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Brief predator sound exposure elicits behavioral and neuronal long-term sensitization in the olfactory system of an insect

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Abbreviations: AL antennal lobe; fe female equivalent; MGC macroglomerular complex; ORN olfactory receptor neuron; OG ordinary glomeruli; *ZE*-9,11-14:OAc (*Z,E*)-9,11-tetradecadienyl acetate

Abstract

Modulation of sensitivity to sensory cues by experience is essential for animals to adapt to a changing environment. Sensitization and adaptation to signals of the same modality as a function of experience have been shown in many cases and some of the neurobiological mechanisms underlying these processes have been described. However, the influence of sensory signals on the sensitivity of a different modality is largely unknown. In males of the noctuid moth, *Spodoptera littoralis*, the sensitivity to the female-produced sex pheromone increases 24 h after a brief pre-exposure with pheromone at the behavioral and central nervous level. Here we show that this effect is not confined to the same sensory modality: the sensitivity of olfactory neurons can also be modulated by exposure to a different sensory stimulus, *i.e.* a pulsed stimulus mimicking echolocating sounds from attacking insectivorous bats. We tested responses of pre-exposed male moths in a walking bioassay and recorded from neurons in the primary olfactory centre, the antennal lobe. We show that brief exposure to a bat call, but not to a behaviorally irrelevant tone, increases the behavioral sensitivity of male moths to sex pheromone 24 h later in the same way as exposure to the sex pheromone itself. The observed behavioral modification is accompanied by an increase in the sensitivity of olfactory neurons in the antennal lobe. Our data provide thus evidence for cross-modal experience-dependent plasticity not only on the behavioral, but also on the central nervous level in an insect.

Key words: sexual behavior, sex pheromone, bat sound, antennal lobe, intracellular recording

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Animals live in an ever-changing environment and must be able to adapt their behavior in response to highly varying sensory cues. One way to achieve such adaptive behavior is through plasticity of the nervous system, where modifications can be induced depending on sensory input. Previous studies have shown that exposure to environmental signals during development and in early adult life might influence the precise design and sensitivity of the targeted sensory system (1-3). The development of the peripheral and central visual, auditory, somatosensory, and olfactory systems in vertebrates has been shown to be highly influenced by experience (*e.g.* 4-11) and regulated by neurogenesis and network re-modeling with *e.g.* axon growth, the increase of the number of synaptic connections and the strengthening of existing synapses (1 and references therein, 12).

Different strategies to achieve high sensitivity for abundant and/or important signals have evolved. One way is limited attention, where an animal focuses on a specific sensory system, or even on a specific type of sensory signal, whereas other, simultaneously occurring information signals might be less attended (13, 14). Experience of sensory input with a behaviorally important stimulus could also elicit long-term changes, which improve the insect's ability to respond to that specific stimulus (*e.g.* 15). Furthermore, brief sensory experience could contribute to the sensitization or maturation of related or even unrelated, but behaviorally relevant, sensory systems. Sensitization has been originally defined as a situation where a sudden aversive stimulus leads to increased responses to the same and even unrelated stimuli (studies on the marine mollusk *Aplysia*; 16 and references therein) but can also lead to a higher sensitivity for an attractive stimulus. Such phenomena have been found in vertebrates, including humans, where cross-modal sensory input influences the central processing of stimuli perceived through different input channels (17, 18).

A brief experience with female-emitted sex pheromones increases male responses in both rodents (*e.g.* 19-21) and in the noctuid moth *Spodoptera littoralis* Boisd. (22, 23). It is not known, however, if this pre-exposure effect is specific for sex pheromones, or if other incoming sensory information has a similar effect on sex pheromone responses.

In *S. littoralis*, as in many other moth species, males orient towards extremely low doses of female-emitted sex pheromones. The sex pheromone of moths normally consists of several compounds, and the species-specificity depends on the combination of compounds and/or the ratio of these. In *S. littoralis*, the pheromone consists of several compounds with (*Z,E*)-9,11-tetradecadienyl acetate (*ZE*-9,11-14:OAc) as the main component (24, 25). *S. littoralis* males use a highly specialized and very sensitive olfactory system to detect and discriminate sex pheromones in their environment. Plant-emitted volatiles serve in parallel among other functions to detect food sources in both sexes (26). The sex pheromone information is received by a large number of specific olfactory receptor neurons (ORNs) and then transmitted via their axons to a male-specific area (the macroglomerular complex, MGC) within the primary olfactory centre, the antennal lobe (AL) (27, 28). Plant-related odors are received by different ORNs, with their axons projecting to the so-called "ordinary" glomeruli (OG) within the AL, which are present in both males and females (28). When flying towards the sex pheromone at night, male moths are often exposed to ultrasounds, emitted by hunting insectivorous bats (29). Moths use a "simple" thoracic ear, consisting of two sensory neurons attached to a tympanic membrane, to detect predator sounds (30). Flying moths may respond to bat sound by eliciting different evasive maneuvers, while walking moths on the vegetation will "freeze" (31, 32). However, the threshold for eliciting these responses to auditory stimuli can be modulated by other sensory signals (33). In case of simultaneous stimulation with bat sound and sex pheromone, the relative strength of the two stimuli determines how the moth responds (33).

