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Perfusion with cAMP analogue affects pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner

Christian Flecke[1,2], Andreas Nolte[2] and Monika Stengl[2,*]

[1]Biologie, Tierphysiologie, Philipps-Universität Marburg, 35032 Marburg, Germany and [2]Biologie, Tierphysiologie, Universität Kassel, 34132 Kassel, Germany

*Author for correspondence (stengl@uni-kassel.de)

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SUMMARY

Octopamine causes time-dependent disadaptation of pheromone-sensitive olfactory receptor neurons (ORNs) of *Manduca sexta*. Because the majority of insect octopamine receptors are positively coupled to adenylyl cyclases we examined whether cyclic adenosine monophosphate (cAMP) mimics octopamine-dependent modulation of pheromone transduction in a time-dependent manner. Long-term tip recordings of single trichoid sensilla of *Manduca sexta* were performed during three zeitgeber times (ZTs, ZT 0=lights on), while stimulating the sensilla with two doses of the main pheromone component bombykal in a non-adapting protocol. The membrane-permeable cAMP analogue 8bcAMP increased the normalized sensillar potential amplitude in a time- and bombykal dose-dependent way. At the higher bombykal dose only, the applied 8bcAMP antagonized an endogenous decrease in the mean sensillar potential amplitude at ZT 1–4 and ZT 8–11 when ORNs were adapted but not at ZT 22–1, when ORNs were sensitized. In contrast to octopamine, 8bcAMP did not consistently affect the initial pheromone-dependent action potential frequency, the phasic/tonic response pattern, or the time-dependent shift to lower mean action potential frequencies at ZT 8–11. Furthermore, 8bcAMP increased the spontaneous action potential frequency time dependently, but differently from octopamine. In conclusion, our results show that cAMP only partly mimics the octopamine-dependent disadaptation of olfactory receptor neurons during photophase, apparently due to another missing octopamine-dependent synergistic factor such as defined intracellular calcium levels.

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Key words: insect olfaction, pheromone transduction, cyclic AMP, octopamine, time-dependent differences.

INTRODUCTION

The second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) is involved in both invertebrate and vertebrate olfaction. Whilst cAMP signalling is best understood in olfactory receptor neurons (ORNs) of vertebrates, less is known about its function in invertebrate olfaction. The stimulation of olfactory receptors in the main olfactory epithelium of vertebrates leads to the activation of G proteins, which then stimulate adenylyl cyclases (Nakamura, 2000). In invertebrate ORNs phospholipase C-dependent transduction cascades were shown to be activated after odour stimulation (Ache and Young, 2005; Boekhoff et al., 1994; Stengl, 1994; Stengl et al., 1999). Only a few studies investigated the role of cAMP in invertebrate olfaction. In lobster ORNs the cAMP pathway leads to hyperpolarization and codes inhibitory responses (Boekhoff et al., 1994; Doolin and Ache, 2005; Hatt and Ache, 1994). In antennae of *Antherea pernyi* perfusion with cAMP increased electroantennogram (EAG) amplitude in responses to stimulation with pheromone (Villet, 1978). In addition, overexpression of the cAMP phosphodiesterase encoding *dunce* gene in olfactory organs of *Drosophila melanogaster* affected both the behaviour in Y-maze experiments (Gomez-Diaz et al., 2004) and EAGs recorded from the antennae (Martín et al., 2001). Recently it was shown that heterologously expressed insect olfactory receptors form cAMP-sensitive ligand-gated ion channel complexes, with the highly conserved chaperone protein OR83b (Sato et al., 2008; Wicher et al., 2008). Furthermore, cyclic nucleotide-sensitive ion channels were characterized in patch clamp recordings of cultured ORNs of *Manduca sexta* (Dolzer et al., 2008; Krannich and Stengl, 2008). Preliminary biochemical experiments indicate that cyclic nucleotide concentrations express time-dependent rhythms in insect antennae (W. Peters, T. Schendzielorz and M.S., unpublished).

Several studies have shown that diurnal production and release of pheromones and calling behaviour of female moths are controlled by unknown circadian pacemakers (Baker and Cardé, 1979; Choi et al., 1998; Rosén, 2002). Also, male moths express circadian rhythms in their responsiveness to pheromone with maxima in the scotophase (Linn et al., 1992; Linn et al., 1996; Rosén et al., 2003). For *M. sexta* it was shown that the maxima in calling behaviour of females (Itagaki and Conner, 1988) correlated with maximal male flight activity in the scotophase (Lingren et al., 1977; Sasaki and Riddiford, 1984). Furthermore, ORNs of *M. sexta* adapt at the beginning of the photophase and respond to pheromone stimulation with lower mean action potential (AP) frequencies at the middle of the photophase (Flecke and Stengl, 2009).

The biogenic amine octopamine (OA) was shown to modulate the sensitivity of pheromone-sensitive ORNs and is thought to affect temporal encoding of pheromone pulses time dependently (Flecke and Stengl, 2009). Injections of OA into the haemolymph enhanced the responsiveness of male moths to pheromone in behavioural experiments (Linn and Roelofs, 1986; Linn and Roelofs, 1992; Linn et al., 1992) and sensitized ORNs of different moth species during tip recordings of pheromone-sensitive trichoid sensilla (Grosmaitre et al., 2001; Pophof, 2000; Pophof, 2002). Furthermore, the OA-

dependent disadaptation is time dependent suggesting a circadian control of OA action (Flecke and Stengl, 2009). Consistent with this assumption is the finding that circadian changes of OA concentration in the haemolymph and brain correlate with circadian rhythms in mating behaviour (Linn et al., 1994; Linn et al., 1996; Lehman, 1990). Since most OA receptors are positively coupled to adenylyl cyclases (Evans and Maqueira, 2005; Farooqui, 2007), it is likely that the OA effects are mediated by increases in the cAMP concentration.

