Chem. Senses 32: 483–491, 2007
doi:10.1093/chemse/bjm017
Advance Access publication May 17, 2007

Increased Behavioral and Neuronal Sensitivity to Sex Pheromone after Brief Odor Experience in a Moth

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Abstract

Plasticity in the response to stimuli related to food and oviposition cues is well documented in insects. However, responses to cues related to reproduction, for example, sex pheromones, are considered to be innate and thus not affected by experience. Here we show that brief preexposure to sex pheromones, without ensuing reward, lowers the threshold for behavioral response and augments the sensitivity in antennal lobe interneurons to pheromone compared with naive male moths. Thus, the sex pheromone system in insects can be modulated by experience. In addition, we show that the behavioral attraction to sex pheromone increases after preexposure in a time-dependent manner: a short-term effect, possibly a form of sensitization, and a long-term effect after more than 24 h. The behavioral long-term effect is paralleled by an increase in sensitivity of interneurons in the primary olfactory center, whereas the peripheral olfactory system does not change its sensitivity. We hypothesize that short-term sensitization to sex pheromone serves as a kind of alert system, whereas the long-term effect improves male performance when reproductively active females are present.

Key words: antennal lobe, behavioral modulation, experience, neural modulation, olfactory interneurons

Introduction

Experience has been shown to modulate animal behavior in many ways. There are many examples showing that prior experience of olfactory cues affects behaviors associated with search for food and oviposition sites and influences the function of neural circuits (e.g., Vinson 1984; Menzel et al. 1996; Hudson 1999; Grubb and Thompson 2004). In a varying environment, this is a beneficial trait that allows animals to exploit available resources more efficiently. However, for signals linked to reproduction, the situation is different. An accurate identification of a suitable partner is essential to avoid a waste of time and energy and to decrease the risk for predation. This would predict that reproductive signals should be more stable and less affected by experience. However, there is still not much known how experience affects the perception of reproductive signals (Dukas 2006).

Many insects depend on sex pheromones when finding a partner. One sex releases a combination of compounds that initiates mate search and upwind motion in the other sex. In moths, the importance of these signals in mate search is reflected in the large antenna and neuroanatomical adaptations of central olfactory neuropil in males (Greenfield 1981). In general, the sex pheromone in moths consists of

a mixture of several compounds, where species specificity is accomplished by using unique combinations or ratios of several compounds. The main component in the sex pheromone of the moth *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) is (*Z,E*)-9,11-tetradecadienyl acetate (ZE-9,11-14:OAc) (Campion et al. 1980). The minor pheromone compound (*Z,E*)-9,12-tetradecadienyl acetate (ZE-9,12-14:OAc) enhances trap catches, when added at 1% to the major compound (Kehat et al. 1976). It is not known, if additional compounds identified from the female pheromone gland could further improve attraction of *S. littoralis* males. (Z)-9-tetradecenol (Z9-14:OH) is known as a behavioral antagonist (Campion et al. 1980). Different olfactory receptor neurons (ORNs), situated in trichoid sensilla on the antenna of *S. littoralis* males, have been shown to be specifically tuned to the compounds of the sex pheromone blend and to the behavioral antagonist (Ljungberg et al. 1993). The axons of each type of pheromone-specific ORNs project to a specific part of the primary olfactory center (antennal lobe, AL), that is, compartments of the macroglomerular complex (MGC) (Ochieng et al. 1995). Local interneurons connect the different parts of the AL, whereas projection neurons transfer olfactory information to higher brain centers (Anton and Homberg 1999 and references therein).

Studies on different moth species have shown that long-term preexposure to high doses of sex pheromone compounds reduces the behavioral response to the pheromone (e.g., Bartell and Roelofs 1973; Figueredo and Baker 1992; Stelinski et al. 2003; Judd et al. 2005). However, in a previous study we found that *S. littoralis* males briefly preexposed to female sex pheromone mimicking a natural situation showed higher sensitivity in behavioral assays the night following preexposure (Anderson et al. 2003). The method used for the preexposure in our study excluded associative learning, as no reward was given, suggesting that a different mechanism than in classical conditioning is involved (Anderson et al. 2003). Also, in rodents, preexposure to pheromone odors from females in combination with nonvolatile compounds have been shown to increase male responses to these odors in subsequent trials (Meredith 1986; Fewell and Meredith 2002; Moncho-Bogania et al. 2002).