Hence, behavior depends on a trade-off situation, which results from bimodal sensory information integration.

The well-described behavior in response to specific olfactory and auditory signals in *S. littoralis* and its well-known sensory apparatus and central nervous system, make this noctuid moth an excellent model to investigate cross-modality effects of pre-exposure at the behavioral and central nervous level. We investigated here, whether the effect of pre-exposure is specific to the sex pheromone system or if stimulation through another sensory modality, i.e. synthesized bat sound, will modulate the sensitivity of the two olfactory sub-systems, *i.e*. the pheromone and the plant odor-processing system. Furthermore, we also investigated if the effects of exposure are restricted to behaviorally relevant stimuli, *i.e* if a pulsed bat sound has the same effect as a non-pulsed tone with the same frequency. We show that auditory stimulation increases olfactory sensitivity in *S. littoralis* males and discuss the results as a case of cross-modal sensitization.

Results

Behavioral response to sex pheromone after pre-exposure to sex pheromone or bat sound. Confirming earlier results (22, 23), males pre-exposed to 1 female equivalent of a pheromone gland extract approximately 24 hours prior to testing showed a significantly higher response to a lower pheromone dose in the walking bioassay compared to naïve males (control). A variation in the sensitivity to female sex pheromone, manifested in differing attraction rates, was found over the experimental period (Fig. 1). However, a pre-exposure effect was always recorded irrespectively of the "absolute" sensitivity of the males.

Of the males pre-exposed to sex pheromone (n=186), 56 % walked up within 5 cm to the odor source, compared to 32 % of the naïve males (n=186) ($p< 0.001$) (Fig. 1). Also males pre-exposed to a pulsed bat-like sound showed a significantly higher response to sex pheromone than naïve males (Fig. 1). Of these pre-exposed males (n=106), 45 % walked up within 5 cm to the odor source compared to 30% of the naïve males (n=106) ($p=0.023$). A slightly lower proportion of bat-sound exposed males responded in the tests compared to pheromone-exposed males, but this difference was not significant ($p=0.30$).

There was no significant difference in the response to sex pheromone between males pre-exposed to a tone and naïve males (Fig. 1). Twenty percent of the tone pre-exposed males (n=88) and 22% of the naïve males (n=88) reached within 5 cm to the odor source ($p=0.85$, *n.s.*).

Simultaneous pre-exposure to sex pheromone and bat sound did not elicit a stronger response than pre-exposure to sex pheromone alone. In this series of experiments, 9% (n=33) of the naïve males were attracted to the sex pheromone, while 39 % (n=31) of the males pre-exposed to sex pheromone and 41 % (n=29) of the males pre-exposed simultaneously to bat sound and sex pheromone walked up to the pheromone source. Both exposure to sex pheromone ($p=0.0052$) and the simultaneous pre-exposure to sex pheromone and bat sound ($p=0.0031$) were different from naïve control males, while no difference between the two treatments was found ($p=0.83$, *n.s.*).

Response thresholds of MGC neurons after pre-exposure to sex pheromone or bat sound. Only neurons responding to at least one of the tested doses of *ZE*-9,11-14:OAc were used for data analysis. We recorded from 66 MGC neurons in 37 pheromone-exposed males, 75 MGC neurons in 32 bat-sound-exposed males (see example in Fig. 2a), 61 MGC neurons in 27 tone-exposed males (see example in Fig. 2a) and 101 MGC neurons in 50