To determine whether cAMP indeed mimics OA actions in *M. sexta* in a time- and pheromone dose-dependent way, long-term tip recordings were performed during three different zeitgeber times (ZTs: ZT 22–1, ZT 1–4 and ZT 8–11) at two pheromone doses. The membrane-permeable cAMP analogue 8-bromo 3′,5′-cyclic adenosine monophosphate (8bcAMP) was applied *via* slow diffusion over the tip recording electrode, which was slipped over the cut tip of a trichoid sensillum. The trichoid sensilla were stimulated with the main pheromone component bombykal (BAL) in a non-adapting stimulation protocol at two BAL doses (1 or 10 μg BAL, interstimulus interval 5 min). Several time- and dose-dependent effects of 8bcAMP on the sensillar potential (SP) and on the spontaneous AP activity were detected.

MATERIALS AND METHODS

Animals and preparation

In the experiments adult male *M. sexta* (L.) were used from our breeding facilities at the University of Marburg and at the University of Kassel. *Manduca sexta* were raised from eggs and the larvae were fed on an artificial diet (modified from Bell and Joachim, 1976). The animals were kept under long-day photoperiod conditions (L:D 17 h:7 h) at 24–27°C and 40–60% relative humidity. The preparation, recording conditions and digitization have been described previously (Flecke et al., 2006). Since light sources with an emission frequency above 600 nm do not function as a zeitgeber (Pittendrigh et al., 1970) and to avoid light-dependent phase shifts, red light emitting LEDs with a narrow frequency spectrum were used for illumination of the preparation and the set-up during the scotophase (Kingbright LKR 530100, Kingbright Electronics, Issum, Germany). For experiments in the scotophase animals were taken out of the rearing area and handled under red light conditions till the lights were switched on at ZT 0, which is the transition from scotophase to photophase. The experiments were performed from ZT 22–1, ZT 1–4 or ZT 8–11. All experiments comply with the German Animal Protection Law.

Application of 8bcAMP and pheromone stimulation

The second messenger 8bcAMP at a concentration of 10 mmol l^{-1} or 100 μmol l^{-1} was applied by perfusion over the recording electrode as described previously (Flecke et al., 2006). Therefore, altered sensillum lymph Ringer solutions were employed. 8bcAMP perfusion started at the beginning of the recordings. The high 8bcAMP concentration of 10 mmol l^{-1} used in most of the recordings was necessary to obtain significant diffusion during the first hour of the recordings and is equal to the concentration of 8-bromo 3′,5′-cyclic guanosine monophosphate employed in an earlier study (Flecke et al., 2006). In a subset of recordings the biogenic amine OA (1 mmol l^{-1}) was applied in the same way (Flecke and Stengl, 2009). The Ringer solutions were prepared with Hepes (all chemicals from Sigma, Deisenhofen, Germany). The pH of the sensillum lymph and haemolymph Ringer solution was adjusted to 6.5. The osmolarity was adjusted to 475 mosmol l^{-1} for sensillum lymph Ringer solution and to 450 mosmol l^{-1} for the haemolymph Ringer solution using mannitol. Details of the pheromone stimulation have been given previously (Flecke et al., 2006). Stimuli with doses of 10 or 1 μg of synthetic BAL [(*E,Z*)-10,12-hexadecadienal], generously provided by T. Christensen (University of Arizona, Tucson, AZ, USA) and J. Krieger (University of Hohenheim, Stuttgart, Germany), were applied in a non-adapting stimulation protocol (stimulus duration: 50 ms) in intervals of 5 min for 180 min of the recording duration.

Acquisition protocols

In *M. sexta* each sensillum trichodeum houses two ORNs. Only one of them is responsive to the main pheromone component BAL. The BAL-sensitive ORNs generate APs with larger peak-to-peak amplitudes, which allow the discrimination of APs of both ORNs (Dolzer et al., 2001). Different protocols were used for digitization of the signal during recording of the pheromone responses and the spontaneous activity of the ORNs. The BAL responses were recorded in sweeps of approximately 5 s duration at a continuous sampling rate of 20 kHz (Clampex 8, episodic stimulation mode; Molecular Devices, Sunnyvale, CA, USA) and the spontaneous activity between stimulations was continuously recorded with a sampling frequency of 19.6 kHz (Clampex 8, fixed-length events).

Data analysis and statistics

The pheromone responses and the spontaneous activity were evaluated with Clampfit 8 (Molecular Devices, Sunnyvale, CA, USA) and the Microsoft Excel Add-in XtraCell (Dolzer, 2002). All analyses were performed using the direct-current-coupled signal. To evaluate the SP, the responses were low-pass filtered at a cutoff frequency of 50 Hz (Clampfit, Gaussian filter). Afterwards, different parameters of the SP response including the maximal SP amplitude were evaluated as previously described (Flecke et al., 2006). Prior to analysis of the APs the responses were pseudo-high-pass filtered as described before (Flecke et al., 2006). The main parameter AP frequency was calculated from the first five interspike intervals (ISI). Because of the high variability between the recordings the time courses of the two parameters were each normalized to the first value and then binned to 5 min intervals. For statistical comparison of the normalized and binned time courses of 8bcAMP and control recordings at each ZT the Mann–Whitney test or Student's *t*-test was employed, depending on the distribution of the data. To analyse changes in the time course of single parameters the time courses were divided into three or four intervals and analyzed with a one-way ANOVA followed by the Tukey HSD *post-hoc* test or with the Mann–Whitney test. Furthermore, the mean SP amplitudes and mean AP frequencies computed for all responses recorded at each ZT were evaluated. To compare different ZTs of 8bcAMP, OA or control recordings, the complete data set for each ZT was compared with that for other ZTs using the Mann–Whitney test. For evaluation of the distribution of APs in responses post-stimulus time histograms (PSTHs; bin length 10 ms, t=0 is the start of the SP) for all recordings at one ZT were created for three intervals of the recording duration (0–20 min, 80–100 min and 160–180 min of the recording). PSTHs of 8bcAMP and control recordings at different ZTs were compared using the Mann–Whitney test. In addition the normalized mean of APs occurring during the first 100 ms of the responses was evaluated and plotted for the recording duration of 180 min. Subsequently changes in this parameter were statistically analyzed by dividing its time course into three intervals followed by a one-way ANOVA with the Tukey HSD *post-hoc* test or the Mann–Whitney test.

