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Mating-induced differential coding of plant odour and sex pheromone in a male moth

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Abstract

Innate behaviours in animals can be influenced by several factors, such as the environment, experience, or physiological status. This behavioural plasticity originates from changes in the underlying neuronal substrate. A well-described form of plasticity is induced by mating. In both vertebrates and invertebrates, males experience a post-ejaculatory refractory period, during which they avoid new females. In the male moth *Agrotis ipsilon*, mating induces a transient inhibition of responses to the female-produced sex pheromone. To understand the neural bases of this inhibition and its possible odour specificity, we carried out a detailed analysis of the response characteristics of the different neuron types from the periphery to the central level. We examined the response patterns of pheromone-sensitive and plant volatile-sensitive neurons in virgin and mated male moths. By using intracellular recordings, we showed that mating changes the response characteristics of pheromone-sensitive antennal lobe (AL) neurons, and thus decreases their sensitivity to sex pheromone. Individual olfactory receptor neuron (ORN) recordings and calcium imaging experiments indicated that pheromone sensory input remains constant. On the other hand, calcium responses to non-pheromonal odours (plant volatiles) increased after mating, as reflected by increased firing frequencies of plant-sensitive AL neurons, although ORN responses to heptanal remained unchanged. We suggest that differential processing of pheromone and plant odours allows mated males to transiently block their central pheromone detection system, and increase non-pheromonal odour detection in order to efficiently locate food sources.

Introduction

During their lifetime, animals undergo marked switches in their innate behavioural patterns. Such switches are attractive models with which to explore the neural control of innate behaviours. A typical form of behavioural switch found in many animal species is the mating-induced change in behaviour. In many vertebrates and invertebrates, newly mated females become unreceptive to the sexual partner (Ogawa & Makino, 1984; Kelliher & Baum, 2002; Kubli, 2003; Chapman, 2008). Likewise, males of many vertebrate species exhibit a behavioural post-copulatory refractory period, which can last from a few seconds to days in some mammals (e.g. Sachs & Barfield, 1970; Aversa *et al.*, 2000; Kumashiro *et al.*, 2003; Phillips-Farfán & Fernández-Guasti, 2009). In male insects, however, although mating-induced changes in sexual behaviour are probably common, there are very few documented examples. Males of the cricket *Gryllus bimaculatus* show a behavioural refractory period of 1 h following mating, which seems to be controlled by the terminal abdominal ganglion (Sakai *et al.*, 1995).

In the noctuid moth *Agrotis ipsilon*, there is transient post-mating inhibition of behavioural and central nervous responses to sex pheromone (Gadenne *et al.*, 2001; Barrozo *et al.*, 2010a, 2010b). Wind tunnel experiments with newly mated males revealed complete inhibition of oriented flight behaviour. Examination of the global response of the antenna to sex pheromone, by means of electroantennogram (EAG) recordings, did not reveal changes in males' responses after mating. However, the sensitivity of antennal lobe (AL) neurons to pheromone dropped drastically after mating, and was restored the next night.

Such behavioural and neural plasticity might be achieved by functional remodelling of the olfactory system. In insects, odour information detected by male antennal olfactory receptor neurons (ORNs) is integrated first in the ALs, which are divided into functional subunits, the olfactory glomeruli (Anton & Homberg, 1999). 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 & Homberg, 1999; Homberg & Müller, 1999). The ALs of male moths consist of two olfactory subsystems, the

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macroglomerular complex (MGC) and the ordinary glomeruli (OG). The MGC receives almost exclusive input from sex pheromone receptor neurons, whereas general odours, such as the plant volatiles commonly used to locate food sources or host plants, are processed in the OG (Koontz & Schneider, 1987; Hansson, 1995; Anton & Homberg, 1999).

The aim of this study was to better understand how the neural network is remodelled in order to modulate the olfactory system after mating, using a multi-level approach. We examined possible changes in the response characteristics of peripheral and central neurons linked to the mating status of males, by comparing individual ORN and AL neuron responses, as well as global calcium signals, in ALs of virgin and mated males to both pheromonal and non-pheromonal (i.e. plant volatiles) odour information.

Materials and methods

Insects

Adult *A. ipsilon* males originating from a laboratory colony in Bordeaux (France) were used throughout the experiments. Animals were reared on an artificial diet in individual cups until pupation. Pupae were sexed, and males and females were kept in separate rooms under a 16-h light/8-h dark photoperiod at 22 °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. Sexually mature virgin males (5 days old) and females (3 days old) were paired in plastic containers, allowing them to copulate (Gadenne *et al.*, 2001; Barrozo *et al.*, 2010a, 2010b). Every 30 min, couples were checked to record the start and the end of copulation, which lasts for more than 30 min in this species. Mated males were quickly removed from the pairing box and used for experiments within the remainder of the scotophase. All mated females were checked for the presence of the spermatophore, in order to confirm that mating was successful.

Stimuli

The behaviourally active sex pheromone of *A. ipsilon* consists of three components: (*Z*)-7-dodecen-1-yl acetate (Z7-12:OAc), (*Z*)-9-tetradecen-1-yl acetate, and (*Z*)-11-hexadecen-1-yl acetate (Picimbon *et al.*, 1997; Gemeno & Haynes, 1998). Because ORNs are tuned to individual pheromone compounds, we used only the major pheromone component, Z7-12:OAc, for single-sensillum recordings. The pheromone blend at a ratio of 4 : 1 : 4 of the above-mentioned three components (Picimbon *et al.*, 1997) was used for optical imaging and intracellular AL neuron recordings to monitor responses to the natural signal. Decadic dilutions of the pheromone blend were prepared in hexane, and amounts of 0.001–100 ng (in 10 μL of hexane) were applied on a filter paper in a Pasteur pipette. For calcium imaging experiments, only one concentration of the pheromone blend was used (10 ng in 10 μL of hexane, applied on a filter paper), to limit the total number of olfactory stimulations.

Heptanal has been identified as a component of blooming linden (*Tilia* spp.), which is an attractive plant for *A. ipsilon*, and is used as a food source (Wynne *et al.*, 1991). Heptanal elicits responses in the antenna and in AL neurons within the OG of *A. ipsilon* (Zhu *et al.*, 1993; Greiner *et al.*, 2002). In all experiments (including calcium imaging), we used four doses of heptanal (1, 10, 100 and 1000 μg) diluted in 10 μL of mineral oil on a filter paper as a non-pheromonal odour. All compounds were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France).