naïve males, serving as control. The observed responses of MGC neurons exhibited the same characteristics as in a previous study (22) and response patterns were rather uniform and highly similar to response patterns of projection neurons in other noctuid moths (34). The response threshold, *i.e.* the lowest concentration, which elicited an odor response exceeding the hexane response by at least 10% (see Material and Methods) was established for all neurons investigated. MGC neurons with response thresholds between 0.01 pg and 1 µg were found in all pre-exposed treatment groups and in naïve moths. By analyzing the cumulative frequency of responding MGC neurons as a function of the tested doses, we observed clear differences among curves (Fig. 3a). Neurons in pheromone pre-exposed males showed a steep curve, reaching rapidly the plateau, with almost 90% of the neurons responding to doses of 10^{-1} ng. On the other hand, the vast majority of neurons in naïve moths responded only to doses of 10^{1} ng or higher, and thus the cumulative response frequencies for naïve males follow a flat curve from 10^{-5} to 10^{0} ng (Fig. 3a). Cumulative response frequencies in bat sound-exposed males showed an intermediate curve, revealing a group of highly sensitive neurons (i.e. no statistical differences were found between bat- and pheromone-exposed males at 10^{-4} ng (see Fig. 3a)), but also a group of neurons with a low sensitivity level, illustrated by a steep curve between 10^{-1} and 10^{1} ng, thus showing a bimodal distribution of sensitivities. Neurons in bat sound-exposed males showed nevertheless more sensitive neurons than both control groups for almost all doses tested (10^{-4} to 10^{1} ng, $p < 0.008$ in all cases). Neurons in males exposed to a tone also had significantly more sensitive MGC neurons for certain doses than the control group (10^{-1} and 10^{0} ng , $p < 0.008$) (Fig. 3a).

We additionally determined the dose at which the 50 % of neurons responded (D50) for each treatment, we found a D50 of about 0.1 pg and *ca* 30 pg for pheromone- and bat

sound-exposed males respectively. For tone-exposed and naïves males the D50 was several orders of magnitude higher than for pre-exposed groups, i.e. 1 and *ca* 9 ng respectively.

Response thresholds of flower odor-responding neurons after pre-exposure to bat sound. To test if the effect of bat sound exposure is specific for the sex pheromone system or has a more general effect on olfactory neurons, we recorded from 61 AL neurons within the array of OG in 15 naïve males (see example in Fig. 2b) and from 59 OG neurons in 18 bat sound-exposed males (see example in Fig. 2b). Also for these neurons, response patterns were typical for projection neurons. Only neurons responding to at least one of the three tested flower volatiles, linalool, geraniol and heptanal were taken into account. As some neurons responded to more than one of the tested compounds, for each neuron, the response to the compound with the lowest threshold was used in the data analysis. Thus, for each neuron the lowest threshold for the best-tuned compound was determined. In bat sound-exposed males the cumulative response frequencies of neurons in OG to flower odors were significantly higher than in control males for all doses analyzed ($p < 0.01$, doses: 10^0, 10^1, 10^2 µg) (Fig. 3b). The D50 of neurons in bat sound-exposed males was 3 µg of plant odor and for the control group the D50 was 30 µg.

Discussion

In the present paper we show that a brief experience with different sensory stimuli during early adult life can lower the response threshold for sex pheromone both at the behavioral and central nervous level in a male moth within 24 hours. Pre-exposure to a bat sound increases behavioral sensitivity to sex pheromone and the sensitivity of central olfactory neurons responding to either sex pheromone or flower volatiles. This indicates that the olfactory system can be shaped by experience-driven plasticity and cross-modal effects.

Sensory input during early adult life has been shown to be crucial for maturation of sensory systems (e.g. 1, 3). In both vertebrates and invertebrates a critical period early in life has been found, where genetically determined sensory responses can be modified by sensory input (35). This plasticity allows the animal to adapt its responses to the local external environment and to organize the immature neural network accordingly. Long-lasting exposure to sensory signals changes behavioral performance and structure and function of sensory pathways. Maturation of the adult olfactory neural circuitry and odor discrimination ability in rats is enhanced by complex sensory experience (36). Sensory systems may thus show a high degree of experience-dependent plasticity either increasing or decreasing their sensitivity.

In our study, we exposed sensory inexperienced males briefly to sex pheromone and to auditory signals in early adult life, mimicking the type of sensory input newly emerged male moths would encounter in their natural habitat. Interestingly, we found a modulation of the sensitivity of the olfactory system at the behavioral level within 24 hours in both cases. The behavioral sensitivity of the olfactory system increased not only after exposure to the sex pheromone (22, 23), but also across modalities, *i.e.* after exposure to a bat-mimicking sound, but not to a behaviorally irrelevant tone. Thus, we found that both sex pheromone and bat sound individually induced changes in sensitivity to sex pheromone. When pre-exposing males to sex pheromone combined with a bat sound no additive effect on the behavioral response was found compared to pre-exposure to the sex pheromone alone. This may indicate that the pre-exposure effect obtained by the female extract elicits already the maximally possible increase in sensitivity. We found that both AL-neurons responding to the sex pheromone and to flower odors showed increased sensitivity after pre-exposure to bat sound. This indicates that the exposure to bat sound elicits a general

sensitization of the olfactory system and not specifically targets central sex pheromone processing.

