

## RESEARCH ARTICLE

# Plant odour stimuli reshape pheromonal representation in neurons of the antennal lobe macroglomerular complex of a male moth

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### SUMMARY

Male moths are confronted with complex odour mixtures in a natural environment when flying towards a female-emitted sex pheromone source. Whereas synergistic effects of sex pheromones and plant odours have been observed at the behavioural level, most investigations at the peripheral level have shown an inhibition of pheromone responses by plant volatiles, suggesting a potential role of the central nervous system in reshaping the peripheral information. We thus investigated the interactions between sex pheromone and a behaviourally active plant volatile, heptanal, and their effects on responses of neurons in the pheromone-processing centre of the antennal lobe, the macroglomerular complex, in the moth *Agrotis ipsilon*. Our results show that most of these pheromone-sensitive neurons responded to the plant odour. Most neurons responded to the pheromone with a multiphasic pattern and were anatomically identified as projection neurons. They responded either with excitation or pure inhibition to heptanal, and the response to the mixture pheromone + heptanal was generally weaker than to the pheromone alone, showing a suppressive effect of heptanal. However, these neurons responded with a better resolution to pulsed stimuli. The other neurons with either purely excitatory or inhibitory responses to all three stimuli did not exhibit significant differences in responses between stimuli. Although the suppression of the pheromone responses in AL neurons by the plant odour is counter-intuitive at first glance, the observed better resolution of pulsed stimuli is probably more important than high sensitivity to the localization of a calling female.

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Key words: olfaction, antennal lobe, mixture interaction, odour pulse, intracellular recording, extracellular recording.

### INTRODUCTION

In a natural environment, animals encounter complex sensory stimuli composed of signals from different origins. To respond to behaviourally relevant cues, they therefore have to analyze combined sensory stimuli, whose information content can be different from the mere sum of their components. This is especially true in olfaction, where animals constantly receive mixtures of different olfactory cues in their natural habitat: (1) environmental odours (e.g. food, habitat and shelter), which are themselves often complex combinations of various compounds, and (2) pheromones, also often multicomponent blends, which trigger intraspecific behavioural responses. Although interactions between components of the same class of odours have been relatively well studied, the reception and coding of mixtures from the two different odour classes represent a challenging problem. Insects, relying on olfaction for recognition and location of mates, food and host plants, are an ideal model system to study the reception, central processing and behavioural output of complex odour mixtures, because they have a relatively simple nervous system and stereotyped odour-guided behaviour.

In insects, odour information detected by antennal olfactory receptor neurons (ORNs) is integrated first in the antennal lobe (AL), which is divided into functional subunits, the olfactory glomeruli (Anton and Homberg, 1999; Rospars, 1988). There, ORNs make synaptic contact with intrinsic AL neurons, the local interneurons (LNs), and with AL output neurons, the projection neurons (PNs), which transmit processed information to higher-order brain centres (Anton and Homberg, 1999; Hansson and Christensen, 1999). The ALs of male moths consist of two olfactory subsystems: the macroglomerular complex (MGC) and the ordinary glomeruli (OG). The MGC processes sex pheromone information, whereas general odours, such as plant volatiles commonly used to locate food sources or host plants, are processed in OG (Koontz and Schneider, 1987; Hansson, 1995; Anton and Homberg, 1999).

Numerous studies analyzing how mixtures of odours within an odour class are perceived and/or processed at different neuronal levels have been performed in different insect and vertebrate species with electrophysiological and optical imaging methods. A widely used definition of interaction types arises from frog ORN recordings

(Duchamp-Viret et al., 2003; Rospars et al., 2008): hypoadditivity is described as a response to the mixture, which is equal to or a little lower than the response to the strongest component; a response to the mixture lower than the strongest component is defined as suppression, and synergism is defined as a response to the mixture that is stronger than the response to the strongest component (Duchamp-Viret et al., 2003). All three interaction types have been found in insects at different neuronal levels from the analysis of responses to mixtures of general odorants and mixtures of pheromone components [honeybee (Deisig et al., 2006); moths (Martin and Hildebrand, 2010; Kuebler et al., 2011; Hillier and Vickers, 2011); *Drosophila* (Silbering and Galizia, 2007)]. Most interactions of mixtures between plant odours and sex pheromones have been described at the behavioural and peripheral levels (reviewed by Reddy and Guerrero, 2004; Party et al., 2009; von Arx et al., 2011).

At the behavioural level, synergistic effects have been observed when adding plant odours to sub-optimal amounts of sex pheromone in many insect species, including male moths (e.g. Deng et al., 2004; Schmidt-Büsser et al., 2009; Barrozo et al., 2010; Varela et al., 2011a). Such synergistic interactions may reflect ecological advantages by regulating and mediating sexual communication in phytophagous insect species (Landolt, 1997). The neuronal basis leading to such odour mixture effects on insect behaviour might occur at the peripheral and/or the central nervous level.

Responses of sex-pheromone-specific ORNs to mixtures of pheromone and plant volatile compounds have been investigated in various insect species demonstrating different interaction types. Although a few examples of synergistic effects of plant odours added to sex pheromone have been reported (Ochieng et al., 2002; Plettner and Gries, 2010), the most frequently observed effect consisted of a suppression of the pheromonal response when a plant odour is added (Schneider et al., 1964; Den Otter et al., 1978; Kaissling et al., 1989; Pophof and van der Goes van Naters, 2002; Party et al., 2009).

In the AL, interactions of sex pheromone compounds with plant odours have only been described in a few moth species so far. In the silk moth, *Bombyx mori*, synergistic effects of plant compounds on pheromone responses in PNs of the pheromone-specific MGC have been described, whereas plant compound responses of neurons in the OG were not modified when adding the sex pheromone (Namiki et al., 2008). In virgin males of the noctuid moth *Agrotis ipsilon*, an increase of responses to the flower volatile heptanal was found in AL neurons of the OG when adding the sex pheromone (Barrozo et al., 2010). Most of these studies [except on the peripheral level (Party et al., 2009; Rouyar et al., 2011)] on mixtures of sex pheromone and plant odours were performed using individual stimulations of odours, thus revealing static aspects (e.g. synergy and inhibition) of odour mixture perception. However, in a natural environment, moths encounter odorants in short temporal succession because of odour plume discontinuity (Vickers et al., 2001), so these dynamic aspects may play an important role in odour mixture perception and have not been analyzed so far.