To be able to investigate the neural mechanisms behind the observed behavioral effects, the aim of the present study was to describe the time course of the preexposure effect in detail and to locate the neuronal level within the olfactory pathway at which changes may take place following preexposure.

Materials and methods

Insects

Spodoptera littoralis males were obtained from a laboratory culture that has been supplemented with wild-collected moths annually for the last 8 years. The larvae were reared on a semiartificial diet (Hinks and Byers 1976). The pupae were sexed, separated, and kept at 23 °C, 70% relative humidity, 16/8 h day/night rhythm in 2 different rearing chambers. Emerging males were collected daily.

Odor stimuli

To make the female gland extracts, pheromone glands from 20 females were dissected approximately 3 h into the scotophase, at the peak of the female calling (Dunkelblum et al. 1987) and extracted for 10 min in 20 µl redistilled hexane. Three different extracts were combined to one large extract that was used for all behavioral experiments. Two such combined extracts were used during the experiments. The combined female gland extracts were analyzed on a gas chromatograph (Jönsson and Anderson 1999) and found to contain about 20 ng of ZE-9,11-14 OAc per female equivalent (FE). Each combined gland extract was diluted with hexane to a concentration of 2 ng/µl of the main component, and one FE was established in the experiment as 10 µl of the gland extract. This extract was further diluted in hexane in decadic steps to 1/1000 FE. For the intracellular recordings, dilutions in decadic steps from 0.01 pg to 1 µg of ZE-9,11-14 OAc were used. All extracts and dilutions were kept at −18° C when not used.

Olfactometer

Preexposure and male attraction to odors were performed in an open arena walking olfactometer (60 × 60 cm), with a charcoal purified and humidified laminar airflow of approximately 0.5 m/s. The experiments were performed in red light at 17 °C–18 °C, 40–60% relative humidity. We have previously shown that the behavior of male *S. littoralis* to sex pheromone in the olfactometer is comparable to similar wind tunnel experiments (Anderson et al. 2003).

Preexposure procedure

Two-day old males were placed individually in a glass tube and brought to the experimental room 1 h before the onset of the scotophase. They were allowed to acclimatize before the experiment started. The males were individually preexposed to 1 FE in the olfactometer at the onset of the scotophase. At this time, female calling and male activity starts (Dunkelblum et al. 1987; Silvegren et al. 2005). This stimulus concentration was chosen because it gives a good estimate of what a female emits and at the same time gives a high response rate. In our previous study, about 95% of all males responded to 1 FE (Anderson et al. 2003). The stimulus was applied on a piece of filter paper (5 × 10 mm) placed at the upwind end of the arena. The filter paper was replaced every 15 min. One at a time, the males were placed in the middle of the arena, 60 cm downwind from the stimulus source. The males were allowed to walk up within 10 cm of the stimulus source before they were removed from the olfactometer. Thus, the males were never in contact with the stimulus source. Males that did not respond to the sex pheromone within 2 min were discarded from the experiment

(6% of the tested males). Thus, the males were maximally exposed to pheromone for 2 min, but for most males the exposure time was less than 10 s as they responded immediately and started to approach the pheromone source.

Males tested the same day as the pretreatment were kept in an area of the olfactometer room ventilated with clean air to avoid contamination until tested in the experiments. For the following behavioral and electrophysiological experiments, the males were brought back to the rearing room and returned to the experimental room just before the start of the scotophase on the day of testing. At every testing occasion, naive males, treated in the same way as the experimental males except that they were not exposed to pheromone, served as control specimens.

Behavioral experiments

The protocol used for the bioassay was the same as that used for the preexposure except that males were allowed to walk all the way up to the odor source. The number of males reaching within 2 cm from the odor source was scored. Each male was given 2 min to get activated before it was noted as not responding.

In the experiments, naive and preexposed males were tested to 0.01 FE of gland extract at 7 different times following the preexposure: 15, 30, 45, 90, and 180 min and 27 and 51 h. This dose made it possible to monitor increases in response rate as well as decreases. The selected test dose activated about 30% of the tested naive males in our previous study (Anderson et al. 2003). Two to three different treatments, with 8–10 preexposed and 8–10 control males in each, were tested on each experimental day depending on the number of males available. Each treatment was tested in one sequence, and preexposed and naive males were tested alternately within each treatment. For the shorter time intervals (15–45 min), the males were tested in the same order as they were preexposed in order to keep the time interval between preexposure and test as accurately as possible. Naive males were tested against 1 FE after the end of the experiments, and those not activated were excluded from the experiment (8%). The hatching of each batch of insects allowed 3 experimental days per week. Thus, each treatment was tested on three to four occasions to minimize effects of day-to-day variation and variation between different batches of moths.

