Pheromone Modulates Plant Odor Responses in the Antennal Lobe of a Moth

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Abstract

In nature, male moths are exposed to a complex plant odorant environment when they fly upwind to a sex pheromone source in their search for mates. Plant odors have been shown to affect responses to pheromone at various levels but how does pheromone affects plant odor perception? We recorded responses from neurons within the non-pheromonal “ordinary glomeruli” of the primary olfactory center, the antennal lobe (AL), to single and pulsed stimulations with the plant odorant heptanal, the pheromone, and their mixture in the male moth Agrotis ipsilon. We identified 3 physiological types of neurons according to their activity patterns combining excitatory and inhibitory phases. Both local and projection neurons were identified in each physiological type. Neurons with excitatory responses to heptanal responded also frequently to the pheromone and showed additive responses to the mixture. Moreover, the neuron’s ability of resolving successive pulses generally improved with the mixture. Only some neurons with combined excitatory/inhibitory, or purely inhibitory responses to heptanal, also responded to the pheromone. Although individual mixture responses were not significantly different from heptanal responses in these neurons, pulse resolution was improved with the mixture as compared with heptanal alone. These results demonstrate that the pheromone and the general odorant subsystems interact more intensely in the moth AL than previously thought.

Key words: Agrotis ipsilon, extracellular recordings, intracellular recordings, mixture interaction, odor pulses, olfaction

Introduction

To accomplish crucial behaviors necessary for their survival, animals face the challenge to extract relevant sensory cues in a complex and changing environment. To recognize and locate their sexual partner, male moths use the sex pheromone released by females. The ability of the male to detect this pheromone from a long distance and generate upwind flight behavior following the intermittent pheromone plume all along to the calling female despite the presence of variable and more concentrated plant volatiles is decisive for reproduction (Rouyar et al. 2011 and references therein).

Moreover, these plant volatiles can be of behavioral relevance in other contexts as moths rely on olfaction for feeding (both sexes) and oviposition (females, but males could use this information as supplementary cue to find females) and therefore must be able to gather information on these plant volatiles also. In different moth species, including the noctuid Agrotis ipsilon, synergistic effects of plant volatiles added to low pheromone doses have been observed (e.g., Light et al. 1993; Barrozo et al. 2010; Reddy and Guerrero 2004; Schmidt-Büßer et al. 2009; Varela et al. 2011), and several
attempts have been made to identify the level in the sensory pathway at which pheromone–plant odor interactions occur. The olfactory system of the male moth is highly specialized to accomplish the specific and sensitive detection of airborne signals necessary to odor-guided behaviors. Specific pheromone-olfactory receptor neurons (Ph-ORNs) on the antennae are narrowly tuned to each component of the pheromone blend (Todd and Baker 1999 and references therein). Their axons project to specific and enlarged glomeruli in the male-specific pheromone processing center, the macroglomerular complex (MGC) in the antennal lobe (AL), the primary olfactory center of insects. General odors and particularly plant volatiles are detected by other ORNs, which project to sexually isomorphic “ordinary” glomeruli (OG) in the AL. Within the AL, olfactory information is integrated and processed by a network of local interneurons (LNs) and projection neurons (PNs), which carry the information to higher brain centers (Anton and Homberg 1999).

Responses of the olfactory system to single odors including pheromones or plant volatiles have been analyzed in many insect species including moths (Visser and de Jong 1988; Galizia et al. 2000). The processing of blends of plant volatiles or blends of pheromone components is also described for the peripheral and central nervous system (Lei and Vickers 2008; Kuebler et al. 2012), but how mixtures of pheromones and plant volatiles are detected and processed in insects is less well understood. Studies in vertebrate ORNs have defined different types of mixture interactions as hypoadditivity, suppression, and additivity, and these terms apply also for insects (Duchamp-Viret et al. 2003; Deisig et al. 2006; Chaffiol et al. 2012; see also Rospars 2013). At the peripheral level, a study in Helicoverpa zea showed an enhancement of the response of Ph-ORNs to the pheromone in presence of plant odors (Ochieng et al. 2002). Most other studies in moths have, however, shown that Ph-ORNs respond less to the pheromone when plant volatiles are added (Spodoptera littoralis, Party et al. 2009; A. ipsilon, Deisig et al. 2012; Heliothis zirens, Hillier and Vickers 2011). A pioneer molecular study of peripheral pheromone–plant odor interactions in H. virescens indicated that plant odors interfere with the signaling process of the pheromone at the receptor level, reducing calcium release upon pheromone stimulation in a heterologous system expressing a H. virescens pheromone OR, rather than affecting binding to pheromone binding proteins (Pregitzer et al. 2012).

At the central level, both suppressive and additive interactions of pheromone and plant odors occur within the pheromone subsystem of different Lepidoptera species. In A. ipsilon, suppressive effects were recorded in the PNs of the MGC in response to mixtures, which largely originate from interactions at the peripheral level (Chaffiol et al. 2012; Deisig et al. 2012). In contrast, PNs of the MGC of Bombyx mori showed an enhanced response to pheromone in presence of the plant odorant Z3-hexenol (Namiki et al. 2008). Within the general odorant subsystem, results concerning the processing of mixtures of pheromone and plant odors are also contradictory among species, whereas A. ipsilon shows evidence for mixture interactions in OGs, B. mori does not. With divergent results from different moth species and uncertainty about their level of interaction (Namiki et al. 2008), how mixtures of pheromone and plant odorants can result in an additive effect at the behavioral level is far from being understood. Furthermore, the temporal aspects of the presentation of the odorants, an important parameter in the ecology of the moth, which encounters odorants in successive pulses due to odor plume discontinuity (Vickers et al. 2001), have not been taken into account in most of these studies.