In our study, we asked three main questions: (1) do MGC neurons process only sex pheromone or can they also serve to process plant odours; (2) how are naturally occurring odour mixtures, which carry relevant information in different, but related behavioural contexts, processed in the MGC; and (3) what are the relative roles of the static and dynamic aspects of the stimulus and, in particular, how do MGC neurons resolve temporal patterns (pulsed stimulations) of odour mixtures *versus* individual stimuli of the two odour classes? More specifically, we studied how a behaviourally active plant

volatile, heptanal, applied in individual or pulsed stimulations, affects responses of neurons within the MGC to the species-specific sex pheromone blend in the noctuid moth *A. ipsilon*, which has a well-studied olfactory-guided behaviour and olfactory system [see Anton et al. (Anton et al., 2007) and references therein] (Barrozo et al., 2010). This is a crucial question in olfactory research, because the AL output delivers highly processed information, used for behavioural decision-making within higher brain centres. The MGC is specifically adapted to treat this question, because it provides massive output concerning a highly specific signal. By means of two complementary electrophysiological approaches, intracellular recordings followed by stainings of individual neurons, and extracellular single- and multi-unit recordings, we investigated the responses of neurons within the MGC to individual odour puffs and pulsed stimulation with the artificial sex pheromone blend, heptanal and their mixture. We discuss the behavioural and ecological relevance of the interactions between sex pheromones and plant odours observed within the AL.

## MATERIALS AND METHODS

### Insect preparation

Experiments were performed with the moth *Agrotis ipsilon* (Hufnagel 1766) (Lepidoptera: Noctuidae). The larvae were reared on an artificial diet (Poitout and Buès, 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 16h:8h 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 and 18:00h on non-anaesthetized virgin 5-day-old (sexually mature) males. They were mounted in a plastic pipette and the head was fixed with dental wax. The head capsule, tracheal sacs, muscles, connective tissues and neurolemma were removed. The preparation was then superfused with Tucson Ringer solution (Christensen and Hildebrand, 1987).

### Olfactory stimulation

The sexual pheromone emitted by female *A. ipsilon* consists of a mixture of (*Z*)-7-dodecen-1-yl acetate, (*Z*)-9-tetradecen-1-yl acetate and (*Z*)-11-hexadecen-yl acetate (Gemeno and Haynes, 1998; Picimbon et al., 1997). The males show the highest attraction to a mixture of the three components at a ratio of 4:1:4 (Causse et al., 1988). Heptanal, a component of linden flower extract, proved to be very attractive on the behavioural level and to evoke strong responses in electroantennographic and intracellular recordings in the AL (Wynne et al., 1991; Zhu et al., 1993; Greiner et al., 2002; Barrozo et al., 2011). Antennae were stimulated with the three-component pheromone blend, heptanal and a mixture of heptanal and the pheromone blend. Stimulation cartridges were prepared by loading a filter paper inserted in a Pasteur pipette with 10µl of the respective odour solution. The pheromone blend and heptanal were dissolved in hexane and mineral oil, respectively, which were used, together with a mixture of both solvents, as control stimuli. All stimuli diluted in hexane were used after a minimum evaporation time of 30 min. We used 1 ng of the blend prepared as in Jarriault et al. (Jarriault et al., 2009), 100µg of heptanal and a mixture of both as stimuli. These doses of the pheromone and heptanal had previously been shown to elicit clear responses in ORNs and AL neurons (Barrozo et al., 2010; Barrozo et al., 2011).

Stimulation was controlled by a CS55 stimulation device (Syntech, Kirchzarten, Germany) with air flow compensation, as

Table 1. Response patterns observed for 74 neurons and classification into four neuron profiles

Profile	Response pattern observed			Number of neurons		
	P	H	M (=P + H)	Intracellular	Extracellular	Total
A1	E1/I/(E2) (27)	E1/I (27)	E1/I/(E2) (27)	15	12	53
A2	E1/I/(E2) (26)	I (24) 0 (2)	E1/I/(E2) (26)	7	19	21
B	E1 (16)	E1 (12) 0 (4)	E1 (16)	5	11	21
C	I (5)	I (4) 0 (1)	I (5)	3	2	5

E1, first excitatory phase; E2, second excitatory phase; I, inhibitory phase; 0, no response; H, heptanal; M, mixture of pheromone and heptanal; P, pheromone. Numbers of neurons are in brackets.

described earlier (Jarriault et al., 2009). Briefly, a continuous air flow of  $0.3\text{ ms}^{-1}$  was blown over the antenna during the whole experiment. Stimuli were delivered for 200 ms at  $7\text{ ml s}^{-1}$ , and consisted of a single odour puff or a sequence of five successive odour puffs at 2 Hz, while part of the continuous airstream was removed to keep the mechanical component of the stimulation to a minimum. Individual odour stimulations were separated by interstimulus intervals of at least 30 s.

The experimental protocol consisted of recording responses of MGC neurons starting with 1 s of spontaneous activity without any olfactory stimulation. Then control stimuli (hexane, mineral oil or hexane/mineral oil) were applied before randomly testing odours (heptanal, pheromone and their mixture). Finally, pulsed stimulations were applied with the three different stimuli. A minimum of two repetitions for every single or pulsed odour stimulation was performed.

#### Extracellular recordings

Recordings were performed using two glass microelectrodes filled with Tucson Ringer solution. Electrodes had a tip diameter of 3–6  $\mu\text{m}$  and a resistance of approximately  $5\text{ M}\Omega$ , measured in the extracellular medium. Signals were amplified using an IDAC 2000 amplifier (Syntech). Glass electrodes were gently inserted into the MGC area of the AL until activity appeared on the two recording sites. The extracellular activity of one or several neurons was monitored using Autospike software (Syntech) and only neurons responding to pheromone stimulation were kept for further analysis.

#### 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 in distilled water (Sigma-Aldrich, Saint-Quentin Fallavier, France), and the shaft was filled with  $2\text{ mol l}^{-1}$  LiCl. Electrode resistance, measured in the extracellular medium, ranged from 120 to  $200\text{ M}\Omega$ . The electrode was inserted in the MGC region close to the antennal nerve until intracellular contact with a neuron was established. Electrical signals were amplified with an AxoClamp-2B (Molecular Devices, Sunnyvale, CA, USA). The neuronal activity was monitored using Autospike software (Syntech).

To anatomically identify recorded neurons, Lucifer Yellow CH was injected iontophoretically using a constant hyperpolarizing current (0.8–1.0 nA) for approximately 10 min. After staining, the brains were dissected out of the head capsules and immersed in a buffered 4% formaldehyde solution for at least 12 h, then washed in saline and incubated in mounting medium (Vectashield H-1000; Vector Laboratories, Burlingame, MA, USA) for tissue clearing. The cleared brains were mounted (whole-mount) and observed under a confocal microscope (SP2 AOBS, Leica, Heidelberg, Germany)

with a  $10\times 0.40$  dry objective using an argon/neon laser for excitation. Image stacks ( $1024\times 1024$  pixels) were analyzed by scrolling through optical sections ( $z$ -step interval =  $1\text{ }\mu\text{m}$ ) to identify the dendritic arborisation area. The obtained image stacks were observed and partial projections were performed with ImageJ (National Institutes of Health, Bethesda, MD, USA). Three-dimensional reconstructions of neurons were carried out with Amira 3.1 and a special skeleton plugin (Visage Imaging, Berlin, Germany).