Electroantennogram recordings

Antennal responses of naive and males preexposed 27 h earlier (corresponding to the time interval with the strongest preexposure effect) were investigated using a standardized electroantennogram (EAG) method (see e.g., Jönsson and Anderson 1999). The antenna was mounted between 2 electrodes containing a Beadle–Euphrussi Ringer's solution and continuously exposed to purified and humidified air (2.0 ml/s). The odor stimuli were prepared by applying the chemicals on a filter paper, in a Pasteur pipette. A puffing device delivered a 0.2-s-long air puff with a flow of 10 ml/s through the Pasteur pipette and into the airstream passing over the antenna. Each antenna was exposed to a series of different stimuli that were presented in fixed order as follows: reference, blank (empty Pasteur pipette), 0.001 FE, reference, 0.01 FE, 0.1 FE, reference, 1 FE, and reference. The plant odor eugenol (10 μg) was used as reference because earlier studies showed reproducible EAG responses (Anderson et al. 1993). Each stimulus was presented at least 1 min after the previous stimulus to avoid adaptation of the antenna. The maximum amplitude of the EAG response was recorded and analyzed with EAG-adapted software (Syntech, Hilversum, the Netherlands). The relative responses were calculated by dividing the stimulus response with the mean value of the reference before and after the test stimulus.

Intracellular recordings

The experiments were performed 22–28 h following preexposure. This time window was chosen to correspond with behavioral and EAG experiments. As intracellular experiments take, however, much more time, we chose a longer period during midscotophase.

Experiments on control and preexposed males were performed each day. Up to 4 neurons were tested in the same male. Male *S. littoralis* were fixed in a plastic pipette tube with the head protruding. The brain was exposed by removing the cuticle, overlaying tissue, and the neural sheath from the AL. The preparation was superfused with a saline solution at pH 6.9 (Christensen and Hildebrand 1987), and the antenna was exposed to a continuous airstream (20 ml/s).

AL neurons in the MGC were penetrated randomly by inserting a recording electrode, filled with 2 M KCl close to the entrance of the antennal nerve. When intracellular contact was established, the ipsilateral antenna was stimulated with a 0.5-s-long air pulse containing clean air, the solvent (hexane), or different doses of ZE-9,11-14:OAc. The pheromone compound was tested at doses from 1 pg to 1 μg, dissolved in hexane on filter paper in a Pasteur pipette in a first series of experiments, and at doses from 0.01 pg to 1 μg in a second series of experiments. Stimulations started at low doses and were repeated, if possible, several times for doses around the threshold for each neuron. If the contact lasted, several doses above the threshold were tested to establish dose–response curves. At least 10 s elapsed between stimuli, the maximal time needed for most neurons to reestablish spontaneous activity at threshold concentrations. Longer interstimulus intervals were applied at higher stimulus doses. A Pasteur pipette containing a filter paper with hexane or an empty pipette was used as blank stimuli. Recordings were stored on digital tape (data recorder, Sony PCM-R300) and analyzed off-line with Autospike 32 software (Syntech).

For the statistical analysis of neuron thresholds, electrophysiological responses were quantified as described

previously (Gadenne and Anton 2000). Briefly, the net number of spikes (number of spikes during a 600-ms period after the stimulus onset minus the number of spikes counted during the preceding 600 ms representing spontaneous activity) produced in response to the blank stimulus was subtracted from the net number of spikes produced in response to the odor stimulus at a given dose to quantify the response. A neuron was classified as responding to a stimulus when the odor response exceeded the blank response by at least 10%.

Statistics

A 2-way analysis of variance (ANOVA) was used to examine differences between treated and naive males in the EAG experiments. The relative EAG values were square root transformed, and logarithmic dose values were used to fulfil ANOVA assumptions. A chi-square test was used in the behavioral experiments to compare the 2 treatments at every time tested. In the intracellular experiments, a *G*-test for heterogeneity was performed to compare the 9 sensitivity groups for preexposed and control males.

