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Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner

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Summary

Pheromone-dependent mate search is under strict circadian control in different moth species. But it remains unknown whether daytime-dependent changes in pheromone sensitivity already occur at the periphery in male moths. Because adapting pheromone stimuli cause rises of cyclic guanosine monophosphate (cGMP) in pheromone-sensitive trichoid sensilla of the night-active hawkmoth *Manduca sexta*, we wanted to determine whether cGMP decreases pheromone-sensitivity of olfactory receptor neurons in a daytime-dependent manner. Long-term tip recordings from trichoid sensilla were performed at the early day (ZT 1–4), when many moths are still active, and at the middle of the day (ZT 8–11), when moths are resting. A non-adapting pheromone-stimulation protocol combined with perfusion of the sensillum lymph with the membrane-permeable cGMP analogue 8bcGMP adapted the action potential response but not the sensillar potential. Perfusion with 8bcGMP decreased the initial action potential frequency, decreased the numbers of action potentials elicited in the first 100 ms of the pheromone response and attenuated the reduction of action potential amplitude. Furthermore, the decrease in 8bcGMP-dependent action potential frequency was stronger in recordings made at ZT 8–11 than at ZT 1–4. In the control recordings during the course of the day the pheromone responses became increasingly tonic and less phasic. At ZT 8–11 only, this daytime-dependent effect was further enhanced by 8bcGMP application. Thus we hypothesize that during the moths' resting phase, elevated cGMP levels underlie a daytime-dependent decrease in pheromone sensitivity and a decline in the temporal resolution of pheromone pulses.

Key words: insect olfaction, pheromone transduction, moth, cyclic nucleotide, antenna, circadian difference.

Introduction

Males of the nocturnal hawkmoth *Manduca sexta* detect the pulsatile release by female moths of the sex pheromone blend using specialized trichoid sensilla. The pheromone-sensitive sensilla are located in large numbers at the leading edge of the antenna and are each innervated by two olfactory receptor neurons (ORNs) (Sanes and Hildebrand, 1976; Keil, 1989; Lee and Strausfeld, 1990). Both ORNs are sensitive to different components of the pheromone blend. Only one of the ORNs, which elicits the larger action potentials (APs), responds to the main sex pheromone component bombykal (BAL) (Starratt et al., 1979; Tumlinson et al., 1989; Kaissling et al., 1989; Dolzer et al., 2001; Kalinová et al., 2001).

Much is already known about the peripheral signal transduction processes underlying the detection of pheromone by the male moth. Pheromone-dependent rises in inositol triphosphate (IP_3) (Breer et al., 1990) cause an influx of Ca^{2+} into insect ORNs (Stengl, 1994). This triggers the opening of Ca^{2+}-dependent ion channels involved in the generation of pheromone-dependent receptor potentials (Stengl, 1993; Stengl, 1994; Zufall and Hatt, 1991; Zufall et al., 1991). In contrast, little is known about the modulation of the pheromone transduction cascade leading to adaptation and sensitization. Previous studies in *Manduca sexta* distinguished desensitization from short-term and long-term adaptation (Dolzer et al., 2003). Desensitization is the decline in excitation, as seen during a phasic–tonic response to a stimulus of long duration, while short-term adaptation is the rapidly (within seconds to minutes) reversible reduction of sensitivity due to prior stimulation. Long-term adaptation is the more slowly occurring and longer persisting (for minutes to hours) reduction of sensitivity due to previous strong stimulation (Ziegelberger et al., 1990; Marion-Poll and Tobin, 1992; Boekhoff et al., 1993; Stengl et al., 2001; Dolzer et al., 2003). Increasing evidence supports the involvement of cyclic guanosine monophosphate (cGMP) in long-term olfactory adaptation and regulatory processes in moths. After strong pheromone stimulation, cGMP levels rise slowly in antennae

and antennal homogenates of *Antherea polyphemus* and *Bombyx mori*, remaining elevated for nearly 30 min and, thus, matching the time course of long-term adaptation (Ziegelberger et al., 1990; Boekhoff et al., 1993). In the silkmoth *Bombyx mori* the perfusion of the sensillar lymph with dibutyryl guanosine 3′,5′-cyclic monophosphate caused a suppression of the sensillar potential and a reduction of the AP response in pheromone-dependent sensilla, mimicking a state of adaptation (Redkozubov, 2000).

In tip recordings of trichoid sensilla of *Manduca sexta*, adapting pheromone stimulation shifted the dose–response curves to higher stimulus intensities (Dolzer et al., 2003). In other moths, as for *Manduca sexta*, this shift is also larger for the AP than for the sensillar potential response, suggesting the presence of more than one adaptation mechanism (Zack, 1979; Kaissling et al., 1986; Kaissling et al., 1987; Dolzer et al., 2003). At least one adaptation mechanism acts on the first step of the olfactory transduction cascade, the generation of the receptor potential, which is assumed to occur in the outer dendritic segment of the ORNs (Kaissling and Thorson, 1980; Stengl et al., 1998; Dolzer et al., 2003). An additional adaptation mechanism apparently acts on the second step of the transduction cascade, the transformation of the amplitude-modulated receptor potential into frequency-modulated APs. The APs are apparently elicited in the soma- or axon-hillock region, as indicated by the polarity of APs and the current necessary to elicit them, and not at a dendritic location, as suggested by other experiments (Kaissling and Thorson, 1980; de Kramer, 1985; de Kramer et al., 1984; Dolzer et al., 2001). The molecular basis and the behavioral consequences of these different mechanisms of insect olfactory adaptation are still not fully understood.

Previous studies have revealed that the responsiveness of male moths to pheromone is daytime-dependent. Males of the nocturnal moths *Trichoplusia ni* and *Agrotis segetum* showed a distinct daily rhythm in their response to pheromone, with a maximum response during the scotophase (Linn et al., 1996; Rosén et al., 2003). Also, daytime-dependent changes in responsiveness to pheromone persist under constant conditions so are controlled by an endogenous circadian clock (Baker and Cardé, 1979; Rosén et al., 2003). Not only male responsiveness, but also calling behavior and pheromone release of female moths, show a diurnal distribution with a maximum at the end of the scotophase for *Manduca sexta* and the middle of the scotophase for *Agrotis segetum* (Itagaki and Conner, 1988; Rosén, 2002). For *Manduca sexta* it was shown that peaks in the calling behaviour of female moths and male flight activity are correlated during the scotophase (Sasaki and Riddiford, 1984). In addition, *Spodoptera littoralis* moths mated significantly less when males and females were raised in different light:dark cycles out of phase (Silvegren et al., 2005). This indicates that circadian clocks rule rhythmic mating preference in male and female moths and that photoperiod and pheromones synchronize the mating behavior of both sexes (Silvegren et al., 2005).

To determine whether daytime-dependent changes in pheromone sensitivity already occur at the periphery in male *Manduca sexta* moths and to determine whether cGMP-dependent mechanisms of long-term adaptation might be employed, we applied the membrane-permeant cGMP analogue 8-bromo guanosine 3′,5′-cyclic monophosphate (8bcGMP) in extracellular tip recordings from trichoid sensilla at two Zeitgebertimes (ZTs; ZT 1–4 and ZT 8–11). ZT 1 is at the beginning of the day (lights on at ZT 0) when the nocturnal moths switch from their active to their inactive phase, and ZT 8 is at the middle of the day when the moths are resting. Pheromone responses to the main pheromone component BAL and spontaneous APs of unstimulated ORNs of the hawkmoth *Manduca sexta* were investigated.

Materials and methods

Animals and preparation for electrophysiological recordings

Hawkmoths *Manduca sexta* (Johannson) (Lepidoptera: Sphingidae) were raised from eggs, feeding on an artificial diet [modified after Bell and Joachim (Bell and Joachim, 1976)]. Male pupae were kept under a long-day photoperiod (17 h:7 h L:D) at 24–27°C and 40–60% relative humidity and were isolated 1 day before emergence, gently cleaned with 70% ethanol, and allowed to hatch without contact with pheromone. After their second dark phase, 30 min prior to the start of each recording, the adults were taken out of the isolation boxes and fixed in a Teflon™ holder. Thus, moths at both ZTs spent the same time in the Teflon™ holder. The flagellum of the right antenna was immobilized with dental wax (Boxing wax, Sybron/Kerr, Romulus, MI, USA), and the 15–16 most apical annuli were clipped off. A glass electrode filled with haemolymph Ringer (Kaissling, 1995), which served as the indifferent electrode, was inserted into the flagellar lumen. To stop the first annuli from drying out the end of the antenna was sealed with ECG electrode gel (PPG, Hellige, Freiburg, Germany) afterwards. The tips of long trichoid sensilla from the apical row on the second remaining annulus were clipped off using sharpened forceps. The recording electrode, filled with sensillum lymph Ringer (Kaissling, 1995), was slipped over one sensillum. To minimize contributions of the electroantennogram, we recorded from an annulus close to the tip of the haemolymph electrode. The connection to the amplifier inputs was established with Ag/AgCl wires immersed in the electrolytes. Signals were amplified about 200-fold in a custom-built amplifier (0 Hz–2 kHz, input impedance 10^{12} Ω), and passed through an anti-aliasing filter with a cut-off frequency of 2 kHz (900C/9L8L, Frequency Devices, Haverhill, MA, USA). For data acquisition, a Digidata 1200 B digitizer and pCLAMP software (version 8) (Axon Instruments, Union City, CA, USA) were used. The electrophysiological signal and a high-pass filtered equivalent (cut-off frequency 5 Hz) were continuously recorded on a strip chart recorder (EasyGraf, Gould, Valley View, OH, USA). Voltage polarity is given with the sensillum lymph electrode in reference to the haemolymph electrode. To be able to record in the photophase we compared the beginning of the day with the

middle of the day to search for ZT-dependent effects of 8bcGMP. All experiments were performed with room lights switched on, starting either at the beginning of the day, 1 h after lights on at ZT1, or at the middle of the day, at ZT8.