#### 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 pheromone-responding neurons were kept for further analysis. Once spike trains from extracellular and intracellular recordings were acquired, spike train analysis and statistics were performed using R (R Foundation for Statistical Computing, Vienna, Austria).

Qualitative analysis was performed for every recorded neuron. We determined whether pheromone (P)-sensitive neurons were also responding to heptanal (H) and the mixture (M), and defined four different neuron response profiles by pooling neurons according to their shared response properties when stimulated with the three odours (see Table 1).

The quantitative analysis consisted of measuring the following parameters for all stimuli. The response latency was determined by measuring the time between the onset of the stimulus (switch of the valve to direct the airstream to the stimulation pipette) and the onset of the response. The onset of responses was defined by the shortest interspike interval of the visually detected excitatory response (Jarriault et al., 2009), and inhibition was defined as a complete cessation of spiking. The response duration [excitatory phase (E) or inhibitory phase (I)] was measured. When the response consisted of more than a single phase [i.e. excitation (E1) followed by inhibition (I), or excitation (E1) followed by inhibition (I) and a second excitation (E2)], the durations of the two first phases were measured separately. During E1, the mean spiking frequency was calculated. For multiphasic responses, the mean spiking frequency following the inhibition phase was also calculated during 1 s (see Results) in order to quantify the E2 phase. For the pheromone, heptanal and mixture stimulation, a mean value of all repetitions per neuron was calculated.

The obtained parameters for the different stimuli were compared within each of the four groups (the four neuron profiles, see Results) using repeated-measures ANOVA, and paired two-sided *t*-test with Holm–Bonferroni corrections were used as *post hoc* tests to compare between pairs of stimuli. Moreover, the E1 spiking frequency for each of the three controls was calculated during a 200 ms window in order to compare it to odour responses values.

Rate-evolution curves and smoothed autocorrelograms and cross-correlograms were calculated using the STAR package (Pouzat and

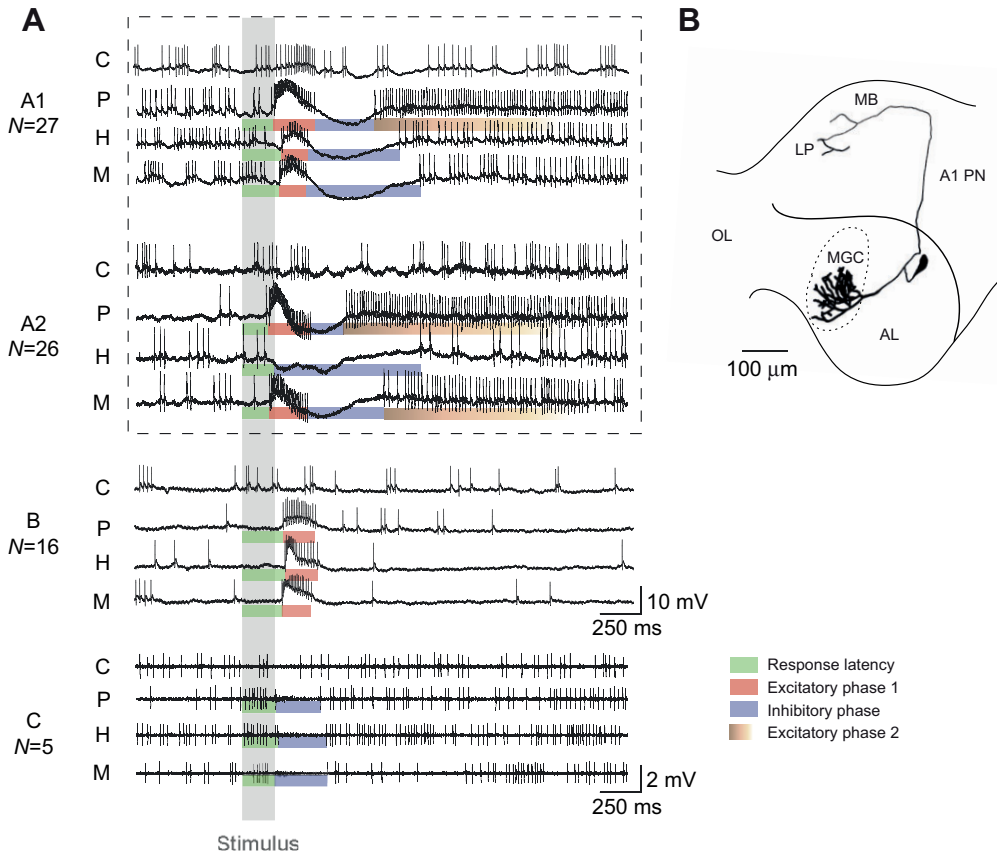


Fig. 1. Physiological responses and anatomy of macroglomerular complex (MGC) neurons in the noctuid moth *Agrotis ipsilon*. (A) Examples of original recording traces, showing four neuron profiles (A1, A2, B and C) in response to pheromone (P), heptanal (H), their mixture (M) and a control (C; hexane + mineral oil). Horizontal coloured bars over the traces show different parameters of the response measured: response latency, excitatory phases 1 and 2, and the inhibitory phase. Grey shading indicates the 200 ms stimulation period. *N*, number of recorded MGC neurons. (B) Anatomical reconstruction (partial Z-projection) of an A1 neuron with dendritic arborisations spreading within the MGC, the cell body and the axon projecting from the AL via the medial antenno-protocerebral tract (mAPT) to the mushroom bodies (MBs), and finally to the lateral horn of the protocerebrum. The black dashed line indicates the outline of the MGC region. AL, antennal lobe; LP, lateral protocerebrum; OL, optic lobe.

Chaffiol, 2009) for R. A spectral analysis using a fast Fourier transform with graphical results shown as smoothed periodograms (spec.pgram function in stats library in 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. Cross-correlograms were calculated in cases where two neurons were simultaneously recorded and autocorrelograms were carried out when comparing the subsequent responses of the same neuron. Both types of correlograms were built from two to three trials of five consecutive stimulus puffs. To check for a potential pulse resolution improvement in MGC neurons, we compared the contrast between responses to P and M on rate-evolution curves and autocorrelograms of all neurons tested with pulsed stimuli and quantified how many neurons per group showed a clear contrast enhancement.