Analysis of spontaneous APs and bursts

For analysis of spontaneous activity, the spontaneous APs recorded between the stimulations were evaluated. APs generated by both ORNs characterized by different peak-to-peak amplitudes were

sorted. Spike sorting of spontaneous APs and calculation of bursts was performed as described before (Dolzer et al., 2001; Flecke and Stengl, 2009). The time courses of the parameters spontaneous AP frequency, percentage of APs in bursts, average APs per burst, number of bursts and number of spikes were normalized to the first value and then binned to 5 min intervals. The data from control recordings were compared with those from 8bcAMP recordings using the Mann–Whitney test or Student's *t*-test. Changes in each time course were analyzed with a one-way ANOVA followed by the Tukey HSD *post-hoc* test or the Mann–Whitney test, depending on the distribution of the data. In addition the mean spontaneous AP frequency, the mean number of bursts per bin and the mean number of spikes per bin calculated from all responses during each ZT were analyzed. These data were not normalized, and represent absolute values. For statistical comparison the whole data set for each ZT was compared with that of other ZTs using the Mann–Whitney test. All statistical calculations were performed with SPSS (version 11; SPSS Inc., Chicago, IL, USA).

RESULTS

Through long-term tip recordings of single pheromone-sensitive trichoid sensilla of the hawkmoth *M. sexta* we examined whether application of the cAMP analogue 8bcAMP mimics OA-dependent modulation of pheromone transduction. To elucidate time-dependent differences in the effects, the experiments were performed at three different ZTs (ZT 22–1, ZT 1–4 and ZT 8–11). During the 3 h recordings the sensillar lymph was perfused with 10 mmol l^{-1} or 100 μmol l^{-1} 8bcAMP and the trichoid sensilla were stimulated with the main pheromone component BAL in a non-adapting stimulation protocol (BAL dose 1 or 10 μg, interstimulus interval 5 min). Different parameters of the SP and AP response were evaluated. A detailed explanation of the parameters analyzed is given elsewhere (Flecke and Stengl, 2009).

Perfusion with 8bcAMP increased the SP amplitude time and BAL dose dependently

In control recordings with 10 μg BAL stimulation the normalized SP amplitude and the normalized initial AP frequency remained stable over the recording duration at ZT 22–1, ZT 1–4 and ZT 8–11 (Fig. 1A,B). In the control recordings with 1 μg BAL the normalized SP amplitude was also not affected at all ZTs (Fig. 2A). However, the normalized AP frequency remained stable at ZT 22–1 and ZT 1–4 and it was slightly but significantly decreased at ZT 8–11 (Fig. 2B) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, P<0.01). In recordings with both 10 and 1 μg BAL stimulation the application of 8bcAMP increased the normalized SP amplitude significantly at ZT 1–4 and ZT 8–11 but not at ZT 22–1 (Fig. 1A, Fig. 2A). The

