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RESEARCH ARTICLE

Experience-dependent modulation of antennal sensitivity and input to antennal lobes in male moths (*Spodoptera littoralis*) pre-exposed to sex pheromone

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SUMMARY

Sex pheromones are intraspecific olfactory signals emitted by one sex to attract a potential mating partner. Behavioural responses to sex pheromones are generally highly stereotyped. However, they can be modulated by experience, as male moths previously exposed to female sex pheromone respond with a lower threshold upon further detection, even after long delays. Here, we address the question of the neural mechanisms underlying such long-term modulation. As previous work has shown increased responses to pheromone in central olfactory neurons, we asked whether brief exposure to the pheromone increases input activity from olfactory receptor neurons. Males pre-exposed to sex pheromone exhibited increased peripheral sensitivity to the main pheromone component. Among nine antennal genes targeted as putatively involved in pheromone reception, one encoding a pheromone-binding protein showed significant upregulation upon exposure. In the primary olfactory centre (antennal lobe), the neural compartment processing the main pheromone component was enlarged after a brief pheromone exposure, thus suggesting enduring structural changes. We hypothesise that higher peripheral sensitivity following pre-exposure leads to increased input to the antennal lobe, thus contributing to the structural and functional reorganization underlying a stable change in behaviour.

Key words: olfactory neuron, pheromone, antennal lobe, plasticity, sensory experience.

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INTRODUCTION

Insects were considered for a long time to be hard-wired organisms capable of only stereotyped, innate behaviours. Contrasting with this view, many recent studies have shown that, like mammals, insects also display a high degree of plasticity (Menzel and Giurfa, 2006; Giurfa, 2007; Groh and Meinertzhagen, 2010). This is particularly well illustrated by the numerous examples of how previous experience of odorants with strong biological significance may lead to adaptive behavioural and neural changes (Vergoz et al., 2007; Vergoz et al., 2009; Arenas et al., 2009; Beggs et al., 2007; Iyengar et al., 2010; Urlacher et al., 2010). Many insect species are capable of olfactory learning, and their olfactory pathways exhibit high degrees of neural plasticity. Whenever the observed changes are stable over days or weeks, they are believed to rely on changes in levels of gene transcription and neural circuits. Indeed, olfactory exposure may lead to structural remodelling of olfactory brain networks as well as changes in gene expression levels (e.g. Devaud et al., 2001; Beggs et al., 2007; Zhou et al., 2009).

The noctuid moth *Spodoptera littoralis* is an excellent system with which to study the effects of olfactory experience. The olfactory pathway of male *S. littoralis* is endowed with dedicated neurons that confer high sensitivity to the female sex pheromone (Hansson and Anton, 2000). First, olfactory receptor neurons (ORNs) housed in antennal trichoid sensilla (Ljungberg et al., 1993) detect the pheromonal components with specific molecular equipment. Pheromone-binding proteins (PBPs) transport odorant pheromone molecules through the sensillar lymph close to the pheromone receptors (PRs), which recognise specific molecules. Pheromone-degrading enzymes (PDEs) contribute to signal deactivation by degrading the ligands (for a review, see Jacquin-Joly and Maïbèche-Coisné, 2009). PR activation requires heterodimerisation with the atypical co-receptor ORco [olfactory receptor co-receptor (Vosshall and Hansson, 2011)]. Input from pheromone-sensitive ORNs reaches the first olfactory centre, the antennal lobe (AL). All axons of one ORN population responding to the same pheromone component target a common compartment (glomerulus) of the male-specific macroglomerular complex (MGC) (Ochieng et al., 1995; Carlsson et al., 2002; Couton et al., 2009). Each of these component-specific MGC glomeruli then sends output through projection neurons (PNs) to higher brain centres in the protocerebrum (mushroom bodies, lateral horn) where olfactory information is further processed (Anton and Hansson, 1995). Such

centres are believed to provide direct or indirect input to the lateral accessory lobes, where an output signal appears to be generated and transmitted to the thoracic ganglia through descending neurons (Kanzaki et al., 1991a; Kanzaki et al., 1991b; Kanzaki and Shibuya, 1992; for a review, see Haupt et al., 2010). Ultimately, the resulting coordinated activity of thoracic flight muscles by motor neurons leads to the characteristic orientation flight towards the pheromone source (Cardé and Willis, 2008). Despite its stereotypy, this behaviour and the underlying processing steps nevertheless admit some degree of plasticity. Indeed, males briefly pre-exposed (1 min) to the female sex pheromone exhibit increased sensitivity to this crucial signal, as they are more likely to fly or walk towards a source of pheromone even 1 day later (Anderson et al., 2003; Anderson et al., 2007). At this time, recordings of AL neurons revealed increased sensitivity to the main pheromone component (Anderson et al., 2007). This suggests that pre-exposure triggers adaptive mechanisms that, given their delay of appearance, may encompass transcriptional regulation of genes encoding for proteins important for peripheral olfactory reception and transduction and/or central synaptic processing of olfactory input, as well as structural modifications of the neural circuits involved in pheromone processing.