**RESULTS**

Intracellular and extracellular recordings of MGC neurons were analyzed. Out of 65 pheromone-responding neurons recorded intracellularly, 30 were tested with the complete stimulus series (individual puffs of control stimuli C, pheromone P, heptanal H and their mixture M). Out of 120 neurons recorded extracellularly, 100 responded to the pheromone, but only 44 displaying the best signal-to-noise ratio were analyzed in order to avoid spike-sorting classification errors. In addition, 26 out of these 44 neurons were also tested with pulsed stimulations, and responses of four pairs of simultaneously recorded neurons were analyzed. As explained below, the response patterns to P allowed us to distinguish three different response profiles – denoted A, B and C – based on the nature and number of phases in the response, A being multiphasic (subsequently divided in A1 and A2 based on the response to H) and the two others monophasic (B, excitatory; C, inhibitory). The

number of neurons tested and qualitative response patterns found are given in Table 1 and illustrated in Fig. 1, the response parameters are quantified in Table 2, and their statistical comparisons are summarized in Tables 2 and 3 and illustrated in Fig. 2. The following subsections describe successively the anatomy of recorded neurons, their responses to single stimuli (C, P and H) and then to the mixture P + H, and finally their ability to resolve periodic pulses.

**Anatomical characteristics of MGC neurons**

We attempted to stain 18 out of 36 intracellularly recorded male MGC neurons, and eight preparations were successful. These eight neurons were responsive to P and H and were all identified as PNs: all stained neurons (five A1, one A2 and two B neurons) arborised exclusively within the MGC glomeruli and projected from the MGC via the medial antenno-protocerebral tract (mAPT) to the mushroom bodies and finally to the lateral horn of the protocerebrum (see example for an A1 neuron in Fig. 1B). No difference in the arborisation patterns in the MGC and projection areas between the eight stained neurons was found.

**Response to control stimulations and single stimuli**

We observed the same response patterns [E1/I/(E2), E1 or I] for extracellularly and intracellularly recorded neurons (Table 1). All recordings were pooled, resulting in a detailed analysis of 74 neurons.

**Response patterns to the pheromone**

The vast majority of the neurons (71%, 53 neurons, profile A) exhibited a multiphasic response pattern while stimulated with a 200 ms pheromone puff (Fig. 1A). This response consisted of an excitatory (E1) phase, followed by an inhibitory (I) and sometimes

Table 2. Response parameter values and results of comparisons between pairs of stimuli for the four neuron profiles

Profile	Response parameter	Odour stimulation			P-value		
		P	H	M	P vs H	H vs M	P vs M
A1	Latency (s)	0.22±0.04	0.31±0.06	0.25±0.07	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.05</b>
	E1 duration (s)	0.22±0.05	0.17±0.07	0.21±0.06	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.642
	I duration (s)	0.50±0.43	0.81±0.93	0.84±0.88	0.11	0.877	<b>&lt;0.01</b>
	E1 frequency (spikes s <sup>-1</sup> )	139±37	96±32	125±35	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.01</b>
A2	E2 frequency (spikes s <sup>-1</sup> )	38±21	22±15	27±19	<b>&lt;0.001</b>	<b>&lt;0.05</b>	<b>&lt;0.001</b>
	Latency (s)	0.23±0.04	0.35±0.09	0.29±0.06	<b>&lt;0.001</b>	<b>&lt;0.01</b>	<b>&lt;0.001</b>
	E1 duration (s)	0.24±0.06	–	0.19±0.06	–	–	<b>&lt;0.001</b>
	I duration (s)	0.38±0.14	0.68±0.44	0.70±0.38	<b>&lt;0.01</b>	0.707	<b>&lt;0.001</b>
B	E1 frequency (spikes s <sup>-1</sup> )	112±30	–	81±24	–	–	<b>&lt;0.001</b>
	E2 frequency (spikes s <sup>-1</sup> )	40±20	–	22±13	–	–	<b>&lt;0.001</b>
	Latency (s)	0.27±0.05	0.32±0.12	0.28±0.06	0.244	0.223	0.354
	E1 duration (s)	0.48±0.42	0.25±0.15	0.47±0.44	0.135	0.113	0.944
C	I duration (s)	–	–	–	–	–	–
	E1 frequency (spikes s <sup>-1</sup> )	71±38	56±32	73±36	0.079	<b>&lt;0.05</b>	0.835
	E2 frequency (spikes s <sup>-1</sup> )	–	–	–	–	–	–
	Latency (s)	0.23±0.03	0.23±0.06	0.23±0.06	0.725	0.781	0.931
	E1 duration (s)	–	–	–	–	–	–
	I duration (s)	0.44±0.18	0.42±0.29	0.38±0.22	0.873	0.53	0.592
	E1 frequency (spikes s <sup>-1</sup> )	–	–	–	–	–	–
	E2 frequency (spikes s <sup>-1</sup> )	–	–	–	–	–	–

E1, first excitatory phase; E2, second excitatory phase; I, inhibitory phase; H, heptanal; M, mixture of pheromone and heptanal; P, pheromone. Data are presented as means ± s.d.

Two-sided paired *t*-tests with Holm-Bonferroni correction; significant *P*-values are denoted in bold ( $\alpha=0.05$ ).

a second excitatory phase (E2), similar to what has been described before as a typical MGC PN response in *A. ipsilon* (Jarriault et al., 2009). Twenty-two percent (16 neurons, profile B) of the neurons exhibited a purely tonic excitatory response and 7% (five neurons, profile C) of the neurons exhibited a purely inhibitory response to the pheromone (Table 1, Fig. 1A, profiles B and C).

#### Response patterns to heptanal

Surprisingly, all MGC neurons (except seven) that responded to the pheromone also responded to heptanal (91%) whereas most neurons responded only weakly to the solvents alone (Fig. 1, Table 3). Among the 67 neurons responding to heptanal, different response patterns were observed: 42% (28 neurons) showed a purely inhibitory response, 40% (27 neurons) showed an excitatory response followed by an inhibitory phase, and 18% (12 neurons) responded with a tonic excitatory phase (Table 1, Fig. 1).

#### Response patterns to the mixture

We basically obtained the same response patterns and with the same proportions with the mixture as with the pheromone alone (Table 1, Fig. 1). However, quantitative analysis revealed significant differences, as described below (see Response to the mixture: effect of heptanal on pheromone responses of MGC neurons).

#### Control responses

While comparing controls (hexane, mineral oil and mixture of hexane and mineral oil) and respective odour responses, we found significantly higher E1 frequency responses to odours (independently of the odour used) than to control stimulations (Table 3, Figs 1, 2).