Results

Behavioral experiments

After 15 min, we found a significantly higher percentage (43.3%) ($P = 0.035$) of preexposed males approaching within 2 cm of the pheromone source compared with naive males (Figure 1). A significant difference was also found 3 h into the scotophase the second (57.1%) ($P = 0.008$) and third (50.0%) ($P = 0.027$) night following preexposure to sex pheromone. During the other times tested, between 30 and 180 min following preexposure, no difference compared with naive males was found. For each time slot in the preexposure experiments, 28–37 males were used, whereas 27–32 males were used for the control experiments.

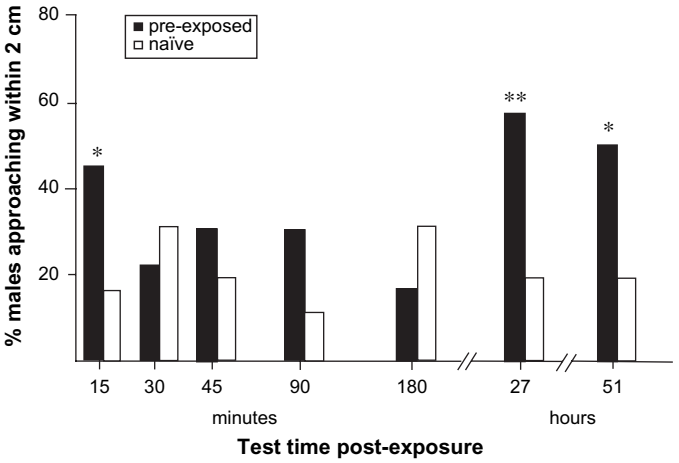

Figure 1 Behavioral responses of male *Spodoptera littoralis* following preexposure to sex pheromone. The percentage of preexposed males responding to the sex pheromone was significantly higher than that of naive males when tested 15 min, 27 h, and 51 h after preexposure (chi-square test, $n = 27–37$. $*P < 0.05$, $**P < 0.01$).

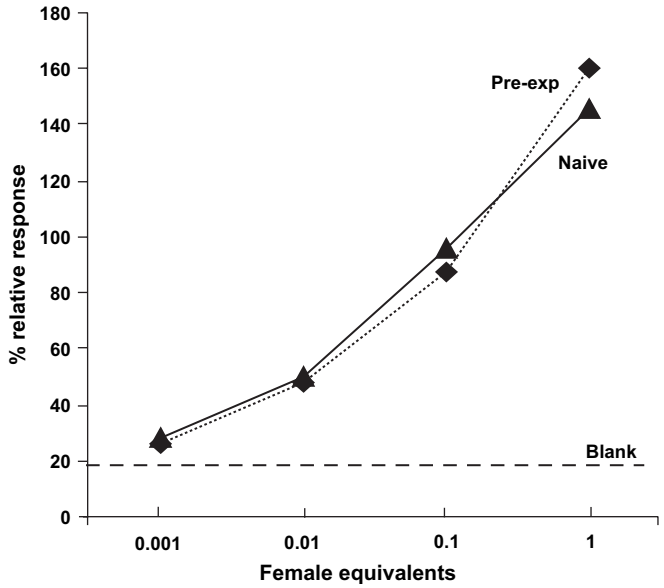

Figure 2 EAG recordings from *Spodoptera littoralis* male antennae, stimulated with sex pheromone in preexposed and naive male moths. No difference in sensitivity was found between the 2 groups (multiple ANOVA, $F = 1.98$, df = 3, $P = 0.12$, $n = 25$).

EAG recordings

When stimulated by pheromone gland extract, no significant difference (multiple ANOVA, $F = 1.98$, df = 3, $P = 0.12$, $n = 25$) in the EAG dose–response curves from male antennae was found between preexposed males and naive males within the concentration range tested (Figure 2).

General physiological characteristics of AL neurons

Only neurons responding to at least one of the doses of ZE-9,11-14:OAc were used for data analysis. We recorded from 66 AL neurons in 37 preexposed males and from 48 neurons in 31 control males. Recorded action potential amplitudes varied between 10 and 30 mV and spontaneous activity between 10 and 80 impulses/s. Above-threshold responses were characterized by an increase in spike rate (Figures 3 and 4). For neurons in which several above-threshold doses were tested, a slight increase in firing rate and a decrease in response delay were observed in most neurons with increasing stimulus loads (Figure 3B).

A few neurons were stained intracellularly after recording and those were all identified as projection neurons (data not shown). This suggests that the majority of the recorded neurons were projection neurons.