Here, we investigated whether early steps of olfactory processing are affected by the brief pre-exposure to sex pheromone, and thus contribute to the increased responses in AL neurons. We first investigated whether peripheral detection of the pheromone is increased at the level of individual pheromone-sensitive ORNs, as electrophysiological recordings of the whole antenna (electroantennograms) had previously failed to reveal any significant difference in sensitivity between naive and pre-exposed males (Anderson et al., 2007). We thus recorded activity specifically from pheromone-sensitive ORNs to detect possible functional changes induced by pre-exposure. We also searched for a possible molecular basis for stable modifications of ORN activity by measuring the expression levels of genes putatively involved in pheromone detection in antennae from pre-exposed and naive males. Because our underlying hypothesis was that enduring changes in ORN sensitivity could lead to increased synaptic input to central neurons, we tested whether structural reorganization could be detected in the AL by measuring potential volume changes in olfactory glomeruli.

MATERIALS AND METHODS

Insects

Spodoptera littoralis Boisduval 1833 (Lepidoptera: Noctuidae) were maintained in an environmental chamber at 22–23°C under a 16 h:8 h light:dark cycle. Newly emerged adults were collected daily in the last hours of the photophase and their wings were cut off after brief CO_2 anaesthesia to prevent flying during the pre-exposure procedure (see below). They were then maintained in plastic cages on a humidified substrate and fed on 10% sugar water. Pre-exposure (see below) took place 1 day later, during their second scotophase. Tissue collection and electrophysiological experiments took place 24 h after pre-exposure, i.e. during the following scotophase.

Pre-exposure

Pre-exposure was performed on a locomotion compensator during the first half of the scotophase under red light. Insects were taken to the experimental room shortly before the exposure. The locomotion compensator was adapted from a previous model (Kramer, 1976). In brief, it consists of a sphere (300 mm diameter) maintained by an aerostatic holder (TrackSphere LC-300, SYNTECH, Kirchzarten, Germany). Servomotors moved the sphere to compensate for the displacement of the animal so as to keep it on the top of the sphere. A wingless male was gently placed on the top of the sphere and exposed to a continuous flow of charcoal-filtered humidifier room air (24 ml s^{-1}) delivered through a horizontal tube (2 cm diameter), the opening of which was placed 10 cm from the insect. A few seconds later, a stimulus controller (CS 55, SYNTECH) switched on an additional airflow (7 ml s^{-1}) to a Pasteur pipette whose tip was inserted in a hole in the main tube, 15 cm from the outlet, and decreased the main flow by 7 ml s^{-1} so that the flow rate over the insect was kept constant during the whole procedure. Insects were pre-exposed for 1 min to one female equivalent of the pheromone gland extract in 10 µl hexane (pre-exposed males) or to 10 µl hexane (naive males) loaded on a filter paper inserted into the pipette. After exposure, males from each group were placed in separate cages and returned to their environmental chamber until further experiments. Natural pheromone extracts were prepared from 2- or 3-day-old females: glands of 20 females were pooled and immersed in 300 µl of hexane for 1 h, and then the hexane extract was separated from the glands by aspiration with a micropipette. Extracts were prepared every week and kept at –20°C for 15 days at most.

Single-sensillum recordings

Recordings were made throughout the entire scotophase. Insects were taken out of the chamber one by one and were then CO_2-anaesthetised and mounted on a Styrofoam block. One antenna was fixed with adhesive tape and oriented to allow an optimal access to the trichoid sensilla. Recordings were carried out with the tip recording technique (Kaissling and Thorson, 1980) from long sensilla trichodea known in *S. littoralis* to respond to the main pheromone component (Ljungberg et al., 1993). The tips of a few hairs were cut off using sharpened forceps. The reference electrode was filled with (in mmol l^{-1}): 6.4 KCl, 340 glucose, 10 Hepes, 12 $MgCl_2$, 1 $CaCl_2$, 12 NaCl, 450 mosmol l^{-1} (glucose) and pH 6.5 (KOH). The recording electrode was filled with: 172 KCl, 22.5 glucose, 10 Hepes, 3 $MgCl_2$, 1 $CaCl_2$, 25 NaCl, 4 EGTA, 425 mosmol l^{-1} (glucose) and pH 6.5 (KOH). To minimise contributions of field potentials, the reference electrode was inserted into an antennal segment close to the recording site. Recorded signals were amplified (×500), sampled at 20 kHz, low-pass filtered at 5 kHz using a Cyberamp 320 amplifier (Molecular Devices, Sunnyvale, CA, USA) and digitised using a Digidata 1200A acquisition board driven by Clampex 8 software (both Molecular Devices).