All stimuli were presented after a minimum evaporation time of 30 min. The same stimulation system was used for single-sensillum and intracellular recordings in the AL. A stimulus controller (CS 55; Syntech, Kirchzarten, Germany) delivered a constant charcoal-filtered and humidified airflow (17 mL/s) on the antenna by means of a glass tube (inner diameter, 8 mm). The antenna was placed in the outlet of this glass tube. Stimuli were applied by inserting a Pasteur pipette containing a filter paper carrying the odour in the glass tube, 20 cm upstream of the antenna, and activating the airflow through the stimulus pipette (air pulse of 7 mL/s) for 200 and 500 ms for ORN and AL neuron recordings, respectively. For optical imaging experiments, an odour-supplying device was placed 2 cm in front of the animal's head positioned under the microscope (Sandoz, 2006). The device was controlled by the imaging acquisition software (TILLVISION). Throughout the recording, a constant clean airflow of 50 mL/s was delivered through a tube (1 cm in diameter). During olfactory stimulation, the airflow was switched from the constant clean airflow to a secondary airstream containing an odour-loaded glass pipette (2.5 mL/s). Hexane and mineral oil solvents were used as control stimuli. In a series of odour presentations, lower stimulus loads were tested first.

Single-sensillum recordings and data analysis

Males were mounted in a styrofoam block holder with the head protruding. The bipectinate antennae were fixed with adhesive tape and oriented to allow optimal access to the trichoid sensilla selected randomly along the stem and the branches in the middle part of the antenna (10th–34th segments from the base). Recordings from pheromone sensilla were carried out with the tip recording technique (Kaissling & Thorson, 1980). The tips of a few hairs were cut off with sharpened forceps. Reference and recording microelectrodes were filled with saline solutions, which approximated, respectively, the ionic composition of the haemolymph and that of the sensillum lymph of moths (Kaissling & Thorson, 1980). The tip of the recording microelectrode was filled with polyvinylpyrrolidone (20% in sensillum saline) to prevent exchange between sensillum lymph and electrolyte. To minimize contributions of field potentials, the reference electrode was inserted into a segment close to the recording site. Recorded signals were amplified (×500), sampled at 20 kHz, low-pass filtered at 5 kHz with a Cyberamp 320 amplifier (Molecular Devices, Sunnyvale, CA, USA), and monitored on a computer with a Digidata 1200A acquisition board (Molecular Devices) driven by CLAMPEX 9 software (Molecular Devices). The recorded signal was high-pass filtered offline (50 Hz) with CLAMPFIT 10. Action potentials (APs) were then detected with a CLAMPFIT routine (threshold search), and sorted according to their amplitude and shape to keep only the APs of the responding ORN (or the APs of the most easily extractable class of APs if two ORNs responded). The maximal spike frequency was calculated by measuring the minimum interspike interval (ISI) in the 2-s period following stimulus onset. We then selected the two ISIs preceding and following the minimum ISI, and calculated the inverse of the mean of these five ISIs (Jarriault *et al.*, 2009). The time course of the changes in spike frequency was evaluated with peristimulus time histograms. We pooled the trains of APs available for each ORN, and calculated the mean frequency per 50-ms bin across all ORNs for each group. As the spontaneous activity in pheromone ORNs was very weak (< 1 AP/s), the threshold was determined as the lowest dose eliciting a 5-Hz increase in the firing frequency as compared with the prestimulus period.

Recordings from plant odour sensilla were carried out with electrolytically sharpened tungsten wires from sensilla selected

randomly along the stem in the middle part of the antenna. The reference electrode was placed within the antenna, and the recording electrode was inserted into the base of the sensillum. Recorded signals were amplified (×500), sampled at 20 kHz, high-pass filtered at 30 Hz, and low-pass filtered at 5 kHz. APs were detected by threshold search, and were pooled for the analysis, because different classes of APs could not be discriminated. Because of the higher spontaneous firing activity in plant odour sensilla than in pheromone sensilla, the analysis of responses was not the same as for pheromone sensilla. Responses were calculated as the frequency of APs during the 1-s period following stimulus end. For peristimulus time histograms, we pooled the trains of APs available for each ORN, and calculated the mean frequency per 50-ms bin across all ORNs for each group. The threshold was determined as the lowest dose eliciting a doubling of the firing frequency as compared with the 1-min prestimulus period.

Statistical analysis of threshold differences between virgin and mated males was performed by means of an $R \times C$ test of independence, using a G-test (physiological status in rows, and odour concentrations in columns) and applying the Williams correction (Sokal & Rohlf, 1995). Two adjacent columns were grouped (doses) when a zero frequency value was present (Sokal & Rohlf, 1995). Dose-dependent effects on the number of spikes in virgin and mated males were evaluated with a two-way ANOVA for repeated measures (Sokal & Rohlf, 1995), in which repeated measures were the different concentrations. Statistical assumption of sphericity was checked and violation was overcome by using Greenhouse–Geisser correction for degrees of freedom (d.f.).

Calcium imaging recording and data analysis

Preparation of insects, Calcium Green 2-AM bath application and *in vivo* calcium imaging recordings were performed as described for honeybees (Deisig *et al.*, 2006). Briefly, recordings were carried out with a TILL Photonics imaging system (Martinsried, Germany). Moths were placed individually under an epifluorescence microscope (Olympus BX-51WI) with a ×10 (NA, 0.3) water-immersion objective (Olympus UMPlanFL). One AL was recorded in each moth. Images were taken with a 640 × 480-pixel 12-bit monochrome CCD camera (TILL Imago) cooled to −12 °C. Each measurement consisted of 100 frames at a rate of five frames per second (integration time for each frame, 10–15 ms). Odour stimuli were given for 1 s. The pixel image size corresponded to approximately 2×2 μm after 4×4 binning on the chip. Monochromatic excitation light at 475 nm was applied with a monochromator (TILL Polychrom V). The filter set on the microscope consisted of a 505-nm dichroic mirror and a 515-nm long-pass emission filter.

As anatomical staining of the AL after imaging experiments was not possible, and seems, in general, to be difficult in moths, individual glomeruli were identified by superposing activity maps obtained for the different olfactory stimulations in each male and comparing these maps between insects. In this way, a total of nine OG could be identified in all AL preparations. As the size of an OG is small in comparison with the complete MGC, three locations were defined for each male in the MGC. Because no difference in the calcium signals was found between these locations, data were pooled.