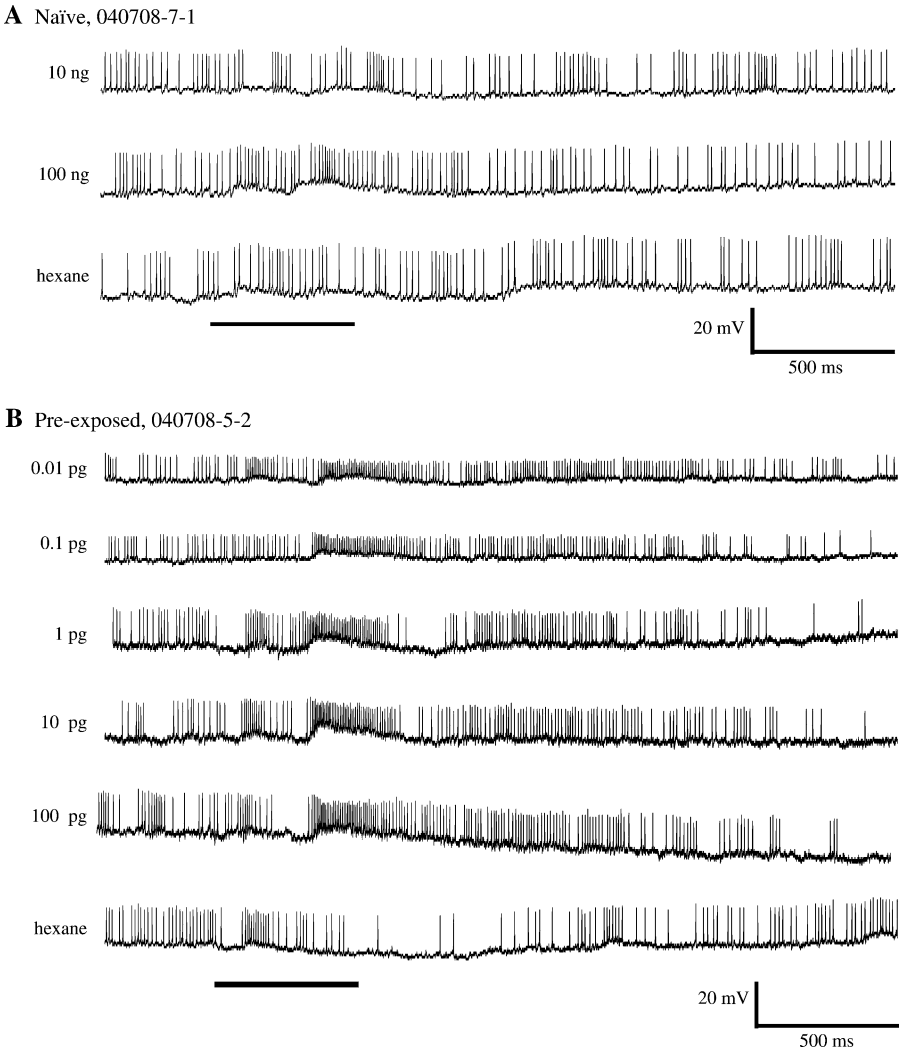

Figure 3 Responses of AL neurons in male *Spodoptera littoralis* to the main sex pheromone component, ZE-9,11-14:OAc. The odor stimulus reaches the antenna at 300 ms. Responses to the solvent hexane are shown as controls. **(A)** Original recording from an AL interneuron with a high response threshold in a naive male. **(B)** Original recording from a neuron with a low threshold in a preexposed male. Note that responses are very similar for different amounts of the pheromone component tested. Bar underneath traces indicates stimulation (500 ms), vertical scale bar 20 mV, horizontal scale bar 500 ms.

Response thresholds of AL neurons in preexposed and naive moths

The response threshold, that is, the lowest concentration, which elicited an odor response exceeding the blank response by at least 10%, was established for all neurons investigated. Neurons with thresholds between 0.01 pg and 1 µg stimulus cartridge loading were found in both preexposed and naive moths. The proportion of neurons with a lower threshold was, however, significantly higher in preexposed than in naive moths (G = 99.19, df = 8, $P < 0.001$) (Figure 5). Thirty-nine percent of all pheromone-responding neurons in preexposed moths responded at the lowest tested dose (0.01 pg), whereas the vast majority of neurons in naive moths (88%) responded only to doses of at least 10 ng (Figure 5). High increase in action potential rate (100–150 impulses/s increase) at threshold for the lowest dose tested (0.01 pg) in preexposed males (Figures 3B and 4B) might indicate that the threshold of these neurons is even lower than 0.01 pg. The increase of firing rate in AL neurons with high threshold in naive males, on the other hand, was in general moderate (increase between 20 and 70 impulses/s) (Figures 3A and 4A).