A humidified and charcoal-filtered airflow (30 l h^{-1}) was continuously directed at the preparation, and an exhaust was placed behind the preparation. Pheromone stimuli were obtained by blowing an air puff (200 ms, 10 l h^{-1}) through a 1.2 mm diameter glass capillary (Blaubrand® intraMark, Wertheim, Germany) placed 3–4 mm from the recording electrode. A new set of stimulus capillaries was prepared daily: a filter paper strip (20×1.2 mm), previously washed in hexane and stored dry in a sealed vial, was impregnated with either 1 µl of hexane (control) or 1 µl of the main pheromone compound, *Z*9,*E*11-tetradecenyl acetate (*Z*9,*E*11-14:Ac) dissolved in hexane, left 30 s under a hood to let the solvent evaporate, and inserted in the stimulus capillary. The pheromone compound was tested over a wide range of doses (10^{-2} to 10^{5} pg), though only those eliciting responses were further analysed (see below). For each insect, 3 min of spontaneous activity were first recorded and then stimuli were applied from low to high pheromone loads. The time interval between two consecutive stimulations was 2 min.

The recorded signal was high-pass filtered offline (50 Hz) using Clampfit 10 (Molecular Devices), and action potentials (APs) were detected by threshold searches. Firing responses correspond to the firing frequency during the 200 ms stimulus. The spontaneous activity (measured as the frequency of APs emitted during the 3 min recording period before stimulating the antenna with pheromone) was compared between naive and pre-exposed males with a Mann–Whitney *U*-test. The effects of dose and treatment (pre-exposed *versus* naive) on the frequency of APs fired in response to pheromone were evaluated using a two-way ANOVA. This was done only for stimuli of at least 10 pg (stimuli eliciting a response). As the spontaneous activity was very weak ($\leq 0.12\,AP\,s^{-1}$), the threshold was determined as the lowest dose eliciting at least five APs during the 400 ms period following stimulation onset (i.e. 200 ms stimulus period + 200 ms post-stimulation period) to take slow responses into account. The minimum stimulus concentration needed for response threshold was compared between pre-exposed and naive males by means of a chi-square test, and individually for each concentration with Fisher's exact tests. All statistical tests were run with R software (R Development Core Team, 2009).

RNA extraction, cDNA synthesis and qPCR experiments

Three different batches of antennae from 15 naive and 15 pre-exposed males were collected, representing three different biological repetitions. In each experiment, antennae (N=30 per group) were dissected and stored at –80°C. RNAs were extracted with the RNeasy® MicroKit (Qiagen, Hilden, Germany), which included a DNase treatment. Concentration and quality were determined using a NanoDrop ND-1000 System (ThermoFisher Scientific, Waltham, MA, USA). Single-stranded cDNAs were synthesised from 1 µg of total RNAs with 200 U of M-MLV reverse transcriptase (Clontech, Mountain View, CA, USA) using buffer and protocol supplied in the Advantage® RT-for-PCR kit (Clontech). Selected genes already described and putatively involved in pheromone detection have been studied here: three genes encoding putative PBPs (*SlitPBP1*, *SlitPBP2* and *SlitPBP3*; GenBank accession numbers EZ982949, EZ981038 and EZ983456, respectively), four candidate PR genes (*SlitPR6*, *SlitPR11*, *SlitPR13* and *SlitPR16*; GenBank accession numbers EZ983328, FQ031000, EZ982777 and EZ981960, respectively) (Legeai et al., 2011), the obligatory co-receptor *SlitORco* (ABQ82137) (Malpel et al., 2008) and a candidate pheromone-degrading enzyme, SlCXE7 (Durand et al., 2010a). Different reference genes were tested for normalisation [*rpL13*, *rpL8*, *GAPDH* and *β-actin*; primers described in Durand et al. (Durand et al., 2010b)]. *rpL8* displayed the most consistent expression in the samples and thus was chosen as the reference gene. Gene-specific primers for *SlitPRs*, *SlitORco* (Legeai et al., 2011) and *SlCXE7* (Durand et al., 2010a) have been previously described.

Gene-specific primers for the three *SlitPBP*s were designed using Beacon Designer 4.0 software (Bio-Rad, Hercules, CA, USA): PBP1 forward primer, ATGGCGAAGAAGTTGGACCTC, PBP1 reverse primer, CTCGTGGATCTTAGTGCGGAAG; PBP2 forward primer, TTCTGGAAGGAAGGCTACGA, PBP2 reverse primer, CAGGGTCTTCATGCAAGGAT; and PBP3 forward primer, GATGGATCGCGTAACGTCTT, PBP3 reverse primer, CTCATGCTTGCATTCTTCCA. The qPCR mix was prepared in a total volume of 12 µl with 6 µl of Absolute QPCR SYBR Green Mix (ThermoFisher Scientific, Epsom, UK), 3 µl of 1/50 diluted cDNA (or no-RT or no-template controls) and 200 nmol l^{-1} of each primer. qPCRs were performed using a Bio-Rad CFX96 System and the following experimental run protocol: denaturation program (95°C for 15 min), amplification and quantification program repeated 40 times (95°C for 10 s, 60°C for 30 s with a single fluorescence measurement), and melting curve program (65–95°C with a heating rate of 0.5°C per 0.5 s). Standard curves were generated using a fivefold dilution series of a cDNA pool to evaluate primer efficiency E ($E=10^{-1/slope}$). All reactions were performed in duplicate. Gene expression levels were calculated relatively to the expression of the *rpL8* control gene and expressed as the ratio $E_{Slitgenes}^{\Delta CTSlitgenes}/E_{rpL8}^{\Delta CTrpL8}$, where CT is cycle threshold (Pfaffl, 2001). Differences in gene expression levels between naive and pre-exposed male antennae were tested using STATISTICA software (StatSoft, Maisons-Alfort, France).