In this study, we investigated how a mixture of a plant volatile and the sex pheromone is processed by the non-pheromonal subsystem, the OG of the AL in A. ipsilon Hufnagel (Lepidoptera: Noctuidae), a model insect, whose olfactory system has been investigated in detail. Moreover, the processing of a plant odorant–pheromone mixture has previously been studied in this species at 3 different levels—antenna, AL output, and behavior (Barrozo et al. 2010; Chaffiol et al. 2012; Deisig et al. 2012). Using extracellular single-unit recordings, or intracellular recordings followed by staining, we analyzed the responses of neurons of the OG to 1) the adult food plant volatile heptanal, 2) an artificial sex pheromone blend, and 3) the mixture of both, presented as single or pulsed stimulations to investigate different aspects of mixture coding.

**Materials and methods**

**Insect preparation**

Agrotis ipsilon larvae were reared on an artificial diet (Poitout and Bués 1974) and kept in individual plastic boxes at 23±1 °C and 50±5% relative humidity until pupation. Pupae were sexed, and males and females were kept in separate rooms under a reversed 16:8 h light: dark photoperiod at 23 °C. Newly emerged adults were separated daily and were provided with a 20% sucrose solution ad libitum. The day of emergence was considered as day 0. Experiments were performed during the scotophase between 10:00 h and 18:00 h on non-anesthetized virgin 5-day-old (sexually mature) males. They were mounted in a plastic pipette cone and the head fixed with dental wax. A small window was opened in the cuticle of the head capsule and the trachea, muscles, connective tissues, and neurolemma were removed. The preparation was then superfused with Tucson Ringer (Christensen and Hildebrand 1987).

**Olfactory stimulation**

Antennae were stimulated with heptanal, an artificial 3-component sexual pheromone blend, and the mixture of heptanal and the pheromone blend. Heptanal is a major...
component of linden flower extract, which is behaviorally attractive to *A. ipsilon* males (Wynne et al. 1991; Zhu et al. 1993) and evokes strong responses in antennal receptor neurons and central olfactory neurons in the AL (Greiner et al. 2002; Barrozo et al. 2010; Deisig et al. 2012). We used a synthetic sex pheromone blend of *A. ipsilon* consisting of a mixture of (Z)-7-dodecen-1-yl acetate, (Z)-9-tetradecean-1-yl acetate and (Z)-11-hexadecen-yl acetate in a ratio of 4:1:4 that has been shown to attract males (Picimbon et al. 1997; Gemeno and Haynes 1998). We used 100 µg of heptanal and 1 ng of the blend, prepared as in Jarriault et al. (2009), and the mixture of both as stimuli. These doses of heptanal and the pheromone have previously been shown to elicit clear responses in ORNs and AL neurons (Barrozo et al. 2010, 2011; Chaffiol et al. 2012; Deisig et al. 2012). Heptanal and the pheromone blend were dissolved in mineral oil and hexane, respectively, to obtain a concentration of 10 µg/µL of heptanal and 0.1 ng/µL of the pheromone blend. Mineral oil, hexane, and their mixture were used as control stimuli. Stimulation cartridges were prepared by loading a filter paper inserted in a Pasteur pipette with 10 µL of the respective odor solution. All stimuli diluted in hexane were used after a minimum evaporation time of 30 min. The amount of 100 µg heptanal corresponds to the behaviorally active dose attracting male *A. ipsilon* in a wind tunnel, similarly to a linden flower extract (Barrozo et al. 2010; Deisig et al. 2012).

Stimulation was controlled by a CS55 stimulation device (Syntech) with airflow compensation, as described previously (Jarriault et al. 2009). A continuous airflow of 0.3 m/s was blown over the antenna during the whole experiment. Stimuli were delivered for 200 ms at 7 mL/s, and consisted of either a single odor puff or a sequence of 5 successive odor puffs at 2 Hz, whereas part of the continuous airstream was removed to keep the mechanical component of the stimulation to a minimum. Individual odor stimulations were separated by interstimulus intervals of at least 10 s or until spontaneous activity was reestablished.

The experimental protocol consisted of recording responses of OG neurons starting with at least 1 s of spontaneous activity without any olfactory stimulation. Then controls (mineral oil, hexane, or mineral oil/hexane) were applied, followed by odor stimuli (heptanal, pheromone, and their mixture) in a random order. Finally, pulsed stimulations were applied with the 3 different stimuli. Every stimulation was replicated at least 2 times.

### Extracellular recordings

Some recordings were performed using glass microelectrodes filled with Tucson Ringer. Electrodes had a tip diameter of 3–6 µm and a resistance of around 5 MΩ, measured in the extracellular medium. Glass electrodes were gently inserted into the OG area of the AL until a clear spontaneous activity appeared. Signals were amplified using an AxoClamp-2B amplifier (Molecular Devices) and were acquired using an IDAC 2000 interface (Syntech). The extracellular activity of one or several neurons was monitored using Autospike software (Syntech). This method generally provided long and stable recordings, but it did not allow staining of the recorded neurons.