#### Classification into neuron response profiles

Because different response patterns to the pheromone (P) and heptanal (H) were obtained, neurons were subsequently classified into distinct profiles by simultaneously taking into account the responses to P and to H. All neurons within the same profile shared similar response patterns to the two stimuli. We classified the 74 neurons into four neuron profiles: A1, A2, B and C (Fig. 1). Neurons with profile A responded to P with a multiphasic pattern: excitation (E1), inhibition (I) and a second excitation (E2). We distinguished two subcategories in A, which differ in their response to heptanal: A1 neurons responded to H with an excitatory phase followed by an inhibitory phase, generally without a second excitatory phase (E1/I), whereas A2 neuron responses to H were purely inhibitory (Fig. 1). In contrast, profiles B and C corresponded to neurons with monophasic excitatory (profile B: E1) or inhibitory (profile C: I) responses for at least one of the two stimuli (Fig. 1). Only two A2,

Table 3. Comparisons between response frequencies of excitatory phase E1 and control stimuli

Profile	E1 frequency (spikes s <sup>-1</sup> )			P-value		
	Hex	MO	Hex-MO	Hex vs P	MO vs H	Hex-MO vs M
A1	77±33	63±35	65±34	<0.001	<0.001	<0.001
A2	43±24	35±27	35±23	<0.001	–	<0.001
B	33±21	33±22	37±25	<0.001	<0.05	<0.05
C	6±7	9±7	8±6	–	–	–

E1, first excitatory phase; H, heptanal; Hex, hexane; Hex-MO, mixture of hexane and mineral oil; M, mixture of pheromone and heptanal; MO, mineral oil; P, pheromone.

Data are presented as means ± s.d.

Two-sided paired *t*-tests with Holm-Bonferroni correction; all *P*-values are significant.

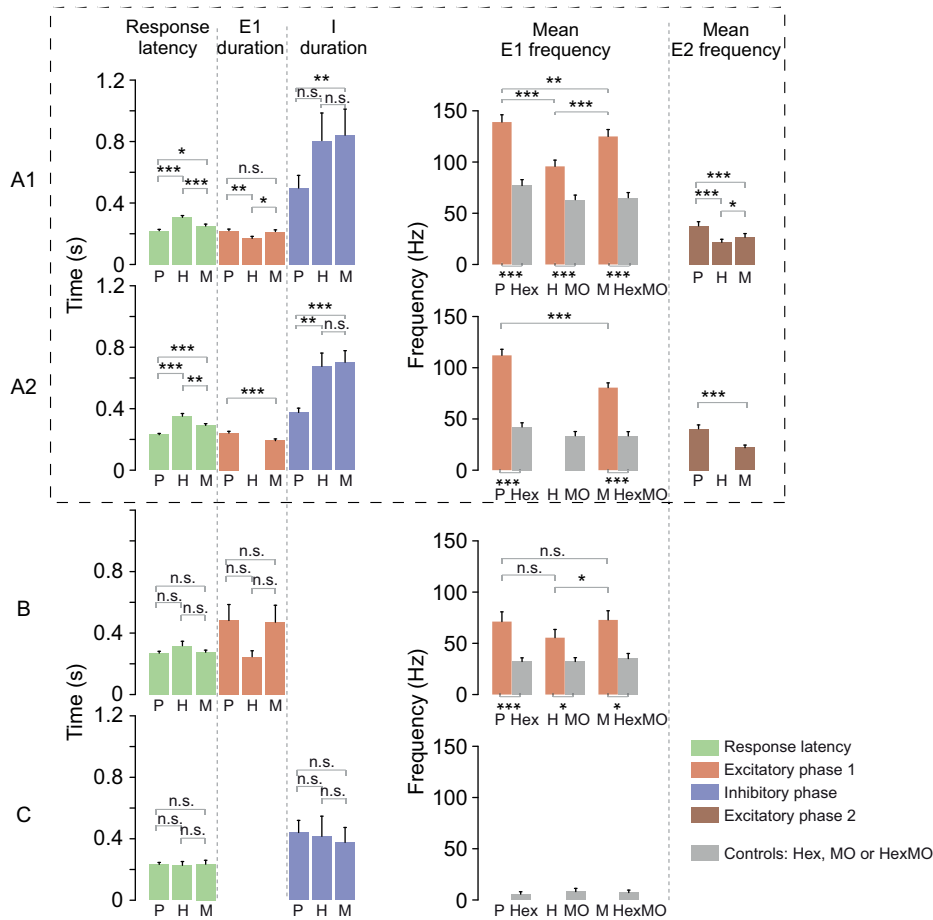


Fig. 2. Quantitative analysis of the response characteristics of MGC neurons to pheromone, heptanal and their mixture for the four response profiles (A1, A2, B and C) in *A. ipsilon*. Data are presented as means  $\pm$  s.e.m.; asterisks indicate significant differences between the groups (two-sided paired *t*-tests with Holm–Bonferroni correction; \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, n.s., not significant). P, pheromone; H, heptanal; M, mixture stimulations; controls: Hex, hexane; MO, mineral oil; HexMO, mixture of hexane and mineral oil.

four B and one C neuron did not respond to heptanal. The various response patterns are detailed in Table 1 and the number of neurons found in each of the four profiles is given. Profile A was the most frequently observed (71%), with the two subcategories A1 and A2 in practically equal proportions (35%); profile B was less frequent (22%) and C was relatively rare (7%).

**Response to the mixture: effect of heptanal on pheromone responses of MGC neurons**

We basically obtained the same response patterns (Fig. 1) and with the same proportions with the mixture as with the pheromone alone (Table 1, response pattern M). However, quantitative analysis revealed significant differences.

**A1 neurons**

The response latency in MGC neurons was significantly higher for H than for P. The duration of the E1 phase as well as E1 and E2 spike frequencies were significantly lower for H than for P (Table 2, Fig. 2). Although A1 neurons were rather uniform in their responses to P and H, responses to M varied slightly. Among the 27 A1 neurons, 25 responded less to M than to P alone, but two neurons responded more strongly (shortened latency and increased firing frequency E1) to M than to either of the two individual compounds (supplementary material Fig. S1). Most A1 neurons thus showed suppressive mixture interactions. When pooling all A1 neurons, most of the analyzed response parameters to M were intermediate between responses to P or H alone (Fig. 2). Response latency was significantly longer for M than for P, but significantly shorter than for H. E1 duration and E1 mean frequency, as well as E2 mean

frequency, were significantly higher for M than for H, but E1 and E2 mean frequency were significantly lower than for P alone. E1 duration was, however, not significantly different between M and P stimulation. I duration did not differ significantly when stimulating with M or H, but was significantly longer than for P stimulation (Table 2, Fig. 2).

**A2 neurons**

The response latency of A2 neurons was significantly longer for H than for P and the duration of the inhibition was significantly longer for H than for P (Table 2, Fig. 2). Most A2 neurons responded less to M than to P, i.e. with lower E1 duration and frequency but also reduced E2 frequency (Figs 1, 2); this could originate from the inhibitory effect of responses to H, thus showing a suppressive effect to the mixture. However, of the 26 A2 neurons, one responded more strongly (shorter latency) to M than to the pheromone (supplementary material Fig. S1). When pooling all A2 neurons, E1 duration and E1 and E2 mean frequency responses to M were significantly smaller than those to P. As in A1 neurons, the latency in response to M was intermediate but significantly different from latencies to P and H as individual compounds. Also, I duration was not statistically different between M and H stimulation, but was significantly longer than in response to P (Table 2, Fig. 2).