Histology and volumetry of antennal lobes

Heads of pre-exposed and naive males were cut off and an opening was made in the cuticle; they were then fixed for 24 h at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS) with 0.2% Triton X-100 (PBS-T). Brains were then removed from head capsules (optic lobes were cut off), rinsed three times in PBS-T and incubated for 5 days at 4°C into a primary antibody solution (anti-synapsin I 1:50 in PBS-T: SYNORF1, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). After rinsing, they were stained with the secondary antibody (Alexa-Fluor-546-conjugated anti-mouse 1:150 in PBS-T; Invitrogen, Abingdon, UK) for 4 days at 4°C. The brains were then rinsed several times in PBS-T and PBS, and then dehydrated in an ethanol series and cleared in methylsalicylate (Sigma-Aldrich, Lyon, France) for at least 48 h at 4°C.

Brains were imaged as wholemounts (in Permount, Sigma-Aldrich) under a laser-scanning microscope (TCS2, Leica, Wetzlar, Germany) with a 20× water immersion objective. Preparations were excited at 561 nm and fluorescence was detected between 570 and 620 nm. For each brain, one complete AL was scanned at intervals of 1 µm with a 4× frame average (stacks of 150–190 frames). Complete stacks of optical sections (512×512 pixels) were imported into Amira 3.1 (Visage Imaging, Berlin, Germany). Reconstructions were performed blindfold respective to the treatment. Estimations of glomerular size were based on a standard protocol previously used in other insect species (Devaud et al., 2001; Devaud et al., 2003; Arenas et al., 2012; Hourcade et al., 2009; Arenas et al., 2012). Individual glomeruli were identified and reconstructed by manually tracing their outlines every three frames and interpolating the surfaces to obtain an estimation of their volume. This was done for five glomeruli in each AL: three belonging to the MGC (glomeruli 17, 18 and 37) and two ordinary glomeruli (4 and 11) [according to the atlas by Couton et al. (Couton et al., 2009)]. Using the same procedure, we obtained the volume of the whole AL in the same preparations. As indicated by a Shapiro–Wilk test, total AL volumes and absolute glomerular volumes did not follow normal distributions; hence a Mann–Whitney *U*-test was used for inter-group comparisons. Glomerular volumes were also normalised to the total volume of the corresponding AL to account for possible effects due to size differences between individual brains (see Results). Such values were distributed normally for all five glomeruli in both groups (Shapiro–Wilk test: in all cases $W>0.902$ and $P>0.06$). Thus, pairwise comparisons between normalised glomerular volumes were performed with a Student's *t*-test, using the Gnumeric spreadsheet (www.gnumeric.org).

RESULTS

Effect of pre-exposure on ORN responses

To test for changes in sensitivity of pheromone-sensitive ORNs following pre-exposure, single-sensillum recordings were performed