### Intracellular recordings and stainings

Recordings were performed according to standard methods (Christensen and Hildebrand 1987; Jarriault et al. 2009). Briefly, the tip of each glass microelectrode was filled with 4% Lucifer Yellow CH (Sigma-Aldrich) or with 2% Neurobiotin (Vector Laboratories) in distilled water, and the shaft was filled with 2 M LiCl. Electrode resistance, measured in the extracellular medium, ranged from 120 to 200 MΩ. The electrode was inserted into the OG area of the AL until intracellular contact with a neuron was established. As mentioned previously, electrical signals were amplified with an AxoClamp-2B amplifier, and the neuronal activity was monitored using Autospike software.

To anatomically identify recorded neurons, Lucifer Yellow CH or Neurobiotin was injected iontophotically using a constant hyperpolarizing current (0.8–1.0 nA) or a depolarizing current (0.3 nA), respectively, for about 3–10 min. After staining, the brains were dissected out of the head capsules and immersed in a buffered fixative solution. Brains injected with Lucifer Yellow CH were fixed in 4% formaldehyde solution for at least 12 h. Brains injected with Neurobiotin were fixed in 4% paraformaldehyde in Millonig's buffer for 4°C for 12 h, and then dehydrated and rehydrated in ethanol and propylene oxide. Neurobiotin was then visualized through incubation in Oregon Green-avidin (Oregon Green 488 conjugate A6374; Molecular Probes). Finally, brains were cleared and mounted in Vectashield medium (Vectashield H-1000; Vector Laboratories) or methyl salicylate. The brains were observed as whole mounts under a confocal microscope (SP2 AOBS; Leica) with a 10×0.40 dry objective using an argon/neon laser for excitation or a confocal microscope (Nikon A1) with a 20×0.75 water immersion objective. Image stacks (512×512 pixels) were analyzed by scrolling through optical sections (z step interval = 1–4 µm) to identify the dendritic arborization area. The obtained image stacks were observed, and partial z-projections were performed with Image J (National Institutes of Health) with maximal intensity parameter and Combine ZP (Image Stacking Software by Alan Hadley) using the pyramid maximum contrast, pyramid-weighted average or weighted average parameter.

### Data analysis

Extracellular recordings monitoring several neurons at the same time required spike-sorting analysis, which was performed by using the R-package SpikeOMatic (Pouzat et al. 2002). Only neurons responding to heptanal, that...
is, exceeding activity obtained with solvent stimulation alone by at least 120% (firing frequency for excited neurons or inhibition duration for inhibited neurons), were kept for further analysis. Once spike trains from extracellular and intracellular recordings were extracted and sorted, their analysis, statistics, and graphics were performed using Autospike (Syntech) and R (The R Foundation for Statistical Computing).

First, a qualitative analysis was performed for every recorded neuron. We defined 3 different physiological neuron types by pooling neurons according to their activity patterns when stimulated with heptanal. Second, we determined first whether these heptanal-sensitive neurons were altering their level of spike firing (i.e., exhibiting an activity pattern) during stimulation with pheromone and the mixture of heptanal and pheromone (see Table 1).

The responses of neurons with an initial excitatory activity pattern were quantified by measuring 4 parameters for all stimuli: response latency, response duration, plus mean and maximum spike frequencies. The response latency was determined by measuring the time between the onset of the stimulus (switch of the valve directing the airstream through the stimulation pipette) and the onset of the response. The onset of responses was defined by the time of occurrence of the spike followed by the shortest interspike interval within the detected excitatory response (Jarriault et al. 2009), and an initial inhibition was defined as a complete cessation of spiking for at least 100 ms. The response duration of either the excitatory phase or the inhibitory phase was measured. When the activity pattern consisted of more than a single phase (excitatory phase followed by inhibitory phase), the durations of the 2 phases were measured separately. For A-neurons (A1 and A2; for definition of neuron types, see Results), the mean and the maximum spiking frequency of excitatory phase were calculated. The maximum spiking frequency was analyzed during the first 50 ms following the response onset. Then, for each of the 3 controls, the mean spiking frequency was calculated during a time-window corresponding to the response duration of the corresponding odor stimuli. We were thus able to compare the mean excitatory phase frequency in response to odors and in controls. For neurons responding with inhibition, we systematically measured during 1 s the postinhibition mean spiking frequency.

Statistics

For each measured parameter, its mean value over all stimulations within the same physiological neuron type was calculated. We first compared activity pattern parameters for each of the 3 physiological neuron types (i.e., the mean excitatory phase frequency for A-neurons and the duration of the inhibitory phase for B-neurons) during stimulation with each odor and its associated solvent alone using paired 2-sided t-tests. Subsequently, responses to heptanal and the mixture were also compared within each physiological neuron type with a paired 2-sided t-test.

To analyze responses to pulsed stimulations, evolution curves of firing rate and smoothed autocorrelograms were calculated using the STAR package for R (Pouzat and Chaffiol 2009). A spectral analysis using a fast Fourier transform with graphical results shown as a power spectrum (spec. pgram function in stats library, R) was also performed in order to check the capacity of the neurons to generate a periodic response correlated with the temporal characteristics of the stimuli. Autocorrelograms were built from 2 to 3 trials of 5 consecutive stimulus puffs to compare the subsequent responses of the same neuron. The evolution of the firing frequency along successive stimulations was analyzed using a Friedman test. We compared the firing frequency between mixture and heptanal for each pulse using pairwise Wilcoxon rank sum tests.