Although cross-modal effects of experience on the behavioral level have been shown in honeybees and crickets using associative learning paradigms (37-39), our study shows in addition a cross-modal effect of experience on the central nervous level in an insect. The observed behavioral increase in sensitivity across modalities seems to originate at least partially from the interaction in a primary sensory integration centre and similar phenomena have so far only been described in higher order brain centers in vertebrates (17, 18). Interestingly, the change in sensitivity of MGC neurons after sound exposure seems to occur in a bimodal manner. Part of the neuron population stays at a low sensitivity level, part of the neuron population switches to a very high sensitivity level and few neurons show intermediate sensitivity. This might indicate that neurons switch between two distinct states of sensitivity. This seems not to be the case for plant odor-responding neurons. However, fewer doses were tested and data for different components were pooled, so it is pre-mature to draw any conclusions from the differences between the olfactory sub-systems.

Whereas neuronal and cellular mechanisms of intra-modal experience effects have been widely described for different sensory systems (1, 3, 12), we can only speculate how auditory stimuli can influence the sensitivity of the olfactory system in male moths. One of the two auditory receptor neurons of the moth ear (the A1-cell) projects its axon to the brain, but it is not known to which area (30). Therefore at least one, most likely several interneurons must be involved in providing a feedback to the AL. We propose that modulatory protocerebral neurons are responsible for the increase in sensitivity of pheromone-responding AL neurons after bat sound exposure. Interestingly, pre-exposure with bat sound seems to sensitize both olfactory sub-systems, which indicates that both

olfactory systems might be modulated by the same protocerebral network. We speculate that this multi-synaptic feedback system might explain why the pre-exposure effect of bat sound is not as strong as the effect of pheromone-exposure, where the area of sensory input and the area in which modulation takes place are identical, even if modulation also in this case might originate from centrifugal feedback from higher brain centers. Although we tried to use stimuli close to a natural situation for pre-exposure, care has to be taken when comparing the magnitude of exposure effects of different sensory modalities. Differences in signal quality, such as duration, intensity, and stimulation frequency of the stimuli used for pre-exposure may influence the effects.

Our data show that exposure to a non-pulsed tone, a sensory signal without a behavioral significance for the male moth, did not elicit a significant increase in the behavioral sensitivity to sex pheromone. However, the sensitivity of the population of neurons in the AL increases even after exposure to a tone. Pulse-repetition is a very important feature of auditory stimuli in many insects and central neurons discriminating pulse repetition rates have been described in the cricket (40) and the locust (41). Neurons tuned to pulsed sound have also been described in the moth central nervous system (42-44) and might be responsible for the stronger effects at the AL level observed when pre-exposing male moths with a bat sound than with a tone. The tone-exposure would, however, not translate into a significant behavioral effect, since a large part of the neuronal activity is filtered out by the pulse repetition rate filter.

Previous studies have shown that male moths briefly pre-exposed with the sex pheromone are more sensitive to the sex pheromone 24 h later compared to naïve males (22, 23). The simultaneous increase in sensitivity of OG neurons to plant odors after pre-exposure to bat sound, however, indicates that we might observe a case of general sensitization rather than a phenomenon of selective attention or selective sensitization.

Material and methods

Further details are provided in SI Materials and Methods.

Insects. Virgin males of *S. littoralis,* 2-4 days old, were used in the study.

Walking bioassay. Experiments were carried out in a 60 x 60 cm open arena olfactometer (23) under red light.

Stimulation. Behaviourally relevant odor and sound stimuli where used during exposure and subsequent tests.

Pre-treatments. The effect of pre-exposure on the subsequent response to sex pheromone was tested in males exposed to four different stimuli:

1. Sex pheromone gland extract (1 fe)
2. Pulsed bat-like sound.
3. A non-pulsed tone.
4. Simultaneous stimulation to sex pheromone gland extract (1 fe) and pulsed bat-like sound as under point 2.

In all experiments naïve males were used as control. These males were handled in the same way as the treated moths except that the stimuli were not switched on in the set-up.

Behavioral tests. The behavioral tests were performed in the same arena as used for the pre-exposure.