**B and C neurons**

B and C neurons responded similarly to individual compounds and their mixture (Figs 1, 2), thus exhibiting a hypoaddivitive mixture interaction. In contrast to A1 and A2 profiles, response parameters to M, H and P were not statistically different in any case when

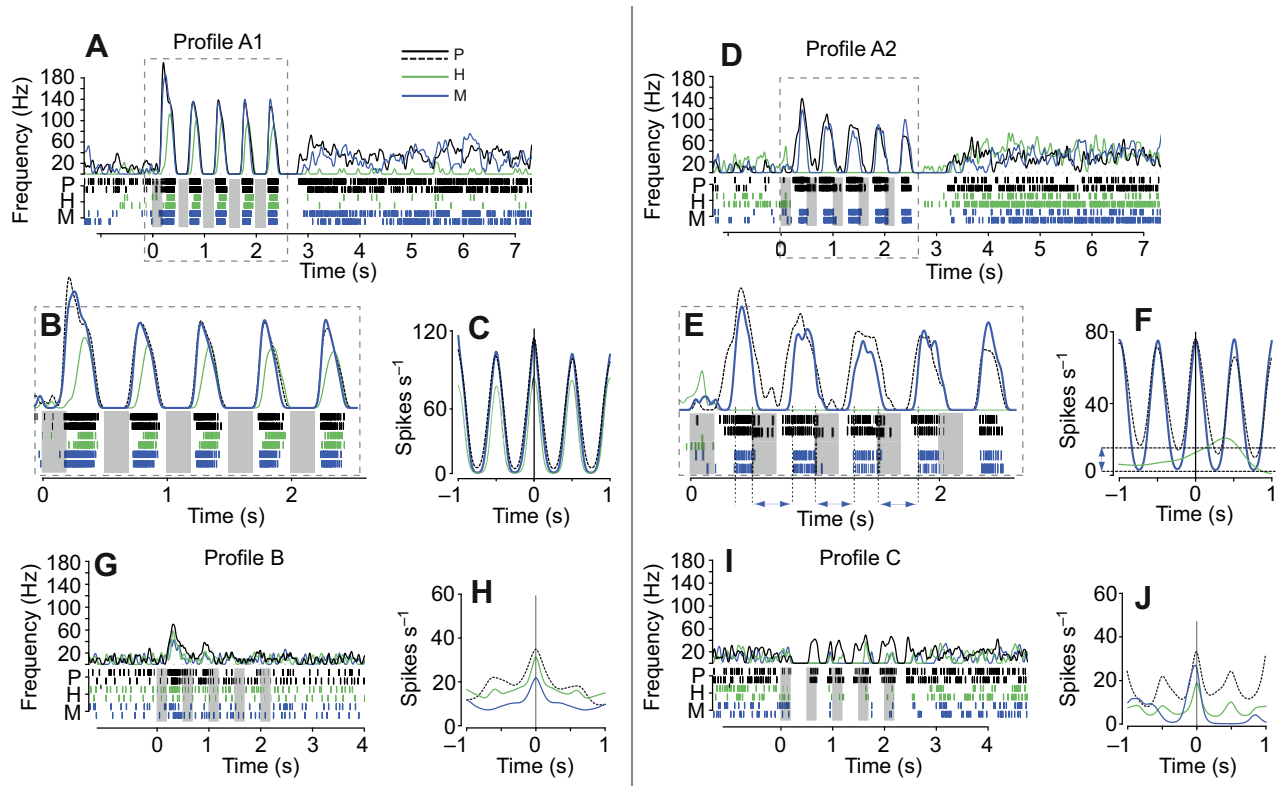


Fig. 3. Response properties of extracellularly recorded MGC neurons to pulsed stimulation in *A. ipsilon*. Neurons were stimulated with five puffs of pheromone (P), heptanal (H) or a mixture of both (M) at 2 Hz. Each panel represents the response of one of the four neuron profiles. Raster plots of action potentials superimposed on discharge rate-evolution curves (built with a 25 ms smoothed Gaussian kernel) are shown for representative example neurons. Correlograms show the periodic component of the neuron response. (A–C) A1 neurons accurately resolved the 2 Hz pulsed stimulation for P, H and M, as can be observed in the raster plots (A,B) and the cross-correlogram (C). (D–F) A2 neurons resolved M stimulation better than P stimulation, as shown in the raster plots, firing rate curves (D,E) and cross-correlogram (F). (G,H) B neurons responded only to the first odour pulse, not resolving subsequent pulsed stimuli. (I,J) C neurons showed a weak ability to follow pulsed stimulation with P, but not with H or M, as shown in the autocorrelogram. Grey bars indicate the stimulus presentation periods (duration: 200 ms; interstimulus interval: 300 ms). Blue arrows (E,F) represent the contrast enhancement between successive responses obtained with the mixture. B and E are magnified areas (indicated by dashed boxes) of A and D, respectively. Black continuous or dotted lines represent P, green lines H and blue lines M.

pooling all neurons of each profile. Only the mean E1 frequency in response to M was significantly higher than to H in B neurons (Table 2, Fig. 2). In two out of 16 B neurons, the response to M exceeded the response to P (longer duration of the excitatory phase), thus showing a synergistic mixture interaction (supplementary material Fig. S1).

#### Stimulus pulse resolution as a function of neuron profile

Typical results are shown in Fig. 3. In A1 neurons, resolution of a 2 Hz pulsed stimulation (tested for 12 neurons) was accurate for P, H and M stimuli, as can be seen in discharge rate-evolution curves (Fig. 3A,B) and cross-correlograms constructed from two simultaneously recorded neurons (Fig. 3C). Cross-correlograms revealed a pronounced peak at a time lag of 0 s, bounded by other peaks at a time lag of 0.5 s, indicating that these neurons produced periodic bursts of spikes at 2 Hz (Fig. 3C). With a longer duration of the inhibitory phase for M than for P, as obtained previously for individual puffs, a better resolution of pulsed stimulation could be expected. However, in these particular repetitive stimulus conditions, only three out of 12 neurons clearly revealed a better resolution of pulses with M as compared with P. Simultaneously recorded neurons ( $N=1$  pair) were already synchronized in their spontaneous activity (supplementary material Fig. S2) and also exhibited synchronized responses to the three stimuli (Fig. 3B). Only the

response to the first stimulus was stronger for both P and M. The latencies of responses to H were clearly higher than for P or M, especially for the first pulse, but this difference in latency considerably decreased with the number of pulses (Fig. 3B).