Data were analysed with custom-made software written in IDL (Research Systems, Boulder, CO, USA). Each recording consisted of three dimensions: two spatial dimensions (x and y pixels of the area of interest) and a temporal dimension (100 frames). Signal calculation was performed as described in Deisig *et al.* (2006). For quantitative and statistical data analysis, only the fast (positive) signal component evoked by odour stimulation was considered, as it is related to intracellular calcium increase, and is supposed to reflect mostly, although not only, presynaptic neuronal activity from ORNs (Galizia *et al.*, 1998; Sachse & Galizia, 2003). For each identified glomerulus, the time course of relative fluorescence change ($\Delta F/F$) was calculated by averaging 25 pixels (5×5) at the centre of each glomerulus. The amplitudes of odour-induced responses were calculated as the mean of three frames at the signal's maximum minus the mean of three frames before the stimulus onset. This value was then used in all computations.

For each moth, 1–3 measurements (according to the number of measured runs) for each olfactory stimulus were averaged pixel-wise, and activity maps were then scaled to a common minimum and maximum for each moth. Calcium signal intensity differences were tested for the four doses of heptanal and the pheromone blend in OG, as well as in the MGC. The control stimuli, solvents and air did not elicit calcium signals. One-way ANOVA or two-way ANOVA for repeated measures were performed.

Intracellular electrophysiology and data analysis

Male moths were immobilized in a cut, disposable pipette tip, the head capsule was opened, and the ALs were exposed, as previously described (Gadenne & Anton, 2000). Standard intracellular recording techniques were used (Christensen & Hildebrand, 1987). A KCl-filled microelectrode was placed in the area of the MGC or into one OG. Electrode resistances measured in saline were about 140–200 MΩ. Once intracellular contact had been established, the stimuli were applied as mentioned above. Interstimulus intervals were at least 10 s. Responses to odour stimulation were amplified with an Axoclamp-2B amplifier (Molecular Devices), stored on a PC, and analysed off-line with AUTOSPIKE 32 software (Syntech, Kirchzarten, Germany) and custom-made programs written in Matlab (The MathWorks, Natick, MA, USA) (see below).

The latency, spike frequency and duration of excitatory and inhibitory phases were quantified as previously described (Jarriault *et al.*, 2009) for virgin and mated males in response to pheromone or to heptanal. Briefly, responses were detected automatically with an algorithm based on the detection of changes in the slope of cumulative AP time distribution (adapted from Blejec, 2005). The values of this slope were compared with its 95th and 5th percentiles calculated during the spontaneous firing activity. The latency (onset of the response) was estimated as the time at which the AP slope exceeded the 95th percentile. The end of the excitatory phase (and onset of the inhibitory phase) was the time at which the AP slope passed under the 5th percentile. The end of the inhibitory phase was the time at which the slope exceeded the 5th percentile (searched from the end of the excitatory phase). Neurons with fewer than five APs in the 1.5-s prestimulation period were discarded from the analysis. The durations of excitatory and inhibitory phases were calculated, respectively, as the time between the onset of the response and the end of excitation, and the time between this last point and the end of the inhibition period. The mean maximal spike frequency was calculated as for ORN responses. For all four measured parameters, statistical differences among reproductive status (virgin and mated) and the doses tested were assessed by means of one-way ANOVA and repeated measures ($P < 0.05$) (Sokal & Rohlf, 1995).

Statistical assumptions of homogeneity of variance (Levene's test), normality and sphericity (Mauchly's test) were checked. Violation of sphericity was overcome by using Greenhouse–Geisser correction for d.f.

Intracellular staining and data analysis

After physiological recordings, AL neurons were injected with a hyperpolarizing current (−1 nA) when the microelectrode was loaded with Lucifer Yellow CH, or with a depolarizing current (0.3 nA) when it was filled with Neurobiotin. Current injections lasted 3–10 min. Subsequently, the brain was excised from the head capsule and immersed in the fixative solution. Brains injected with Lucifer Yellow CH were fixed in 4% formaldehyde solution overnight at room temperature (20–24 °C). Brains injected with Neurobiotin were immersed in 4% paraformaldehyde in Millonig's buffer at 4 °C for 12 h, and then dehydrated and rehydrated in ethanol and propylene oxide. Neurobiotin was then visualized after incubation in Oregon Green–avidin (Oregon Green 488 conjugate A6374; Invitrogen, Carlsbad, CA, USA), with 0.2% Triton X and 1% bovine serum albumin added to the incubation solution, for 12 h at 4 °C. Finally, all brains were rinsed in Millonig's buffer, and then cleared and mounted in Vectashield medium (Vectashield Mounting Medium; Vector Laboratories, ABCYS, Paris, France). Preparations were optically sectioned with a laser scanning confocal microscope (SP2 AOBS; Leica Microsystems, Heidelberg, Germany) equipped with an argon–krypton laser and an HC PL APO CS 10.0 × 0.40 dry objective. Preparations were excited at a wavelength of 488 nm, staining was detected with a 505-nm long-pass filter, and images were scanned at a resolution of 512 × 512 pixels with a step size of 2 μm. Neurons were reconstructed from confocal stacks with ADOBE PHOTOSHOP. Statistical differences among the frequencies of neuron types encountered were assessed with a chi-square test ($P < 0.05$).

Results

Effect of mating on response characteristics of pheromone receptor neurons

ORN responses to the main sex pheromone component (Z7-12:OAc) were very similar between virgin and mated males for pheromone-specific neurons within long trichoid sensilla. Response thresholds did not reveal any significant differences between virgin ($n = 62$) and mated ($n = 52$) insects ($G = 3.03$, d.f. = 3, $P = 0.39$) (Fig. 1A). The spike frequency of ORNs increased with dose for both groups ($F_{2.4,273.2} = 401.17$, $P < 0.0001$), and no significant differences were found between the two groups ($F_{0.8,91.1} = 0.91$, $P = 0.34$) (Fig. 1B). Furthermore, both groups of males displayed similar temporal