Electrophysiology. Intracellular recordings from AL neurons were performed 22 to 28 h after pre-exposure with bat sound, a tone, or the sex pheromone. Control recordings were done with un-experienced males submitted to the same procedure as pre-exposed animals. Males of at least two different treatments were always tested the same experimental day and up to four neurons were screened in an individual moth.

Statistical analyses. For the behavioral tests, an analysis of frequency by using a Chi-square test for independence was carried out to evaluate differences. The difference in the pre-exposure effect between sex pheromone and bat sound was checked by using the approximation of a binomial distribution by a normal distribution and the properties of a linear combination of normally distributed random variables.

For the intracellular recording experiments, we carried out cumulative frequency plots. Statistical differences were evaluated among treatments for each individual dose by means of a G-test for independence and applying the Williams's correction (45). Doses of 10^2 and 10^3 ng for MGC neurons and 10^{-1} and 10^3 µg for OG neurons were not included in the statistical analysis, due to the presence of zeros in frequency values. Pairwise *post hoc* comparisons were carried out for each dose and the experimentalwise error rate was adjusted by using the Dunn-Šidák method (k, number of pairwise comparisons per dose = 6; experimentalwise error (corrected alfa) = 0.008) (45).

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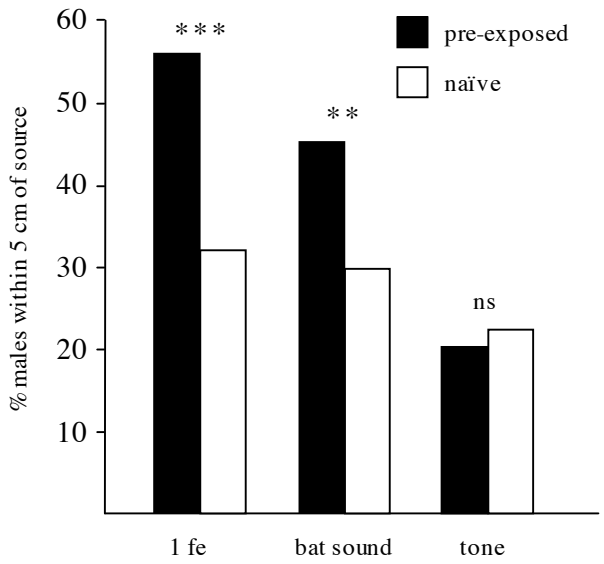
Figure legends

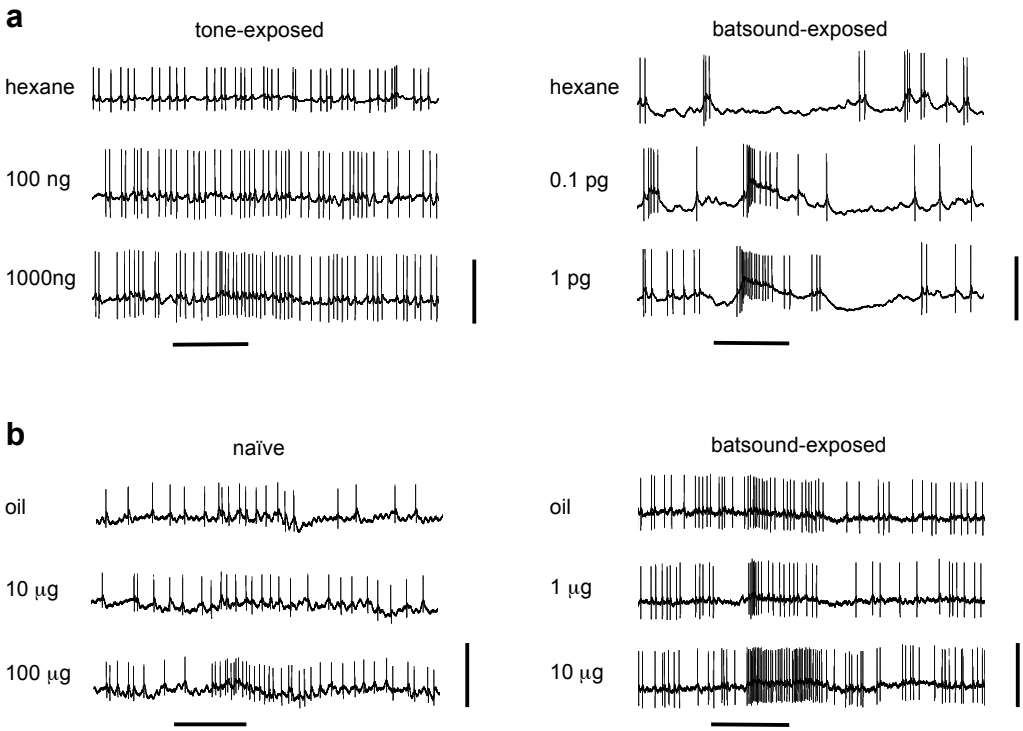
Fig. 1. The percentage of *S. littoralis* males approaching within 5 cm from an odor source of 0.03 female equivalents (fe) of sex pheromone gland extract in a walking bioassay. The response of males pre-exposed to either 1 fe, bat sound or a tone was compared to the response of naïve males. Statistical analysis by a Chi-Square test for independence was done (**=$p<0.01$, ***=$p<0.001$). Numbers in bars indicate numbers of tested males. The higher n-value for 1 fe is due to this treatment being used as a control in parallel with each other treatment.

