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RESEARCH ARTICLE

Ordinary glomeruli in the antennal lobe of male and female tortricid moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) process sex pheromone and host-plant volatiles

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SUMMARY

Both sexes of *Grapholita molesta*, a key pest of stone fruits, are able to detect host-plant volatiles and the sex pheromone emitted by females, and to modify their behaviour accordingly. How olfactory information is processed in the central nervous system is unknown. Intracellular recordings and stainings were used to characterize antennal lobe (AL) neuron responses to single pheromone components, a behaviourally active blend of five peach volatiles and a pear-fruit ester. AL neurons with different response patterns responded to pheromone components and plant volatiles. In males more neurons responded specifically to the main pheromone component than in females, whereas neurons responding to all three pheromone components were more abundant in females. Neurons responding to all three pheromone components often responded also to the tested plant volatiles in both sexes. Responses to all pheromone components were dose dependent in males and females, but dose–response relationships differed between neurons and tested pheromone components. Among the five AL projection neurons identified neuroanatomically in males, no arborizations were observed in the enlarged cumulus (Cu), although all of them responded to pheromone compounds. In one of two stained projection neurons in females, however, the glomerulus, which is thought to be homologous to the Cu, was targeted. The processing of pheromone information by ordinary glomeruli rather than by the macroglomerular complex is thus a striking feature of this species, indicating that pheromone and plant volatile processing are not entirely separate in this tortricid moth AL. However, the absence of recorded pheromone responses in the Cu needs to be confirmed.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/4/637/DC1

Key words: *Grapholita molesta*, olfaction, antennal lobe, pheromone, intracellular recording and staining.

INTRODUCTION

Olfaction is widely used in many animals for intraspecific and interspecific communication. Sex pheromones are crucial cues used when finding a mating partner in such diverse animal groups as mammals and insects (Hildebrand, 1995; Lledo et al., 2005; Mori et al., 1999; Wyatt, 2003), whereas plant volatiles act as feeding attractants or oviposition stimulants, depending on the behavioural response that they elicit in the receiving organism (Schoonhoven et al., 2006). Central nervous processing of sex pheromone information has been studied extensively in a few model organisms (Mori et al., 1999; Kanzaki and Shibuya, 1992; Hildebrand, 1995) and although evidence is accumulating of a number of common principles in the structure and function of the pheromone-processing system, differences in coding mechanisms and structure–function correlations have been found in some of these, for example in moths (Anton and Hansson, 1999; Christensen, 1997; Heinbockel et al., 1999; Mustaparta, 1997). Also plant odour processing has been studied in different insects, including moths (e.g. Anton and Hansson, 1994; Anton and Hansson, 1995; Sadek et al., 2002; Greiner et al., 2004; Masante-Roca et al., 2005; Reisenman et al., 2004; Reisenman et al., 2005; Namiki et al., 2008). In spite of largely separate anatomical pathways for pheromone and plant odour processing in moths, it seems that the principal processing mechanisms are similar (Christensen and Hildebrand, 2002), with each odorant being represented spatiotemporally within the glomerular array (Friedrich, 2002; Galizia and Menzel, 2001; Wilson and Mainen, 2006). Therefore, studying pheromone and plant odour processing in different moth species with a well-known behavioural background can reveal interesting information concerning both general coding mechanisms and specific adaptations to a certain life style.

The olfactory system of insects is known to consist of two subsystems. Axon terminals from olfactory receptor neurons (ORNs) expressing a specific membrane receptor innervate a particular glomerulus in the antennal lobe (AL) responsive to a specific set of odorants (Gao et al., 2000; Vosshall et al., 2000; Hallem and Carlson, 2006; Couto et al., 2005). In moths, plant odour processing occurs mainly in the ordinary glomeruli (Christensen and Hildebrand, 2002; Christensen and White, 2000) and pheromone processing occurs in distinct glomeruli, the macroglomerular complex (MGC) (Anton and Homberg, 1999; Hansson et al., 1992). The two systems are, however, not entirely separate because pheromone responses have also been found in neurons within ordinary glomeruli (Anton and Hansson, 1995; Kanzaki et al., 1989).

Within the AL, local neurons (LNs) interconnect many glomeruli, and projection neurons (PNs) send information to higher order neuropils, such as the mushroom body (MB) and the lateral protocerebrum (LPC) (Anton and Homberg, 1999; Galizia and Roessler, 2010). In contrast to the numerous studies dealing with male moth pheromone detection and processing, female auto-detection of sex pheromone has been demonstrated in only a few moth species, and little is known about central pheromone processing in female moths (e.g. Barnes et al., 1992; DeLury et al., 2005; Palaniswamy and Seabrook, 1985; Schneider et al., 1998; Stelinski et al., 2003; Stelinski et al., 2006; Weissling and Knight, 1996).