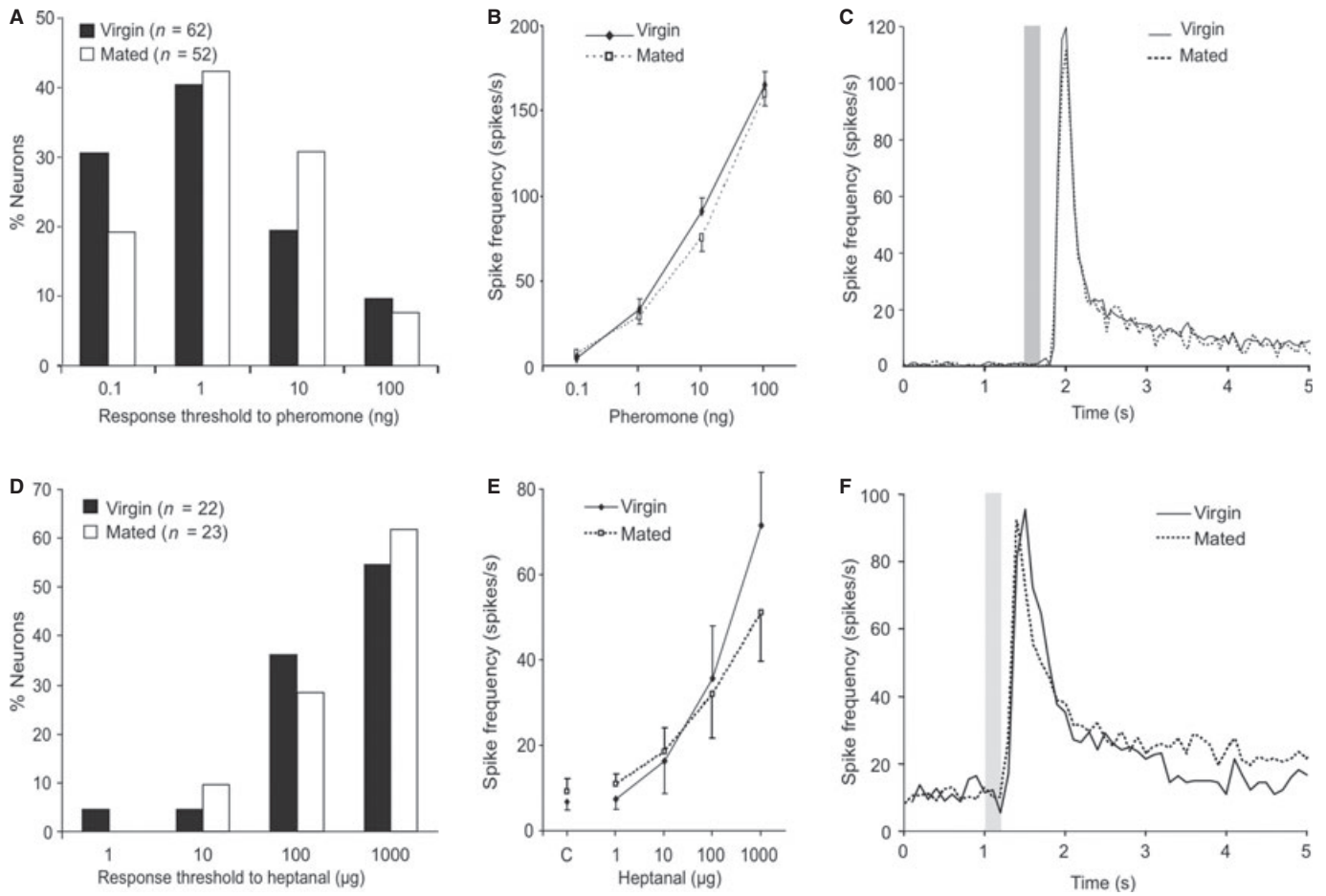

FIG. 1. Extracellularly recorded responses of ORNs to the major pheromone component (Z7-12:OAc) (A–C) and the plant-related odour heptanal (D–F) in virgin and mated *A. ipsilon* males. (A, D) Distribution of response thresholds of ORNs for pheromone and heptanal, respectively. For both odours, response thresholds were not different between the two groups. (B, E) Spike frequency of ORNs as a function of the dose of pheromone and of heptanal, respectively. For both odours, no differences between the two groups were detected. Mating did not change the dynamic range of stimulus dose encoding. Spike frequency (mean ± standard error of the mean) was measured in the phasic part of the response. (C, F) Time courses of responses to 1 ng of Z7-12:OAc and to 1000 μg of heptanal, respectively. The curves are superimposed to illustrate the fact that the temporal pattern of the response in ORNs is not affected by mating. The grey bars in C and F represent the 200-ms stimulation period. *n*, number of neurons tested.

dynamics of the response to 1 ng of Z7-12:OAc, as shown in the time-course curves (Fig. 1C). Thus, none of the evaluated response characteristics indicated that pheromone-responding ORNs are modulated as a function of mating status.

Effect of mating on response characteristics of heptanal receptor neurons

Responses of ORNs tuned to the flower odour heptanal and situated within short trichoid sensilla were also very similar between virgin and mated males. Response thresholds were not significantly different between virgin (n = 22) and mated (n = 23) males (G = 0.29, d.f. = 2, P = 0.86) (Fig. 1D). The spontaneous activity of heptanal-responding ORNs was higher than that of pheromone-responding ORNs, but did not differ between virgin and mated males (Fig. 1E). Spike frequencies of heptanal-responding ORNs were dose-dependent ($F_{1.5,63.3}$ = 57.9, P < 0.00001), but did not differ significantly between virgin and mated males ($F_{1,41}$ = 0.012, P = 0.91) (Fig. 1E), and temporal dynamics of the heptanal responses did not change either (Fig. 1F). Thus, the characteristics of heptanal-responding ORNs did not change significantly after mating.

Effect of mating on the glomerular activation pattern within the AL

As well as the above-described individual ORN response patterns, we performed a more global analysis of the effect of mating on odour representation in the ALs of male moths. This was achieved by analysing calcium signal patterns during stimulation with either the pheromone blend or with the flower-related odour, heptanal, after bath application of the calcium-sensitive dye on the brain.

Activity maps obtained during olfactory stimulation with the plant odour heptanal were restricted to a small number of OG (Fig. 2A, left panels), whereas stimulation with 10 ng of the pheromone blend