Discussion

Previous experience with female sex pheromone increases sensitivity to sex pheromone in 2 distinct time windows in male *S. littoralis*. We found a short-term effect that lasted for less than 30 min and a long-term effect that was found on the second and third night following preexposure. The

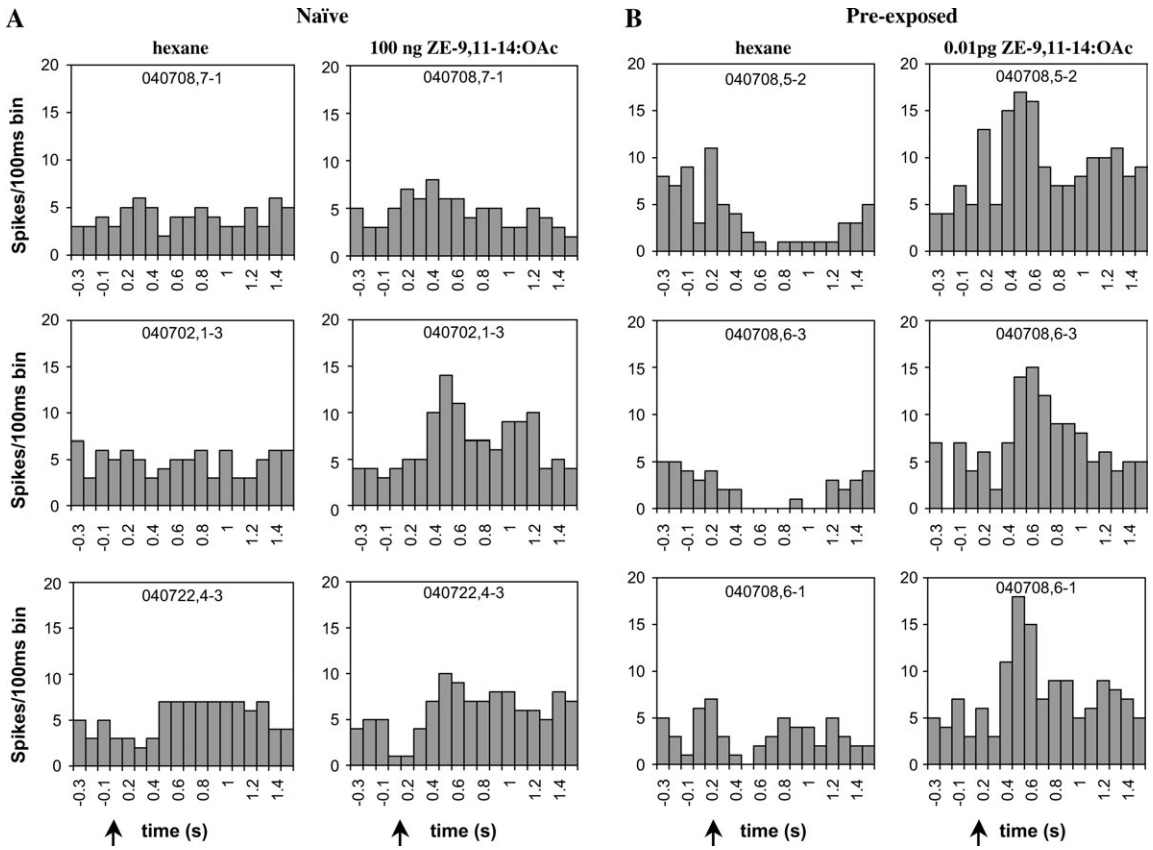

Figure 4 Responses of AL neurons in male *Spodoptera littoralis* to the main sex pheromone component. The odor stimulus takes approximately 300 ms to reach the antenna. Responses to the solvent hexane are shown as controls. **(A)** Responses to a 100-ng dose of the neuron in 3A (upper row) and 2 others in naive males. **(B)** Responses to a 0.01-pg dose of the neuron in 3B and 2 others in preexposed males. Doses indicate amounts of ZE9,11-14:OAc, hexane = solvent (control). Poststimulus–time histograms showing numbers of spikes/100-ms bins. Arrows indicate stimulus onset.

long-term increase in behavioral sensitivity was accompanied by an increased sensitivity to sex pheromone in neurons in the AL, whereas peripheral sensitivity as measured by EAG did not change. Hence, the preexposure effect does not seem to take place at the sensory level, but at the level of perception. Thus, the system is plastic, and the sensitivity at the central level is modulated by experience.