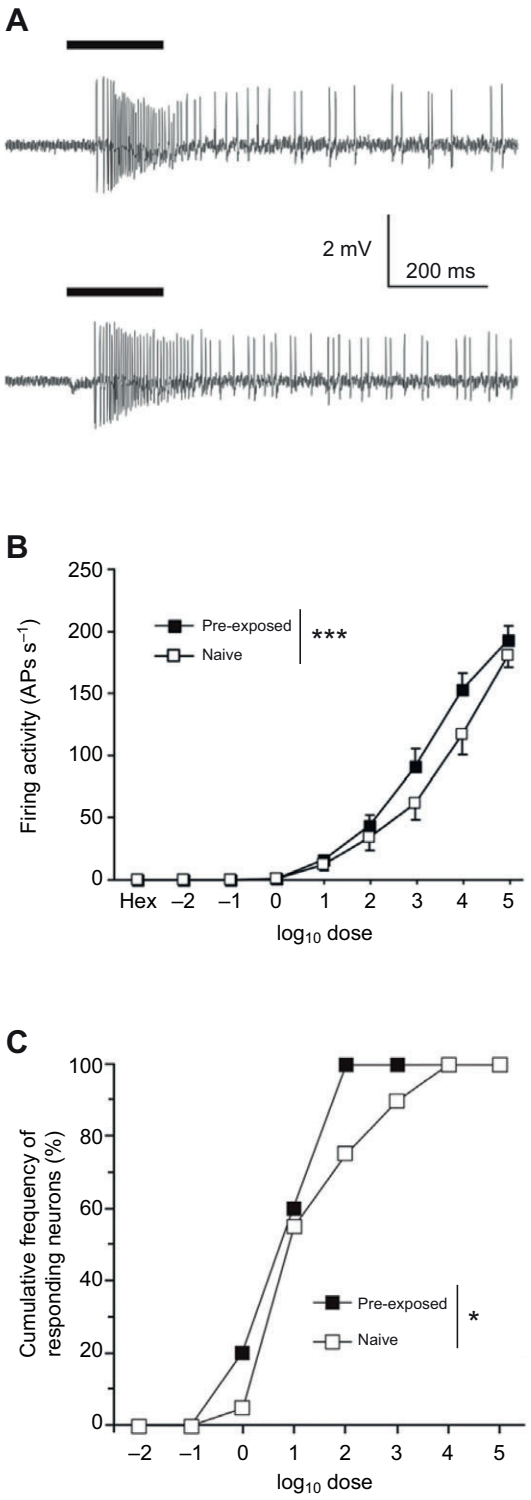

Fig. 1. Olfactory receptor neurons (ORNs) are more sensitive to pheromone after pheromone pre-exposure. (A) Examples of recordings of ORN responses to 10 ng of *Z*9,*E*11-tetradecenyl acetate (*Z*9,*E*11:14:Ac) from a pre-exposed (upper trace) and a naive male *Spodoptera littoralis* (lower trace). (B) Mean (±s.e.m.) firing activity during the 200 ms stimulation period with 10^{-2} to 10^{5} pg of *Z*9,*E*11-14:Ac or Hexane (Hex) as a control in pre-exposed and naive males. The two dose–response curves differ significantly (two-way ANOVA for repeated measures, $P<0.001$). AP, action potential. (C) Cumulative distribution of response thresholds of ORNs in naive and pre-exposed males. Response thresholds were different between the two groups (χ^2, $P<0.05$). $N=20$ pre-exposed males and 20 naive males in B and C.

on antennae from pre-exposed and naive males (Fig. 1). First, as a control we recorded firing activity from pheromone-sensitive ORNs in absence of any stimulation to determine whether pre-exposure could have affected their basal activity. As shown in Fig. 1A, the spontaneous discharge frequency was similar between the two groups (naive: 0.12±0.03 Hz, pre-exposed: 0.11±0.03 Hz; Mann–Whitney *U*-test: $U=209.0$, $P=0.82$). We then recorded responses evoked by increasing doses of pheromone (Fig. 1B). Irrespective of the pre-exposure treatment, no detectable response was recorded for doses below 1 pg, whereas firing activity increased sharply for higher doses, up to approximately 200 Hz when stimulating with 10^5 pg of pheromone. A two-way ANOVA revealed a strongly significant dose effect ($F_{4,147}=170.1$, $P<0.001$). In addition, pre-exposure significantly increased pheromone-evoked responses ($F_{1,147}=16.1$, $P<0.001$), whereas there was no significant interaction between dose and treatment ($F_{4,147}=1.88$, $P>0.05$). Consistent with an increase in sensitivity after pre-exposure, response thresholds were lower in ORNs from pre-exposed than from naive males ($\chi^2=10.03$, d.f.=3, $P<0.05$; Fig. 1C). Indeed, neurons from pre-exposed males responding in the range of 10^2–10^4 pg were more frequent that those from control males. Pairwise comparisons for individual concentrations showed that this trend reached the level of significance for the 10^3 value (Fisher's exact test, $P<0.05$). Thus, sensillar recordings show that pheromone pre-exposure increases the sensitivity of male ORNs to sex pheromone.

Effect of pre-exposure on gene expression in antennae

We then tested whether males pre-exposed to sex pheromone differed from their naive controls in levels of expression of genes related to pheromone detection in ORNs. Fig. 2 reports the comparative expression levels of nine genes targeted as putatively involved in pheromone detection in naive and pre-exposed male antennae. These genes encode three putative PBPs (*SlitPBP*s), four putative PRs (*SlitPR*s), the olfactory co-receptor (*SlitORco*) and a putative PDE (*SlCXE7*). As shown in Fig. 2, we detected significant upregulation for one gene (*PBP3*) in pre-exposed *versus* control males ($U=0.0$, $P<0.05$). We did not detect any significant difference of expression for the other genes tested, probably because of quite variable expression across the three experimental repetitions.

Effect of pre-exposure on AL glomerular volumes

To investigate possible structural changes linked to the increased sensitivity of ORNs (Fig. 1) and AL neurons (Anderson et al., 2007), we investigated whether modifications of the AL architecture might be revealed by volume variations of glomeruli. In particular, we examined whether those glomeruli treating sex pheromone information might be specifically affected, by comparing volumes of specific glomeruli from pre-exposed and control males. First, total AL volumes were measured and compared: average AL volumes were slightly (7%), but not significantly, higher in pre-exposed males (Mann–Whitney *U*-test: $U=154.0$, d.f.=35, $P=0.447$, data not shown). This difference might be due to either an effect of exposure or unequal distribution of larger and smaller brains across the samples, irrespective of treatment. We thus reasoned that we should be able to detect possible effects of treatment on glomerular size, independently of natural size variability, by comparing relative, rather than absolute, glomerular volumes. Therefore, in each animal, individual glomerular volumes were normalised to their AL volume to account for individual size variations. As shown in Fig. 3, all glomeruli from the sample but one remained unaffected by the pre-exposure treatment ($P>0.23$ in all cases). These included 'ordinary'