Results

Response activity patterns to heptanal and classification into physiological neuron types

Intracellular and extracellular recordings were performed from OG neurons and analyzed quantitatively. As we observed the same activity patterns for extracellularly and intracellularly recorded neurons, all recordings were pooled (see Chaffiol et al. 2012). More than 100 neurons were recorded, and out of 49 heptanal-responding neurons analyzed and tested with the complete stimulus series (individual puffs of control stimuli C, heptanal, pheromone, and their mixture pheromone + heptanal), 18 were also tested with pulsed stimulations and 8 neurons were successfully stained. The activity patterns to heptanal allowed us to distinguish 3 different physiological neuron types denoted A1, A2, and B, based on the nature (initial excitation A or inhibition B) and number of phases in the response: A1-neurons being monophasic (tonic excitatory phase), A2 multiphasic (excitation followed by inhibition, excitatory phase/inhibitory phase), and B considered as monophasic (but as the inhibitory phase was often followed by a weak excitatory rebound, we also measured postinhibitory activity systematically). The numbers of neurons tested, of neurons exhibiting qualitative activity patterns and of responses found, as well as numbers of anatomical neuron types are given in Table 1 and illustrated in Figure 1, quantitative data of the pattern parameters are presented in Table 2, and statistical comparisons of activity patterns between stimuli are summarized in Tables 2 and 3 and illustrated in Figure 2. Recording examples and results of the analyses of neuron responses to pulsed stimulations are presented in Figures 3 and 4. The following subsections describe successively, the neurons’ responses to single stimuli (C, heptanal, and pheromone) and to the
mixture heptanal + pheromone, their ability to resolve periodic pulses, and finally the anatomy of the stained neurons.

**Response to single puffs with single odorants**

**Response to heptanal**

Different proportions of the 3 different recorded physiological neuron types were encountered in our recordings. Although stimulated with a 200-ms heptanal puff, the vast majority of the neurons (A-neurons, 36 neurons) displayed an excitatory response, whereas the remaining neurons were inhibited (B-neurons, 13 neurons; Table 1, Figure 1A). Almost half of all recorded neurons (23 neurons, type A1) exhibited a monophasic response pattern, consisting of a purely tonic excitatory response (excitatory phase). Thirteen neurons (type A2) exhibited a biphasic response pattern consisting of an excitatory phase followed by an inhibitory phase (excitatory phase/inhibitory phase). Figure 1A shows intracellular recording examples of neuron responses for the 3 physiological neuron types (A1, A2, and B: rows H), and Figure S1 shows extracellular raw recordings examples.

**Response to the sex pheromone**

Only A1-neurons displayed odor activity patterns significantly different from their solvent activity patterns while stimulated with pheromone (Figure 2 and Table 2). Indeed 17 out of 18 A1-neurons responded to the pheromone. The physiological neuron types A2 and B did not exhibit significant responses to the sex pheromone when comparing average values of the population of neurons, nevertheless a few individual neurons responded to the sex pheromone using the 120% above solvent criterion (5 neurons out of 8 for A2 and 5 neurons out of 12 for B; examples in Figure 1A).

**Response to single puffs of the mixture**

All physiological neuron types responded to the mixture, as illustrated by higher mean excitatory phase frequency for A-neurons, and longer inhibitory durations for B-neurons after mixture stimulation compared with control stimulation. We basically obtained the same 3 response patterns in the same proportions with the mixture pheromone + heptanal as with heptanal alone (Figure 1A, rows M). However, quantitative analysis revealed significant differences for both intensity and temporal parameters, for the different physiological neuron types as described in the two following sections.

**A-neurons**

The response latency was significantly shorter, and the maximum frequency of excitatory phase was significantly higher for the mixture than for heptanal alone in A1-neurons. In turn, the duration of the excitatory phase as well as its mean firing frequency was not different (Figure 2, A1). This suggests additive mixture interactions, at least for the onset of the response, while the more static part of the response was not affected.

The response latency of A2-neurons was significantly shorter for mixture than for heptanal (as found in A1-neurons), and the duration of the excitatory phase was significantly longer for the mixture than for heptanal. However, duration of the inhibitory phase, plus mean and maximum frequencies of the excitatory phase were not statistically different between heptanal and mixture responses (Table 2, A2; Figure 2, A2).

**Table 1** Number of neurons exhibiting activity patterns and odor responses for intra- and extra-cellular recordings and classification into 3 physiological neuron types

<table>
<thead>
<tr>
<th>Response type</th>
<th>Stimulus</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Total number of neurons with activity patterns</th>
<th>Total number of neurons with significant responses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LN</td>
<td>PN</td>
<td>Not stained</td>
<td>Intracellular</td>
</tr>
<tr>
<td>A1 (E)</td>
<td>H</td>
<td>1</td>
<td>2</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1</td>
<td>2</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>A2 (E/I)</td>
<td>H</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>B (I)</td>
<td>H</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

E, excitatory phase; H, heptanal; I, inhibitory phase; M, mixture of heptanal and pheromone; P, pheromone.

aThat is, activity different from spontaneous regime.
bUsing the 120% above solvent criterion, number of neurons displaying odor activity patterns significantly different from their solvent activity patterns.
A2-neurons showed complex mixture interactions; addition of pheromone affects the temporal patterns of the response, but not its intensity parameters.

B-neurons

B-neurons responded similarly to heptanal and the mixture at single stimulations (Figure 2B), thus exhibiting hypoadditive mixture interaction. In contrast to A-neurons, response parameters to mixture and heptanal were not statistically different in any case.