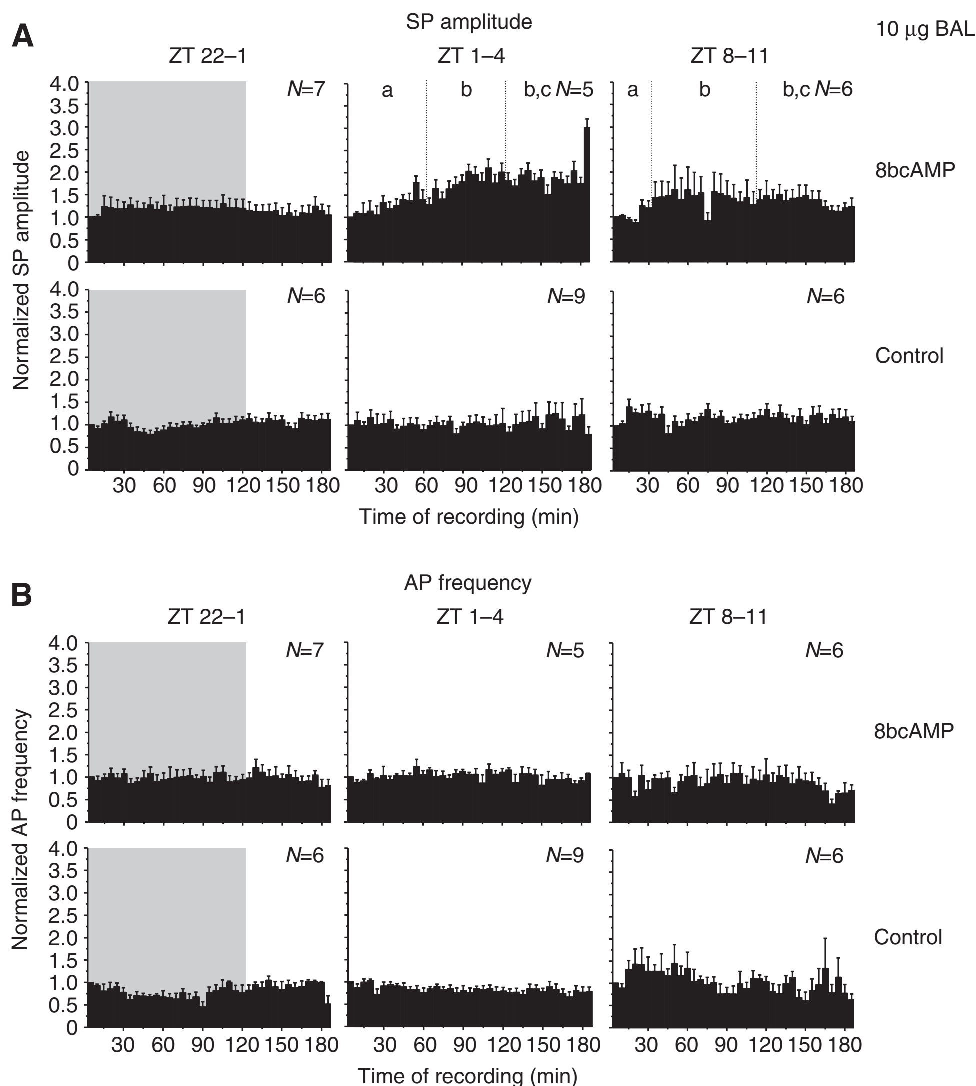

Fig. 1. 8bcAMP increased the SP amplitude in recordings with 10 μg bombykal (BAL) at zeitgeber time (ZT) 1–4 and ZT 8–11. Analysis of the normalized and binned sensillar potential (SP) amplitude (A) and initial action potential (AP) frequency (B) (computed over the first five interstimulus intervals) for recordings with 10 μg BAL stimulation at ZT 22–1, ZT 1–4 and ZT 8–11. Values are means + s.e.m. Perfusion with 10 mmol l^{-1} 8-bromo 3′,5′-cyclic adenosine monophosphate (8bcAMP) increased the normalized SP amplitude significantly at ZT 1–4 and ZT 8–11, when compared with the controls (Student's *t*-test for independent samples, P<0.001 or Mann–Whitney test, P<0.001) and when comparing the intervals within each affected time course (ANOVA and Tukey HSD *post-hoc* test; α=0.001, P<0.001 or Mann–Whitney test, P<0.01). The 8bcAMP-dependent increase at ZT 1–4 was 1.3-fold stronger than at ZT 8–11. Perfusion with 8bcAMP did not affect the normalized initial AP frequency at any ZT. In the control recordings no significant changes in the time courses of the normalized SP amplitude and the normalized AP frequency were observed for any ZT. Same lower case letters denote no significant differences between tested groups of mean values.

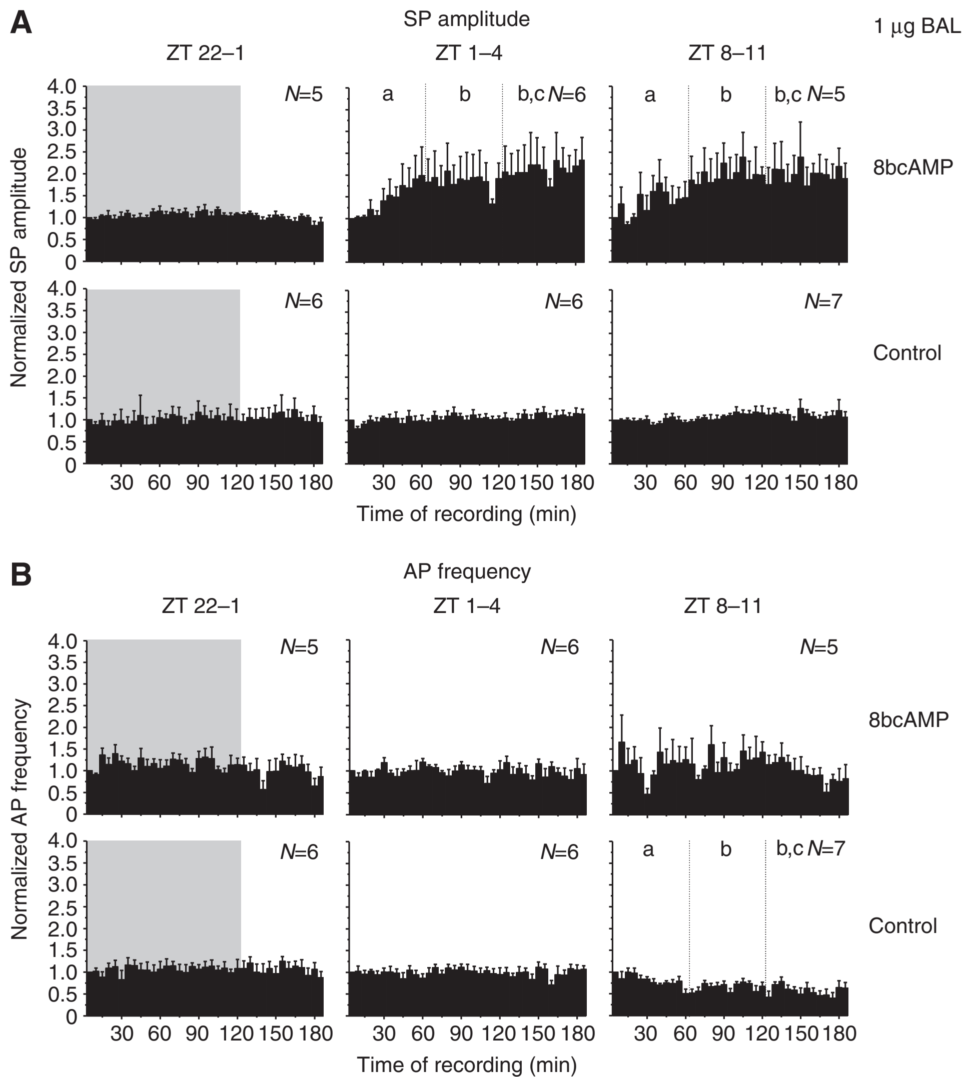

Fig. 2. In recordings with 1 µg BAL, 8bcAMP increased the SP amplitude but only during the photophase. Comparison of the normalized and binned SP amplitude (A) and initial AP frequency (B) for recordings with 1 µg BAL at ZT 22–1, ZT 1–4 and ZT 8–11. Values are means + s.e.m. Perfusion with 10 mmol l^{-1} 8bcAMP increased the normalized SP amplitude with a similar time course and strength at ZT 1–4 and ZT 8–11. The increases were significant in comparison to the controls (Mann–Whitney test, $P<0.001$) and when comparing the intervals within each affected time course (Mann–Whitney test, $P<0.01$). In the control recordings at ZT 22–1, ZT 1–4 and ZT 8–11 no effects on the normalized SP amplitude were observed. In contrast, the normalized AP frequency in the control recordings decreased significantly at ZT 8–11, but not at ZT 22–1 or ZT 1–4 (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). Same lower case letters denote no significant differences between tested groups of mean values.