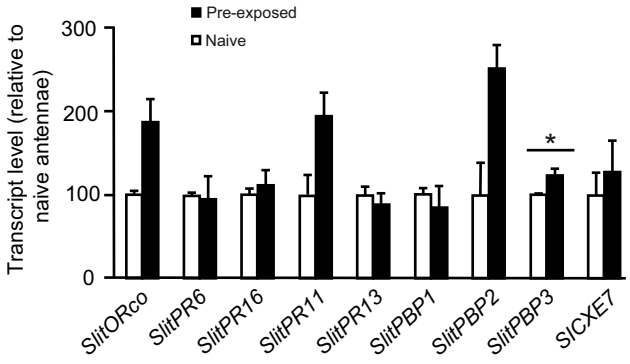

Fig. 2. Comparison of transcript levels between naive and pre-exposed male *Spodoptera littoralis* antennae. Expression levels of genes encoding candidate pheromone-binding proteins (*SlitPBP1, 2, 3*), pheromone receptors (*SlitPR6, PR16, PR13, PR11*), the co-receptor (*SlitORco*) and a candidate pheromone-degrading enzyme (*SlCXE7*), as quantified using real-time PCR on male antennae. Data were obtained from triplicate experiments and are given as means ± s.e.m. for naive and pre-exposed *S. littoralis* males. Expression levels were calculated relative to the expression of *rpL8* and are reported relative to the level in naive antennae (100%). **P*<0.05 (Mann–Whitney *U*-test).

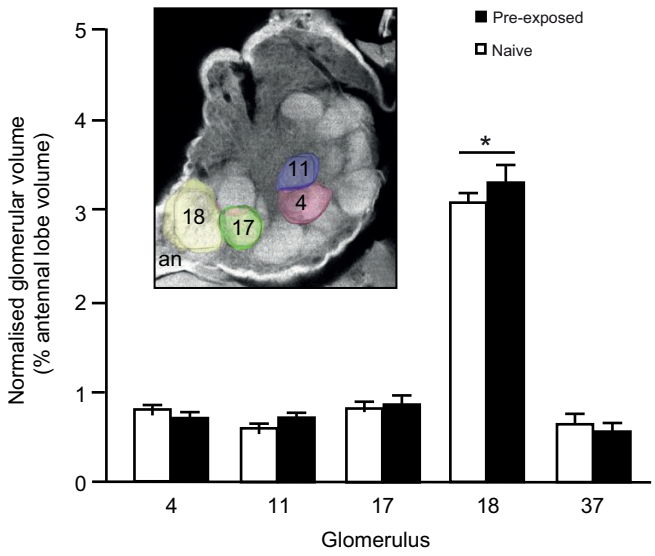

Fig. 3. The cumulus of the macroglomerular complex (MGC) is enlarged in pre-exposed male *Spodoptera littoralis*. Volumes (mean ± s.e.m.) of reconstructed glomeruli in naive (*N*=20) *versus* pre-exposed (*N*=18) males, normalised to the whole AL volume. Macroglomerulus 18 (cumulus) was significantly larger in pre-exposed than in naive males. No significant volume difference was observed in any other reconstructed glomerulus. Inset: confocal optical section of a left antennal lobe, showing the superimposed reconstruction of four of the analyzed glomeruli (glomerulus 37 was in another plane). an, antennal nerve. Scale bar, 50 μm. **P*<0.05 (Mann–Whitney *U*-test).

glomeruli (glomeruli 4 and 11) as well as glomeruli from the MGC that are dedicated to pheromone processing (glomeruli 17 and 37). Interestingly however, glomerulus 18 was significantly larger in pre-exposed animals (+6.9%; t=–1.0648, d.f.=35, P<0.05). Also known as the cumulus, this glomerulus is the part of the MGC known to process the main pheromone component, *Z*9,*E*11-14:Ac (Ochieng et al., 1995; Carlsson et al., 2002). Thus, ALs of pre-exposed males undergo structural changes that appear to be restricted to the part of the MGC processing information on the major pheromone component.

DISCUSSION

Male *S. littoralis* exhibit an enduring change in their behavioural response to sex pheromone, once briefly pre-exposed to it (Anderson et al., 2003; Anderson et al., 2007). Here, we present evidence of functional modifications in the antennae of pre-exposed males, which are likely to contribute to their change in behaviour. Our data indicate that, 24 h following exposure, the peripheral sensitivity to the sex pheromone is increased. We thus tested the hypothesis that the likely sustained increase in pheromone-evoked input to the ALs induces long-term changes at the central level. Accordingly, we show that the glomerulus specifically processing the main pheromone component is enlarged in pre-exposed males. Altogether, our data reveal that a short pre-exposure to a highly significant odorant such as sex pheromone triggers changes at multiple levels of the olfactory pathway.