Response to pulsed stimulations

Typical responses of OG neurons to pulsed stimulation are shown in Figure 3 for each of the 3 physiological neuron types.
Pheromone Modulates Plant Odor Responses

Table 2  Response parameter values and statistics for the 3 physiological neuron types

<table>
<thead>
<tr>
<th>Types</th>
<th>Response parameter</th>
<th>H</th>
<th>P</th>
<th>M</th>
<th>H vs. M (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (E)</td>
<td>Latency</td>
<td>0.29±0.05</td>
<td>0.29±0.07</td>
<td>0.26±0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A1 (E)</td>
<td>E duration</td>
<td>0.39±0.42</td>
<td>0.25±0.21</td>
<td>0.46±0.44</td>
<td>0.118</td>
</tr>
<tr>
<td>A1 (E)</td>
<td>E mean freq</td>
<td>79±26</td>
<td>66±29</td>
<td>81±28</td>
<td>0.553</td>
</tr>
<tr>
<td>A1 (E)</td>
<td>E max freq</td>
<td>102±51</td>
<td>81±39</td>
<td>121±57</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>Latency</td>
<td>0.25±0.04</td>
<td>0.27±0.05</td>
<td>0.23±0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>E duration</td>
<td>0.22±0.09</td>
<td>0.20±0.10</td>
<td>0.25±0.12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>I duration</td>
<td>0.52±0.46</td>
<td>0.33±0.21</td>
<td>0.61±0.46</td>
<td>0.168</td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>E mean freq</td>
<td>91±27</td>
<td>61±14</td>
<td>89±25</td>
<td>0.520</td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>E max freq</td>
<td>121±34</td>
<td>80±21</td>
<td>129±35</td>
<td>0.210</td>
</tr>
<tr>
<td>B (l)</td>
<td>Latency</td>
<td>0.20±0.05</td>
<td>0.23±0.04</td>
<td>0.20±0.05</td>
<td>0.291</td>
</tr>
<tr>
<td>B (l)</td>
<td>I duration</td>
<td>0.51±0.22</td>
<td>0.33±0.16</td>
<td>0.57±0.21</td>
<td>0.141</td>
</tr>
<tr>
<td>B (l)</td>
<td>E mean freq</td>
<td>27±11</td>
<td>27±13</td>
<td>27±10</td>
<td>0.651</td>
</tr>
</tbody>
</table>

E, excitatory phase; H, heptanal; I, inhibitory phase; M, mixture of heptanal and pheromone; P, pheromone. Data are presented as mean ± standard deviation, phase durations are given in seconds, frequencies in spikes/second. Two-sided paired t-tests: significant P-values are denoted in bold letters (α = 0.05).

Table 3  Statistical significance of differences in the activity triggered by odor stimuli versus solvent controls in A (E frequency) and B-neurons (I duration)

<table>
<thead>
<tr>
<th>Neuron types</th>
<th>Nature of control stimulus</th>
<th>MO</th>
<th>Hex</th>
<th>MO/Hex</th>
<th>Statistic tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (E)</td>
<td>MO vs. H</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>A1 (E)</td>
<td>Hex vs. P</td>
<td>P &lt; 0.001</td>
<td>P = 0.081</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>A1 (E)</td>
<td>MO/Hex vs. M</td>
<td>P &lt; 0.01</td>
<td>P = 0.236</td>
<td>P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>MO vs. H</td>
<td></td>
<td></td>
<td></td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>A2 (E/l)</td>
<td>Hex vs. P</td>
<td></td>
<td></td>
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E, excitatory phase; H, heptanal; Hex, hexane; I, inhibitory phase; M, mixture of heptanal and pheromone; MO, mineral oil; MO/Hex, mixture of mineral oil and hexane; P, pheromone. Data are presented as mean ± standard deviation, frequencies are given in spikes/second, inhibitory phase durations in seconds. Two-sided paired t-tests: significant P-values (α = 0.05) are denoted in bold letters.

A1-neurons

In A1-neurons, resolution of a 2-Hz pulsed stimulation was clearly better for the mixture than for its components. Indeed these tonic excitatory neurons reached progressively higher spiking rates and a more robust periodic response during successive stimulations, as can be observed in the raster plots and the firing rate evolution curves (Figure 3A) as well as in the autocorrelogram for the shown neuron example (Figure 3B). With the mixture, the autocorrelogram revealed a more pronounced peak at time lag 0 s bounded by other peaks at time lag 0.5 s, compared with heptanal or pheromone, indicating the presence of stronger bursts of spikes at 2 Hz (Figure 3B). Mean power spectra (calculated from 8 A1-neurons) quantifying the periodicity and strength of the periodic components of the response differed for the 3 odors. Although almost no periodic response was detected for pheromone at the group scale, the strength of the periodic 2-Hz peak was noticeably higher with the mixture than with heptanal alone (Figure 4A, A1).

By analyzing the evolution of the firing frequency between successive heptanal or mixture responses, we found that A1-neurons generally displayed a strong response to the first odor puff followed by a clear adaptation during the next responses (Friedman test: χ² = 23.03, P < 0.001 for heptanal and χ² = 23.03, P < 0.001 for mixture). This adaptation was particularly visible for the transitions from the first to the second pulse (the spike frequency of the first pulse was on average twice as high as that of the second one), as shown in Figure 4B, A1 (N = 8). Except for the overall higher response frequency to the mixture than to heptanal (Wilcoxon test: P < 0.05 for pulse 1, P = ns for pulse 2, P < 0.05 for pulse 3, P < 0.05 for pulse 4, and P = ns for pulse 5), the 2 response adaptation curves have similar shapes.