8bcAMP-dependent increases were significant when compared with the respective controls (Student's *t*-test for independent samples, $P<0.001$ or Mann–Whitney test, $P<0.001$) and in the analysis of differences between the intervals of each affected time course (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.001$, $P<0.001$ or Mann–Whitney test, $P<0.01$). When comparing the effect of 8bcAMP in recordings with stimulation by 10 µg BAL (Fig. 1A), it was observed that the increase of the normalized SP amplitude was 1.3-fold stronger at ZT 1–4 than at ZT 8–11. However, in recordings with 1 µg BAL no significant differences between the 8bcAMP-dependent effects for ZT 1–4 and ZT 8–11 were found (Fig. 2A). The application of 8bcAMP did not affect the normalized initial AP frequency in recordings with either BAL dose and during any ZT (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P>0.01$ or Mann–Whitney test, $P>0.01$) (Fig. 1B, Fig. 2B). However, 8bcAMP counteracted the decrease of the normalized AP frequency found in control recordings with 1 µg BAL stimulation at ZT 8–11 (Fig. 2B) (Student's *t*-test for independent samples, $P<0.001$). Application of a 100-fold lower 8bcAMP concentration (100 µmol l^{-1}) in recordings with 1 µg BAL at ZT 1–4 only weakly, but significantly, increased the normalized SP amplitude (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P>0.01$ and Student's *t*-test for independent samples, $P<0.001$) but not the normalized AP frequency (Fig. 3). The effect of 100 µmol l^{-1} 8bcAMP on the SP amplitude was 0.7-fold weaker than the effect of 10 mmol l^{-1} 8bcAMP. Thus, because of the high variance between the recordings the higher 8bcAMP concentration was employed in most recordings to allow adequate quantitative analysis of the observed effects. No 8bcAMP-dependent effects on the transepithelial potential (TEP) or on the resistance of the preparation were found that correlated with increases in the SP amplitude (data not shown). Moreover no 8bcAMP-dependent effects on the AP amplitude reduction during pheromone responses were observed (data not shown).

To determine the absolute 8bcAMP effects, the non-normalized mean SP amplitude (Fig. 4A, Table 1) and the mean AP frequency (Fig. 4B, Table 2) were calculated from the absolute values of all responses at each ZT. In the control recordings with 10 µg BAL stimulation significant decreases in the mean SP amplitude were observed at ZT 1–4 and ZT 8–11 compared with ZT 22–1 (Fig. 4A) (Mann–Whitney test, $P<0.001$). In contrast, in controls with 1 µg BAL stimulation no significant differences were found between the three ZTs. Perfusion with 8bcAMP in recordings with 10 µg BAL stimulation increased the mean SP amplitude significantly at ZT 1–4 and ZT 8–11 (Fig. 4A) (Mann–Whitney test, $P<0.001$). In contrast, in 8bcAMP recordings employing 1 µg BAL the mean SP amplitude was only increased at ZT 1–4 but decreased at ZT 8–11

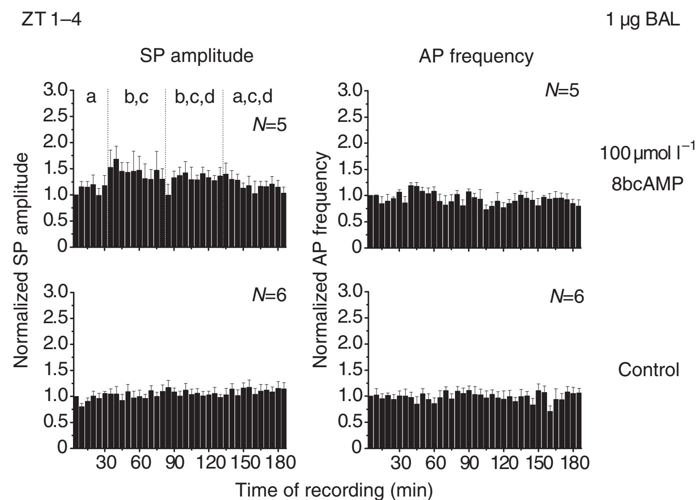

Fig. 3. Perfusion with 100 μmol l^{-1} 8bcAMP in recordings with 1 μg BAL stimulation at ZT 1–4 increased the normalized SP amplitude slightly but significantly (ANOVA and Tukey HSD *post-hoc* test; α=0.01, P<0.01 and Student's *t*-test for independent samples, P<0.001) but had no effect on the normalized AP frequency. Same lower case letters denote no significant differences between tested groups of mean values.