The Oriental fruit moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) is an important pest of peaches, apples and pears worldwide (Hickel and Ducroquet, 1998; Kovanci et al., 2004; Reis et al., 1988). The sex pheromone comprises three components, one major: (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), and two minor components: (*E*)-8 dodecenyl acetate (*E*8-12:Ac) and (*Z*)-8 dodecen-1-ol (*Z*8-12:OH), at a ratio of 100:6:10, respectively (Cardé et al., 1979; Roelofs et al., 1969). Male behaviour has been extensively studied in response to sex pheromone (Baker and Cardé, 1979; Charlton and Cardé, 1981; Figueredo and Baker, 1991; Stelinski et al., 2005). Recently, wind-tunnel tests have demonstrated that the addition of plant volatiles to the *G. molesta* sex pheromone blend has a synergistic effect on the upwind flight of virgin males (N.V., unpublished). In contrast, studies of female pheromone auto-detection found that after perceiving their own pheromone, females advance their calling time (i.e. start to call 2 h earlier) (Stelinski et al., 2006). Also, attraction towards a blend of five plant volatiles ((Z)-3-hexenyl acetate, (Z)-3-hexenol, (E)-2-hexenal, benzaldehyde and benzonitrile) has been recently reported in *G. molesta* females (e.g. Natale et al., 2004; Piñero and Dorn, 2007; Piñero et al., 2008). The response of *G. molesta* to ethyl (E,Z)-2,4-decadienoate (pear-ester: PE), a volatile emitted by ripe pears (Knight and Light, 2004), has also been observed in some apple orchards (F. M. Molinari, personal communication). PE is a well-known attractant of *Cydia pomonella* (Knight et al., 2005) and other tortricids (Schmidt et al., 2007).

Although the behaviour of *G. molesta* has been well studied, the structure and function of its peripheral and central olfactory systems are largely unknown. The wide array of behavioural data available make this species well suited for functional studies of plant volatile and pheromone processing and their plasticity. In the present study we have used intracellular recording and staining techniques to characterize physiologically and morphologically central neurons of virgin *G. molesta*. Antennal stimulation with plant volatiles and the three sex pheromone components of this species, at behaviourally relevant doses, were used in order to investigate how pheromone and plant volatile information are integrated in the AL. Neurobiotin and Lucifer Yellow staining permitted the identification of the recorded neuron types and of target glomeruli with the help of a three-dimensional (3-D) map of the AL (Varela et al., 2009).

MATERIALS AND METHODS

Insect preparation

Grapholita molesta larvae were reared on a semiartificial diet modified from that of Ivaldi-Sender (Ivaldi-Sender, 1974) under a 16 h:8 h L:D photoperiod at 25±1°C. Pupae were sexed, and males and females were placed in separate environmental chambers at 23°C under the same photoperiod. Emergence was checked daily. Two- to three-day old individuals were restrained in a plastic micropipette tip, with only the head protruding. The head was immobilized with dental wax and the brain was exposed by removing cuticle, tracheal sacs and muscles. The preparation was superfused with a constant flow of saline solution containing 150 mmol l^{-1} NaCl, 3 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} KCl, 10 mmol l^{-1} *N*-Tris-methyl-2-aminoethanesulfonic acid buffer and 25 mmol l^{-1} sucrose (pH 6.9) (Christensen and Hildebrand, 1987) in order to prevent desiccation of the tissues.

Stimulation

The single components of the sex pheromone of *G. molesta*: *Z*8-12:Ac, *E*8-12:Ac and *Z*8-12:OH, were used at doses of 0.001, 0.01, 0.1, 1.0, 10 and 100 ng. The blend of the three components, at a ratio of 100:6:10 respectively, was used at a dose of 1 ng, which is attractive in wind tunnel conditions (N.V., personal observation). In a second series of experiments a blend of five plant volatiles (5VB) [(*Z*)-3-hexenyl-1-yl acetate, (*Z*)-3-hexenol, benzaldehyde, (*E*)-2-hexenal and benzonitrile, at a ratio of 70:14:13:2:1, respectively] was tested, as well as the PE, (*E*,*Z*)-2,4-decadienoate, both at a dose of 1000 ng. This dose was selected on the basis of observations of their behavioural activity (N.V., personal observation). The odour doses reaching the antenna in the electrophysiological experiments should be similar to concentrations experienced by the males close to the odour source in the wind tunnel, as the stimulus application was the same in both cases (on a filter paper). 10 µl of the volatile solutions, dissolved in hexane, were applied on a filter paper, which was placed inside a glass Pasteur pipette. At the time of stimulation the tip of the stimulus pipette was inserted midway in a 7-mm internal diameter glass tube, through a hole in its wall, 20 cm away from the antenna. The downwind end of the tube was 5 mm from the antennal preparation and the other end was connected to a charcoal-filtered and humidified airflow, which was constantly blown over the antenna at a velocity of 0.3 m s^{-1}. A 0.5-s air pulse (7 ml s^{-1}) was blown through the stimulus pipette using a stimulation device (CS 55, Syntech, Kirchzarten, Germany) and the continuous flow was switched off during stimulation to minimize mechanical stimulation of the antenna. The stimulus reached the antenna approx. 150 ms after the stimulus onset trigger. As a blank stimulus, a filter paper containing only hexane was used. For neurons tested only with pheromone, the tested series always started with two stimulations of hexane, followed by the pheromone blend and then low doses of the three pheromone components were tested before higher doses. In neurons tested with both pheromone and plant volatiles, the latter were always tested before the individual pheromone components. Otherwise, the odours were presented randomly, separated by inter-stimulus intervals of at least 10 s.