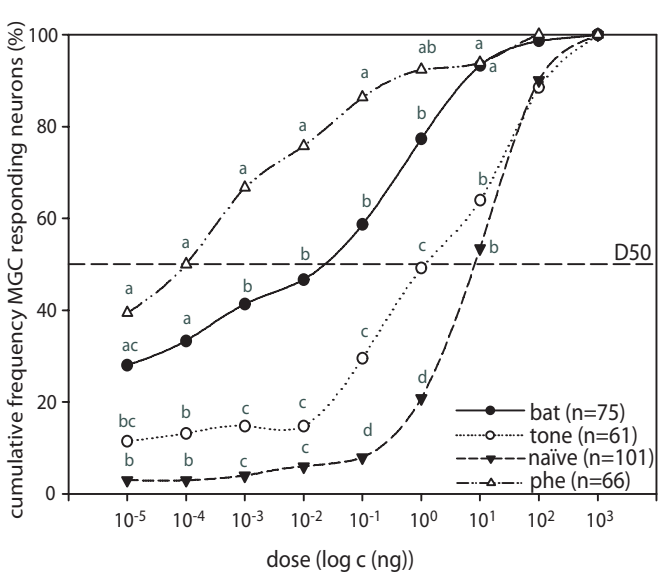
Fig. 2. Responses of AL neurons in male *S. littoralis* to the main pheromone component, *ZE*-9,11-14:OAc and its solvent hexane (**a**) and to the plant odor linalool and its solvent mineral oil (**b**). The odor stimulus reaches the antenna at around 250 ms. **a)** Typical recordings from MGC neurons in a tone-exposed male (left) with responses only to high pheromone doses (1000 ng) and in a batsound-exposed male (right) with responses to very low pheromone doses (0.1 pg). **b)** Typical recordings from OG neurons in a naïve male (left), responding to high doses of linalool (100 µg) and in a batsound-exposed male (right), responding to lower doses of linalool (1 µg). The horizontal black bar underneath the traces indicates stimulation duration (500 ms). Vertical scale bars 20 mV in **a**, 10 mV in **b**.

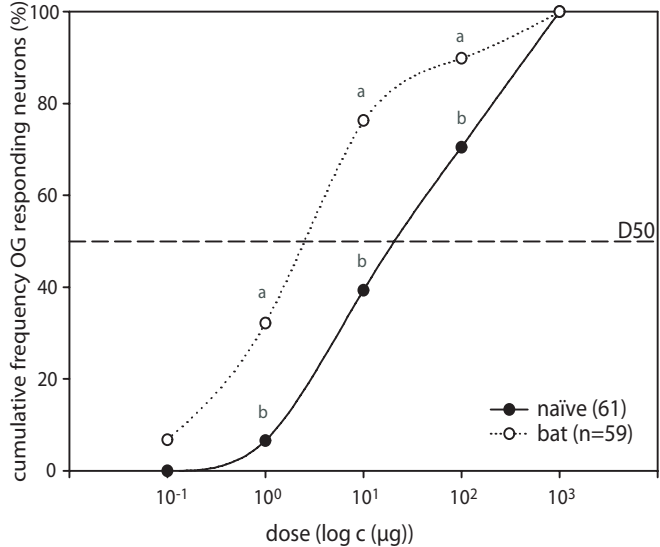
Fig. 3. Cumulative frequency curves of response thresholds of AL neurons in *S. littoralis* males. **a)** Cumulative response threshold distribution of MGC neurons to the main pheromone component, *EZ-9,*11-14:OAc. Mainly neurons with a high threshold were found in naïve and tone-exposed males, while lower threshold neurons were found in males that had been pre-exposed to either pheromone or bat sound. The D50, dose at which the 50%

