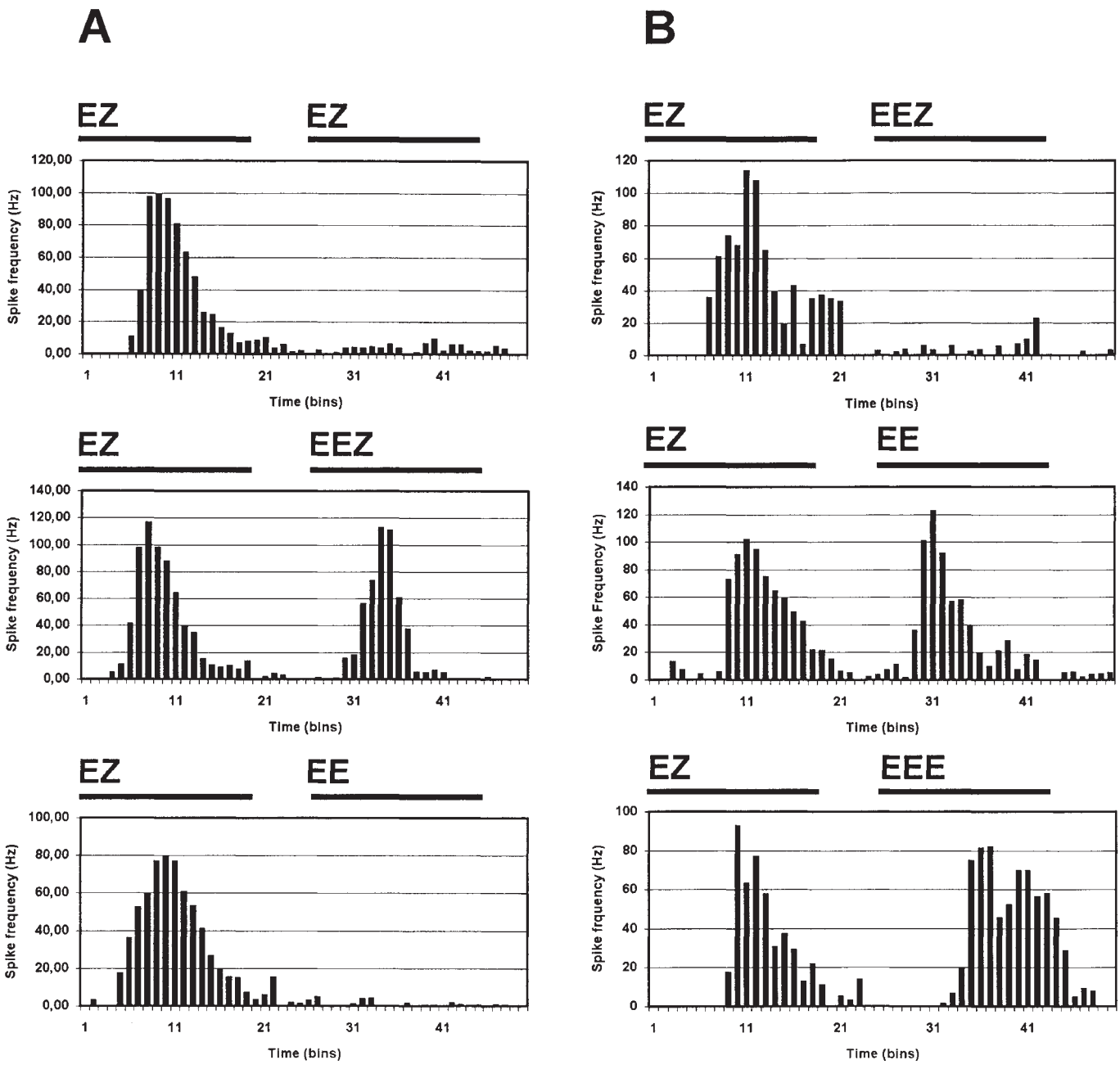

Figure 5 The frequency histograms obtained from sensilla trichodea type I with EZ and EEZ **(A)** or EZ and EE ORNs **(B)**. Each graph represents neuronal responses to two successive stimuli (blocking and testing), specificity of which are displayed above the stimulation markers on the top of each graph. The time (*x* axis) is expressed in bins (1 bin = 25 ms). During each bin the neuron spiking frequency was evaluated and plotted against *y* axis (Hz). Each graph represents averaged values obtained from 12 (A) and four (B) different sensilla.