Intracellular recording and staining

Intracellular recordings were performed according to standard methods (Christensen and Hildebrand, 1987). The tip of each glass microelectrode was filled with 0.5% Neurobiotin™ (Abcys, Paris, France) in 0.25 mol l^{-1} KCl or with 1% Lucifer Yellow (Sigma-Aldrich, Saint-Quentin Fallavier, France), and the shaft was filled with 3 mol l^{-1} KCl or with 2 mol l^{-1} $LiCl_2$, respectively. The AL was randomly penetrated with the microelectrode until intracellular contact was established. After a contact was established, the activity of the neuron was monitored before, during and after stimulation using Autospike software (Syntech, Kirchzarten, Germany). The recorded signals were amplified with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA), and subsequently digitized and analyzed with Autospike. For staining, a 0.03 nA depolarizing current was applied for 2 min to the electrode in order to inject Neurobiotin iontophoretically into the recorded neuron, or a 0.5 nA hyperpolarizing current was applied for 5 min to the electrode to inject Lucifer Yellow.

Data analysis

The number of spikes during each part of the response of a given neuron was manually counted. The number of spikes during the same duration of spontaneous activity preceding the response was subtracted from the number of spikes during the excitatory period of each response, resulting in a net spike number. As the duration of the excitatory period of odour responses varied between neurons, the analyzed spiking period was different for different neurons. For some neurons a response to hexane was observed, and therefore the neuron was considered to be responding when the net number of spikes following stimulation exceeded, by at least 100%, the net spike number elicited by hexane stimulation. The net spike number elicited by hexane stimulation was subtracted from the net spike number in response to the pheromone stimulus. The most common shapes of dose–response curves were then chosen to calculate average curves. In males, such an average curve was calculated for *Z*8-12:Ac and *Z*8-12:OH from neurons that showed an increase in spike number with increasing stimulus dose. An average curve for *E*8-12:Ac was obtained from neurons that showed an increase followed by a decrease in the net spike number. In females, for *Z*8-12:Ac an average curve was calculated from neurons that showed an increase in spike number with increasing stimulus dose, and for *E*8-12:Ac and *Z*8-12:OH an average curve was obtained from neurons that showed an increase followed by a decrease of the net spike number.

A neuron was classified as 'component specific' when it responded only to one of the tested compounds at any of the tested doses, and as 'generalist' when it responded to more than one of the compounds tested.

Statistical comparisons of neuron responses between the sexes were analyzed with 2×2 contingency tables and Fisher Exact test.

Histology

To facilitate dissection the head was fixed in 4% formaldehyde in Millonig's buffer (pH 7.2) with 0.25% Triton X-100 for at least 3 h at room temperature. The head was then dissected and the brain was fixed in 4% buffered formaldehyde in Millonig's buffer overnight at room temperature. Once fixed, the brains were washed in Millonig's buffer and then dehydrated and rehydrated in an ethanol series and propylene oxide to make membranes more permeable. The brains were washed again in Millonig's buffer and synapsin-antibody staining was used to reveal the glomerular structure of the AL (for details, see Varela et al., 2009). Neurobiotin-stained brains were incubated for 24 h at 4°C in a 4:2.5:96 solution of anti-synapsin (Klagges et al., 1996): Oregon Green–Avidin 488 (stock solution 1 mg ml^{-1} to visualize Neurobiotin) (Molecular Probes, Cergy-Pontoise, France): Millonig's buffer with 0.25% Triton X-100 and 1% bovine serum albumin. Lucifer-Yellow-stained brains were incubated in a 4:96 solution of anti-synapsin: Millonig's buffer with 0.25% Triton X-100 and 1% bovine serum albumin. The brains were then washed in Millonig's buffer and incubated in the secondary antibody, Alexa Fluor 633 rabbit anti-mouse (Invitrogen, Barcelona, Spain; ref A21063), diluted at 1.5:250 in Millonig's buffer at 4°C for 24 h to visualize synapsin staining. Finally, the brains were washed, cleared and mounted in Vectashield mounting medium (Vectashield H-1000, Vector Laboratories, Burlingame, USA).

Laser scanning confocal microscopy and 3-D reconstruction

The brains were viewed as wholemounts on a Leica laser-scanning confocal microscope (SP2 AOBS, Leica, Heidelberg, Germany) equipped with a HC PL APO CS 20.0×0.40UV air objective. The Oregon Green antibody, used to observe the Neurobiotin staining, was excited at a wavelength of 488 nm. For the Alexa Fluor antibody staining, used for glomeruli visualization, the excitation wavelength was 633 nm. Each brain was scanned frontally with a step size of 1 µm for detailed imaging. Image stacks were analysed by scrolling through the optical sections to identify the arborization areas. Maximum projection images were constructed using ImageJ (National Institutes of Health, MD, USA) and individual neurons and glomeruli were reconstructed using Reconstruct (Fiala, 2005).

RESULTS

General physiological characteristics of olfactory neurons

Intracellular recordings with a complete stimulus series tested in males were obtained from 62 neurons responding to at least one pheromone stimulus, and from 26 neurons tested with both pheromone and plant volatiles (supplementary material Tables S1 and S2). For females we obtained recordings from 18 neurons that responded to at least one pheromone component, and 22 neurons tested with both pheromone and plant volatiles (supplementary material Tables S3 and S4). Out of the 128 responding neurons, 92 showed spike amplitudes from 8 to 45 mV, and 36 showed spike amplitudes of ~5 mV.

Neurons responding to sex pheromone

For both sexes the most frequently occurring response type was tonic excitatory, but other response patterns were also observed (Fig. 1). In most cases the excitatory response exceeded the duration of the stimulation (Fig. 1A,B,D,E). In a few of these neurons the excitatory phase was followed or preceded by an inhibitory phase (Fig. 1C,F). A large variability of latencies of the excitatory period and response durations was observed (Fig. 1). Latency was usually the same for the different pheromone components in an individual neuron (Fig. 1A,D,E,F), but occasionally different latencies for different components were found in male neurons (Fig. 1B,C).