of neurons responded, is indicated as a dashed line. Neurons in pheromone- and bat-exposed males reached the D50 at lower doses than in tone-exposed and naïves males. bat: neurons in bat sound-exposed males, tone: neurons in tone-exposed males, phe: neurons in pheromone-exposed males. **b)** Cumulative response threshold distribution of OG neurons to flower odors. For each neuron the lowest threshold for the best-tuned compound out of the three tested odors (linalool, geraniol and heptanal) was determined. Neurons in bat-exposed males had lower thresholds than neurons in control males. Statistical differences among treatments were assessed for each individual dose by means of a G-test for independence and pairwise *post hoc* comparisons. Numbers in brackets indicate numbers of tested neurons. Different letters denote statistical differences ($\alpha' = 0.008$).


60
50
40
30
20
10
% males within 5 cm of source
* * *
* *
ns
pre-exposed
naïve
1 fe
bat sound
tone


a
tone-exposed
batsound-exposed
hexane
100 ng
1000ng
hexane
0.1 pg
1 pg
b
naïve
batsound-exposed
oil
10 μg
100 μg
oil
1 μg
10 μg


a
cumulative frequency MGC responding neurons (%)
100
80
60
40
20
0
D50
bat (n=75)
tone (n=61)
naïve (n=101)
phe (n=66)
10-5 10-4 10-3 10-2 10-1 100 101 102 103
dose (log c (ng))


b
cumulative frequency OG responding neurons (%)
100
80
60
40
20
0
D50
naïve (61)
bat (n=59)
10-1 100 101 102 103
dose (log c (µg))

Supporting Information

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SI Material and Methods

Insects. The insects were reared for several generations in the laboratory and the culture was supplemented yearly with wild collected insects. Larvae were reared on a potato-based diet (1). Pupae were separated according to sex and kept in separate rearing chambers at 23°C, 70% RH and a 16 h/8 h light/dark cycle.

Walking bioassay. In the open arena olfactometer (2), Air was pushed through a baffle with spaced 2 mm holes while an exhaust in the other end sucked out contaminated air, resulting in a laminar flow of 0.5 m/s. Experiments were done 1½-4 h into the scotophase in red light (3 lux) at 18-20°C. During the photophase males were placed individually in glass tubes with one end covered with a mesh net and transported to the experimental room before the start of the scotophase. The moths were allowed to acclimatize in the testing room for about 2 h before the onset of the experiments and experienced the onset of the scotophase in the experimental room.

Odor stimuli. In *S. littoralis* the pheromone blend composition shows geographical variation (3, 4). The main pheromone compound is always *ZE*-9,11-14:OAc, and in addition a number of minor compounds have been identified, but the minor components differ in both presence and ratio between different populations. In the population of insects we use, the complete sex pheromone blend composition has not yet been fully identified. For this reason, we used female sex pheromone gland extracts for our behavioral

experiments. For each extract, 20-30 glands from 2-day-old virgin females were dissected and extracted in 100 µl hexane 2-3 hours into the scotophase. For storage the extract was transferred to a small glass vial and diluted to 1 female equivalent (1 fe) per 10 µl. Gas-chromatographic analysis showed that 1 fe corresponded to approximately 20 ng of *ZE*-9,11-14:OAc. For electrophysiological experiments, dilutions in decadic steps from 0.01 pg to 1 µg of *ZE*-9,11-14:OAc (>97% purity checked by gas chromatography [GC], CAS 50767-79-8, was synthesized in the laboratory in Versailles: courtesy of Martine Lettere) in hexane and from 0.1 µg to 1000 µg of linalool (Sigma-Aldrich, racemic, 97% purity,), geraniol (Sigma-Aldrich, 96% purity), and heptanal (Sigma-Aldrich, 95% purity) in mineral oil (Sigma-Aldrich, 95% purity) were used. The three plant-related compounds have previously been shown to be emitted by flowers and to be detected by *S. littoralis* and other noctuid moth antennal ORNs (5-8).

Sound stimuli. Sound stimuli were generated by multiplying square wave signals from a pulse generator (Berkeley Nucleonics Corporation 555) with sine wave signals from a function generator (Agilent 33120A) in a custom-built trapeze modulator. The signal was attenuated (Kay 865 step attenuator), amplified (UltraSoundAdvice S55) and broadcasted through an electrostatic loudspeaker (UltraSoundAdvice S56). The loudspeaker was placed close to the surface of the olfactometer floor. The sound intensity was measured by a ¼ inch microphone (G.R.A.S.) that was calibrated against a G.R.A.S. sound calibrator. The sound pressure was measured at several points in the middle of the arena in front of the

loudspeaker at the surface level and according to these results, an area was marked in which the sound pressure did not vary by more than ±3dB around the mean of 102 dB SPL.