Application of 8bcGMP

Drug was applied to pheromone sensilla during tip recordings by perfusion with water-soluble agents, as first suggested by Kaissling et al. (Kaissling et al., 1991). Long-term recordings from intact animals revealed no damage of the sensilla when continued for up to several days (Dolzer et al., 2001). So we allowed the sensillum lymph ringer to passively perfuse the sensillum lymph cavity and did not apply any pressure or suction. In recordings of stimulated trichoid sensilla, 8bcGMP diluted in sensillum lymph ringer was applied over the recording electrode. Therefore, we used an altered sensillum lymph ringer with a concentration of 10 mmol l^{-1} 8bcGMP. This application method is further referred to as sensillum lymph perfusion. Biogenic amines injected into the head capsule near the base of the antenna influenced oscillations of the transepithelial potential (TEP) of trichoid sensilla (Dolzer et al., 2001). Thus, the agents were transported into the antenna after injection. In recordings of the spontaneous activity of ORNs, 8bcGMP diluted in haemolymph ringer was injected through a hole in the head capsule, which was pierced with a syringe needle approximately 1 mm dorsocaudal to the right antennal base. We injected a minimum of 3 μl of 10 mmol l^{-1} 8bcGMP solution and a maximum of 5 μl of 100 mmol l^{-1} solution, resulting in a concentration of 30–500 nmol l^{-1} 8bcGMP. Because an adult moth contains approximately 1 ml of haemolymph the final 8bcGMP concentration in the haemolymph was between 30–500 μmol l^{-1}. This application mode is further referred to as haemolymph injection. The ringers used for sensillum lymph perfusion and the respective controls were prepared with *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethane sulfonic acid) (Hepes; all chemicals from Sigma, Deisenhofen, Germany); the ringers used for haemolymph injection were prepared with a phosphate buffer (monopotassium phosphate). The pH was adjusted to 6.5, and osmolality was adjusted to 475 mosmol l^{-1} with mannitol for sensillum lymph ringer and to 450 mosmol l^{-1} for the haemolymph ringer.

Pheromone stimulation

All recordings were performed at room temperature (18–23°C). Charcoal-filtered and moistened air was permanently blown over the preparation through a glass cartridge (13 l min^{-1}). The air stream could be redirected through cartridges containing a piece of filter paper (about 1 cm^2) loaded with synthetic bombykal (E,Z-10,12-hexadecadienal; BAL) generously provided by T. Christensen (Tucson, AZ, USA). The air stream was switched between the cartridges using solenoid valves (JFMH-5-PK and MFH-5-1/8, Festo, Esslingen, Germany) controlled by the computer. Doses of 10 μg BAL dissolved in *n*-hexane (Merck, Frankfurt, Germany) were applied to the filter papers (10 μl per paper), and the solvent was allowed to evaporate. Stimulus intensity is always given in terms of the BAL dose applied to the filter paper. The cartridges were placed in the outlet in a distance of 4.5–6 cm from the recording site about 25 s prior to stimulation. Stimuli of 10 μg BAL and 50 ms duration were applied every 5 min during a recording session of 3 h. A suction tube of 10 cm diameter was placed below the animal for rapid removal of the pheromone component after stimulation, and to avoid uncontrolled stimulation due to BAL leaking out of the stimulus cartridges. Between the recording sessions, the cartridges were stored at –20°C in individual glass scintillation vials. Control cartridges loaded with hexane alone were prepared and treated the same way. A set of stimulus cartridges was used for 5–10 recording sessions; the control cartridges were used for up to 30 recordings.

Acquisition protocols and data analysis

Each sensillum trichodeum contains two ORNs, both generating spontaneous APs and responses to different compounds of the pheromone blend. The APs of both ORNs can be distinguished by their amplitude. The spontaneous activity of the ORNs was acquired in segments of about 10 min with a sampling frequency of 19.6 kHz (Clampex 8, fixed-length events). Each AP triggered a sweep of duration 12.75 ms, and the high-pass filtered signal served as a trigger channel only. All analyses were performed using the direct-current-coupled signal. The mean voltage during the initial 2.5 ms was defined as the baseline and used to measure the TEP. The baseline of all AP sweeps was then adjusted to 0 mV to identify sweeps that were triggered by artifacts. To evaluate the shape of APs, the waveforms of both classes were averaged for intervals of 10 min. The pheromone responses were recorded in sweeps of 5161 ms duration at a continuous sampling rate of 20 kHz (Clampex 8, Episodic Stimulation Mode) with a pre-trigger portion of 180.6 ms and a post-trigger portion of 4930.2 ms. The spontaneous activity between the stimuli was recorded in segments of approximately 5 min in fixed length events, as described above. The recordings of the pheromone responses were evaluated using the Microsoft Excel Add-in XtraCell (Dolzer, 2002) and Clampfit 8. For analysis of the sensillar potential, the responses were low-pass filtered at a cut-off frequency of 50 Hz (Clampfit, Gaussian filter). The evaluated parameters of the sensillar potential (SP), as illustrated in Fig. 1A,B, were: (1) the overall amplitude (SP amplitude), (2) the initial slope between the onset of the DC response and the half-maximal SP amplitude (initial slope) and (3) the half-time of the rising phase ($t_{1/2rise}$). The second portion of the rising phase of the sensillar potential was described by an exponentional fit of first order, using only the time constant (τ). The fitting was performed with a non-iterative Chebyshev algorithm

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C$$

including 4-point smoothing. For analysis of the APs (Fig. 1C), the low pass-filtered trace was subtracted from the original response. This pseudo-high-pass filtering procedure, in contrast

to actual high-pass filters, does not distort the shape of the APs, and therefore allows the analysis of their amplitude and waveform. The time and positive amplitude of every AP occurring during the 5161 ms-long sweep recorded during the stimulations was counted. When APs of both amplitude classes were observed during the response, they were analyzed separately. The AP response was characterized by: (1) the peak frequency computed from the first five interspike intervals (AP frequency; Fig. 1C); (2) the latency between the beginning of the DC response and the occurrence of the first AP (AP latency; Fig. 1B); and (3) the positive AP amplitude. The peak frequency represented the phasic part of the phasic–tonic AP

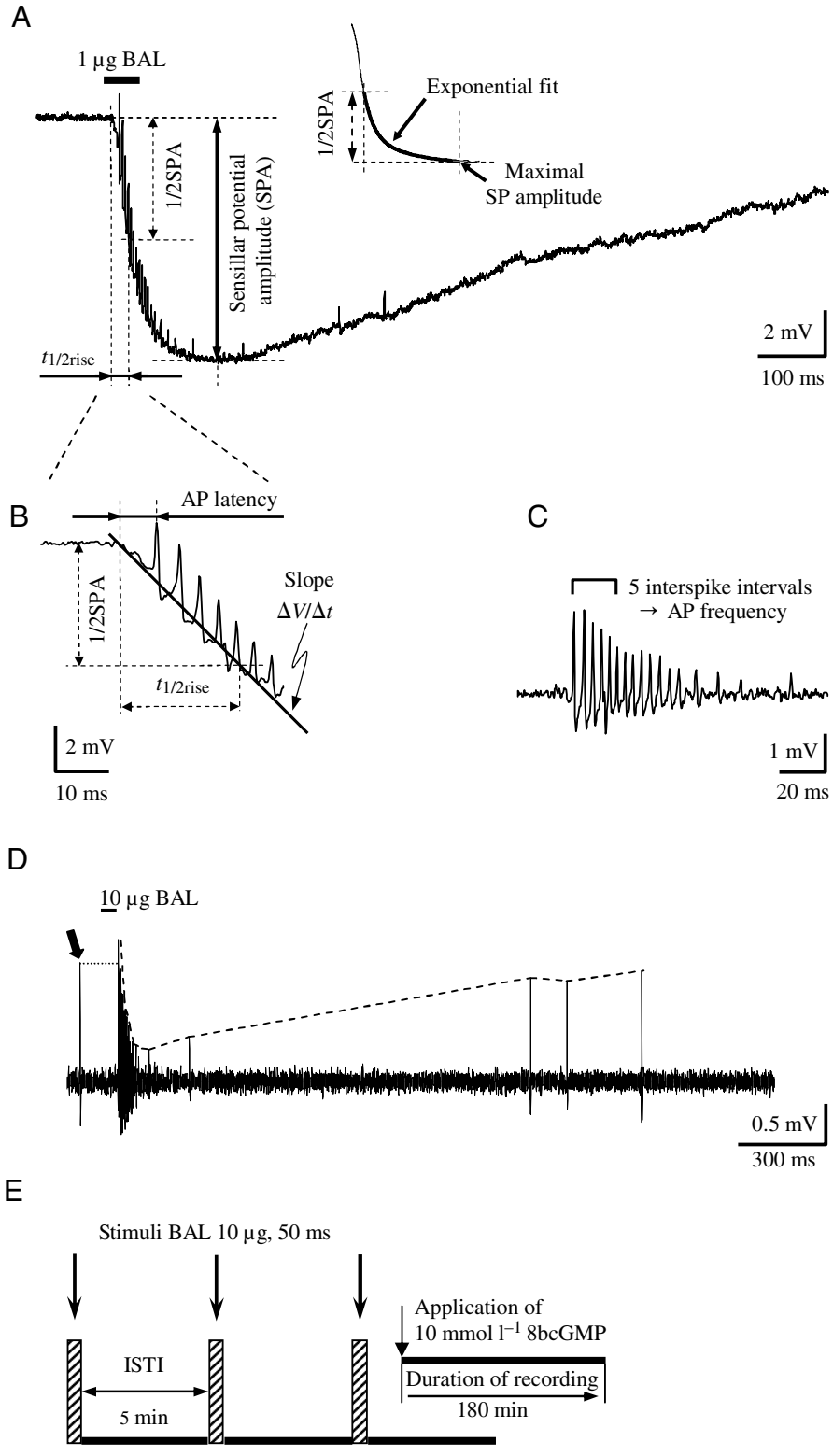

Fig. 1. The pheromone response is characterized by six parameters. (A) A non-filtered (DC) response to a 50 ms stimulus of 1 µg bombykal (BAL). Action potentials are superimposed on the negative deflection of the transepithelial potential, the sensillar potential (SP) response. The maximal SP amplitude (SPA) is measured between the baseline before the response and the negative peak of the SP. The half-time of the rising phase ($t_{1/2rise}$) is determined between the onset of the SP and the time the potential has reached 50% of the SPA (½SPA). The second portion of the rising phase is described by an exponential fit of first order, using only the time constant (τ). For the analysis of all parameters describing the SP, the responses were low-pass filtered at 50 Hz. (B) The initial phase of the response at an enlarged time scale. The initial slope is determined by dividing ½SPA by $t_{1/2rise}$. The AP latency is measured between the onset of the sensillar potential and the peak of the first action potential. (C) For the analysis of action potentials, the low-pass filtered response is subtracted from the original trace, yielding a straight baseline. The initial action potential frequency (AP frequency) is computed over the first five interspike intervals. (D) Pseudo-high-pass filtered (AC) response to a 50 ms stimulus of 10 µg BAL. The amplitudes of the large action potentials are reduced after strong BAL stimuli and regain their original amplitude in the course of several seconds (broken line). A spontaneous action potential of the non-BAL cell occurred before (filled arrow) the response. APs of the non-BAL cell can be separated from the BAL-APs by their lower and steady amplitude after stimulation with BAL. (E) Non-adapting stimulus-protocol: 50 ms long stimuli of 10 µg BAL per filter paper were applied with an interstimulus-interval (ISTI) of 5 min over a period of 180 min. 8bcGMP at 10 mmol l^{-1} was applied by perfusion over the recording electrode with the beginning of the recording [modified after (Dolzer, 2003)].

response and was calculated according to $f=N\times1000/\Sigma ISI$ [N=number of interspike intervals (ISI)]. Due to the high variability of the evaluated response parameters of each recording, the parameters were normalized to the first response of a recording. Subsequently the data for each parameter was binned to intervals of 5 min and the mean was plotted against the time of recording.