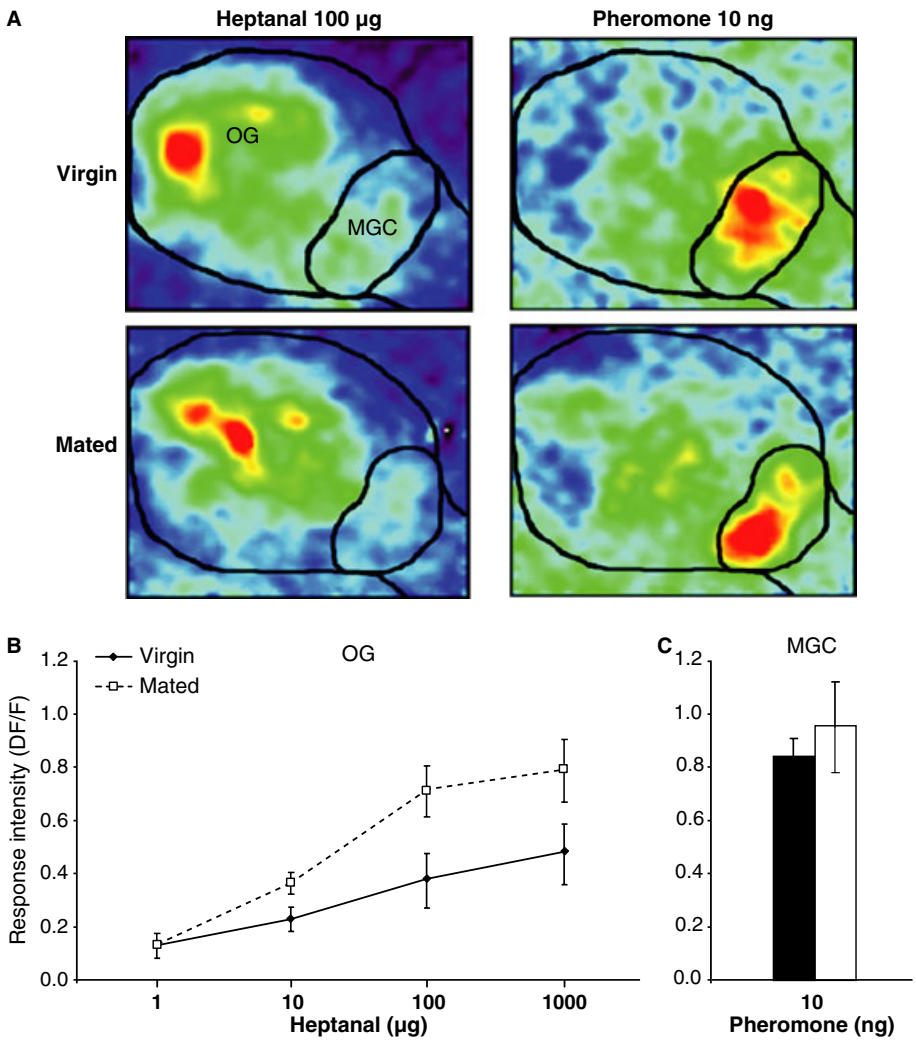

FIG. 2. Odour-evoked activity in the AL measured by calcium imaging in virgin and mated *A. ipsilon* males. (A) Example of optical imaging of responses to the plant odour heptanal and to the sex pheromone blend in a virgin *A. ipsilon* male and in a mated *A. ipsilon* male. The AL and MGC are outlined in black. The amplitude of odour responses is represented in a false-colour code, from dark blue (background signal) to red (maximum signal). The area of the MGC is activated following pheromone blend stimulation, whereas the region of the ordinary glomeruli (OG) is activated following plant odour stimulation. (B) Dose-dependent glomerular response intensities measured in the OG in virgin (n = 9) and mated (n = 8) males. Increasing doses of the plant odour heptanal induced increasing response intensities, which were, overall, higher in mated than in virgin males. (C) Intensities of the MGC response to 10 ng of the sex pheromone blend in virgin and mated males were not different. Mean response intensities ($\Delta F/F$) ± standard deviations are given in B and C.

induced calcium signals located exclusively in the MGC (Fig. 2A, right panels) in both virgin and mated males. All heptanal and pheromone stimulations evoked calcium signals in the ALs of both virgin and mated males. Stimulation with the control stimuli hexane, mineral oil and air produced no activity (data not shown). Signals were highly reproducible between individuals (data not shown).

Increasing doses of heptanal induced significantly increased response intensities in OG in mated ($n = 8$) and virgin ($n = 9$) males ($F_{3,45} = 26.0$, $P < 0.01$). An overall two-way ANOVA for repeated measures revealed significantly higher OG response intensities in mated males than in virgin males ($F_{1,15} = 6.0$, $P = 0.03$) (Fig. 2B). In contrast, virgin ($n = 9$) and mated ($n = 8$) males did not differ in their MGC pheromone response intensities ($F_{1,15} = 2.3$, $P = 0.09$) (Fig. 2C).

Effect of mating on AL neuron responses to pheromone

Independently of the mating status of males, different neuron types responding to the sex pheromone blend were identified on the basis of their physiological response patterns (Fig. 3A). A typical response observed in the majority of pheromone-sensitive neurons in the MGC comprised a biphasic pattern, with an excitatory phase followed by an inhibitory phase, before spontaneous activity resumed. This pattern (type 1; Fig. 3A) was conserved in 86% (i.e. 52 of 60) of the recorded MGC neurons in virgin males and in 81% (29 of 36 neurons) of the recorded neurons in mated males. This response pattern is characteristic of PNs in *A. ipsilon* (Jarriault *et al.*, 2009). The identification of such type 1 neurons as PNs was further confirmed in four successfully stained neurons, all arborizing within the MGC, with axons leaving the ALs and with arborizations in the protocerebrum (Fig. 3A). The remaining neurons (14% in virgin moths and 19% in mated moths) showed a long and tonic excitatory phase without an inhibitory phase (type 2; Fig. 3A). In these neurons, the spontaneous activity recovered after only a few seconds. The percentages of type 1 and 2 neurons were independent of the mating status ($\chi^2_{(1)} = 0.26$, $P = 0.61$).

Effect of mating on AL neuron responses to plant odour

Among the 76 OG neurons tested with four doses of heptanal, we identified four types of neuron, which showed different response patterns independently of the reproductive status of the males ($\chi^2_{(3)} = 2.61$, $P = 0.45$) (Fig. 3B). Types 1 and 2 presented the response profiles already described for MGC neurons responding to sex pheromone (see above, Fig. 3B). Type 3 neurons exhibited a triphasic pattern, consisting of a membrane hyperpolarization without APs, followed by a strong depolarization with a high AP frequency, again followed by a second inhibitory phase during which all firing was suppressed (Fig. 3B). Type 4 neurons showed a biphasic response pattern, comprising an inhibitory phase without spikes and a delayed tonic excitation that lasted for several seconds before the spontaneous activity was recovered (Fig. 3B). Type 1 neurons accounted for 57% of the recorded OG neurons in virgin males (25 of 44 neurons) and 47% in mated males (15 of 32 neurons). Type 2 neurons accounted for 27% in virgin males (12 of 44 neurons) and 37% in mated males (12 of 32 neurons). Type 3 and 4 neurons were less frequently observed in both virgin and mated insects (type 3 was found in 7% of virgin males and in 13% of mated males; and type 4 was observed in 9% of virgin males and in 3% of mated males). Not all recorded neurons could be stained after physiological characterization, owing to a generally low success rate of staining (15%), probably resulting from the variable quality of intracellular contact. However, we could morphologically identify at least one neuron of three of the four physiologically described types defined above. Two type 1 neurons were identified as PNs innervating mainly one OG and then projecting to the protocerebrum, similarly to MGC type 1 neurons responding to sex pheromone (Fig. 3B). No type 2 neuron was stained, and type 3 and 4 neurons were anatomically identified as LNs (Fig. 3B).