A2 neurons followed pulsed stimulation (tested for 10 neurons) with P and M, but the resolution of pulses was more accurate with M, as can be seen in the zoom of discharge rate-evolution curves and raster plots (Fig. 3D,E), as well as in the cross-correlogram constructed from two simultaneously recorded neurons (Fig. 3F). With a shorter duration of the E1 phase and a longer duration of the inhibitory phase for M than for P, it is even more likely than for A1 neurons that pulse resolution is better for M than for P. Indeed, eight out of 10 neurons showed a better resolution of pulsed stimuli with M than with P. As for A1 neurons, simultaneously recorded A2 neurons ( $N=3$  pairs) were already synchronized during spontaneous activity (supplementary material Fig. S2). However, the cross-correlogram shows that a better contrast between puffs was achieved with M than with P alone: the pronounced peak at a time lag of 0 s is bounded by deeper troughs for M than for P (Fig. 3F). The improved resolution can be explained by shorter E1 durations in response to M as compared to P, and by a better burst synchronization (Fig. 3D,E). Moreover, the difference in mean E1 response frequency observed between P and M during the first stimulation of a series tends to disappear with subsequent

stimulations (Fig. 3D,E). An example of an original intracellular recording of an A2 neuron following pulsed stimulation with P and M is given in supplementary material Fig. S3.

B neurons only responded to the first pulse of P in a series of periodic stimulations, and this did not change when H was added (Fig. 3G). The only C neuron that could be tested with pulsed stimulations weakly followed pulsed stimuli to P but not to H or M (Fig. 3I). Autocorrelograms for B and C neurons confirm the poor if not absent resolution of all pulsed stimuli (Fig. 3H,J).

Mean periodograms calculated for A1 ( $N=12$ ), A2 ( $N=10$ ), B ( $N=3$ ) and C ( $N=1$ ) neurons confirmed at the population scale what was shown in the four examples in Fig. 3 (supplementary material Fig. S4).

## DISCUSSION

In this study, we show that most recorded pheromone-sensitive neurons also responded to a plant odour, heptanal, either with a response pattern very similar to the pheromone blend, or with an inhibition, independently of the response pattern they exhibit to the pheromone. When stimulating with the mixture of pheromone and heptanal, neuron responses resembled more closely the pheromone than the heptanal response, but were in most cases decreased. Although a lower response to the mixture than to the pheromone alone might at first glance seem counter-intuitive, the resolution of pulsed stimuli was improved because of the suppressive effect within the mixture, which is crucial for oriented flight behaviour in moths (Vickers, 2001; Mafra-Neto and Cardé, 1998).

### MGC neurons show a multiphasic response to pheromone

In most recordings of MGC neurons, we found a multiphasic response pattern, with an initial excitation followed by an inhibition to the pheromone, as previously described for MGC PNs in *A. ipsilon* (Jarriault et al., 2009), but often with an additional second excitatory period following the inhibition. This pattern is present in many other noctuid moth species, including *Agrotis segetum* (Lei and Hansson, 1999), *Spodoptera littoralis* (Han et al., 2005) and *Trichoplusia ni* (Anton and Hansson, 1999). *Manduca sexta* pheromone responses differ slightly from *A. ipsilon* pheromone responses as they exhibit a triphasic response with a very short inhibitory phase before the first excitatory phase (Heinbockel et al., 1999). Tonic excitatory pheromone responses as observed in B profile neurons have been reported for several other moth species, e.g. *B. mori* (Kanzaki et al., 2003; Namiki et al., 2008), *Helicoverpa (Heliothis) zea* (Christensen et al., 1991) and *Ostrinia nubilalis* (Anton et al., 1997). Inhibition of MGC PNs after pheromone stimulation as in C profile neurons has so far only been described for stimulation with the minor pheromone component in *M. sexta* (Christensen et al., 1989; Heinbockel et al., 1999; Heinbockel et al., 2004; Lei et al., 2002). We found that *A. ipsilon* MGC neurons display a small repertoire of distinct response pattern profiles and different classes of neurons may thus serve different functions. Unfortunately, no anatomical data of C profile neurons were obtained in this study. However, AL neurons with inhibitory responses to heptanal were previously identified as LNs in *A. ipsilon* and inhibitory responses to pheromone might also originate from LNs (Barrozo et al., 2011). For all other neuron profiles (A1, A2 and B), all obtained stainings showed PNs innervating the MGC.

### MGC neurons are sensitive to a behaviourally relevant plant odour

Unexpectedly, almost all pheromone-sensitive MGC neurons of *A. ipsilon* were found to respond to heptanal with response

patterns very similar to the pheromone response patterns or with an inhibitory response. Our data show that the separation of pheromone and general odour processing in the two parts of the AL is not as complete as previously thought (Christensen and Hildebrand, 2002). Excitatory responses of MGC PNs to plant odours have also been reported for *S. littoralis* (Anton and Hansson, 1995), and inhibitory responses of MGC PNs to plant odours were described in *M. sexta* (Reisenman et al., 2008). Similar evidence has been found in the tortricid moths *Cydia pomonella* and *Cydia molesta*, where responses to plant odours were found in MGC neurons and pheromone responses in PNs within other glomeruli (Trona et al., 2010; Varela et al., 2011b). Such results are in agreement with data from vertebrates, where both 'common' odours and pheromones can activate the main olfactory bulb and the accessory olfactory bulb (Xu et al., 2005), and thus overlapping processing of both odour types occurs in both compartments of the primary olfactory centre.

As information on pheromones and plant odours enters the AL via different input channels and responses of ORNs are predominantly excitatory, excitatory and inhibitory responses to the plant odour in MGC neurons probably originate from different connectivities and thus different ways of processing within the AL network.

### The reshaped mixture response is dominated by the pheromone response

Although mixture stimulation elicited in most cases a weaker response than pheromone stimulation (A1 and A2 neurons), responses to the mixture were always more similar to the pheromone response than to the heptanal response in our study. According to the definition of interactions described by Duchamp-Viret et al. (Duchamp-Viret et al., 2003), A1 and A2 neurons showed a 'suppression' type of mixture interaction. Purely excitatory or purely inhibitory responses of B and C neurons to the pheromone were not affected when heptanal was added, thus these neurons exhibited a 'hypoadditivity' type of interaction.