[compare four EEE ORNs out of 431 contacts in our study (0.9%) and three EEE ORNs out of 50 contacts (6%) (Kaissling *et al.*, 1989)]. Such a discrepancy could be explained by the fact that different laboratory colonies of *M. sexta* were used. However, since in earlier investigation the EE was not tested, we can also speculate that some previously identified EEE neurons might be identical to the EE ones. Some EE neurons impaled in our experiments were indeed also sensitive to EEE and selective blocking experiments showed undoubtedly that EE and EEE stimulated the same ORN.

Surprisingly, a relatively large variation in specificity among ORNs associated with male-specific type I sensilla trichodea was observed. Some ORNs responded highly specifically only to the key compound, even at elevated doses, while others were quite sensitive also to other stimuli

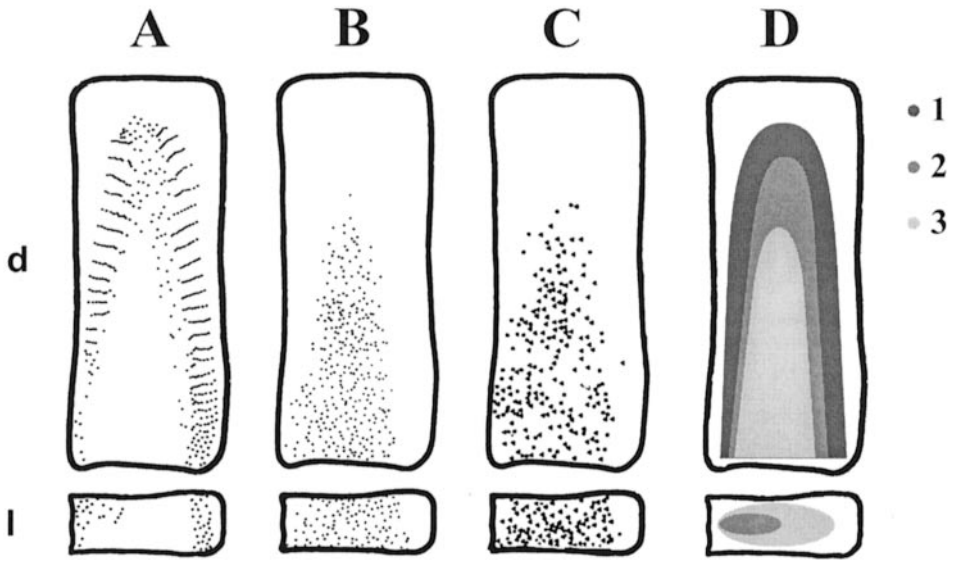

Figure 6 **(A, B, C)** The spatial distribution of different morphological types of olfactory sensilla on dorsal surface (d) and leading edge (l) of one antennal annulus on the antenna of a male *M. sexta*. (A) Sensilla trichodea type I, (B) sensilla trichodea type II, (C) sensilla basiconica [adapted from Lee and Strausfeld (Lee and Strausfeld 1990); each dot represents an individual sensillum]. **(D)** The distribution of different physiological types of ORNs found on the male antenna—1: the distribution of EZ-, EE-, EEZ-sensitive neurons within phallanxes; 2: the distribution of EZ-, EE-, EEZ-sensitive neurons outside phallanxes; 3: the distribution of ORNs sensitive to host odours and Z11.