The data of the evaluated parameters of 8bcGMP and associated control recordings in each time slot were compared using the Student's *t*-test. For statistical analysis of changes in several parameters over the recording duration, its time course was divided into three intervals and then analyzed using a one-way ANOVA followed by the Tukey HSD *post-hoc* test. For analysis of the AP amplitude reduction, action potentials of three consecutive responses within an interval (from 1 min before to 2 min after the stimulus) were binned to 10 ms intervals and plotted against time. To measure changes in the amplitude reduction, the ratio of the minimal and maximal AP amplitudes was calculated and the normalized parameter for each recording plotted against the time of recording. To evaluate changes in the AP frequency or distribution in time, the AP response characteristics were analyzed using post-stimulus-time histograms; the AP responses were added up, binned to 10 ms intervals and plotted over time, with t=0 being the start of the DC response.

Results

In extracellular tip recordings of trichoid sensilla of the hawkmoth *Manduca sexta*, we investigated daytime-dependent effects of 8bcGMP perfusion on stimulated ORNs. The recordings were performed over the course of 3 h. Recordings started either at the beginning of the day at ZT 1, when the nocturnal moths switch from their active to their inactive phase, or at the middle of the day at ZT 8, when the moths are resting. Slow, amplitude-modulated BAL-dependent sensillar potentials with superimposed, fast, frequency-modulated APs were quantified (Fig. 1A–C). BAL stimuli were applied in a non-adapting protocol with an interstimulus interval of 5 min (Fig. 1E). In addition, the effects of 8bcGMP on spontaneous APs of unstimulated trichoid sensilla were examined.

Pheromone responses

To search for cGMP-dependent modulation of BAL responses, we evaluated various parameters of the BAL-dependent sensillar potential and AP response (Fig. 1). Parameters describing the sensillar potential are the maximum sensillar potential amplitude, the initial slope characterizing the first half, and fit τ describing the second half of the rising phase of the sensillar potential (Fig. 1A,B). Parameters describing the phasic part of the AP response are the AP frequency computed over the first five interspike intervals, the AP latency in relation to the onset of the sensillar potential, and the stimulus-dependent amplitude reduction (Fig. 1B–D). The beginning and end of the recordings were compared (Fig. 2). In both time slots from ZT 1–4 and ZT 8–11, tip recordings with perfusion of the sensillar lymph with 10 mmol l^{-1} 8bcGMP applied over the recording electrode were compared to control recordings without cyclic nucleotides. In control recordings at both ZTs, no changes in

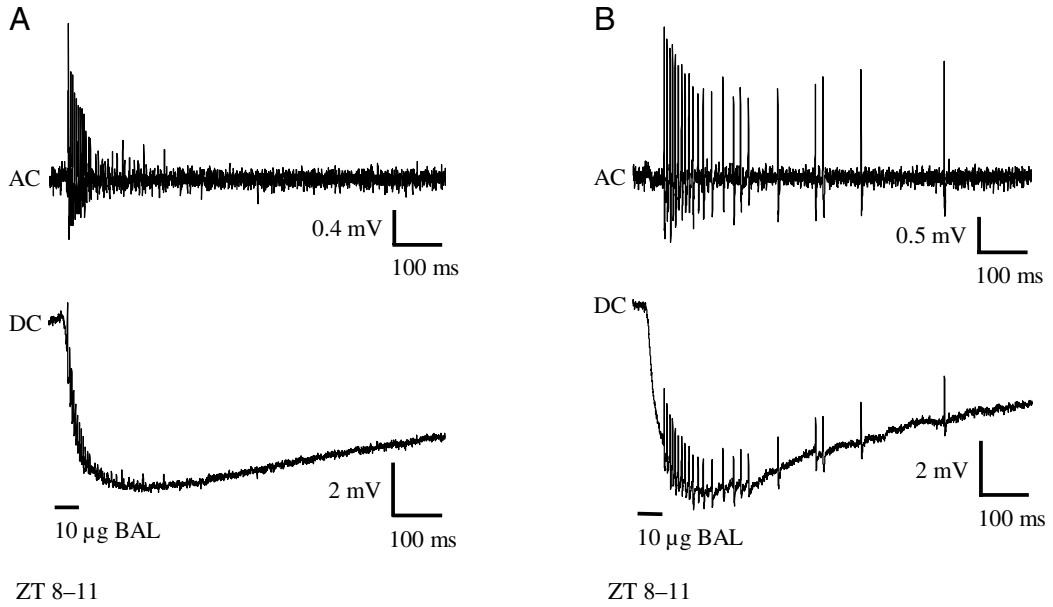

Fig. 2. Sensillum lymph perfusion with 10 mmol l^{-1} 8bcGMP at ZT 8–11 reduced the action potential (AP; top recordings) response, but left the sensillar potential (SP; bottom recordings) response unaltered. (A) At the beginning of the recording, the AP frequency in response to bombykal stimulation was 245 Hz. After 158 min (B) the SP was unaltered, but the AP frequency was reduced to 147 Hz. Also, the latency to occurrence of the first AP (AP latency) was prolonged. The effect on the AP latency was characterized by high variability between recordings. AC, pseudo-high-pass filtered signal; DC, non-filtered signal.

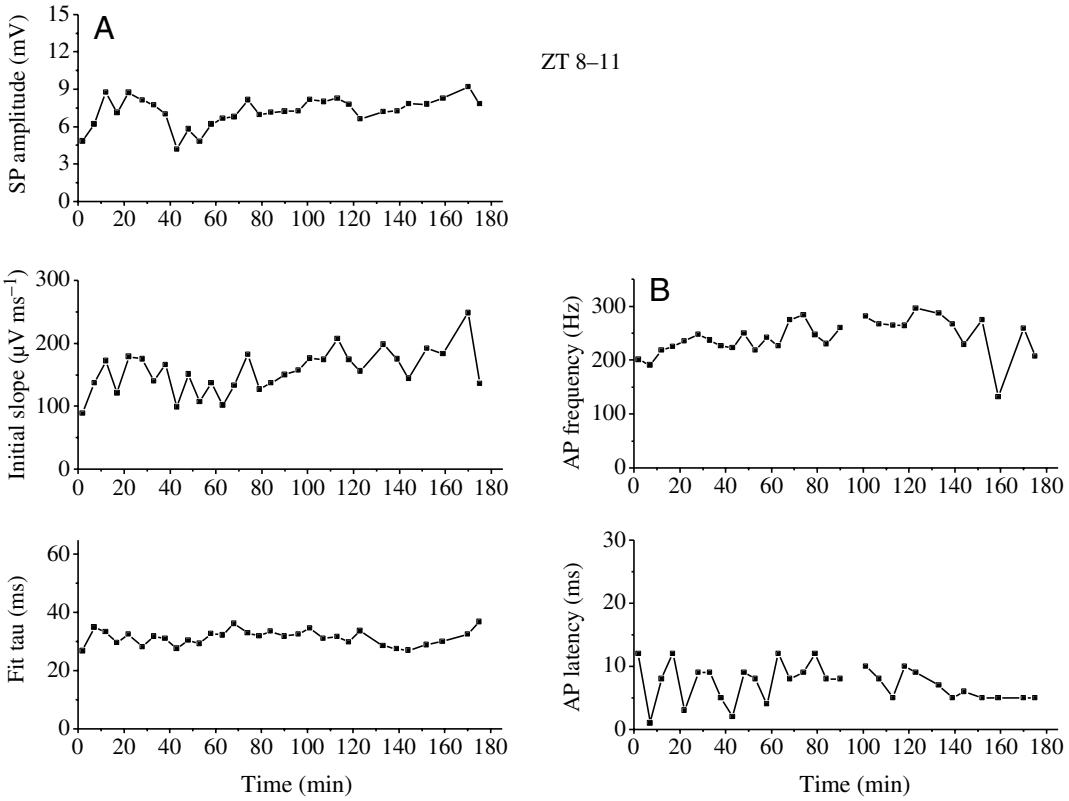

Fig. 3. Example time course of sensillar potential (A) and action potential (B) parameters for one recording under control conditions at ZT 8–11. The pheromone responses remained virtually constant during 3 h of repetitive stimulation. A bombykal stimulus of 10 μg dose and 50 ms duration was applied every 5 min.

the evaluated parameters could be observed throughout the 3 h of recordings (Figs 3, 5). In the presence of 8bcGMP the AP frequency was continuously decreased, and in a few recordings the latency between the onset of the sensillar potential and the occurrence of the first AP increased (Figs 2, 4). All other parameters that describe the AP and the sensillar potential response remained constant over the time of recording (Fig. 4). For recordings from ZT 8–11 (Fig. 5), significant differences were found in both the comparison of the AP frequency of 8bcGMP and control recordings (Student's *t*-test for independent samples, $P<0.01$) and also within the time course of the AP frequency (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). No significant difference was found between intervals 2 and 3 of the time course. In contrast no significant differences were found for the sensillar potential amplitude. Due to the high variability for the AP latencies no significant difference for recordings with or without 8bcGMP was found (data not shown).

As in recordings from ZT 8–11, the AP frequency of recordings from ZT 1–4 (Fig. 5) showed a significant decrease when 10 mmol l^{-1} 8bcGMP was included in the recording electrode (Students *t*-test for independent samples, $P<0.01$), but no significant difference was found between groups 2 and 3 of the time course (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). The sensillar potential amplitude and the evaluated parameters of control recordings (Fig. 5) did not change over time ($N=9$). Comparison of the AP frequency depletion between both time slots revealed a 12% stronger decrease for recordings at ZT 8–11.