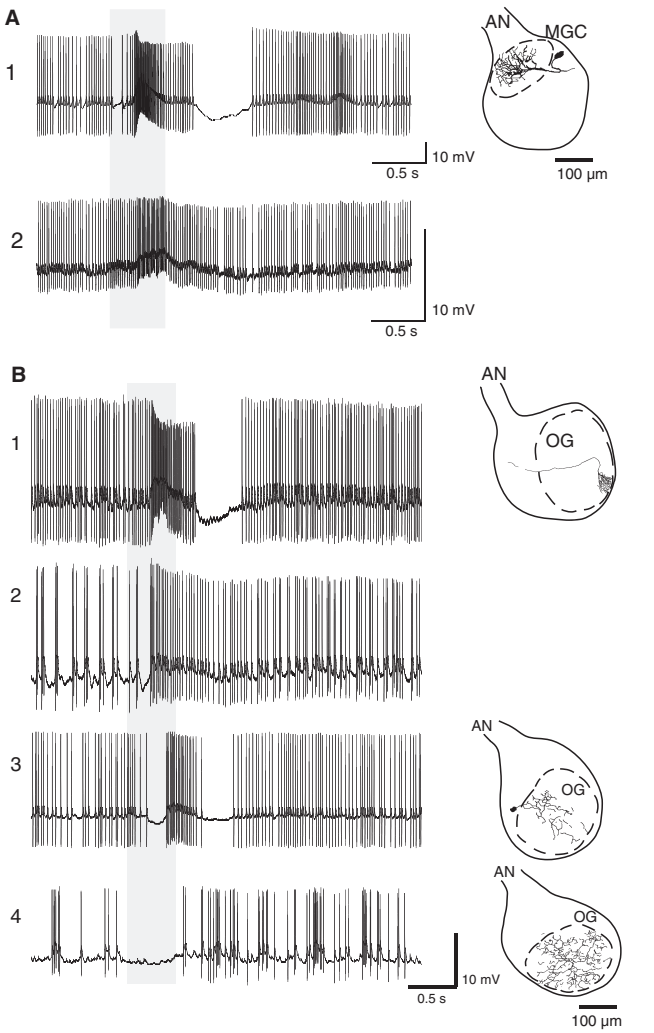

FIG. 3. AL neuron response types for (A) the sex pheromone blend and (B) the plant odour heptanal. All neuron types occur in both virgin and mated *A. ipsilon* males. The corresponding anatomy of neurons with the different response types are shown next to the recordings. (A) A majority of neurons responded to the pheromone blend with a biphasic pattern (1) consisting of an excitatory phase followed by an inhibitory phase. A few neurons did not show the inhibitory phase (2). (B) In response to plant odours, four patterns were observed: patterns 1 and 2, similar to patterns 1 and 2 observed in response to the pheromone blend; a triphasic pattern (3) with an inhibitory phase followed by an excitatory phase and an inhibitory phase; and a biphasic pattern (4) with an inhibitory phase followed by an excitatory phase. Grey bars represent the stimulation period. AN, antennal nerve.

Effect of mating on response characteristics of pheromone-responding AL neurons

A detailed analysis of different response parameters was carried out in MGC type 1 neurons (Fig. 4). The spike frequency of MGC neurons during the excitatory phase in both virgin (27 neurons) and mated (18 neurons) males increased with increasing doses of the pheromone