of pheromonal origin. The excitability of impaled cells could in some cases be affected by the penetration of the electrode. However, the observed variability might reflect real differences in ORN physiology. The wide range of lengths of type I trichoids may represent the various physiological subclasses (Lee and Strausfeld, 1990). This suggestion was proposed by Kanaujia and Kaissling who studied sensillar physiology in *Antheraea polyphemus* (Kanaujia and Kaissling, 1985). Their study implies that different lengths of sensilla, and hence of dendrites, may indicate specific functional roles amongst members of the respective classes. The different trichoid lengths may confer different biophysical properties relating to sensitivity and transduction (Kanaujia and Kaissling, 1985). It would be interesting to know if there is any systematic correlation between physiology of the ORNs and the sensillar position on antennal annuli in *M. sexta.*

The selective blocking technique proved to be an efficient tool in discrimination of different ORNs responding with similar spike amplitudes in *M. sexta*. We have chosen to name the technique selective blocking, as we cannot be sure if the observed effect is a result of an adaptation process or of a depolarization block. Both mechanisms might contribute in this case. Adaptation could be argued to allow doubt regarding the specificity of the neuron adapted, as a second receptor type could theoretically be expressed in the dendritic membrane of the same neuron. Under such circumstances a single, adapted neuron could still respond to a second component. Depolarization block would provide an unambiguous result, as all responses of the affected neuron would be abolished.

The second new ORN type found in our study was specific to Z11. Neurons sensitive to Z11 were discovered in short sensilla of the free space between phalanxes spotted among sensilla sensitive to plant-related odour. In this area, sensilla trichodea type II and sensilla basiconica are found. These sensilla are supposed to carry information about non-pheromonal odours (Christensen *et al.*, 1995), since axons of associated ORNs target glomeruli outside the macroglomerular complex (MGC)—the structure where all pheromone-specific ORNs have traditionally been considered to project (Christensen and Hildebrand, 1987). Our finding that among these sensillar types do exist ORNs sensitive to one of the pheromone components is noticeable and raises some interesting questions. Could these sensilla be identical to those expressing the pheromone-binding protein (PBP) in free space between phalanxes (Vogt *et al.*, 2002)? Do neural circuits outside the MGC process some features of the pheromone signal? Recent findings that pheromone responses can indeed be recorded from antennal lobe neurons restricting their arbors to ordinary glomeruli (Anton and Hansson, 1999) support such a possibility. However, further studies are needed to answer these questions and to understand entirely pheromone processing in *M. sexta.*

We did not find ORNs specific to E11, Z9 or 16:Ald. Considering the number of sensilla present on each antennal annulus and the number of sensilla contacted in this study, we cannot conclude whether these receptor types exist or not. If very few specific ORNs are present on each antennal annuli, the possibility of contacting them is low. In the turnip moth, *Agrotis segetum*, ORNs tuned to one of the

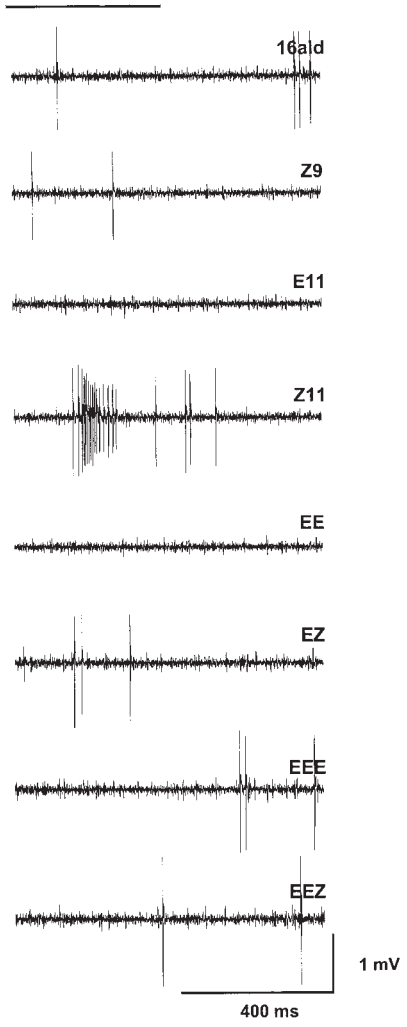

Figure 7 Physiological responses of ORNs recorded from pheromone-sensitive sensilla of female sphinx moth *M. sexta* to Z11. Stimulus bar = 0.5 s. The compounds were tested at 100 ng doses.