It was next determined whether there are any daytime-dependent differences in the distribution of APs in response to stimulation with BAL in controls or in the presence of 8bcGMP. To analyse the distribution of APs occurring during the first 1000 ms, for responses from the beginning (0–20 min), middle (80–100 min) and end (160–180 min) of the recordings, the counted APs were binned to 10 ms intervals and plotted as post-stimulus-time histograms (Figs 6, 7). In control recordings from ZT 1–4 during the first 100 ms of the responses the response peak occurred later and the number of APs in the first 100 ms decreased significantly over the 3 h recording, leading to a slightly less phasic characteristic of the responses (Fig. 6, insert in C). Under the influence of 8bcGMP a weak decline in the number of APs over the course of the 3 h recording was also recognizable (Fig. 6A–C); owing to high variability, however, the decrease over the first 100 ms of the responses was not significant (Fig. 6C, insert).

In control recordings from ZT 8–11 the distribution of APs changed to a more tonic spike pattern (Fig. 7), which was much more pronounced than in control recordings at ZT 1–4. Also, the number of APs over the first 100 ms of the BAL

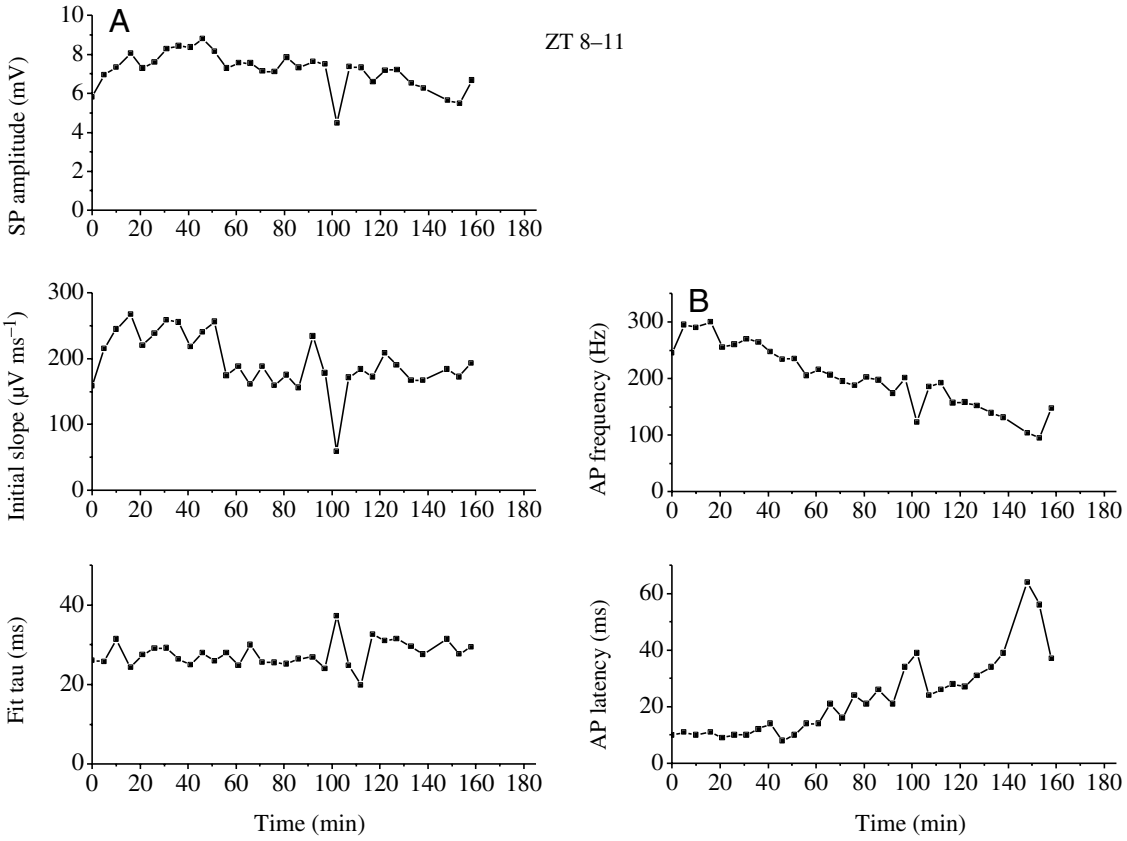

Fig. 4. Time course of sensillar potential (SP; A) and action potential (AP; B) parameters under the influence of 8bcGMP perfusion at ZT 8–11. Shown is one representative recording. During sensillum perfusion with 10 mmol l^{-1} 8bcGMP the AP frequency continuously decreased and the AP latency increased, while the parameters that describe the SP remained virtually constant.

responses significantly decreased over the course of the recordings. The numbers of APs were much more strongly decreased in control recordings at ZT 8–11 than at ZT 1–4 (Fig. 6C, Fig. 7C, inserts). In the presence of 8bcGMP the change to a more tonic spike pattern, as well as the decline in the number of APs in the first 100 ms of the BAL responses, were even more pronounced than in the controls at ZT 1–4 and at ZT 8–11.

Effects on the amplitude reduction

Characteristic for responses to BAL stimuli of higher dose is the reduction of the AP amplitude (Fig. 1C,D), which lasts for seconds to minutes. Especially in responses to strong stimuli with BAL concentrations higher than 1 μg per filter paper, the reduction of the peak-to-peak amplitude in the phasic portion is very strong and, in many cases, after about 150 ms the APs cannot be distinguished from noise. When comparing the beginning and end of a recording (Fig. 2), or the positive amplitude of APs in responses during an interval of 1 min before to 2 min after stimulation, several effects of 8bcGMP on the amplitude reduction can be observed (Fig. 8A). At the beginning of the recordings the amplitude reduction is very strong, but it gradually attenuates over the time of the recording until the reduction is only very weak and transient in the presence of 8bcGMP (Fig. 8A). In control recordings no strong changes in the amplitude reduction are recognizable (Fig. 8A). In addition to the cGMP-dependent attenuation of the decline of the AP amplitude, the kinetics of the amplitude attenuation also changed (data not shown). At the beginning of the recording the APs did not return to their prestimulus amplitude until 2 min after the stimuli had been applied. Under the influence of 8bcGMP the recovery phase was gradually attenuated until it disappeared. Also the minimal positive AP amplitude linearly increased over the course of the recording (data not shown). Furthermore, the normalized ratio of the minimal and maximal positive AP amplitude (Fig. 8B) (as a mean of the strength of the amplitude reduction) shows that 8bcGMP perfusion attenuates the AP amplitude reduction more strongly than in the controls at both ZTs. In addition, there are differences between both ZTs in the control recordings and in those with 8bcGMP perfusion. At ZT 8–11 with application of 8bcGMP, an attenuation of the amplitude reduction was recognizable in all of the recordings, whereas in the associated controls the strength of the amplitude reduction was reduced in only one recording over the entire time course of the recordings. At ZT 1–4 the recordings were more unequally distributed. In the control recordings there was higher variance compared to

recordings at ZT 8–11, which also increased with the duration of the recording. Under the influence of 8bcGMP 2 populations of recordings were recognizable. In one population that describes the majority of the recordings, the amplitude reduction increased only slightly and this increase did not start until 90 min after the beginning of the recording. In the second population a very strong attenuation of the amplitude reduction was present from the beginning. Changes in AP amplitudes of spontaneous APs between BAL responses were not found.