Neurons responding to both sex pheromone and plant volatiles

In neurons responding to both pheromone and plant volatiles a tonic excitatory response was encountered most frequently for both sexes (Fig. 2B,C,D). Inhibition after the excitatory period was observed for most phasic-tonically and tonically responding neurons (Fig. 2). In females, this inhibition period was always longest in response to PE (Fig. 2C). Differences in latency were observed between sexes and among the different stimuli. In males, the shortest latency was always to the 5VB stimulation, followed by the pheromone blend response (Fig. 2A,B). In females, both the pheromone blend and the 5VB response showed approximately the same latency (Fig. 2C,D). For both sexes, the PE stimulation always elicited the longest latency (Fig. 2). Two neurons in males showed an excitatory response to the pheromone blend, and an inhibitory response of the 5VB stimulus (example Fig. 2A). In these two neurons the PE response was also excitatory.

Neurons responding only to plant volatiles

Five neurons in each sex responded only to plant volatiles (supplementary material Tables S3 and S4) with similar response patterns to neurons responding to both pheromone and plant volatiles.

Response specificity of AL neurons

When stimulated with pheromone and plant volatiles, we found, for both sexes, pheromone specialist neurons (male, 39%; female, 13%), pheromone–plant generalist neurons (male, 42%; female, 64%) and

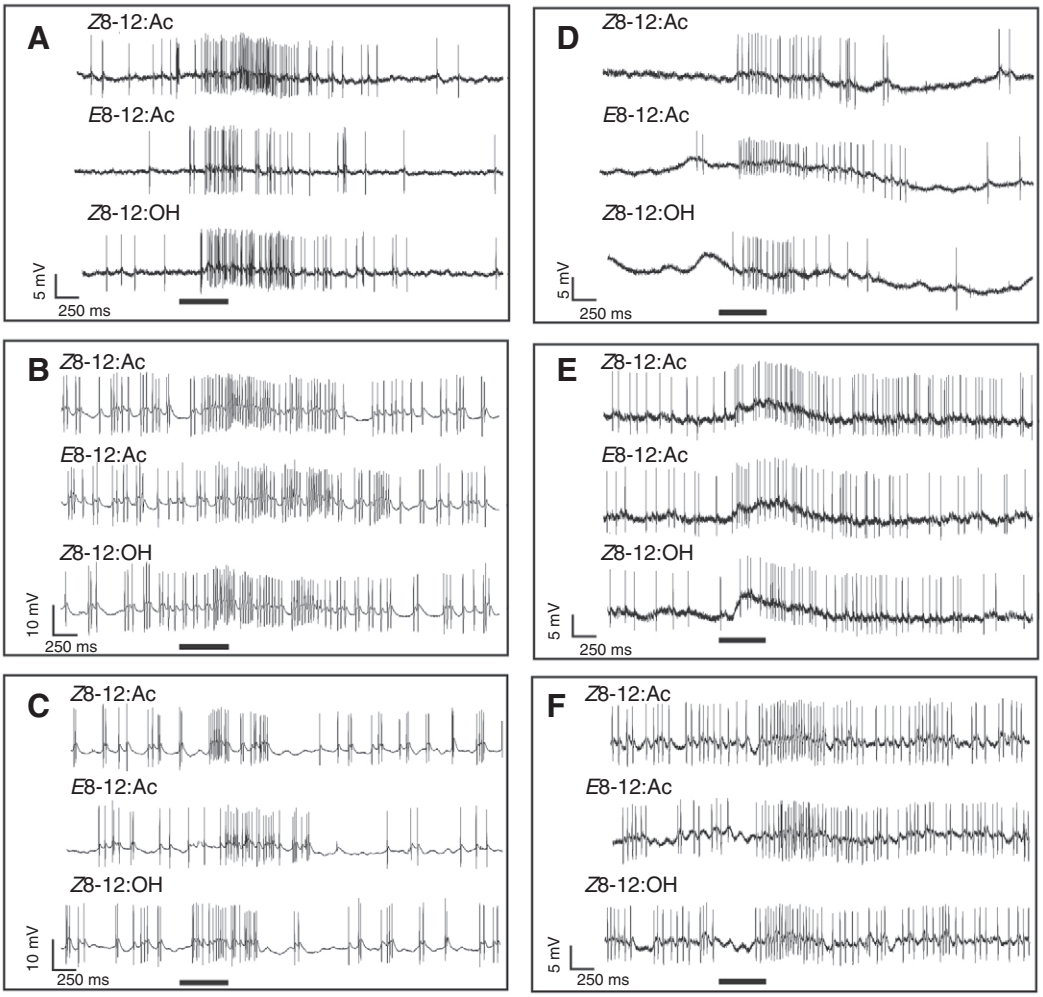

Fig. 1. Examples of the different response patterns found in antennal lobe neurons of *Grapholita molesta* males and females responding to the three individual pheromone components (*Z*8-12:Ac, *E*8-12:Ac and *Z*8-12:OH) at 1 ng. (A–C) Neurons in males. (D–F) Neurons in females. (A) Male neuron responding with the same latency to different pheromone components. (B,C) Male neurons responding with different latencies to different pheromone components. (D,E) Female neurons responding with short latency to all pheromone components. (F) Female neuron responding with long latency to all pheromone components. Horizontal dark bars indicate the time during which the stimulus was applied (500 ms).

plant specialist neurons (male, 19%; female, 23%; supplementary material Tables S3 and S4). The proportions of these different neuron classes were not significantly different between sexes (P=0.204).