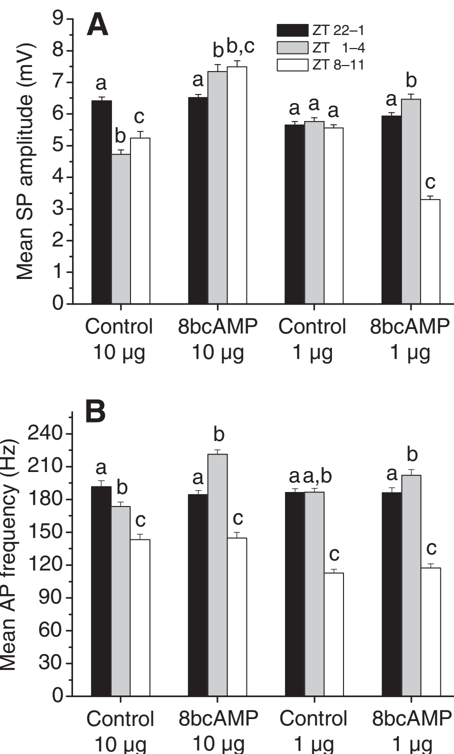

Fig. 4. Time- and pheromone dose-dependent changes for control and 8bcAMP recordings were observed in the non-normalized mean SP amplitude (A) and mean AP frequency (B) calculated from all responses for ZT 22–1, ZT 1–4 and ZT 8–11. Values are means + s.e.m. The mean SP amplitude in control recordings with 10 μg BAL stimulation decreased significantly at ZT 1–4 and ZT 8–11, when compared with that at ZT 22–1 (Mann–Whitney test, P<0.001). In controls employing 1 μg BAL stimulation no differences were found between the three ZTs. The application of 8bcAMP increased the mean SP amplitude in recordings with 10 μg BAL stimulation at ZT 1–4 and ZT 8–11 (Mann–Whitney test, P<0.001). Also, in 8bcAMP recordings with 1 μg BAL stimulation a significant increase in the SP amplitude was found at ZT 1–4; at ZT 8–11, however, a strong decrease in the mean SP amplitude was observed (Mann–Whitney test, P<0.001). As shown previously (Flecke and Stengl, 2009), in controls with both 10 and 1 μg BAL stimulation a significant decrease in the mean AP frequency (B) was found at ZT 8–11 when compared with that at ZT 22–1 (Mann–Whitney test, P<0.001). Also, in the recordings with 8bcAMP application significant decreases in the mean AP frequency were observed at ZT 8–11 for recordings employing 10 and 1 μg BAL (Mann–Whitney test, P<0.001) when compared with the other ZTs. In contrast, 8bcAMP increased the mean AP frequency at ZT 1–4 for recordings with 10 and 1 μg BAL stimulation (Mann–Whitney test, P<0.001). Same lower case letters denote no significant differences between tested groups of mean values.

(Mann–Whitney test, P<0.001). As shown previously (Flecke and Stengl, 2009), in controls with 10 and 1 μg BAL stimulation a significant decrease in the mean AP frequency occurred at ZT 8–11 (Fig. 4B) (Mann–Whitney test, P<0.001). Also, in the presence of 8bcAMP with 10 and 1 μg BAL stimulation a decrease in the mean AP frequency was found at ZT 8–11 (Fig. 4B) (Mann–Whitney test, P<0.001), confirming the lack of 8bcAMP-dependent effects on the AP response observed for the normalized time courses. In addition, in recordings with 10 and 1 μg BAL stimulation 8bcAMP significantly increased the mean AP frequency at ZT 1–4 (Mann–Whitney test, P<0.001). Time-dependent changes in the mean SP amplitude were not always followed by consistent changes in the mean AP frequency, especially at ZT 8–11. In 8bcAMP recordings with 10 μg BAL stimulation, although an increase in the mean SP amplitude was observed at ZT 8–11, the shift to lower mean AP frequencies at ZT 8–11 was not antagonized by this increase. Also, in control recordings with 1 μg BAL stimulation the mean AP frequency was significantly decreased at ZT 8–11 (Fig. 4B) without a consistent decrease in the mean SP amplitude at this ZT (Fig. 4A). The absolute values of the mean SP amplitude and the mean AP frequency measured at ZT 22–1 were in the same range for all 8bcAMP and control recordings of equal BAL dose (Tables 1, 2), confirming similar conditions during the experiments.

Effects on the AP distribution in BAL responses

OA was shown to antagonize an endogenous shift in the response patterns of BAL responses in recordings at ZT 8–11 (Flecke and Stengl, 2009). To investigate whether 8bcAMP also mimics the OA-dependent effects on the AP distribution in BAL responses, PSTHs for the first 1000 ms of the responses were created for the beginning (0–20 min), middle (80–100 min) and end (160–180 min) of the recording duration. As shown previously (Flecke et al., 2006), in control recordings with 10 μg BAL stimulation the responses at ZT 8–11 were shifted from a phasic to a tonic response pattern (Fig. 5A) and the number of APs in the first 100 ms decreased slightly at ZT 1–4 (supplementary material Fig. S1) and strongly at ZT 8–11 (Fig. 5A,B) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, P<0.01). Also, in recordings with 1 μg BAL stimulation similar shifts in the AP distribution and significant decreases in the number of APs during the first 100 ms of the responses were found at ZT 1–4 and ZT 8–11 (supplementary material Fig. S1, Fig. 5A,B) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, P<0.01 or Mann–Whitney test, P<0.01). Application of 8bcAMP did not have a significant effect on the AP distribution either in recordings at ZT 1–4 (supplementary material Fig. S1) or in recordings at ZT 8–11 (Fig. 5A,B). In the control recordings and in the presence of 10 mmol l^{-1} 8bcAMP at ZT 8–11 the 10 μg BAL-dependent AP distribution was shifted from a phasic to a tonic

Table 1. Mean SP amplitude (±s.e.m.) in BAL responses for three ZTs

	10 μg BAL		1 μg BAL	
	Control	8bcAMP	Control	8bcAMP
ZT 22–1	6.39±0.13 (*N*=212)	6.52±0.11 (*N*=246)	5.64±0.11 (*N*=187)	5.92±0.11 (*N*=187)
ZT 1–4	4.72±0.12 (*N*=201)	7.34±0.21 (*N*=132)	5.76±0.11 (*N*=179)	6.47±0.14 (*N*=206)
ZT 8–11	5.24±0.2 (*N*=196)	7.49±0.19 (*N*=207)	5.57±0.1 (*N*=217)	3.28±0.12 (*N*=165)

BAL, bombykal; ZT, zeitgeber time.

response pattern (Fig. 5A) and the number of APs in the first 100 ms of the responses decreased significantly (Fig. 5B) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, *P*<0.01). The PSTHs of 8bcAMP and control recordings with 10 μg BAL stimulation at the end of the recordings did not differ significantly from each other (Fig. 5A) (Mann–Whitney test, *P*>0.05). In addition, no significant differences were found between the time courses of the mean number of APs in the first 100 ms of 8bcAMP and control recordings with 10 μg BAL (Fig. 5B) (Student's *t*-test for independent samples, *P*>0.05). In contrast, in 8bcAMP recordings with 1 μg BAL stimulation at ZT 8–11 no clear shift from phasic to tonic responses was measured (Fig. 5A). Although comparison of the PSTHs of 8bcAMP and control recordings from the end of the recording duration revealed no significant differences (Mann–Whitney test, *P*>0.05), no decrease in the number of APs in the first 100 ms was found in 8bcAMP recordings with 1 μg BAL. Nonetheless, consistently, all PSTHs from the end of the recordings at ZT 8–11 were found to show less phasic AP distributions.