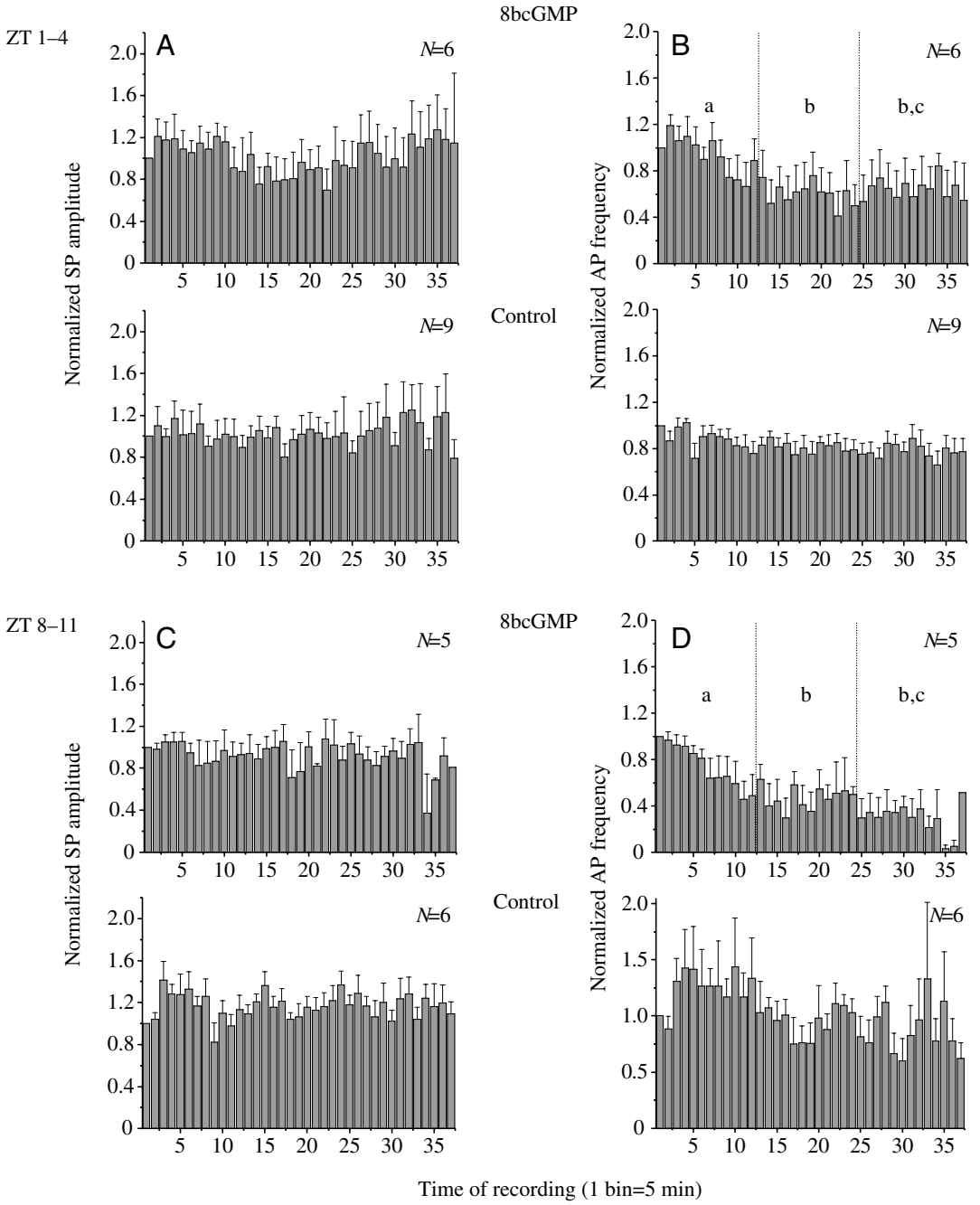

Fig. 5. The normalized and binned sensillar potential (SP) amplitude (A,C) and action potential (AP) frequency (B,D) parameters over 185 min (1 bin=5 min) for recordings from ZT 1–4 (A,B) and ZT 8–11 (C,D). Values are means + s.e.m. (B,D) Under the influence of 8bcGMP (top recordings) a significant decrease of the AP frequency can be recognized for both ZTs when compared to the respective controls (bottom recordings) (Student's *t*-test for independent samples, $P<0.01$) and when comparing the three intervals within each time course (separated by dotted lines). Different lower case letters denote significant differences between tested groups of means (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). The means in the second (b) and third time interval (c) are not significantly different (b,c) for both ZTs. The decrease of the action potential frequency at ZT8–11 is 12% stronger than in recordings from ZT1–4. (A,C) In contrast, the SP amplitude remained unchanged.

Spontaneous APs

In addition to the effects of 8bcGMP on pheromone responses, 8bcGMP also had an effect on the waveform of spontaneous APs of unstimulated sensilla trichoidea of isolated male moths that had never experienced pheromone. Changes in the waveform of both AP classes were observed after 6 of 8 haemolymph injections of 30–500 nmol 8bcGMP for several hours. In one recording (Fig. 9) the time course of the AP waveform could be monitored almost continuously for 6 h after the injection at the base of the antenna. The waveforms of both AP classes were influenced independently, suggesting that the 8bcGMP effect took place within the individual ORNs (N=6). After injection of 8bcGMP, two aspects of the waveform were altered: the peak-to-peak amplitude increased and the negative phase of the APs was prolonged. These effects then reverted and reappeared later, suggesting the presence of feedback-coupled mechanisms that were triggered by 8bcGMP injection. A consistent change in the frequency or burst behaviour of the APs was not observed. In recordings of stimulated olfactory neurons no effects of 8bcGMP on the waveform of spontaneous APs between the stimuli were found (data not shown).

Discussion

Using long-term tip recordings from pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*, we tested whether cGMP affects responses to stimulation with the main pheromone component BAL in a daytime-dependent manner. Perfusion of the sensillum lymph with 10 mmol l^{-1} of the membrane-permeable cGMP analogue 8bcGMP reduced the AP frequency of ORNs in response to BAL stimulation in a daytime-dependent manner. The reduction was 12% stronger at the middle of the day (ZT 8–11) than in recordings starting at ZT 1. Also, in control recordings daytime-dependent differences in the AP distribution of the pheromone responses were observed. Pheromone responses at ZT 8–11 became increasingly tonic and fewer APs were elicited within the first 100 ms of the responses. At ZT 8–11 only, 8bcGMP also strongly decreased the number of APs occurring during the first 100 ms of the response, leading to a more tonic AP response. The 8bcGMP perfusion also attenuated the reduction of the AP amplitude in most of the recordings from ZT 8–11 and in a subpopulation of recordings from ZT 1–4. Furthermore, the injection of 8bcGMP into the haemolymph altered the

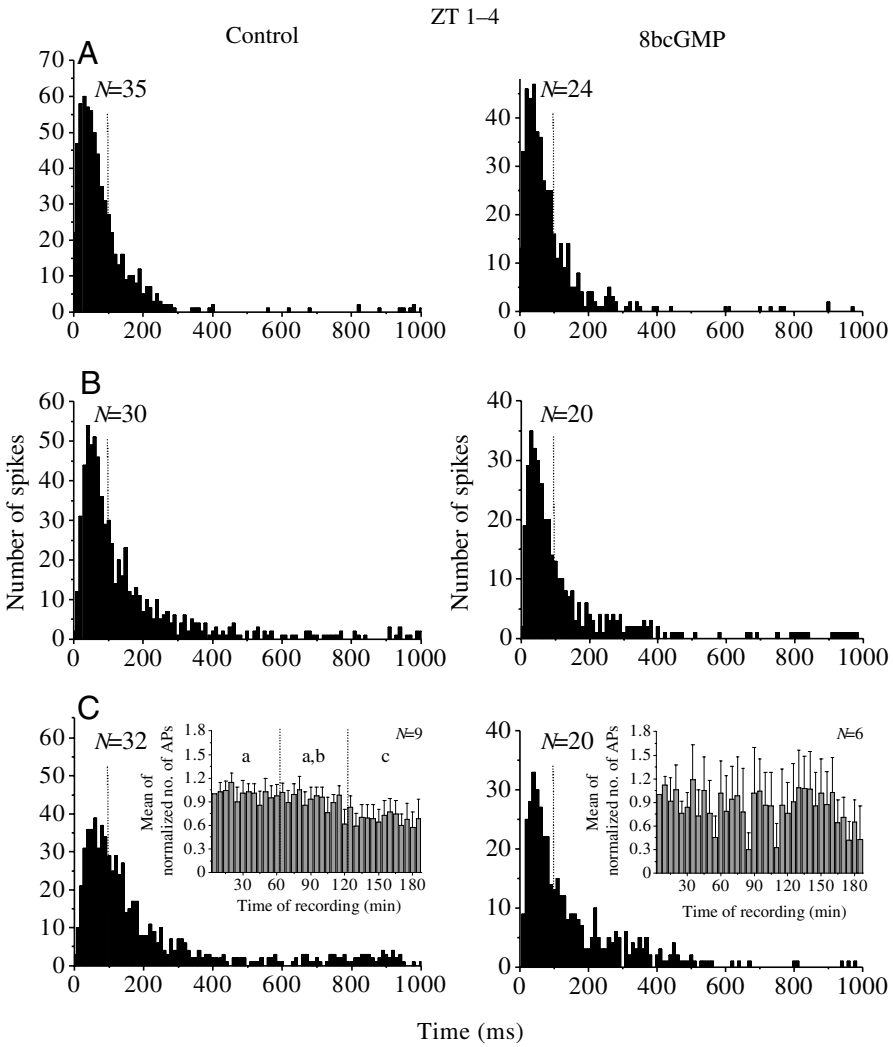

Fig. 6. Action potential (AP) distribution in responses to stimulation with 10 μg bombykal. Post-stimulus-time histograms (binwidth= 10 ms) for recordings from ZT 1–4 for the beginning (A; 0–20 min), middle (B; 80–100 min), and end of the recordings (C; 160–180 min). In the control recordings (left) the numbers of APs in the first part of the phasic response decreased. Also, the number of APs over the first 100 ms of the responses (insert in C) showed a slight but significant decline at the end of the recording duration. Under the influence of 8bcGMP (right) no changes are recognizable. Values in inserts are means + s.e.m. Since the sample sizes differed for the different time windows all y-axes were scaled to $y=n\times2$ (dotted line=100 ms after the onset of the sensillar potential). Different lower case letters denote significant differences between tested groups of means (separated by dotted lines in the insert) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, P<0.01).

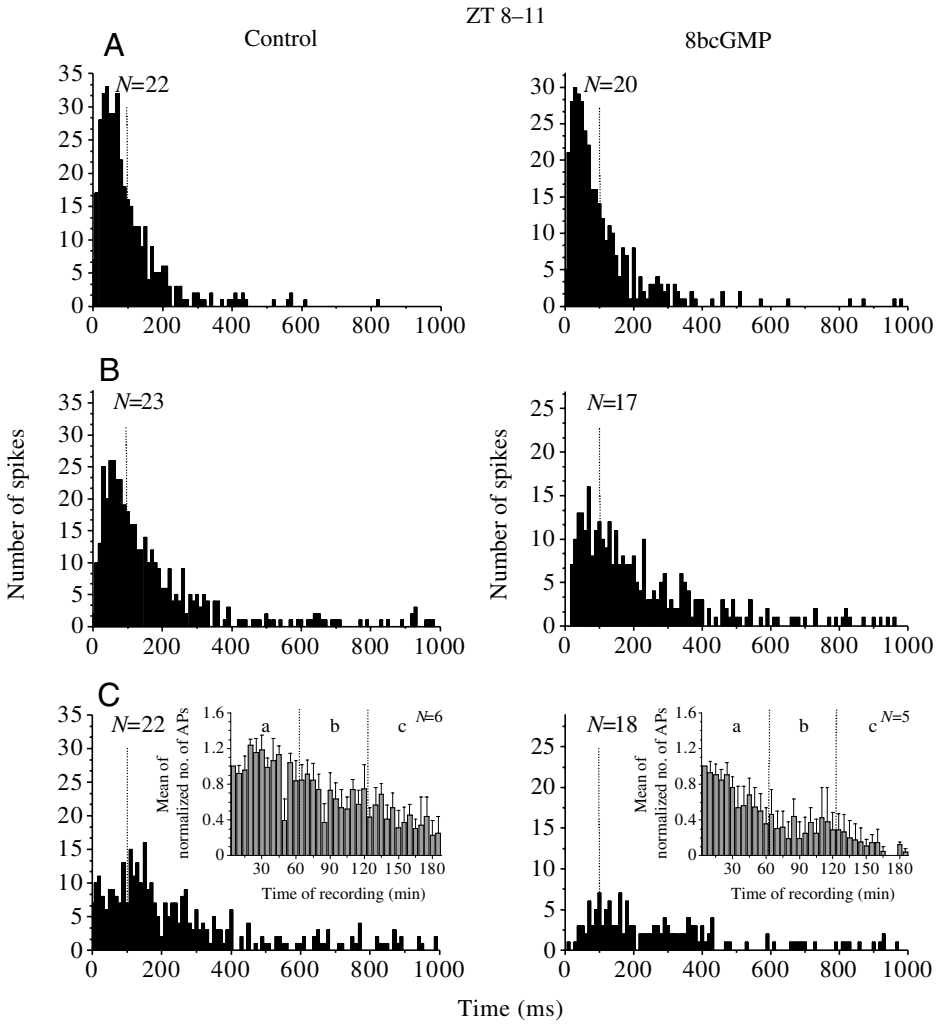

Fig. 7. Action potential (AP) distribution in responses to stimulation with 10 μg bombykal. Post-stimulus-time histograms (binwidth=10 ms) for recordings from ZT 8–11 for the beginning (A; 0–20 min), middle (B; 80–100 min) and end of the recordings (C; 160–180 min). Both in the controls (left) as well as under the influence of 8bcGMP (right) the first phasic part of the AP response declined, leading to a more tonic response. Also, the numbers of APs over the first 100 ms (insert in C) showed a significant decrease both with 8bcGMP and in the controls. This decline was stronger in the presence of 8bcGMP. Since the sample sizes differed for the different time windows all *y*-axes were scaled to $y=n\times1.6$ (dotted line=100 ms after the onset of the sensillar potential). Different lower case letters in the inserts denote significant differences between tested groups of means, which are separated by dotted lines (ANOVA and Tukey HSD post-hoc-test; $\alpha=0.01$, $P<0.01$).

waveform of spontaneous APs of unstimulated ORNs. The sensillar potential in contrast remained unaffected at all ZTs.