Pheromone-specific neurons

This section includes all neurons that responded to the pheromone independently of whether they were also stimulated with plant volatiles. Pheromone-compound specialists (responded only to one pheromone compound), and generalist neurons (responded to two or three pheromone compounds) were found for both sexes (supplementary material Tables S1 to S4). A similar number of male generalist neurons responded to two or three compounds, whereas in females more neurons responded to all three compounds. This proportion was significantly affected by sex (P<0.001). In addition the proportion of the specialist neurons that responded to the major pheromone component, *Z*8-12:Ac, was larger in males (27 out of 30) than in females (4 out of 7; P=0.019; supplementary material Tables S1 and S3). For neurons in both sexes that were challenged with pheromone and plant volatiles, the probability of being pheromone-compound specialist or generalist was independent of whether the neuron also responded to plant volatiles (P=0.99).

Plant-odour-specific neurons

This section includes the neurons that, when exposed to pheromone and plant volatiles, responded to at least one plant volatile. Of the neurons that responded to plant volatiles, 43% in males and 47% in females were plant-volatile specialists (responding only to PE or only to 5VB; supplementary material Tables S3 and S4). Approximately half of these neurons responded to 5VB or to PE in both sexes. The proportion of both plant specialists and 5VB- or PE-specialist neurons were independent of sex (P=0.71 and P=0.49, respectively).

Response threshold and dose–response characteristics

For all pheromone components, a high percentage of recorded neurons had a response threshold at the lowest dose tested (0.001 ng), thus showing a high sensitivity (supplementary material Tables S1 and S2). In males, most of the recorded neurons that responded to more than one component (20 out of 63) showed the lowest threshold to the main pheromone component (*Z*8-12:Ac; supplementary material Table S1). A large proportion of the neurons had a high response threshold (100 ng) to *Z*8-12:OH (supplementary material Tables S1 and S2).

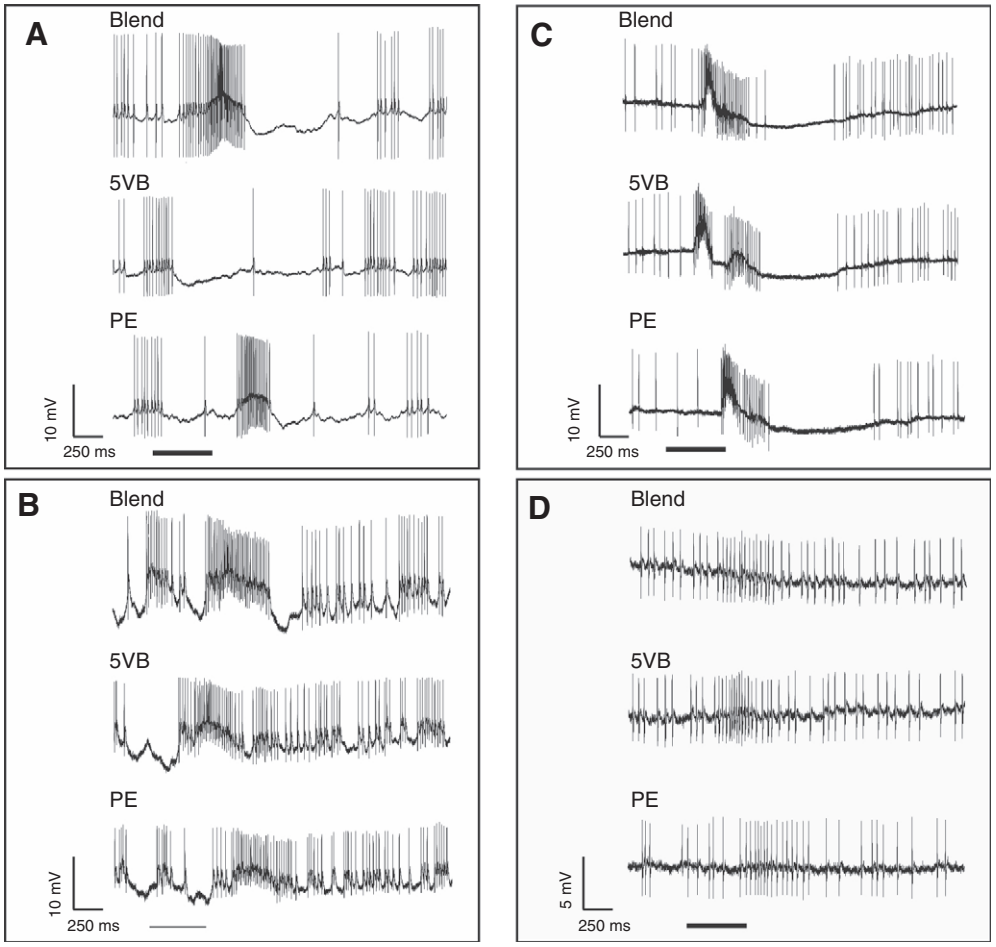

Fig. 2. Examples of the different response patterns found in antennal lobe neurons of *Grapholita molesta* males and females responding to the pheromone blend (blend: *Z*8-12:Ac, *E*8-12:Ac and *Z*8-12:OH at a ratio of 100:6:10) at 1 ng, to a blend of five peach volatiles (5VB) and to ethyl-(*E*,*Z*)-2,4-decadienoate (pear ester; PE), both at 1000 ng. (A,B) Neurons in males. (C,D) Neurons in females. (A) Male neuron responding with different patterns to the different stimuli. (B) Male neuron responding with similar patterns, but different latencies to the different stimuli. (C) Female neuron responding with an inhibitory phase after stimulation with all stimuli. (D) Female neuron responding without an inhibitory phase to all stimuli. Horizontal dark bars indicate the time during which the stimulus was applied (500 ms).