Pre-exposure increases sensitivity at the detection level

Our results show that ORNs respond to the sex pheromone at lower concentrations and with higher firing frequencies in pre-exposed males. Interestingly, the effects of pre-exposure were observed in responses to behaviourally relevant doses (1–10 ng of *Z*9,*E*11-14:Ac), because the extract from a single female gland (female equivalent) contains 20 ng of the main pheromone component (Anderson et al., 2007). Previous recordings of whole antennal responses (electroantennograms) had reported no significant effect of pre-exposure within this concentration range (Anderson et al., 2007). This discrepancy is most likely due to the greater sensitivity of single-sensillum recordings compared with electroantennography (Lucas and Renou, 1989), which can detect changes in particular subtypes of ORNs – such as those responding to pheromone compounds – that would otherwise go undetected when recording from the whole ORN population. This lower response threshold was observed without any change in ORN spontaneous activity, thus indicating that it was not due to any change in basal firing probability. Along the same lines, it was shown recently that young fruitflies exposed early in life to a given odorant later displayed increased ORN sensitivity to this odorant (Iyengar et al., 2010). Our findings that pre-exposure to the pheromone leads to increased ORN responses and an increase in the volume of the main MGC glomerulus indicate that an increased input to the AL may contribute to the reduced response threshold found in AL neurons after pre-exposure in a previous study (Anderson et al., 2007).

Such modified odorant-induced responses were observed after a delay (24 h) that allows transcription to take place. Hence, we questioned whether changes in mRNA levels could be detected in antennae of pre-exposed males. In a first attempt to identify upregulated antennal genes upon pre-exposure, we targeted nine transcripts putatively involved in pheromone reception. We reasoned that differences in expression of such elements between pre-exposed and naive males may account for different pheromone sensitivities, as experience has been shown to modulate olfactory receptor expression in other insect species (Fox et al., 2001; Zhou et al., 2009; Khong et al., 2010). Accordingly, pre-exposure induced an overall increase in gene expression. However, only one gene, encoding PBP3, showed a statistically significant small upregulation upon pre-exposure, the other genes exhibiting wide variations in their expression level between repetitions. Because we targeted only nine genes among the 130 genes previously described in *S. littoralis* antennae as putatively involved in odorant detection (Legeai et al.,

2011), we may have missed highly upregulated candidates. In particular, none of the three *SlitPBP*s and none of the four *SlitPR*s studied here have been functionally characterised yet. It cannot be excluded that none is involved in *Z*9,*E*11-14:Ac detection, which might thus explain the lack of important change in their expression upon pre-exposure to this component. Alternatively, it may be that a slight increase in transcript level, as observed for *PBP3*, would be enough to account for the slight increase observed in antennal sensitivity. In this case, the cumulated twofold increases observed in one *SlitPBP*, one *SlitPR* and the co-receptor *SlitORco* may, in concert, enable a more efficient transport and a better detection of *Z*9,*E*11-14:Ac, leading to a higher antennal sensitivity to this component. Further functional characterisation of *SlitPBP2*, *SlitPBP3* and *SlitPR11* is now needed to determine their respective involvement in *Z*9,*E*11-14:Ac transport and reception and test this last hypothesis.

Pre-exposure leads to structural changes at the central level

A stable increase in peripheral sensitivity may be sufficient to explain the observed increase in behavioural and central nervous responses to pheromone after pre-exposure (Anderson et al., 2007). However, previous studies have shown that exposure to odorants can also induce changes in AL structure, as observed from changes in glomerular volumes (Devaud et al., 2001; Devaud et al., 2003; Sachse et al., 2007). Thus, we investigated whether pre-exposure also leads to a structural reorganization of the central AL network, particularly in those glomeruli belonging to the MGC. Indeed, our results reveal a significant enlargement of one such glomerulus (18) in pre-exposed *versus* naive males. Interestingly, this glomerulus is dedicated to the processing of the main sex pheromone component, *Z*9,*E*11-14:Ac (Ochieng et al., 1995; Carlsson et al., 2002). This is in accordance with the enlargement of those glomeruli normally activated by the odorant to which the animal was exposed, as observed in *Drosophila melanogaster* (Sachse et al., 2007). We hypothesise that the enlarged glomerular volume may originate from reorganization of the neural network (e.g. changes in synapse number), as discussed earlier (Hourcade et al., 2009), but other mechanisms, such as glial growth cannot be included (Brown et al., 2002).