The role of cGMP in insect olfactory adaptation

It was known from extracellular tip recordings from different moth species that strong, long or high frequency pheromone stimuli can cause desensitization, short- or long-term adaptation to pheromone responses depending on stimulus strength and time course (Zack, 1979; Dolzer et al., 2003; Marion-Poll and Tobin, 1992). In *Manduca sexta* it was shown that, depending on BAL stimulus, length and strength parameters of the sensillar potential and AP response adapt differentially (Dolzer et al., 2003). After adapting to pheromone stimuli, the time of rise of the sensillar potential did not increase after short-, only after long-adapting stimuli, whereas both stimulation schemes shifted the dose–response curves of the sensillar potential amplitude, as well as the initial slope of its rising phase, to higher stimulus concentrations. The shift in the dose–response curve of the AP response was larger compared to the shift in dose–response curve of the sensillar potential response, indicating that depending on the properties of the stimulus unknown mechanisms of adaptation occur at different levels of the transduction process (Dolzer et al., 2003).

Biochemical experiments implicated that rises in cGMP concentration are involved in at least one mechanism of long-term adaptation, because strong and long pheromone stimuli caused delayed and sustained rises in intracellular cGMP concentrations in antennal homogenates of *Antherea polyphemus* and *Bombyx mori*. These increased levels of cGMP concentration matched the time courses of long-term adaptations (Ziegelberger et al., 1990; Boekhoff et al., 1993). In immunocytochemical experiments and *in situ* hybridizations it could be shown that at least subpopulations of pheromone-sensitive ORNs of male *Manduca sexta* upregulate cGMP concentrations after minute-long (but not seconds-long) exposure to female pheromones (Stengl et al., 2001). These cGMP rises were augmented *via* exposure to NO donors in the presence of a pheromone-inducible NO-synthase-like enzyme in ORNs, but not *via* stimulation of a soluble guanylyl cyclase (Stengl and Zintl, 1996; Stengl et al., 2001). Thus, adapting, long and strong pheromone stimuli, as occur in close vicinity to the calling female, cause long-lasting rises in cGMP concentrations. But it remained unknown which antennal

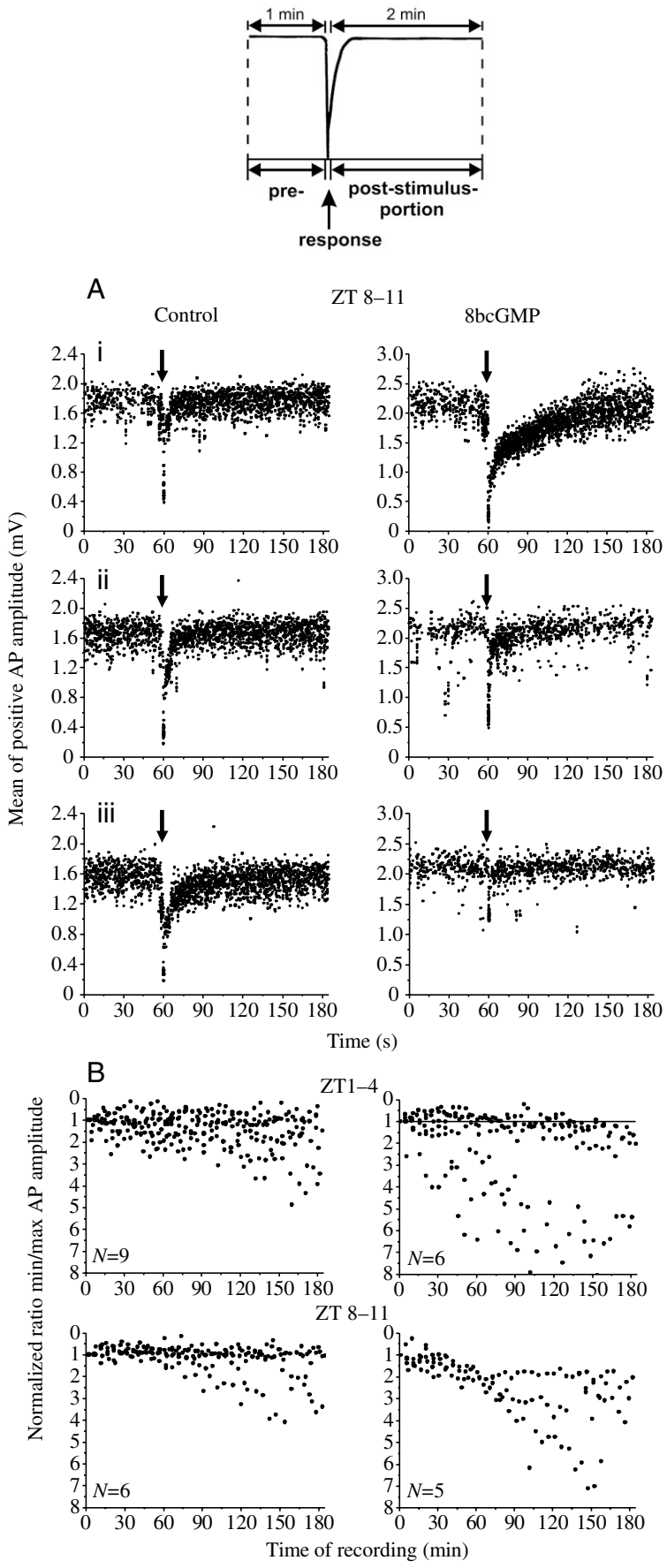

Fig. 8. Analysis of the amplitude reduction of action potentials (APs). (A) The 8bcGMP-dependent AP amplitude reduction for the beginning (i), the middle (ii) and the end (iii) of one recording at ZT 8–11. Plots show the mean of the positive amplitude of APs that occurred during an interval of 1 min before to 2 min after the stimulation. Arrows indicate the time of stimulation. Each plot consists of the binned (binwidth=10 ms) and averaged AP amplitudes of three consecutive responses to a stimulus of 10 μg BAL. For the first three responses of the recording the AP amplitudes were strongly reduced. The APs returned to its pre-stimulus amplitude not until about 2 min after the stimuli were applied. After about 90 min the slow portion of the recovering phase disappeared. Also, the amplitude reduction showed a slight decrease. At the end of the recording a strong 8bcGMP-dependent decrease in the amplitude reduction was observed. The amplitude reduction was only weak and transient. In the control recordings no or only weak fluctuations in the reduction of positive APs were found. In contrast to the recording with 8bcGMP the slowly recovering phase is even more prominent at the end of the recording. (Due to the non-linear change of the BAL-AP amplitude in the post-stimulus portion BAL- and non-BAL APs could not be distinguished). (B) Normalized ratio between the minimal and maximal positive AP amplitude of a response as a mean of the strength of the amplitude reduction. Each recording was normalized to the first value. Values >1 represent a decrease in the reduction of the AP amplitude. In recordings from ZT 1–4 with 10 mmol l^{-1} 8bcGMP diluted in the sensillum lymph ringer (top right), two populations of recordings can be observed. One population showed a very strong increase from the beginning on, the other one resembled the time course of the reduction in control recordings. For control recordings from ZT 1–4 (top left), most of the data points showed a cumulative composition at the beginning followed by an increasing variance leading to a continuous broadening in the distribution later in the recordings. Under the influence of 8bcGMP all of the recordings from ZT 8–11 (bottom right) showed an increase, whereas in the associated controls (bottom left) most data points were located in a relatively distinct band around 1.