As male moths normally are involved in scramble competition for females (Greenfield 1981), an innate high sensitivity to female sex pheromone would be expected. We found that males do indeed have a high innate sensitivity to female sex pheromone, but this sensitivity is further increased by prior experience. We hypothesize that it could be beneficial for naive males to increase sensitivity to sex pheromones only after experience.

A higher sensitivity to pheromone may imply costs for the males that are associated with fitness. In *Drosophila melanogaster*, the formation and maintenance of neural structures associated with a long-term memory decreased the longevity of flies compared with control flies that were not subjected to associative learning (Mery and Kawecki 2005). It is possible that the exposure to pheromone in a similar way may affect fitness-related cues in *S. littoralis.* In that case it would be advantageous to have high sensitivity only when receptive females are present and the key stimulus for assessing their presence is the sex pheromone.

Furthermore, the amount of information that can be processed simultaneously by an individual is limited. Mechanisms of differential attention to cope with this constraint are ubiquitous among animals (Dukas 1998). Focusing attention on one cue enhances neuronal and behavioral performance compared with dividing attention among several cues (Dukas 2004). However, this leads to lower attention to other cues in the environment. Thus, a male moth that has high sensitivity to sex pheromone could become more vulnerable to predation by devoting less attention to predator cues. In *S. littoralis*, a trade-off between the response to sex pheromone and predator cues, bat sounds, has been described (Skals et al. 2005). The study by Skals et al. (2005) showed that male moths devote less attention to predator sound when they are simultaneously exposed to female sex pheromones. This indicates that there is plasticity in the response to sex pheromone that depends on other stimuli.

Our behavioral experiments indicate that 2 different mechanisms may increase the sensitivity to sex pheromone in *S. littoralis*. The short-term effect could be due to a form

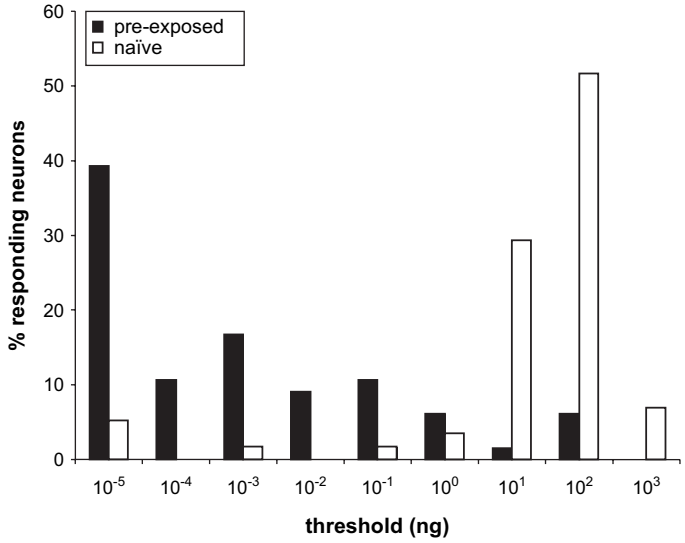

Figure 5 Experience-dependent sensitivity of AL neurons in *Spodoptera littoralis* males to the main pheromone component, ZE-9,11-14:OAc. The proportion of neurons with a specific threshold in naive (n = 58 neurons) and preexposed males (n = 66 neurons) is given in the histogram. Whereas mainly neurons with a high threshold were found in naive males, mainly low-threshold neurons were observed in preexposed males. Doses indicate amounts of ZE-9,11-14:OAc.

of sensitization that could either be specific to sex pheromone or more general concerning different olfactory stimuli or even different sensory modalities. Further studies need to investigate if the sensitization is specific to sex pheromone or can occur also with preexposure to other stimuli. In addition to the short-term effect, there is a stable, long-term effect lasting at least 2 nights following preexposure, confirming the indications of a similar effect found in an earlier study (Anderson et al. 2003). An adaptive explanation for this effect may lie in the narrow circadian time window of activity and the short life span of night-active moths. Both males and females have an activity peak in the middle of the scotophase (Dunkelblum et al. 1987, Silvegren et al. 2005). It does therefore make sense that increased attention of males is retained during the following nights as females tend to be stationary. The long-term effect must clearly be due to mechanisms different from associative learning because no reward is given in our experiments. During or following the preexposure, the males were never in contact with either the pheromone source or females.