Pre-treatments. In the experiments, males were submitted to different pre-treatments before the trial. During all pre-treatments the moths were kept inside glass tubes that were covered by a mesh on both sides and were individually placed in the center of the olfactometer. We have earlier shown that upwind movement towards the pheromone source is not necessary for obtaining pre-exposure effects (2). During pre-exposure to the pheromone, 1 fe of gland extract was placed at the upwind end of the olfactometer and the glass tubes with one male each, were individually put 40 cm downwind in the pheromone plume. During the exposure time of 10 s, a large majority of the males showed activation and upwind movement within the glass tube. For sound exposure moths were placed in the marked area on the arena where sound pressure did not vary more than ±3dB. After a short time allowing the moths to settle, the sound was turned on manually for 10 s.

The following stimuli were used for pre-exposure:

1. Sex pheromone gland extract (1 fe)

2. Pulsed bat-like sound. The sound consisted of pulse trains consisting of 20 trapezoid shaped pulses. Each pulse was 4.7 ms long with a carrier frequency of 30 kHz. The pulse trains were repeated ten times, resulting in a total exposure time of 940 ms. Sound intensity was 102 dB SPL (sound pressure level). This stimulus elicited a consistent behavioral response. 30 kHz was chosen because it is within the moths' best frequency of hearing (9) and since many bats, including gleaners, emit echolocation signals including 30 kHz. The temporal structure of our stimuli corresponds roughly to the signals emitted during the

search phase of many bats (10-12). Furthermore, these stimulus parameters evoke maximum silencing response in the acoustically signaling moth, *Achroia grisella* (13).

3. A tone. The tone consisted of a continuous 940 ms 30 kHz signal at 102 dB SPL.

4. Simultaneous stimulation to sex pheromone gland extract (1 fe) and pulsed bat-like sound as under point 2.

After the pre-treatments both treated and control males were kept individually in the glass tubes and transported back to the rearing chamber. The next day, just before the onset of the scotophase, they were returned to the experimental room for test trials. Each treatment was tested on several occasions to minimize the effects of variations in conditions between days and variation between different batches of moths. Males were tested 24 to 27 h after pre-exposure and normally pheromone exposure and pheromone tests were done on different days. In the rare cases where pre-exposure with pheromone was done the same day as subsequent behavioral tests of males from the previous day, the airflow of the olfactometer was allowed to run at least for 1 hour to clear any remaining pheromone traces.

Behavioral tests. The behavioral tests were performed in the same arena as used for the pre-exposure. In an earlier study we have shown that male moth behavior in the walking assay and in a wind tunnel were similar, even if response thresholds differed (2). Pheromone solutions at an amount of 0.03 fe were applied on pieces of filter paper, which were placed upwind at the center of the arena in front of the baffle. This dose was chosen because it resulted in a low percentage of responses of the naïve males and allowed us to observe a clear effect of pre-exposure. The filter paper was changed every 20 minutes.

Males were introduced individually in the arena at the center of the odor plume 40 cm downwind from the odor source. Moths were given 120 s to respond and the males, which reached within 5 cm from the odor source or made source contact were scored.

Electrophysiology. Intracellular recordings of neurons situated in the MGC and in OG of the AL were performed according to standard methods (14). The stimulation procedure and experimental protocol of intracellular recordings have been described in detail earlier (15). Briefly, thresholds of AL neurons were determined by stimulating the ipsilateral antenna with increasing doses of sex pheromone or plant compounds with at least 10 s between individual stimulations or until spontaneous activity recovered. For stimulation, an airflow (7 ml/s) carrying the odor stimulus was inserted for 500 ms into a continuous airflow (17 ml/s) using a stimulus controller (CS 55, Syntech, Kirchzarten, Germany). Recordings were stored and analyzed on a PC with Autospike 32 software (Syntech, Kirchzarten, Germany). For the statistical analysis of neuron thresholds, electrophysiological responses were quantified as described previously (16). Briefly, spontaneous activity during the 500 ms preceding odor stimulation was subtracted from the response during 500 ms during stimulation to determine a response. Since most AL neurons responded also to the solvent hexane (or to the mechanical stimulus during the switch of airflows), a neuron was classified as responding to a stimulus when the odor response exceeded the response to a hexane stimulus by at least 10%. Data are presented as cumulative threshold curves as a function of stimulus dose threshold distributions.

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