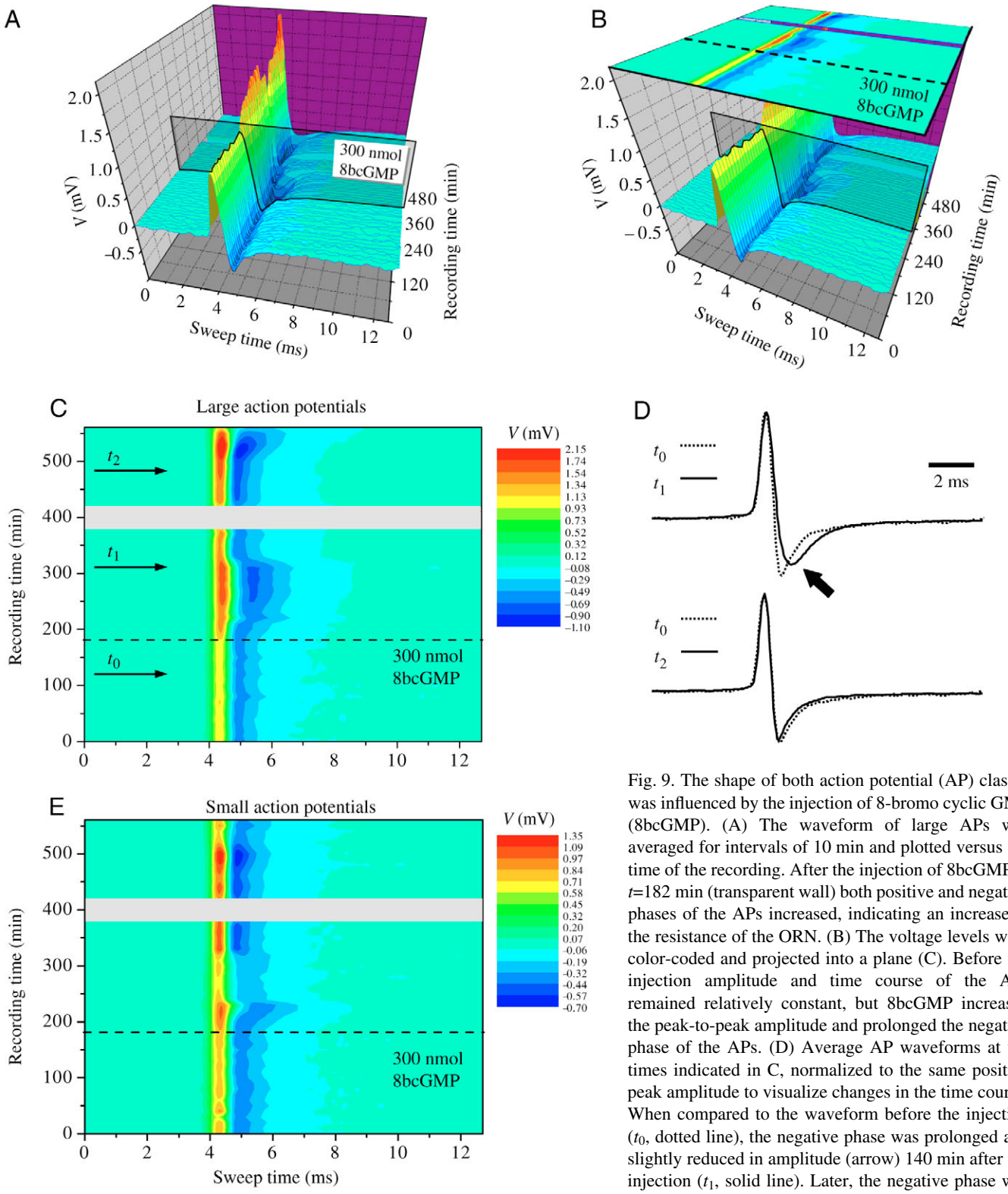

Fig. 9. The shape of both action potential (AP) classes was influenced by the injection of 8-bromo cyclic GMP (8bcGMP). (A) The waveform of large APs was averaged for intervals of 10 min and plotted versus the time of the recording. After the injection of 8bcGMP at t=182 min (transparent wall) both positive and negative phases of the APs increased, indicating an increase in the resistance of the ORN. (B) The voltage levels were color-coded and projected into a plane (C). Before the injection amplitude and time course of the APs remained relatively constant, but 8bcGMP increased the peak-to-peak amplitude and prolonged the negative phase of the APs. (D) Average AP waveforms at the times indicated in C, normalized to the same positive peak amplitude to visualize changes in the time course. When compared to the waveform before the injection (t_0, dotted line), the negative phase was prolonged and slightly reduced in amplitude (arrow) 140 min after the injection (t_1, solid line). Later, the negative phase was shortened again, and 300 min after the injection the time course of the averaged and normalized waveform (t_2, solid line) was identical to the pre-injection waveform (t_0, dotted line). (E) Changes in the waveform of the small APs also showed increases of the peak-to-peak amplitude and a prolongation of the negative phase, but over a different time course. The prolongation of the negative phase reversed after 50 min, while with the large APs the reversal occurred after 150 min. The peak-to-peak amplitude of the small APs was transiently reduced back to the pre-injection level between 70 and 120 min after the injection, while the large APs reached their highest peak-to-peak amplitude at the same time. During the gap in the data (gray areas in C and E), small and large APs could not be distinguished.

targets are affected *via* cGMP and whether rises in cGMP concentration underlie at least one mechanism of long-term olfactory adaptation.

In the present study we have shown in tip recordings of trichoid sensilla of the hawkmoth *Manduca sexta* that brief but strong pheromone stimuli applied in a non-adapting protocol with interstimulus intervals of 5 min cause no changes in the pheromone response, either in the sensillar potential or in the AP response. Only during long-term exposure to the cGMP analogue 8bcGMP was the AP frequency decreased over the 3 h of the recordings; the sensillar potential response remained unchanged. This selective decrease in the AP frequency distinguishes one of the different forms of adaptation in *Manduca sexta* for the first time. Interestingly, injection of dibutyryl guanosine 3′,5′-cyclic monophosphate into the antennae of *Bombyx mori* decreased the AP frequency as well as the sensillar potential amplitude (Redkozubov, 2000). The recording methods and the cGMP analogues employed differed between both moth species, as well as the stimulation scheme. While only very brief strong pheromone stimuli were applied in our recordings, Redkozubov employed either 10 times longer and stronger pheromone stimuli, or presented continuous pheromone stimuli of a lower dose. Most likely, these differing pheromone stimulation protocols were responsible for the differences observed.

The decrease in the AP frequency, the decrease in the number of APs in the first 100 ms of the response, and the observed cGMP-dependent slowdown of the negative phase of spontaneous APs, could all be explained by a closure of K^+-channels. Also, the increase in the peak-to-peak amplitude of spontaneous APs could be explained by the closure of K^+-channels resulting in an increased resistance. This increase in the resistance of the preparation could also be responsible for the 8bcGMP-dependent attenuation of the AP amplitude reduction. In *Manduca sexta*, a pheromone-activated, cGMP-blockable K^+-channel was described without its function being understood (Zufall et al., 1991; Stengl et al., 1992). Based on our findings and on FURA-measurements with cultured ORNs from *Manduca sexta* (M.S., unpublished observations), we assume that strong or long pheromone stimulation resulted in long-term rises of intracellular Ca^{2+} levels. These Ca^{2+} elevations then appeared to cause rises of intracellular cGMP levels, which then close the fast BAL-dependent cGMP-dependent K^+-channel. Whether other cGMP-gated ion channels that were described in patch clamp recordings in *Manduca sexta* (Dolzer, 2002) are also involved in the observed adaptations of the action potential response remains to be shown.

Daytime-dependent differences in the cGMP effects

In addition to daytime-dependent differences in the control recordings, several daytime-dependent effects of cGMP were found. 8bcGMP-dependent distribution of APs became more tonic and the number of APs during the first 100 ms of the BAL response only decreased at ZT 8–11. Furthermore, the 8bcGMP-dependent decrease of the AP frequency at ZT 8–11 was 12% stronger than in recordings at ZT 1–4. Also, the 8bcGMP-dependent attenuation of the reduction of the positive AP amplitude was more prominent at ZT 8–11. In addition to daytime-dependent differences in the effects of 8bcGMP, a different distribution in the timing of APs in control recordings between both ZTs were observed. In recordings from ZT 8–11 the pheromone-dependent AP response became less phasic and more tonic, and also fewer numbers of APs were elicited during the first 100 ms of the BAL response, than at ZT 1–4. Thus, the main differences between the responses of the recordings at ZT 1–4 and ZT 8–11 are the distribution and numbers of APs elicited in the phasic portion of the response. An ORN with more tonic responses would very likely be less able to resolve pheromone filaments of high frequencies.

The effects of the plume structure on behavioural and flight responses of male *Cadra cautella* to pheromone were investigated in wind tunnel experiments (Mafra-Neto and Cardé, 1995a; Mafra-Neto and Cardé, 1995b). Faster frequencies of pulses were followed by a higher percentage of males responding by shorter latencies and less time spent in the search behaviour. Because the variation of the fine structure of the plume had more influence on the flight pattern of males than a 1000-fold increase in the pheromone dosage, the temporal resolution of ORNs appears to be very crucial. In addition, it was shown that the antennal lobe network is strongly modulated by the temporal pattern of the stimulus (Christensen et al., 1998a; Christensen et al., 1998b). The antennal lobe is tuned to fast temporal discrimination of pheromone pulses, which appears to be necessary for odor blend discrimination (Christensen and Hildebrand, 1997; Stopfer et al., 1997). Whether the decrease of the numbers of APs within the first 100 ms represents a further adaptation mechanism that decreases the ability of the ORNs to encode pulsed pheromone signals and therefore blend discrimination remains to be shown in future investigations.

Our results suggest for the first time that there might be daytime-dependent differences in the sensitivity of moth ORNs to pheromone. Whether the observed differences are controlled by an endogenous circadian clock that regulates the sensitivity of ORNs remains to be studied. So far, it has been assumed that daytime-dependent rhythms in male responsiveness to pheromone occur at the level of the antennal lobe and midbrain (Payne et al., 1969; Worster and Seabrook, 1988; Rosén et al., 2003). In addition, investigations of circadian sensitivity changes to food odours in *Leucophaea maderae* indicated that these rhythms were driven by a circadian pacemaker in the optic lobes but not in the ORNs (Page and Koelling, 2003), while circadian changes in the electroantennogram (EAG) in *Drosophila melanogaster* suggested that they depend on PER-dependent endogenous circadian pacemakers within the antenna (Krishnan et al., 1999; Tanoue et al., 2004; Zhou et al., 2005). The main difference between our experiments and the studies mentioned is that we performed tip- instead of EAG-recordings, which allowed us to analyse the AP response of a single ORN with high temporal and spatial resolution. The shift from a phasic-tonic to a tonic response pattern as an effect of 8bcGMP application and circadian differences has never been

shown before because it cannot be resolved by the EAG-recording technique.

It is still unknown whether *Manduca sexta* males are maximally sensitive to their intermittently pulsed pheromone signals during the late night when the females are calling. Also, it is unresolved whether there is a circadian rhythm that might adapt the ORNs in the photophase when no pheromone is released by the females and when the moths are inactive. To investigate rhythms in the sensitivity of ORNs to pheromone and to challenge the hypothesis that the blend discrimination is affected *via* a cGMP-dependent mechanism, long-term recordings starting at the late scotophase, which comprises the shift from scoto- to photophase, will be performed. In addition, it will be investigated in behavioral studies whether male *Manduca sexta* show rhythmic changes in their responsiveness to pheromone. Furthermore, current biochemical experiments examine whether there are differences in cyclic nucleotide concentrations in the moth antenna at different ZTs depending on differing stimulation schemes (K. Riedinger and M.S., unpublished).