Dose–response relationships varied among neurons and within the same neuron for different components, but were similar in males and females (for examples of dose–response relationships see supplementary material Fig. S1). The most common dose–response relationship for the main pheromone component, *Z*8-12:Ac, consisted of an increase in the number of spikes with increasing doses (Fig. 3A). In males the same pattern was observed for the minor component *Z*8-12:OH, however, in females, the increase in spike response to this compound was followed by a decrease at higher doses (Fig. 3C). This last relationship was also observed for the other minor component *E*8-12:Ac, in both sexes, until a maximum of 0.1 or 1.0 ng followed by a decrease of the spike number at higher doses (Fig. 3B).

Anatomical characteristics

In males, 21 out of 32 preparations were successfully stained. Five preparations were classified as projection neurons (PNs; Fig. 4A–C), three as local neurons (LNs), four as protocerebral (PC) neurons, and in nine multiple neurons were stained, all using Neurobiotin. Preparations with more than one neuron type stained were not further analyzed; however, none of the neurons arborized within the cumulus (Cu; data not shown). We will not further describe LNs and PC neurons in the present paper.

In females, only two preparations were successfully stained out of 19 attempts. Both preparations were classified as PNs (Fig. 4D,E). In all seven PN preparations from males and females, only uniglomerular arborizations were observed. All anatomically characterized PNs showed a response to 1 ng of the sex pheromone blend (Fig. 4, supplementary material Tables S1 and S3).

In males, PN arborizations targeted three different glomeruli, all located in the most posterior area of the AL, and identified as glomeruli 31, 42 and 35 (Fig. 4A–C). The incompletely stained PN that arborized in glomerulus 31 responded to all three pheromone components (Fig. 4A, no. 36). The PN that branched in glomerulus 42 was also incompletely stained and responded only to the main pheromone component (*Z*8-12:Ac; Fig. 4B, no. 21). From the three stained PNs that branched in glomerulus 35, one preparation showed the axon leaving the AL *via* the medial antenno-cerebral tract (mACT) and projecting into the calyces of the MB and the LPC (not shown), and this neuron responded only to the main pheromone component (*Z*8-12:Ac; no. 22 in Fig. 4C). The other two stainings were incomplete and the neurons responded to all three pheromone components at the lowest dose tested and to the 5VB, respectively (nos 64 and 86 in Fig. 4C). None of the stained pheromone-responding PNs branched in the large glomerulus situated at the entrance of the antennal nerve (AN), the Cu.

In females, the two PN stainings revealed two different glomeruli, identified as 35 and 44 (Fig. 4D,E); glomerulus 44 being the female homologue of the Cu in males. The axon leaving the AL was identified in both cases, and in one preparation it left the AL *via* the inner antenno-cerebral tract (iACT), but no staining was visible in the PC (no. 6 in Fig. 4D). In the second brain, the axon left the AL through the medial antenno-cerebral tract (mACT) and had arborizations in the MBs (no. 7 in Fig. 4E). Both stained neurons responded to all three pheromone components at 0.001 ng.

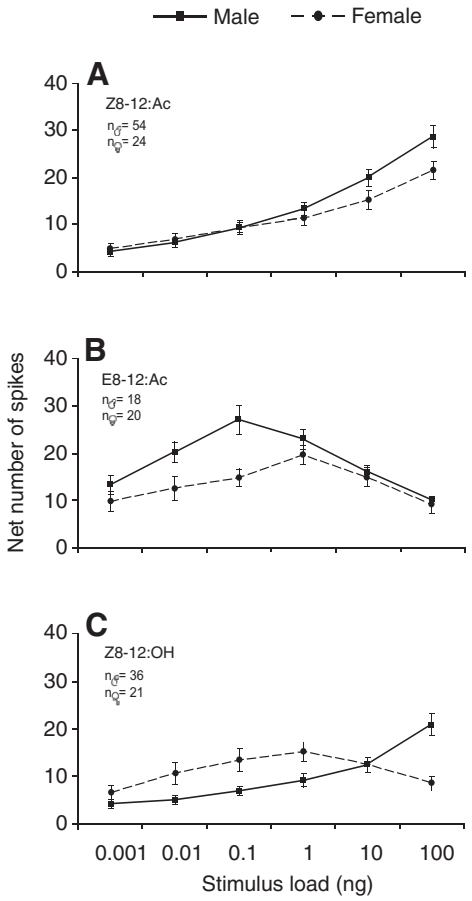

Fig. 3. Average dose–response curves for the most common curve shapes, obtained for antennal lobe neurons in both sexes of *Grapholita molesta* in response to the three pheromone components. Values are means ± standard deviation. (A) Increasing spike numbers with increasing doses. (B) Bell-shaped curve with increasing responses at lower doses and decreasing responses at higher doses in both sexes. (C) Increasing spike numbers with increasing doses in males and bell-shaped curve in females.