The main interaction of the pheromone blend and heptanal observed in this study (A1 and A2 profiles), suppression of the pheromone response, has, to our knowledge, not been reported as major interaction type for any other moth species. Synergism of pheromone and plant odour has previously been reported for MGC neurons in *B. mori* (Namiki et al., 2008). In *C. pomonella* PNs, both synergism and suppression were found in response to mixtures of pheromone and plant odours, but no dominant interaction type was identified (Trona et al., 2010). In PNs arborising in the OG of *A. ipsilon*, synergistic interactions of pheromone and heptanal were found in virgin males, whereas suppression of the heptanal response was found in newly mated males, when the pheromone was added (Barrozo et al., 2010). We are currently investigating whether the interactions of heptanal and sex pheromone also change within the MGC as a function of the mating state in male *A. ipsilon*. This question is important as it will help us to identify the pathways involved in olfactory plasticity and understand the underlying mechanisms.

The origin of the observed mixture interactions might be different depending on the different neuron types. Evidence for inhibitory effects of plant compounds on pheromone responses in antennal sensilla responding exclusively to pheromone compounds has been found in several moth species (e.g. Pophof and van der Goes van Naters, 2002; Party et al., 2009), including *A. ipsilon*, using single sensillum recordings (Deisig et al., 2012). Optical imaging studies have also shown that the input of pheromone information to the AL



is reduced when adding plant odours (Deisig et al., 2012). Although plant odours down-modulate pheromone detection at the peripheral level (Deisig et al., 2012), the response in most MGC neurons to heptanal observed in this study indicates that input through independent information channels reaches the AL, and that the AL network must be involved in the mixture interactions observed in AL output neurons. We assume that the suppression of the pheromone response upon stimulation with the mixture in the AL is caused by GABAergic inhibition *via* LNs, but the precise mechanisms still need to be unveiled. We aim to identify the sources of the inhibition of PNs during the pheromone response and heptanal stimulation by pharmacological blocking of GABA receptors or blocking of Ca<sup>2+</sup>-activated K<sup>+</sup> channels.

The few observed synergistic odour interactions might originate from synergistic interactions on peripheral neurons, as reported for *H. zea* (Ochieng et al., 2002), by different wiring within the AL, involving, for example, excitatory LNs, or by signal processing *via* multiple inhibitory LNs (disinhibition pathways).

#### Plant odour enhances the response contrast during pulsed pheromonal stimulation

In *A. ipsilon*, only MGC neurons exhibiting multi-phasic response patterns to the pheromone were able to follow pulsed stimulation (i.e. A1 and A2 neurons), as previously described for other moth species (Christensen and Hildebrand, 1988; Lei and Hansson, 1999; Lei and Vickers, 2008). The resolution of pulsed pheromone stimuli further improved when heptanal was added, especially for A2 neurons, because of the shortening of the E1 phase and the lengthening of the following inhibition. In *S. littoralis*, an improvement of pulse resolution in pheromone specific ORNs was also found when pheromone stimuli were applied in a plant odour background (Party et al., 2009; Rouyar et al., 2011). The contrast enhancement between the single spike trains emitted in response to the discontinuous mixture signal in A2 neurons in *A. ipsilon* is amplified at the neuron population scale. Indeed, we recorded synchronized neuron pairs belonging to the same profiles and they showed almost identical response patterns. Heptanal-induced inhibition of the MGC neurons through the LN network might thus synchronize MGC PNs and provide a more coincident input to upstream neurons in the mushroom bodies. There, the Kenyon cells are thought to need simultaneous input from several PNs, because they have been described as sparse coding cells in different insect species (Demmer and Kloppenburg, 2009; Ito et al., 2008; Jortner et al., 2007; Luo et al., 2010; Perez-Orive et al., 2002; Szyszka et al., 2005; Wang et al., 2004).

#### Behavioural relevance of mixture coding properties in the AL

As multiple odours coincide in a natural environment, it may be advantageous for *A. ipsilon* males to respond to a mixture of pheromone and heptanal, a component emitted by linden flowers (Zhu et al., 1993), because this food source might represent an additional cue indicating the presence of females. Behavioural pheromone responses of *A. ipsilon* males in a wind tunnel were improved when heptanal was added, and this synergistic effect was particularly evident at the dose we used in the present study (Barrozo et al., 2010). In contrast, the main effect of the plant odour on the pheromone responses within neurons in the MGC in this study was suppressive; more specifically, the presence of heptanal prolonged the inhibitory phase. Previous work has shown that the inhibitory phase duration of MGC PNs in *A. ipsilon* is not affected by the dose (Jarriault et al., 2009). We show here, however, that the inhibitory phase can be modulated by the presence of a plant odour. The

suppressed neuron response to the mixture compared with the response to the pheromone alone is thus different from the response to a lower dose of pheromone. A male moth should, as a consequence, be able to differentiate between different doses of the pheromone compared with the presence of pheromone–plant odour mixtures, because different parameters of the neuron responses are affected. It was previously shown that *M. sexta* males lose their ability to fly towards a pheromone source when the inhibitory phase following the excitatory phase in AL neuron responses is pharmacologically blocked (Lei et al., 2009). The improved resolution of the temporal stimulus pattern in MGC neurons for the mixture as compared with pheromone alone, originating from a more pronounced inhibitory phase, might thus allow males to optimally track an intermittent pheromone plume. The importance of the intermittence of pheromone plumes has been described for many moth species (e.g. Baker et al., 1985; Kennedy, 1983; Vickers and Baker, 1994).

#### Conclusions and perspectives

In the moth *A. ipsilon*, strong and variable interactions between pheromones and plant volatiles occur in MGC neurons of the AL. The different MGC neuron profiles revealed in this study may represent distinct subpopulations of pheromone-sensitive cells serving different functions and conveying complementary information about mixture characteristics to higher brain centres. Although the prolonged inhibitory phase caused by heptanal addition to the pheromone in A-type neurons might contribute to improve tracking of intermittent pheromone plumes, we are still far from understanding how odour interactions precisely contribute to improved orientation behaviour of male moths. In any case, analyzing mixture effects within the AL with simultaneous application of the compounds and a single dose is only a first step, even if the doses were chosen on the basis of previous behavioural and electrophysiological data (Barrozo et al., 2010). Orientation behaviour of male moths towards pheromone is generally more affected by plant odours when suboptimal pheromone doses are used (Deng et al., 2004; Schmidt-Büsser et al., 2009); therefore, it seems important to test different pheromone doses in the future. In a natural environment, pheromones and plant odours will not be emitted by the same source and their temporal patterns will vary independently. Therefore, our next step is to test the effects of stimulations with different temporal patterns of the two stimuli. Another important issue is to analyze how the pheromone and plant odour pathways communicate in the AL during odour mixture stimulation and transfer this information to higher brain centres to finally generate appropriate motor activity.

#### LIST OF ABBREVIATIONS

AL	antennal lobe
E1	first excitatory phase
E2	second excitatory phase
H	heptanal
I	inhibitory phase
LN	local interneuron
M	pheromone/heptanal mixture
MGC	macroglomerular complex
OG	ordinary glomeruli
ORN	olfactory receptor neuron
P	sex pheromone blend
PN	projection neuron

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