List of abbreviations

AP	action potential
BAL	bombykal
cGMP	cyclic guanosine monophosphate
EAG	electroantennogram
IP_3	inositol triphosphate
ISI	interspike interval
ORN	olfactory receptor neuron
SP	sensillar potential
SPA	SP amplitude
TEP	transepithelial potential
ZT	Zeitgebertime

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References

Baker, T. C. and Cardé, R. T. (1979). Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta* (Busck). *J. Insect Physiol.* **25**, 943-950.

Bell, R. A. and Joachim, F. A. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Ent. Soc. Am.* **69**, 365-373.

Boekhoff, I., Seifert, E., Göggerle, S., Lindemann, M., Krüger, B. W. and Breer, H. (1993). Pheromone-induced second-messenger signaling in insect antennae. *Insect Biochem. Mol. Biol.* **23**, 757-762.

Breer, H., Boekhoff, I. and Tareilus, E. (1990). Rapid kinetics of second messenger formation in olfactory transduction. *Nature* **395**, 65-68.

Christensen, T. A. and Hildebrand, J. G. (1997). Coincident stimulation with pheromone components improves temporal pattern resolution in central olfactory neurons. *J. Neurophysiol.* **77**, 775-781.

Christensen, T. A., Waldrop, B. R. and Hildebrand, J. G. (1998a). GABAergic mechanisms that shape the temporal response to odors in moth olfactory projection neurons. *Ann. NY Acad. Sci.* **855**, 475-481.

Christensen, T. A., Waldrop, B. R. and Hildebrand, J. G. (1998b). Multitasking in the olfactory system: context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *J. Neurosci.* **18**, 5999-6008.

de Kramer, J. J. (1985). The electrical circuitry of an olfactory sensillum in *Antheraea polyphemus*. *J. Neurosci.* **5**, 2484-2493.

de Kramer, J. J., Kaissling, K.-E. and Keil, T. A. (1984). Passive electrical properties of insect olfactory sensilla may produce the biphasic shape of spikes. *Chem. Senses* **8**, 289-295.

Dolzer, J. (2002). Mechanisms of modulation and adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta.* PhD thesis, Philipps-Universität, Marburg, Germany. http://archiv.ub.uni-marburg.de/diss/z2002/0185.

Dolzer, J., Krannich, S., Fischer, K. and Stengl, M. (2001). Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* **204**, 2781-2794.

Dolzer, J., Fischer, K. and Stengl, M. (2003). Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **206**, 1575-1588.

Itagaki, H. and Connor, W. E. (1988). Calling behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. *Ann. Entomol. Soc. Am.* **81**, 798-807.

Kaissling, K.-E. (1995). Single unit and electroantennogram recordings in insect olfactory organs. In *Experimental Cell Biology of Taste and Olfaction* (ed. A. I. Spielmann and J. G. Brand), pp. 361-377. Boca Raton, NY: CRC Press.

Kaissling, K.-E. and Thorson, J. (1980). Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. In *Receptors for Neurotransmitters, Hormones and Pheromones in Insects* (ed. D. B. Satelle, L. M. Hall and J. G. Hildebrand), pp. 261-282. Amsterdam: Elsevier, North-Holland.

Kaissling, K.-E., Zack-Strausfeld, C. and Rumbo, E. R. (1986). Adaptation processes in insect olfactory receptors: their relation to transduction and orientation. *Chem. Senses* **11**, 574.

Kaissling, K.-E., Zack-Strausfeld, C. and Rumbo, E. R. (1987). Adaptation processes in insect olfactory receptors. Mechanisms and behavioral significance. *Ann. NY Acad. Sci.* **510**, 104-112.

Kaissling, K.-E., Hildebrand, J. G. and Tumlinson, J. H. (1989). Pheromone receptor cells in the male moth *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **10**, 273-279.

Kaissling, K.-E., Keil, T. A. and Williams, J. L. D. (1991). Pheromone stimulation in perfused sensory hairs of the moth *Antheraea polyphemus*. *J. Insect Physiol.* **37**, 71-78.

Kalinová, B., Hoskovec, M., Liblikas, I., Unelius, C. R. and Hansson, B. S. (2001). Detection of sex pheromone components in *Manduca sexta* (L.). *Chem. Senses* **26**, 1175-1186.

Keil, T. A. (1989). Fine structure of the pheromone-sensitive sensilla on the antenna of the hawkmoth, *Manduca sexta*. *Tissue Cell* **21**, 139-151.

Krishnan, B., Dryer, S. E. and Hardin, P. E. (1999). Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature* **400**, 375-378.

Lee, J. K. and Strausfeld, N. J. (1990). Structure, distribution, and number of surface sensilla and their receptor cells on the antennal flagellum of the male sphinx moth *Manduca sexta*. *J. Neurocytol.* **19**, 519-538.

Linn, C. E., Campbell, M. G., Poole, K. R., Wu, W.-Q. and Roelofs, W. L. (1996). Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*. *J. Insect Physiol.* **42**, 881-891.

Mafra-Neto, A. and Cardé, R. T. (1995a). Influence of plume structure and pheromone concentration on upwind flight of *Cadra cautella* males. *Physiol. Entomol.* **20**, 117-133.

Mafra-Neto, A. and Cardé, R. T. (1995b). Effect of the fine-scale structure of pheromone plumes: pulse frequency modulates activation and upwind flight of almond moth males. *Physiol. Entomol.* **20**, 229-242.

Marion-Poll, F. and Tobin, T. R. (1992). Temporal coding of pheromone pulses and trains in *Manduca sexta*. *J. Comp. Physiol A* **171**, 505-512.

Page, T. L. and Koelling, E. (2003). Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. *J. Insect Physiol.* **49**, 697-707.

Payne, T. L., Shorey, H. H. and Gaston, L. K. (1969). Sex pheromones of noctuid moths: Factors influencing antennal responsiveness in males of *Trichoplusia ni. J. Insect Physiol.* **16**, 1043-1055.

Redkozubov, A. (2000). Guanosine 3′,5′-cyclic monophosphate reduces the response of the Moth's olfactory receptor neuron to pheromone. *Chem. Senses* **25**, 381-385.

Rosén, W. Q. (2002). Endogenous control of circadian rhythms of pheromone production in the turnip moth *Agrotis segetum*. *Arch. Insect Biochem. Physiol.* **50**, 21-30.

Rosén, W. Q., Han, G. B. and Löfstedt, C. (2003). The circadian rhythm of the sex-pheromone-mediated behavioral response in the turnip moth, *Agrotis segetum*, is not controlled at the peripheral level. *J. Biol. Rhythms* **18**, 402-408.

Sanes, J. R. and Hildebrand, J. G. (1976). Origin and morphogenesis of sensory neurons in an insect antenna. *Dev. Biol.* **51**, 300-319.

Sasaki, M. and Riddiford, L. M. (1984). Regulation of reproductive behaviour and egg maturation in the tobacco hawk moth, *Manduca sexta*. *Physiol. Entomol.* **9**, 315-327.

Silvegren, G., Löfstedt, C. and Rosén, W. Q. (2005). Circadian mating activity and effect of pheromone pre-exposure on pheromone response rhythms in the moth *Spodoptera littoralis*. *J. Insect Physiol.* **51**, 277-286.

Starratt, A. N., Dahm, K. H., Allen, N., Hildebrand, J. G., Payne, T. L. and Röller, H. (1979). Bombykal, a sex pheromone of the sphinx moth *Manduca sexta*. *Z. Naturforsch.* **34**, 9-12.

Stengl, M. (1993). Intracellular-messenger-mediated cation channels in cultured olfactory receptor neurons. *J. Exp. Biol.* **178**, 125-147.

Stengl, M. (1994). Inositol-trisphosphate-dependent calcium currents precede cation currents in insect olfactory receptor neurons in vitro. *J. Comp. Physiol. A* **174**, 187-194.

Stengl, M. and Zintl, R. (1996). NADPH diaphorase activity in the antennae of the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **199**, 1063-1072.

Stengl, M., Zufall, F., Hatt, H. and Hildebrand, J. G. (1992). Olfactory receptor neurons from antennae of developing male *Manduca sexta* respond to components of the species-specific sex pheromone *in vitro*. *J. Neurosci.* **12**, 2523-2531.

Stengl, M., Ziegelberger, G., Boekhoff, I. and Krieger, J. (1998). Perireceptor events and transduction mechanisms in insect olfaction. In *Insect Olfaction* (ed. B. S. Hansson), pp. 49-66. Berlin, New York, Heidelberg: Springer.

Stengl, M., Zintl, R., de Vente, J. and Nighorn, A. (2001). Localization of cGMP immunoreactivity and of soluble guanylyl cyclase in antennal sensilla of the hawkmoth *Manduca sexta*. *Cell Tissue Res.* **304**, 409-421.

Stopfer, M., Bhagavan, S., Smith, B. H. and Laurent, G. (1997). Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature* **390**, 70-74.

Tanoue, S., Krishnan, T., Krishnan, B., Dryer, S. E. and Hardin, P. E. (2004). Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. *Curr. Biol.* **14**, 638-649.

Tumlinson, J. H., Brennan, M. M., Doolittle, R. E., Mitchell, E. R., Brabham, A., Mazomemos, B. E., Baumhover, A. H. and Jackson, D. M. (1989). Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. *Arch. Insect Biochem. Physiol.* **10**, 255-271.

Worster, A. S. and Seabrook, W. D. (1988). Electrophysiological investigation of diel variations in the antennal sensitivity of the male spruce budworm moth *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *J. Insect Physiol.* **35**, 1-5.

Zack, C. (1979). Sensory adaptation in the sex pheromone receptor cells of saturniid moths. PhD thesis, Ludwig-Maximilians-Universität, München, Germany.

Zhou, X., Yuan, C. and Guo, A. (2005). *Drosophila* olfactory response rhythms require clock genes but not pigment dispersing factor or lateral neurons. *J. Biol. Rhythms* **20**, 237-244.

Ziegelberger, G., van den Berg, M. J., Kaissling, K.-E., Klumpp, S. and Schultz, J. E. (1990). Cyclic GMP levels and guanylate cyclase activity in pheromone-sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *J. Neurosci.* **10**, 1217-1225.

Zufall, F. and Hatt, H. (1991). Dual activation of a sex pheromone-dependent ion channel from insect olfactory dendrites by protein kinase C activators and cyclic GMP. *Proc. Natl. Acad. Sci. USA* **88**, 8520-8524.

Zufall, F., Stengl, M., Franke, C., Hildebrand, J. G. and Hatt, H. (1991). Ionic currents of cultured olfactory receptor neurons from antennae of male *Manduca sexta*. *J. Neurosci.* **11**, 956-965.