A2-neurons

A2-neurons were better odor pulse followers than A1-neurons because they always displayed a postexcitatory inhibition...
phase after stimulation with each puff, as shown before for single puff stimulations (Figure 1A). This was demonstrated for heptanal and the mixture with clear periodic responses (Figure 3C). A2-neurons sometimes resolved mixture stimulation slightly better than heptanal stimulation as shown in the raster plots and firing rate curves (Figure 3C) and autocorrelograms (Figure 3D) in the example, but on average (N = 4), the periodicity and strength of the periodic components of their response for heptanal and the mixture were similar (see power spectra on Figure 4A, A2). Compared with A1-neurons, the strength of the periodic components detected by the power spectrum was much higher (because of the higher contrast between responses), and responses were more robust and similar when comparing heptanal and the mixture (Wilcoxon test: \( P = \text{ns} \) for all pulses) and less sensitive to adaptation (Friedman test: \( \chi^2 = 9.06, P = \text{ns} \) for heptanal and \( \chi^2 = 6.05, P = \text{ns} \) for mixture; Figure 4B, A2).

### B-neurons

B-neurons, whose responses to every odor start with an inhibition, should display a weak ability to follow pulsed stimulation. But noticeably, when neuron spontaneous activity was strong enough, a postinhibition firing activity could be observed before the next pulse response (4 out of 6 neurons), reaching a higher frequency than original spontaneous activity and allowing the neurons to follow 2-Hz pulses with the 3 odors as illustrated by the neuron example and shown in the autocorrelograms with a peak at time lag 0 s bounded by other peaks at time lag 0.5 s (Figure 3E, F). On average, a stronger periodic response was observed with the mixture than with heptanal alone as shown for B-neurons in Figure 4A (N = 6) as well as a higher postinhibition firing frequency tendency with the mixture compared with heptanal (Wilcoxon test, \( P = \text{ns} \) for all pulses), but their 2 response adaptation curves looked very similar (Figure 4B, B) with no sign of adaptation (Friedman test \( \chi^2 = 0.86, P = \text{ns} \) for heptanal and \( \chi^2 = 2.67, P = \text{ns} \) for the mixture).

### Anatomical characteristics of OG neurons

We attempted to stain intracellularly OG neurons recorded in 12 male moths, and 8 preparations were successful. These 8
neurons responded to heptanal and generally more weakly to pheromone and were identified as PNs or LNs. Five stained PNs (2 A1, 1 A2, and 2 B) arborized exclusively within 1 or several OGs and projected from the AL via the medial antenno-protocerebral tract to the calyces of the mushroom bodies and finally innervated the lateral protocerebrum (Figure 1B). The 3 stained LNs (1 A1, 1 A2, and 1 B) branched widely within different glomeruli, including the MGC (see examples for PNs and LNs in Figure 1B for each type). The A1- and A2-LNs had heterogeneous branching patterns, whereas the B-LN had a homogeneous branching pattern in OG with sparse branches in the MGC (Figure 1B). Because both PNs and LNs were found for each physiological neuron type, we cannot correlate anatomical neuron category (LN or PN and targeted glomeruli) with specific physiological neuron types.

Discussion

In natural conditions, the chemical information received by animals is often complex mixtures of volatiles that emanate from distinct ecological sources. Insects, depending on their internal state and needs, have to extract relevant features from these bouquets that will guide them for finding a mate or food sources. In this study, we describe how the responses from these bouquets that will guide them for finding a mate or food sources. In this study, we describe how the responses from these bouquets that will guide them for finding a mate or food sources. In this study, we describe how the responses from these bouquets that will guide them for finding a mate or food sources.

Figure 3  Typical response properties of OG neurons to pulsed stimulation according to their physiological type. Neurons were stimulated with 5 puffs of heptanal (H), pheromone (P), or a mixture of both (M) at 2 Hz. Each panel shows representative responses of 1 of the 3 neuron types. Raster plots of action potentials superimposed with discharge rate evolution curves (build with a 20-ms smoothed Gaussian kernel) are shown for all neurons (A, C, and E). Smoothed autocorrelograms (B, D, and F) show the temporal patterns of the spike discharges of the neuron response displayed on the raster plots. (A and B) A1-neurons resolved the 2-Hz pulsed stimulation better with M than with H alone, with higher spiking frequency for each pulse and a more robust periodic response, as can be observed in the raster plots and rate curves (A) and in the autocorrelogram (B). (C and D) A2-neurons, showing postexcitatory inhibitions after every pulse, are excellent neuron candidates for fine temporal tuning. They followed accurately H and M pulses but not P pulses. They adapted less than A1-neurons with increasing pulse numbers. They generally resolved H and M stimulations in a similar way, even if in this example, the M response is slightly stronger than with H especially after the second pulse, as shown in the raster plots, firing rate curves (C), and autocorrelograms (D). (E and F) B-neurons responding to every stimulation with pure inhibition, showed generally an ability to follow pulsed stimulation if they displayed a significant postinhibitory rebound, as in the shown example with all 3 odors. The gray bars indicate the stimulus presentation periods (duration: 200 ms, interstimulus interval: 300 ms). Boxed areas in A, C, and E are magnified in the panel underneath.