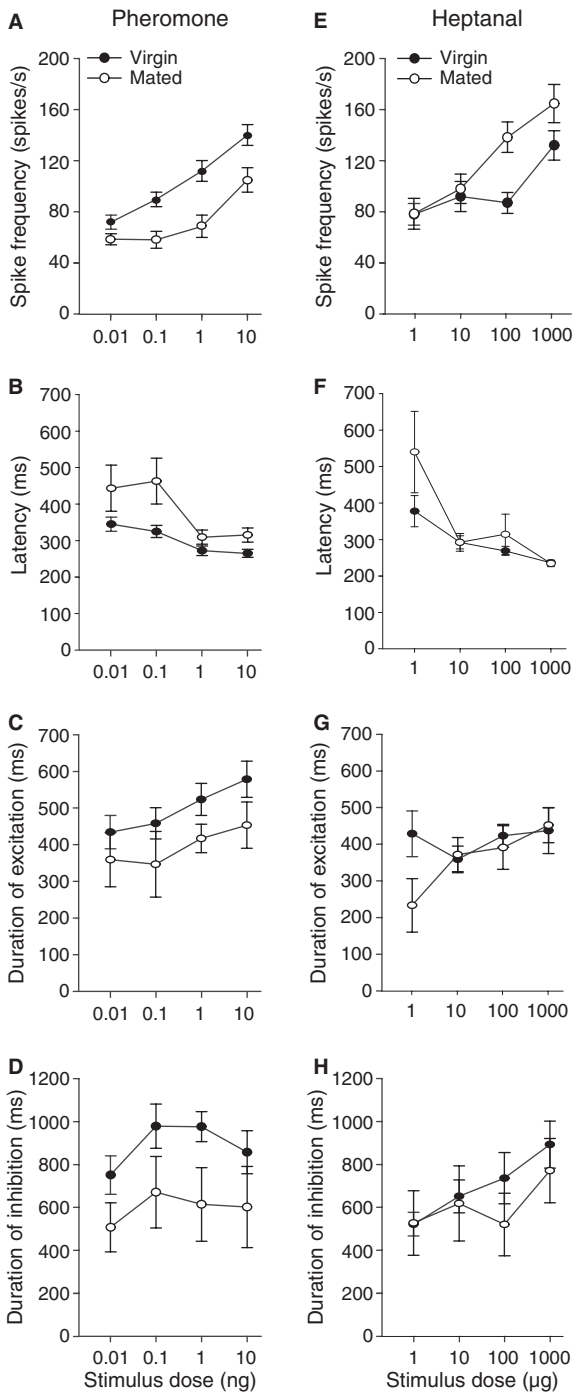

FIG. 4. Characteristics of the responses of AL neurons to the sex pheromone blend (A–D) and the plant odour heptanal (E–H) in virgin and mated *A. ipsilon* males. (A, E) Spike frequency. Mating affects the response of AL neurons within both the MGC and the OG, but in opposite directions. MGC neurons in mated males ($n = 18$) show lower firing rates than those in virgin males ($n = 27$). In contrast, OG neurons in mated males ($n = 13$) responded with higher spike frequencies than those in virgin males ($n = 21$). (B, F) Response latency. MGC neurons responded with a lower latency in virgin ($n = 25$) than in mated ($n = 17$) males. No differences were observed between OG neurons of virgin ($n = 19$) and mated ($n = 14$) males. (C, G) Duration of the excitatory phase. MGC neurons responded with a longer excitatory phase in virgin ($n = 23$) than in mated ($n = 17$) males. No differences were observed between OG neurons of virgin ($n = 17$) and mated ($n = 14$) males. (D, H) Duration of the inhibitory phase. MGC neurons responded with a longer inhibitory phase in virgin ($n = 23$) than in mated ($n = 16$) males. No differences were observed between OG neurons of virgin ($n = 15$) and mated ($n = 13$) males.

blend ($F_{2.4,90.5} = 42$, $P < 0.00001$) (Fig. 4A). However, the firing rate was significantly reduced after mating ($F_{1,43} = 11.7$, $P = 0.0014$) – in mated males, the mean maximal spike frequency was 72 ± 6 spikes/s, and in virgin males it was 100 ± 6 spikes/s (Fig. 4A).

The response latency also varied with the dose of the pheromone blend, decreasing with increasing stimulus loads in both groups of males ($F_{2.2,49.2} = 3.66$, $P = 0.028$) (Fig. 4B). MGC neurons from virgin males exhibited a significantly shorter latency in response to the pheromone blend (308 ± 12 ms; 25 neurons) than those from mated males (379 ± 25 ms; 17 neurons) ($F_{1,40} = 7.8$, $P = 0.008$) (Fig. 4B).

The durations of the excitatory and inhibitory phases in MGC neurons from virgin males (23 neurons) in response to the pheromone blend were, on average, 507 ± 30 and 883 ± 65 ms, respectively. On the other hand, MGC neurons from mated males showed mean values of the excitatory (17 neurons) and inhibitory (16 neurons) phases of 395 ± 38 and 583 ± 101 ms, respectively. For both parameters, there was no dose-dependent effect ($F_{1.6,29.1} = 0.63$, $P = 0.51$ for the duration of excitation; $F_{3,36} = 1.75$, $P = 0.17$ for the duration of the inhibitory phase), although we found statistical differences between virgin and mated males (Fig. 4C and D). Mated males showed not only shorter excitatory phases than virgin males ($F_{1,38} = 5.5$, $P = 0.02$) (Fig. 4C), but also shorter inhibitory phases than virgin males ($F_{1,37} = 6.8$, $P = 0.013$) (Fig. 4D).

Effect of mating on response characteristics of plant odour-responding AL neurons

We analysed the response characteristics of the most common OG type 1 neurons as a function of the mating status of male moths. Figure 4E shows the spike frequencies of OG neurons measured at different heptanal loads in virgin (21 neurons) and mated (13 neurons) males. In both groups, increasing doses elicited an increasing firing response during the excitatory phase ($F_{2.1,60.4} = 12.9$, $P = 0.00001$) (Fig. 4E), a decreasing response latency ($F_{1.4,16.6} = 10.95$, $P = 0.002$) (Fig. 4F), and an increasing duration of excitation ($F_{3,18} = 8$, $P = 0.001$) (Fig. 4G). In contrast, although the duration of inhibition globally increased with the doses, no statistical difference was found for it ($F_{1.6,9.7} = 3.13$, $P = 0.09$) (Fig. 4H). The spike frequency of OG neurons responding to heptanal was significantly higher in mated (125 ± 9 spikes/s) than in virgin (96 ± 6 spikes/s) males ($F_{1,32} = 6.3$, $P = 0.017$) (Fig. 4E). There was no difference in the response latency between OG neurons from virgin (289 ± 15 ms; 19 neurons) and mated (304 ± 35 ms; 14 neurons) males (Fig. 4F) ($F_{1,31} = 0.2$, $P = 0.65$). The average duration of the excitatory phase was also not statistically different between virgin (411 ± 40 ms; 17 neurons) and mated (392 ± 41 ms; 14 neurons) males ($F_{1,29} = 0.09$, $P = 0.75$) (Fig. 4G). Furthermore, no statistical differences were found for the duration of the inhibitory phase between the two groups of males (virgin, 740 ± 85 ms, 15 neurons; and mated, 736 ± 130 ms, 13 neurons) ($F_{1,26} = 0.0007$, $P = 0.97$) (Fig. 4H).

Discussion

Our analysis of the effect of mating on the processing of both sex pheromones and plant odours at the peripheral (antennal) and central (AL) levels of the olfactory system of *A. ipsilon* male moths revealed that sex pheromone processing is only modulated at the central nervous level, where pheromone sensitivity decreases massively after mating, as reflected by changes in coding properties of MGC neurons. Plant odour processing, on the other hand, seems to depend less on mating status. However, an increase in spike frequencies of OG neurons in

response to heptanal and increased calcium signals in OG of mated males indicate that up-modulation of flower odour sensitivity might occur within the AL. Our recordings of heptanal-responding ORNs in mated males show that the observed increase in sensitivity within the AL does not originate from modifications at the peripheral level.

Mating-independent peripheral detection of sex pheromone

We observed no post-mating difference in the general response pattern of ORNs to sex pheromone. So far, our results from single ORN recordings and optical imaging experiments suggest that the absence of behavioural responsiveness to the sex pheromone after mating cannot be attributed to differences in the sensitivity of ORNs to pheromone in mated moths. A preliminary study of the effect of mating at the antennal level, by means of EAG recordings, revealed an invariant response with regard to mating status (Gadenne *et al.*, 2001). However, an EAG signal is supposedly a sum of receptor potentials (Kaissling, 1995), whereas AP activity can only be assessed with single-sensillum recordings. We now show that the AP responses of individual pheromone-sensitive receptor neurons, measured with single-sensillum recordings, do not reveal any difference between virgin and mated males, and thus confirm that pheromone input from the peripheral level remains constant. In contrast to our study, peripheral plasticity of the olfactory system in response to pheromones was shown in other model systems. There is peripheral plasticity in human (men and women) odour responses to androstenone (Wang *et al.*, 2004). Peripheral plasticity for chemical stimuli was also observed with age in moths (Seabrook *et al.*, 1979; Domingue *et al.*, 2006) and flies (Rees, 1970; Crnjar *et al.*, 1990). In the long-lived moth *Caloptilla fraxinella*, reproductively inactive adult males (summer and autumn) show a lower antennal response than reproductively active males (spring) (Lemmen & Evenden, 2009). Peripheral modulation of odour sensitivity has also been shown in *Xenopus laevis* larvae (Breunig *et al.*, 2010).