Increased sensitivity to sex pheromone compounds following preexposure has also been observed in vertebrates, but in these cases, learning mechanisms are involved (Meredith 1986; Moncho-Bogania et al. 2002). In previous studies on insects, male moths were found to respond less to sex pheromones following exposure (e.g., Bartell and Roelofs 1973; Figueredo and Baker 1992; Daly and Figueredo 2000; Stelinski et al. 2003; Judd et al. 2005). The aim in these experiments was, however, primarily to investigate the possibility to use sex pheromones in insect control, and the moths were intentionally exposed to doses of pheromone that are much higher than emitted by females in nature and/or at much longer durations than in this study. The reduced response to sex pheromone in these studies was most likely due to adaptation or habituation to the odor (Daly and Figueredo 2000; Stelinski et al. 2003; Judd et al. 2005).

EAG recordings showed that peripheral responses do not increase in parallel with an increased behavioral sensitivity. These results coincide with earlier studies on male moths, where no differences in EAG responses were found between different physiological states (Gadenne et al. 1993, 2001). However, we cannot exclude that certain individual ORNs change their sensitivity following preexposure in *S. littoralis*, but a strong general effect can be out ruled. A general increase in sensitivity that coincided with increased behavioral sensitivity was on the other hand found in the population of interneurons investigated in the AL. The lower behavioral response threshold established in long-term preexposed males may be explained by changes occurring in the brain of the insect after experience of the sex pheromone. Whereas the mechanisms causing this type of plasticity are unknown so far, there is strong evidence for changes in central representation of odors and synaptic recruitment within insect olfactory centers during another form of plasticity, associative learning (Faber et al. 1999; Daly et al. 2004; Yu et al. 2004). In all 3 studies, changes in the spatiotemporal representation pattern of odors after associative learning have been described. Also in mammals, experience of sex pheromones can lead to enhanced activity in brain regions receiving input from the vomeronasal/accessory olfactory system (Fewell and Meredith 2002; Westberry and Meredith 2003).

The shift in sensitivity of the neuron population found in the present study corresponds to what has been observed in olfactory plasticity linked with changes in the physiological state of insects (Gadenne et al. 1993; Gadenne and Anton 2000 and references therein). AL neurons were shown to increase in sensitivity with age and juvenile hormone level in parallel with a lower behavioral response threshold to sex pheromones on one hand (Anton and Gadenne 1999), whereas neurons showed a rapid decrease in sensitivity after mating (Gadenne et al. 2001). Sensitivity of AL neurons responding to plant odors, on the other hand, did not change in male moths of different age (Greiner et al. 2002), showing a specificity of the plasticity for the pheromone system. We need, of course, to test if the preexposure effect observed in *S. littoralis* is equally specific for the pheromone system or if it can be extended to other odors or other sensory modalities.

Neuromodulators, such as octopamine and serotonin, have been implemented as agents causing changes of behavioral sensitivity in different insects (e.g., Linn et al. 1992; Barron et al. 2002) and changes of sensitivity within different levels of the olfactory system to pheromones (e.g., Linn 1997; Kloppenburg et al. 1999; Pophof 2000; Gatellier et al. 2004).

One of our future goals is to examine if neuromodulators are involved in sensitivity changes caused by experience. We also need to test if experience to sex pheromone will alter the response to other sensory cues in male moths and consequently if olfactory stimuli different from pheromones or other sensory cues affect male moth response to sex pheromone.

Acknowledgements

We are very grateful to Elisabeth Marling for rearing moths, to Drs Fredrik Schlyter and Christophe Gadenne for statistical help and fruitful discussions, and to Dr Rickard Ignell for valuable comments on earlier versions of the manuscript. This study was supported by a Linnaeus Grant (Formas, Sweden), Institut National de la Recherche Agronomique (France), and The Danish Natural Science Research Council.

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Accepted March 7, 2007