A large proportion of OG neurons responding to a specific plant odorant also respond to the sex pheromone

The most abundant physiological neuron type within OG neurons, responding with pure excitation to heptanal (A1), also responded significantly to the sex pheromone. Although no significant response to the pheromone was found at the population level for physiological neuron types A2 and B, some individual neurons responded nevertheless to the pheromone. Electrophysiological responses of OG neurons to sex pheromones have also been reported to occur occasionally in another noctuid moth species (Anton and Hansson 1995) and more frequently in males of 2 tortricid species (Trona et al. 2010; Varela et al. 2011). However, these responses have so far not been attributed to specific physiological neuron types, that is, LNs or PNs. Interestingly, general odorant ORNs in A. ipsilon do not respond to the pheromone (Deisig et al. 2012) at the dose that elicited responses in central OG neurons, suggesting that the pheromone-elicited activity in the OGs results from indirect input processing within the AL network.

Processing of the pheromone–heptanal mixture differs between the MGC and OG system and is species dependent

Detailed quantitative analysis of responses to mixtures of heptanal and the sex pheromone revealed additive interactions...
for AL neurons within OGs with excitatory response types. Inhibitory responses to heptanal in B-neurons, in turn, were not modified by adding the sex pheromone. These data confirm preliminary results of an earlier study in *A. ipsilon*, where synergy of heptanal and sex pheromone was detected in about 50% of the recorded OG neurons in virgin males (Barrozo et al. 2010).

With recently accumulated detailed knowledge on pheromone–heptanal mixture interactions in the peripheral and central olfactory system of *A. ipsilon*, both for the pheromone- and the plant odor-subsystems, we can now propose a simple model of peripheral (ORNs) and central (AL neurons) interactions between heptanal and the sex pheromone (Figure 5). At the peripheral level, plant odor-specific ORNs do not respond to the sex pheromone and their responses to heptanal are not modulated by the sex pheromone (Deisig et al. 2012). On the other hand, Ph-ORNs respond to heptanal alone at the tested doses, and responses to the sex pheromone are suppressed and/or masked by the plant odorant (Deisig et al. 2012; Figure 5). At the central level, a differential processing of the mixture exists among OG neurons within the AL (Barrozo et al. 2010; Deisig et al. 2012; this study), with a large proportion of neurons showing additive responses (Figure 5). These additive responses might originate from information transfer from the MGC through excitatory LNs or a disinhibitory pathway involving several LNs (Figure 5, Namiki et al. 2008). Our earlier work showed that pheromone responses of the most common physiological types of MGC neurons are strongly suppressed (longer latencies, lower spiking frequencies and shorter excitatory phases) when adding heptanal. Since heptanal is a partial agonist of pheromone at the Ph-ORN level, reduction in MGC neuron activity is partially due to peripheral mixture suppression, but LNs receiving input from plant odor glo-meruli might also enhance the suppression in the AL, possibly by inhibiting MGC PNs (Chaffiol et al. 2012 and Figure 5 in this work). Hence, pheromone–heptanal mixture processing is different in the pheromone-specific part of the AL.

Figure 4  Periodicity and evolution of responses to pulsed stimulation over time. (A) Periodicity and strength of neuron responses to pulsed stimulation displayed in power spectra. Each neuron-type panel represents 2 power spectra calculated from responses to the 2 different odors and quantifies the strength and frequency of the periodic components at the mean group level (for A1-neurons, N = 8; A2-neurons, N = 4, and B-neurons, N = 6). The strength of the heptanal (H) periodic peak can be modulated by the addition of pheromone to the mixture, even if the pheromone alone produced only a weak periodic response. Overall, the strongest periodic components were obtained with the A2-neuron type. A1- and B-neurons clearly showed a higher periodic 2-Hz peak in response to the mixture, compared with what was observed with heptanal alone, indicating an activity locked more closely to the frequency of the stimulation pulses with the mixture. For A2-neurons, the mixture did not elicit a stronger periodic response compared with heptanal alone. The small circle on each power spectrum represents the mixture peak detected automatically as a local maximum on the graph, values for this peak are given next to it (freq, frequency of the detected periodic component; power spectrum, strength of the periodic component). (B) Effect of periodic stimulations on the evolution of the maximum spiking frequency of responses for A1-, A2-, and B-neurons. A1-neurons maintained a higher spiking frequency with mixture (M) during all successive responses compared with H alone. Except the higher frequency, the 2 response curves are very similar, with the same adaptation characteristics. The adaptation phenomenon was particularly visible between the first and second pulses. A2-neuron response evolution is very similar for H and M, and globally A2-neurons were less sensitive to frequency adaptation than A1-neurons. B-neurons did not show adaptation to successive stimulation, and no significant difference was observed between H and M. P1–P5 successive stimulation pulses. The gray dashed horizontal lines on the 3 graphs correspond to the mean spontaneous activity.
and in the OG, even though we can at this point only speculate on the origin of these differences. Lateral interactions between the MGC and OG subsystems have been previously observed, for example, in Manduca sexta (Lei and Vickers 2008; Reisenman et al. 2008; Riffell et al. 2009). Excitatory LNs and a disinhibition pathway through multiple LNs have been described in the general odorant processing system of the fruitfly (Yaksi and Wilson 2010).