Effects on the spontaneous AP frequency

In addition to the effects on the responses to stimulation with BAL, 8bcAMP also affected the generation of spontaneous APs recorded between the stimulations. In recordings at ZT 8–11 and with 1 μg BAL stimulation, perfusion with 8bcAMP was followed by a fast increase in the normalized spontaneous AP frequency (Fig. 6) (Mann–Whitney test, *P*<0.01). The 8bcAMP-dependent increase in the normalized AP frequency was caused by equal increases in the number of bursts per bin and the number of spikes per bin (Fig. 6) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, *P*<0.01), without affecting the percentage of APs occurring in bursts or the average number of APs per burst. All 8bcAMP-dependent effects were significant in comparison to the controls (Mann–Whitney test, *P*<0.001 or Student's *t*-test for independent samples, *P*<0.001). At both BAL doses [Fig. 6 (Flecke and Stengl, 2009), supplementary material Fig. S2] the normalized spontaneous AP frequency, the number of bursts per bin, and the number of spikes per bin decreased slightly but significantly in the control recordings (Mann–Whitney test, *P*<0.01 or ANOVA and Tukey HSD *post-hoc* test; α=0.01, *P*<0.01). In recordings employing 10 μg BAL no significant 8bcAMP-dependent increase was found for the normalized spontaneous AP frequency (supplementary material Fig. S2). However, the 8bcAMP-dependent, stable time courses of the normalized AP frequency and the number of spikes per bin differed significantly from the parameters of the associated controls (supplementary material Fig. S2) (Mann–Whitney test, *P*<0.01).

To determine whether 8bcAMP affected the absolute (non-normalized) values of the mean spontaneous AP frequency calculated from the whole data set at each ZT, different ZTs of control and 8bcAMP recordings were compared (Fig. 7). In the control recordings at both BAL doses significant and gradual decreases in the mean spontaneous AP frequency occurred at ZT 1–4 and ZT 8–11 (Fig. 7A) (Mann–Whitney test, *P*<0.001). These adaptations were antagonized by 8bcAMP perfusion. In addition, OA counteracted the decrease to a lower mean AP frequency at ZT 1–4 and significantly increased the mean spontaneous AP frequency at ZT 8–11 (Fig. 7A) (Mann–Whitney test, *P*<0.001). In accordance with the shift of the mean AP frequency the mean number of bursts per bin (Fig. 7B) and the mean number of spikes per bin (Fig. 7C) decreased significantly in control recordings with 1 μg BAL at ZT 1–4 and ZT 8–11 (Mann–Whitney test, *P*<0.001). In control recordings with 10 μg BAL the decrease in the mean number of bursts and spikes was only significant at ZT 8–11 (Mann–Whitney test, *P*<0.001). The application of 8bcAMP antagonized shifts to a lower mean number of bursts (Fig. 7B) and a lower mean number of spikes per bin (Fig. 7C) at both BAL doses. Furthermore, OA counteracted the shift to a lower mean number of bursts (Fig. 7B) and a lower mean number of spikes per bin (Fig. 7C) at ZT 1–4 and significantly increased both parameters at ZT 8–11 (Mann–Whitney test, *P*<0.001). The OA-dependent increase in the mean number of bursts was 1.4-fold stronger than the increase in the mean number of spikes. No significant differences between the ZTs were found for the mean average spikes per burst and the percentage of APs in bursts (data not shown).

DISCUSSION

To examine whether cAMP application mimics OA effects, a membrane-permeable cAMP analogue was applied *via* the tip recording electrode during long-term extracellular recordings of pheromone-sensitive trichoid sensilla. Because 8bcAMP increased the SP amplitude in a time-dependent manner without consistently affecting the AP responses 8bcAMP does not mimic all OA effects. Interestingly, differences in the 8bcAMP-dependent modulation at different BAL doses hinted interactions of cAMP with other factors such as intracellular calcium levels.

Table 2. Mean AP frequency (±s.e.m.) in BAL responses for three ZTs

	10 μg BAL		1 μg BAL	
	Control	8bcAMP	Control	8bcAMP
ZT 22–1	191.65±5.54 (*N*=188)	184.35±3.83 (*N*=243)	186.38±3.45 (*N*=225)	186.1±4.47 (*N*=181)
ZT 1–4	173.57±3.87 (*N*=283)	221.34±3.91 (*N*=144)	186.61±3.51 (*N*=216)	202.1±5.24 (*N*=200)
ZT 8–11	143.37±4.68 (*N*=191)	144.85±5.18 (*N*=204)	112.87±3.29 (*N*=251)	117.23±4.12 (*N*=164)