major pheromone components occur only in 0.1% of the sensilla (Hansson *et al*., 1990). If a similar relationship is present in *M. sexta*, a sample of 1000 sensilla would statistically be required to encounter all types.

The processing of minor pheromone components in the male brain of M. sexta has been investigated in the deutocerebrum (Christensen *et al.*, 1989), where the activity of antennal lobe interneurons was recorded intracellularly. These experiments proved that minor components have some physiological effect in the male brain, however, the neural substrate for their detection remains unknown. Our study shows that male-specific ORNs tuned to major pheromone components responds to other pheromone components only when elevated doses are used and with high certainty do not represent a relevant channel to the brain regarding their detection. On the other hand, the newly identified ORNs specifically tuned to EE and Z11 undoubtedly delineate the previously unknown sensory pathway.

The role of minor pheromone components in sexual communication in *M. sexta* is not yet fully understood. One of the difficulties in working with the pheromone of this species is the instability and unavailability of the triene aldehydes. In wind tunnel experiments it has been shown that from all components produced by female sex pheromone glands, a blend of two components, EZ and EEZ, is essential to elicit male precopulation behaviour (Tumlinson *et al*., 1989). Further tests in the wind tunnel suggested, but did not clearly demonstrate, that other components of the gland rinse played a role in mating communication in this species. These experiments also showed that a four-component blend (EZ, EEZ, EE and EEE) was less effective than either the two-component blend or the full component blend (Tumlinson *et al.*, 1989, 1994). Field experiments showed that the synthetic full component blend is attractive for males in the field. Addition of one or more of the saturated and monounsaturated components to EZ and EEZ improved the male response. The authors of the study suggested that all eight 16-carbon aldehydes are active (Tumlinson *et al.*, 1994). In the male brain, all the 16-carbon aldehydes found in the pheromone gland elicit some form of response in olfactory interneurons (Christensen *et al.* 1989), but EZ, EEZ and EEE evoke the greatest responses. Our finding of two new ORN types tuned to EE and Z11 suggests that except EZ, EEZ and EEE also EE and Z11 play an active role in sexual communication of *M. sexta.* However, their exact roles must be further investigated.

Females of *M. sexta* have been consistently noted not to respond physiologically or behaviourally to sex pheromone (Schweitzer *et al*., 1976; Hildebrand, 1996). In spite of this, the expression of 'male-specific' pheromone binding protein (PBP) in antennae of female *M. sexta* (Györgyi *et al*., 1988; Vogt *et al*., 1991, 2002) and in some other species, females of which have been previously considered as pheromone anosmic, have been reported (Steinbrecht *et al*., 1992; Nagnan-Le Meillour *et al*., 1996; Maibeche-Coisné *et al*., 1997; Callahan *et al*., 2000). Immunological and histological studies have shown that PBP is expressed at a low level compared with that in male antennae and the expression is associated with a small number of specific, but otherwise uncharacterized, group of olfactory sensilla (Steinbrecht *et al*., 1992, 1995; Laue and Steinbrecht, 1997; Vogt *et al.*, 2002). Our data bring the first physiological evidence that *M. sexta* females do respond to at least one pheromone component. Our sensilla associated with ORNs specifically tuned to Z11, found in a small number on female antennae, may be among those showing expression of PBP. The behavioural meaning of female ability to detect Z11 is not known and should be further investigated.

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