Finally, higher olfactory centers contribute further to the behavioral output upon pheromone-heptanal stimulation by integrating the outputs from the 2 subsystems (Figure 5). So far we know that linden essential oil, which contains large amounts of heptanal (Zhu et al. 1993), enhances behavioral responses to the sex pheromone in wind tunnel experiments in virgin A. ipsilon males (Barrozo et al. 2010). Heptanal itself triggers a moderate level of upwind flights in male A. ipsilon (Limousin D, Wycke MA, personal communication) and flight responses are raised when pheromone is added to heptanal, but not above the level of response triggered by pheromone alone. Scoring the percentage of oriented flights did not allow to determine whether males were increasing their response to heptanal as a food signal or rather responding to the sexual signal. However, the influence of sex pheromone on behavioral responses to heptanal can be evaluated in newly mated A. ipsilon males, which do not respond to the pheromone alone. In these newly mated males, responses to heptanal are suppressed when adding high doses of sex pheromone (Barrozo et al. 2010), indicating a strong salience of pheromone over heptanal.

It was shown in a recent study how sex and habitat chemosensory signals could be integrated as a blend in the AL of Cydia pomonella (Trona et al. 2013). Interestingly, the authors also found a synergy at the behavioral level, but correlated it with a synergy in MGC neurons and a mixture suppression in OG neurons, which is exactly the opposite of our observations in A. ipsilon. Similar asymmetric interactions have previously been described in B. mori, where the plant odorant (Z)3-hexen-1-ol enhances responses of pheromone-sensitive PNs within the MGC when added to the sex pheromone, but plant odor responses of OG PNs are not modified by the sex pheromone (Namiki et al. 2008). These results from different species indicate that behavioral responses to mixtures of intra- (i.e., sex pheromone) and inter- (i.e., plant volatile) specific signals might be due to their different processing by pheromonal and general odorant subsystems within the AL, independently from their further integration in higher olfactory centers (Clifford and Riffell 2013) and underline the importance of parallel processing of sensory information. Other subpopulations of neurons that do not display mixture interactions may be involved in more elemental processing and could be particularly important by contributing to maintain specificity between sexual and plant information (Hansson and Christensen 1999).

Processing of sensory information from different modalities along parallel pathways is a basic feature in the nervous system (Young 1998). However, little is known about parallel processing of different signals in the olfactory system. Recent studies in the honeybee show that 2 different tracts of AL PNs with similar input may extract differential features from 1 complex olfactory stimulus (Galizia and Rössler 2010; Sandoz 2011; Carcaud et al. 2012; Brill et al. 2013; Rössler and Brill 2013). The sex pheromone subsystem in moths is considered as a classical example of parallel olfactory systems because of the clear anatomical and functional segregation of the MGC from the remaining AL (Galizia and Rössler 2010). However, evidence is accumulating that MGC/OG subsystems are not fully segregated (Namiki et al. 2008; Barrozo et al. 2010; Chaffiol et al. 2012; Trona et al. 2013).

**Mixture stimulation improves pulse resolution for certain neuron types**

In natural conditions, olfactory stimuli are temporally and spatially dynamic (Riffell et al. 2008). Odor stimuli will occur with

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**Figure 5** Simple model of processing of the plant odor/pheromone mixture throughout the olfactory pathway. Although heptanal activates the Ph-ORNs by itself and suppresses responses in Ph-ORNs to the sex pheromone, plant odor ORN (Hep ORNs) responses to heptanal are not altered by the sex pheromone. Differential responses of PNs within the 2 subsystems of the AL to the mixture might originate from lateral interactions between the MGC and OG through different types of LNs. Although mixture interactions within the AL correlate partially with behavioral responses, processing in higher brain centers should contribute additionally to behavioral output. Arrowheads indicate flow of information; + excitatory input; − inhibitory input.
pulse intervals depending on wind conditions and the distance to the odor source. Mixtures of odors might, therefore, not only influence the qualitative and quantitative representations in the central nervous system but also alter coding of temporal features of the stimuli. The AL processing of the pheromone–heptanal mixture is particularly interesting because it offers the opportunity to address mixture effects on coding of the temporal structure of signals with different behavioral significances that have been shown to individually be sufficient to elicit an oriented flight. In MGC neurons, using the same stimulation protocols (Chaffiol et al. 2012), we found that the resolution of pulsed pheromone stimuli, which is crucial for oriented flight behavior in moths (Mafra-Neto and Cardé 1998; Vickers et al. 2001), was improved when adding heptanal due to its effects on latency, spiking frequency, and duration of the excitatory phases. In this study, heptanal-responding OG neurons with monophasic responses (A1- and B-neurons) also improved odor pulse resolution following pheromone addition, due to contrast enhancement, whereas A2-neurons with their post-excitatory inhibition resolved odor pulses already well with heptanal stimulation alone. This contrast between successive odor puff responses makes these neurons good candidates to follow complex fragmented odor plumes, as their typical On/Off behavior may guide action selection based on signaling presence or loss of the plant odor as it was recently proposed for typical MGC PNs (Martinez et al. 2013).

Conclusions

Our findings support the hypothesis that relevant mixtures composed by elements that may represent distinct ecological cues (like feeding, oviposition sites, or mating) are not processed by entirely segregated channels but by interacting parallel channels probably extracting different features of the stimuli. We have shown together with others that 2 different subsystems interact already in the primary olfactory center in moths more intensely than previously thought. How the information from these parallel pathways in interaction is read out in higher brain centers and translated into ecologically coherent behavior remains to be clarified. In natural conditions, different odor cues emanate from different sources and may even display different temporal features due to different microturbulences. In future studies, this will have to be taken into account in order to understand odor-guided behavior in moths in a complex environment. Also, different stimulus concentrations will have to be taken into account, as mixture interaction is concentration dependent (Rospars et al. 2008). Moreover, to clarify the contribution of LNs to the suppressive/additive effects in the AL, it will be necessary to pharmacologically modify their activity.

Supplementary material

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