It should be noted that this effect of pre-exposure was measured on the normalised glomerular volume, i.e. independently of any possible effect of inter-individual variations in brain and/or AL sizes. The fact that AL volumes tended to be higher in males of the pre-exposed group may be indeed an effect of such variability, or alternatively may reflect a general impact of pre-exposure. In any case, our results show that, even if the treatment might affect many glomeruli, the size of the cumulus was particularly affected. Consistently, no relative volume change was detected in the ordinary glomeruli (which process non-pheromonal odorants) measured here. Though we cannot exclude that other ordinary glomeruli may also be enlarged, it is unlikely that this would be the general rule because this would necessarily affect the overall AL volume, which was not the case in our study. This is indeed what would be expected if upregulation of ORco in all sensilla led to a general increase in sensitivity to all odorants. Rather, this specific volume change most likely reflects an adaptation restricted to the pheromone-dedicated pathway, and more specifically to the glomerulus processing information on the major pheromone component, which represents 99% of the pheromone blend in our strain of *S. littoralis*. Therefore, it is not surprising that the other MGC glomeruli displayed no volume changes, although they may have undergone structural changes without detectable volume modification. This may also reflect a lower plasticity of neurons processing minor pheromone components, in line with earlier observations of stronger age-dependent changes in sensitivity for MGC neurons tuned to the major pheromone component than for minor components in the moth *Agrotis ipsilon* (Gadenne and Anton, 2000). The probable increase in ORN input to the AL and potential increase in synaptic connections, reflected by a volume increase for the glomerulus treating information on the main pheromone component, may thus contribute to the increased sensitivity of AL neurons after pre-exposure, as described previously (Anderson et al., 2007).

Plasticity occurs at multiple levels of the olfactory pathway

Taken together, our results show that even a brief exposure to sex pheromone, a stimulus with a high biological significance, leads to adaptive changes at multiple levels of the olfactory pathway. This is consistent with previous studies that have shown long-term effects of olfactory exposure on odorant receptor expression (Zhou et al., 2009), odorant-evoked responses of ORNs (Iyengar et al., 2010) or glomerular volume (Devaud et al., 2001; Sachse et al., 2007). However, to our knowledge this is the first report of multi-level changes in a given model species.

A possible explanation for such changes would imply that peripheral sensitivity results from upregulated expression of genes involved in pheromone detection. We show here that at least one such gene is significantly affected among those tested; more genes need to be examined to test this hypothesis. As a result, input from the corresponding ORNs to the MGC would be increased, possibly leading to activity-dependent structural rearrangements in the MGC network. The latter may contribute to the higher sensitivity observed in AL neurons responding to pheromone components (Anderson et al., 2007). As they require some time, these changes would not account for variations of the behavioural response threshold on much smaller time scales (e.g. Charlton et al., 1993).

Among the possible effectors at the basis of the observed regulations are the biogenic amines. For instance, octopamine and dopamine are neuromodulators known to regulate activity both in sensory and central olfactory neurons of insects, in particular following exposure to pheromones (Grosmaître et al., 2001; Dolzer et al., 2001; Perk and Mercer, 2006; Vergoz et al., 2009). Studies on several moth species have revealed that octopamine lowers the behavioural response threshold to pheromone (Linn et al., 1986; Linn et al., 1992) and increases the sensitivity of pheromone-responsive AL neurons (Jarriault et al., 2009). Interestingly, an octopamine/tyramine receptor is expressed in the ORNs of the noctuid moth *Mamestra brassicae* (Brigaud et al., 2009), suggesting that biogenic amines target the peripheral olfactory neurons and may participate in regulating their transcriptional or electrical activity. An increase in the sensitivity of ORNs and AL neurons may finally lead to a lower behavioural response threshold, because PNs project from the AL to higher brain centres – including the mushroom bodies – that may undergo synaptic reorganization, as shown after olfactory learning in bees (Hourcade et al., 2010). Because the mushroom bodies are multimodal integration centres (Gronenberg, 2001; Wessnitzer and Webb, 2006), structural reorganization of their network may also be involved in the cross-modal effects of pre-exposure (Anton et al., 2011). Thus, in the future it will be of interest to search for possible physiological and anatomical changes in the mushroom bodies, upstream of the AL.

In summary, our results provide a striking example of the high degree of plasticity of the insect olfactory system, even in the subsystem dedicated to rather stereotyped responses to pheromones. It is remarkable that a short (1 min) exposure to pheromone may

yield modifications in the olfactory pathway leading to a stable (at least 24 h) change in the behavioural response. What might be their adaptive value? A key point here is that males are not allowed to mate during pre-exposure. Hence, a shift in sensitivity lasting for some time would increase the probability of successful mating upon subsequent encounters with the pheromone, particularly at low concentrations. It has been proposed that, as an energy-consuming action, pheromone-induced flight may be accompanied by a stress response (possibly involving octopamine release) (Dolzer et al., 2001). It is thus possible that pheromone detection without mating may keep the organism in a state enabling a better mating chance for some time. In contrast, sensitivity to the pheromone has been shown to decrease in the olfactory system of mated males of *Agrotis ipsilon* (Barrozo et al., 2010; Barrozo et al., 2011), consistently with a strong capacity of the moth olfactory system to adjust its sensitivity to sex pheromone depending on the situation.

LIST OF ABBREVIATIONS

AL	antennal lobe
AP	action potential
MGC	macroglomerular complex
ORN	olfactory receptor neuron
PBP	pheromone-binding protein
PDE	pheromone-degrading enzyme
PN	projection neuron
PR	pheromone receptor
*Z*9,*E*11-14:Ac	*Z*9,*E*11-tetradecenyl acetate

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