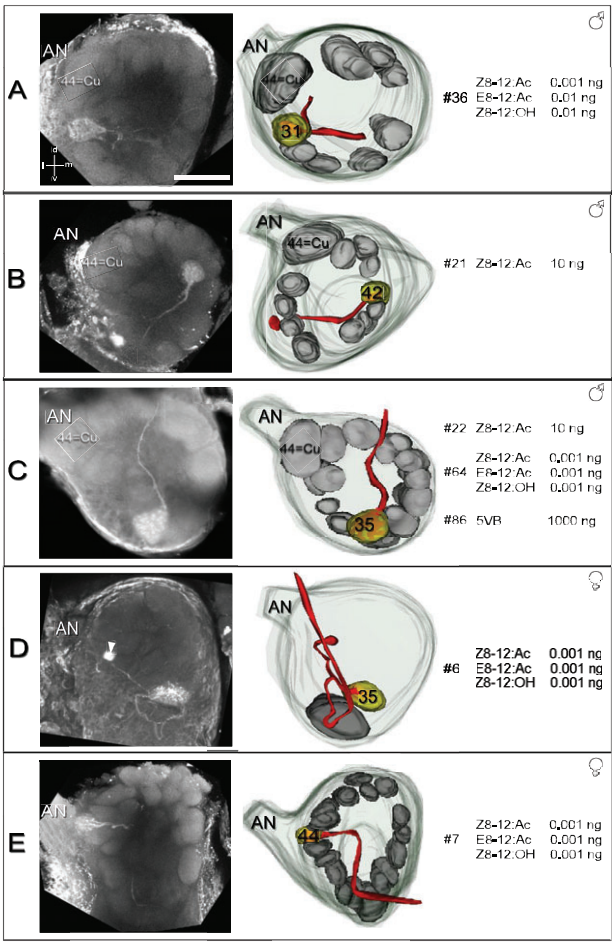

Fig. 4. Morphological and physiological characteristics of projection neurons stained with Neurobiotin and Lucifer Yellow. (A–C) Male and (D–E) female maximum projections of optical sections through the antennal lobe, showing the stained PN with arborizations in one individual glomerulus and the cell body (arrowhead in D). On the right of each panel is a 3-D reconstruction of the AL with the target glomerulus in yellow and the stained neuron in red. The pheromone components and threshold doses, to which the neuron responded are also given. AN, antennal nerve; CB, cell body cluster; Cu, cumulus; d, dorsal; l, lateral; m, medial; v, ventral. Scale bar, 50 μm.

DISCUSSION

We have described the responses of AL neurons in the tortricid moth *G. molesta* to sex pheromone components and plant volatiles. Although we cannot exclude that some of the anatomically unidentified neurons are PC neurons, we assume that the majority of the characterized neurons are AL neurons. Adults of both sexes are able to detect and respond to the pheromone emitted by the females, a feature that is rarely reported for moth species. Many characteristics of *G. molesta* AL neurons are similar to what has been found in other moth species. Response patterns and dose–response relationships were, however, highly variable between neurons and even within individual neurons, to a larger extent than usually described (e.g. Christensen and Hildebrand, 1987; Christensen et al., 1995; Jarriault et al., 2009). The most striking difference, when compared with what is known, is that pheromone and plant-odour processing pathways seem to be less separated in *G. molesta* than in other moths.

Odour quality coding

Neurons responding only to one, to two or all three pheromone components were recorded in the present study. We also found neurons only responding or not responding to the pheromone blend. All these types of neurons have been described before as specialists, generalists and blend specialists, respectively (e.g. Anton and Hansson, 1994; Anton et al., 1997; Christensen et al., 1991; Heinbockel et al., 2004; Wu et al., 1996). Male specialist and generalist (responding to three pheromone components) neurons tuned to the main pheromone component were in approximately equal proportions, but in females, generalist neurons were more abundant. In the sex pheromone of *G. molesta* the main component – *Z*8-12:Ac – is present at a much higher concentration than the minor ones, therefore when over 90% of the studied neurons showed a response to *Z*8-12:Ac, for both sexes, and almost one third of the male neurons responded specifically to it, the essential role of *Z*8-12:Ac in the sexual communication of this species was corroborated. A similar correlation between behavioural and physiological data has been described in male *Agrotis ipsilon*, where the majority of AL neurons respond to the major pheromone component (Jarriault et al., 2009).

For females it might be that individual components are less important than for males. In males, information on *Z*8-12:Ac might

indicate the presence of females, even if all three components are needed for male orientation and upwind flight (Charlton and Cardé, 1981; Sanders and Lucuik, 1996). In females, broadly tuned neurons might be sufficient to indicate the presence of other females in the neighbourhood. However, the importance of the main component has been shown in females of other insect species (Groot et al., 2005; Ochieng et al., 1995; Schneider et al., 1998).

Most neurons that responded to plant volatiles also responded to pheromone components and in this case often to all of them. This demonstrates that pheromone and plant volatile processing is not completely separate in the AL of *G. molesta*. The high percentage of AL neurons responding to 5VB underlines its importance in guiding host-finding behaviour not only in females (Natale et al., 2004; Piñero and Dorn, 2007), but also in males (N.V., personal observation). Fewer neurons responding to PE might be due to the fact that pears are only a secondary host plant for *G. molesta,* unlike its related species *C. pomonella* (Knight et al., 2005).