The mating-independent peripheral detection of pheromone was further confirmed by our calcium imaging results, which did not show any significant changes after mating in the activation patterns of the MGC. Although our imaging experiments performed in the AL reflect neural activity of the brain, the calcium response patterns after bath application of the dye supposedly originate mainly, although not exclusively, from ORNs (Galizia *et al.*, 1998; Sachse & Galizia, 2003; Galizia & Vetter, 2005; Heil *et al.*, 2007).

Mating-induced down-modulation of central processing of sex pheromone

Our present detailed data analysis shows that changes in pheromone coding properties in the MGC after mating are in accordance with our previously demonstrated decrease in sensitivity (Gadenne *et al.*, 2001; Barrozo *et al.*, 2010a). Following mating, an MGC neuron is slower to respond and generates fewer APs during a shorter excitatory period, and the duration of the following inhibitory period decreases. Modulation of the AL network by centrifugal neurons and/or LNs might be at the origin of these changes in male MGC neuron responses after mating, as ORN responses did not change with mating status. Mating might modulate the membrane excitability of PNs and LNs within the AL. This type of modulation might be caused by neuromodulators such as serotonin, which have been shown to change membrane excitability in the ALs of *Manduca sexta* (Kloppenburg & Hildebrand, 1995). Another possible explanation for the changes in MGC neuron responses of mated *A. ipsilon* males could be an inhibitory action of LNs, which could mediate the gain control of the olfactory circuit, as shown in *Drosophila* (Olsen & Wilson, 2008).

Although biogenic amines have been shown to modulate the central olfactory system in both vertebrates and invertebrates (Mercer *et al.*, 1996; Kloppenburg *et al.*, 1999; Perk & Mercer, 2006; Dacks *et al.*, 2008; Serguera *et al.*, 2008), it is unlikely that they are involved in the post-mating refractory period in *A. ipsilon* males, because biogenic amine treatments did not restore the behavioural response to sex pheromone after mating (Barrozo *et al.*, 2010b).

Mating-independent peripheral detection of plant odour

Our results show that detection of the plant odour heptanal in antennal ORNs does not change after mating. So far, to our knowledge, no systematic comparison of plant odour-responding ORNs between different physiological states or as a function of experience has been undertaken. This is essentially because of the difficulties in recording from large numbers of ORNs responding to the same plant-derived compound. Our study is a first attempt to fill this knowledge gap, but systematic investigations will be needed with a larger panel of plant compounds.

Mating-induced partial up-modulation of AL processing of plant odour

The general pattern of responses of AL neurons in the OG to heptanal was very similar to that in our previous studies in *A. ipsilon* (Greiner *et al.*, 2002). In *A. ipsilon* males, mating did not significantly affect the latency or the duration of the excitatory and inhibitory phases of OG neurons during heptanal stimulation, corroborating previous results showing that response thresholds for plant volatiles are similar in OG neurons in virgin and mated *A. ipsilon* males (Barrozo *et al.*, 2010a) and in the grapevine moth, *Lobesia botrana* (Masante-Roca *et al.*, 2002). The majority of our results are consistent with the behavioural responses observed in virgin and mated *A. ipsilon* males to a linden flower extract – both groups showed similar oriented flights to the plant extract in wind tunnel experiments (Barrozo *et al.*, 2010a). However, our analysis of the spike frequency of OG neurons revealed a significant difference between virgin and mated males. These differences might have been hidden in our previous, less detailed, studies. Our parallel studies of the neural bases of age-dependent olfactory plasticity in *A. ipsilon* males showed that the AL neuron response thresholds for different plant compounds, including heptanal, did not change as a function of age, but no detailed analysis of the data was performed (Greiner *et al.*, 2002; Anton *et al.*, 2007).

In *A. ipsilon*, the absence of differences in plant odour detection by ORNs between virgin and mated males indicates that the observed modulation found in calcium imaging experiments might be caused by presynaptic modulation at the ORN axon terminals within the AL, or might originate from a contribution of the AL network to the measured calcium signals. Similar differences in odour representation in the AL were shown as a function of age in the honeybee (Wang *et al.*, 2005). The changes at the AL level observed in *A. ipsilon* by optical imaging are, then, also reflected by a change in spike frequencies in OG PN responses to the plant odour after mating.

Conclusions

Newly mated *A. ipsilon* males avoid new sexual encounters during the time when they need to refill their sex glands before being able to re-mate. Here, we show not only that their central olfactory system

reduces its sensitivity to sex pheromone, but also that the response characteristics of AL PNs are modulated. Our results show that strong down-modulation of sex pheromone processing occurring in the central nervous system is paralleled by significant up-modulation of plant odour processing, also originating from modifications within the AL. Therefore, even if males are not attracted to sex pheromone after mating, they conserve or even improve their plant odour perception machinery. This strategy would prevent males from wasting energy searching for females (when they are incapable of copulation), and at the same time promote searching for food sources to rapidly restore reserves. However, as both odour classes – sex pheromones and plant odours – occur together in a natural environment, and recent studies indicate that complex interactions between sex pheromone and plant odour processing systems occur in moths (Christensen & Hildebrand, 2002; Namiki *et al.*, 2008; Schneider, 1964; Ochieng *et al.*, 2002; Party *et al.*, 2009; Barrozo *et al.*, 2010a), it will be important to analyse responses of ORNs and AL neurons to mixtures of plant odour and sex pheromone in the future. A second major challenge is the investigation of pheromone and plant odour responses of LNs as a function of mating status.

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Abbreviations

AL, antennal lobe; AP, action potential; d.f., degrees of freedom; EAG, electroantennogram; ISI, interspike interval; LN, local neuron; MGC, macroglomerular complex; OG, ordinary glomerulus; ORN, olfactory receptor neuron; PN, projection neuron; Z7-12:OAc, (*Z*)-7-dodecen-1-yl acetate.

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