Intensity coding

Central olfactory neurons recorded in this study showed a high variability in their dose–response relationships. This diversity might reflect the distinct functional roles of the neurons in different behavioural contexts or different environmental conditions (Christensen et al., 2000). In our study, individual neurons did not necessarily respond equally to different components, nor did responses to the same component elicit the same dose–response curves in different neurons. In ~70% of the recorded neurons spike frequency increased with higher doses for Z8-12:Ac and *Z*8-12:OH in males, showing that the level of saturation was not reached, which would indicate a large dynamic response range. Increasing spike frequencies with increasing amounts of pheromone have been observed before in AL neurons in other moth species (Anton et al., 1997; Hartlieb et al., 1997; Heinbockel et al., 2004). Most *G. molesta* neurons in both males and females had an equally high sensitivity, which is different from findings in other moth species. In the noctuid moth *Spodoptera littoralis* it was proposed that the behavioural sensitivity of females to their own sex pheromone is lower than in males, because there were fewer female ORNs sensitive to the sex pheromone, even though they are as sensitive in females as in males (Ljungberg et al., 1993). A study of AL neurons in female *S. littoralis* revealed similar sensitivity of ORN and AL neurons to sex pheromone (Anton and Hansson, 1994), whereas *S. littoralis* male AL neurons are much more sensitive than ORNs because of high convergence (e.g. Anton and Hansson, 1995; Hansson and Christensen, 1999).

Temporal coding

The response pattern observed in a given recorded neuron was often the same for the different pheromone components and for all tested doses. However, in some male neurons latency differences were observed, suggesting that the temporal characteristics of a single neuron can give information about the received component (Jarriault et al., 2009). This phenomenon occurred mostly in high frequency responses. PNs within the MGC that respond to pheromone stimuli with this response pattern have been suggested to code for quality and stimulus intensity and to be able to report the onset of the stimulation but not its end (Friedrich and Stopfer, 2001; Laurent et al., 2001). *G. molesta* ORNs can resolve 20-ms pulses, at three pulses per second (Baker et al., 1988). With their long-lasting responses most *G. molesta* neurons seem not to have the ability to transmit rapid changes to higher brain centres, as short excitatory responses have been reported to do (Vickers et al., 2001).

Structure–function correlations

Our study shows that PNs responding to the pheromone components of *G. molesta* had only uniglomerular branching patterns in ordinary glomeruli. Until now, the functional organization, based on anatomical observations of ORNs in male Lepidoptera, has suggested that the number of MGC compartments is correlated to the number of behaviourally active pheromone components (Hansson et al., 1991). Also AL PNs, recorded in male moths and responding to the pheromone components, innervate one or a few glomeruli of the MGC (Heinbockel et al., 1999; Jarriault et al., 2009; Vickers et al., 1998). Nonetheless, neurons responding to pheromone and arborizing in ordinary glomeruli, have been found before (Anton and Hansson, 1995; Kanzaki et al., 1989), showing that the processing of pheromones and plant volatiles is not completely separate in moths. For *G. molesta* males, all stainings of PNs responding to pheromone components revealed ordinary glomeruli. These glomeruli were located in the posterior area of the AL and were identified, using an available AL map (Varela et al., 2009), but are unlikely to form part of a, so far incompletely described, MGC because of their location far from the Cu. Generally it is believed that the number of axons from an ORN population in the antenna is correlated with the size of glomeruli in the AL. Thus, the Cu of male *G. molesta*, being by far the largest glomerulus within the AL, most likely receives a large number of ORN axons. We therefore expected the large Cu of the *G. molesta* male AL to process pheromone information. However, we do not yet know its functional role, and additional studies are needed to clarify its function.

In females, in contrast, one of the stained neurons arborized within glomerulus 44, which is located close to the entrance of the AN and has been identified as the homologue of the male Cu of *G. molesta* ALs (Varela et al., 2009). In females of *S. littoralis*, glomeruli located close to the entrance of the AN also house arborizations of pheromone-sensitive ORNs and PNs (Anton and Hansson, 1994; Ochieng et al., 1995). Glomerulus 35 has been stained in both sexes for PNs responding to the sex pheromone (male and female) or to 5VB (male). This demonstrates that the processing for pheromone and plant volatiles is happening in the same ordinary glomerulus in both sexes.

Conclusions

Although most of our results confirm that *G. molesta* follows central odour processing principles, which are common to different moth species, the AL shows some exceptional features, such as the representation of pheromone information in ordinary rather than in the MGC glomeruli. With our relatively limited dataset, the absence of pheromone response in the Cu might be partially because of the technically difficult recordings in this very small species. However, the lack of arborizations of pheromone-responding neurons in the Cu of the MGC is unusual. This study also shows that pheromone and plant volatile processing are not entirely separated in both sexes of this species and will serve as a starting point for investigations of the neural basis for the well-described mating- and experience-dependent plasticity of pheromone and plant-odour-guided behaviour in this species.

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LIST OF ABBREVIATIONS

5VB	five plant volatile blend
AL	antennal lobe
AN	antennal nerve
Cu	cumulus
*E*8-12:Ac	(*E*)-8 dodecenyl acetate
iACT	inner antenno-cerebral tract
LN	local neuron
LPC	lateral protocerebrum
mACT	medial antenno-cerebral tract
MB	mushroom body
MGC	macroglomerular complex
ORN	olfactory receptor neuron
PC	protocerebrum
PE	pear ester
PN	projection neuron
*Z*8-12:Ac	(*Z*)-8 dodecenyl acetate
*Z*8-12:OH	(*Z*)-8 dodecen-1-ol

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