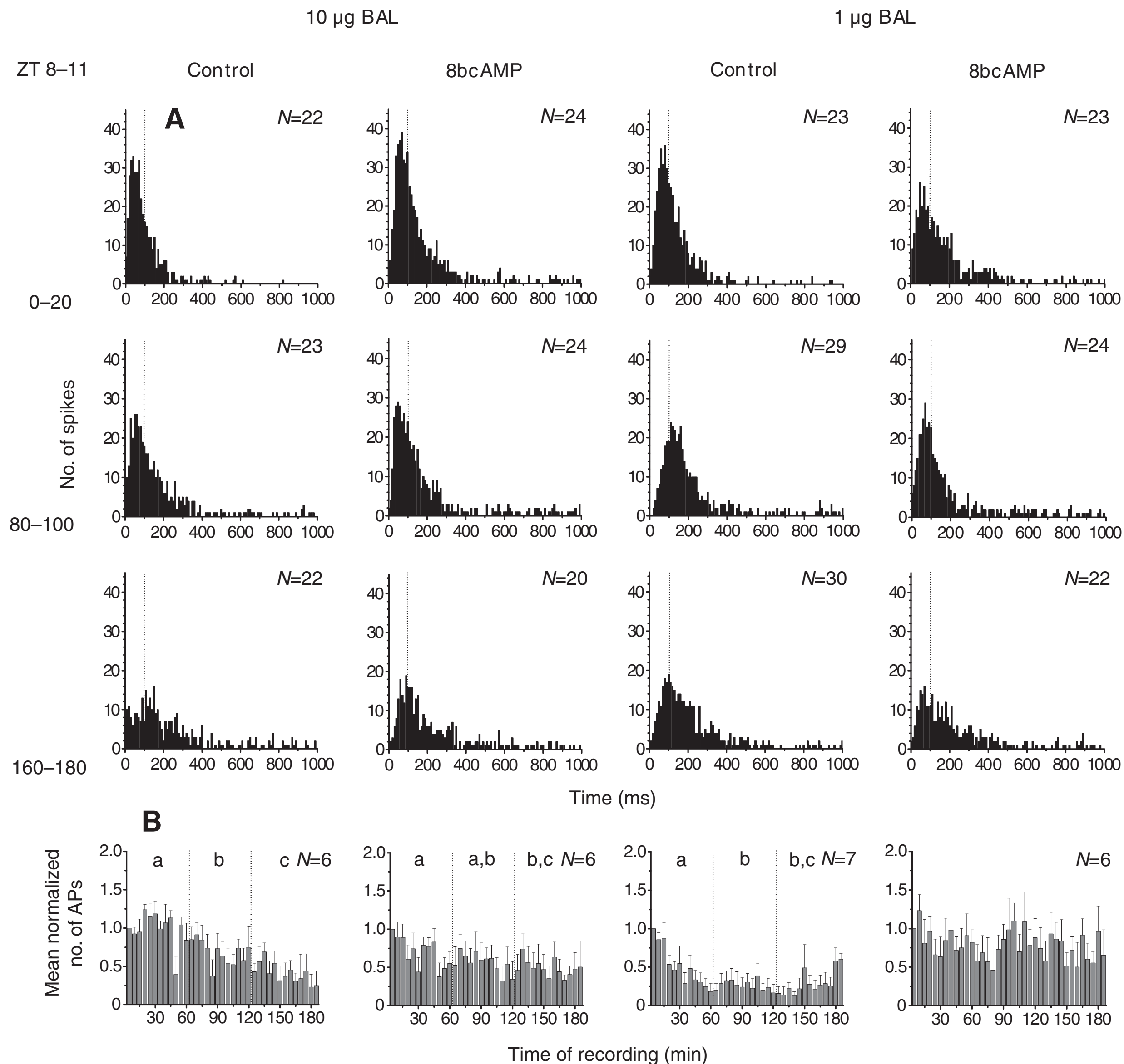

Fig. 5. Perfusion with 8bcAMP only moderately affected the endogenous shift to tonic BAL responses at ZT 8–11. (A) PSTHs from the beginning (0–20 min), middle (80–100 min) and end (160–180 min) of the recording duration for recordings with 10 (left) or 1 μg (right) BAL stimulation at ZT 8–11. In the controls AP responses to both BAL doses showed a strong shift from phasic to tonic response patterns. Also, the normalized mean (+ s.e.m.) number of APs which occurred in the first 100 ms of the responses (B) was significantly decreased (ANOVA and Tukey HSD *post-hoc* test; α=0.01, *P*<0.01 or Mann–Whitney test, *P*<0.01). During 8bcAMP application a comparable and significant shift to tonic responses and a decrease in the number of APs in the first 100 ms was only found in recordings with 10 μg BAL (left) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, *P*<0.01). Although in recordings with 1 μg BAL stimulation the PSTHs of 8bcAMP and control recordings from the end of the recording period were not significantly different (Mann–Whitney test, *P*>0.05), because of the weak responses at the beginning of the 8bcAMP recordings no decrease in the number of APs in the first 100 ms was observed. Same lower case letters denote no significant differences between tested groups of mean values.

8bcAMP affects BAL transduction dose dependently and partly mimics OA effects

We have shown here for the first time that 8bcAMP specifically affects the SP of single pheromone-sensitive trichoid sensilla. Our results are in agreement with a previous study which showed that perfusion with cAMP and its analogues, or with phosphodiesterase inhibitors, increased pheromone-dependent EAG amplitude in antennae of *A. polyphemus* (Villet, 1978). Also, in *D. melanogaster* evidence was found for the cAMP-dependent modulation of insect odour transduction. In *dunce* mutants, which overexpress a cAMP phosphodiesterase, rise-time kinetics in EAG recordings were slowed down (Martín et al., 2001) and the sensitivity to acetone and ethanol in behavioural Y-maze experiments was decreased (Gomez-Diaz et al., 2004). Application of OA *via* the tip recording electrode increased the SP amplitude even more strongly than 8bcAMP but with a similar time course (Flecke and Stengl, 2009). In contrast to 8bcAMP, OA also increased the normalized initial AP frequency and antagonized endogenous shifts to lower mean AP frequencies and to more tonic pheromone responses at ZT 8–11 (Flecke and Stengl, 2009). Thus, the application of 8bcAMP only partly mimics the OA-dependent effects. Because in preliminary tip recording experiments application of 1 $\mathrm{mmol\,l^{-1}}$ serotonin was

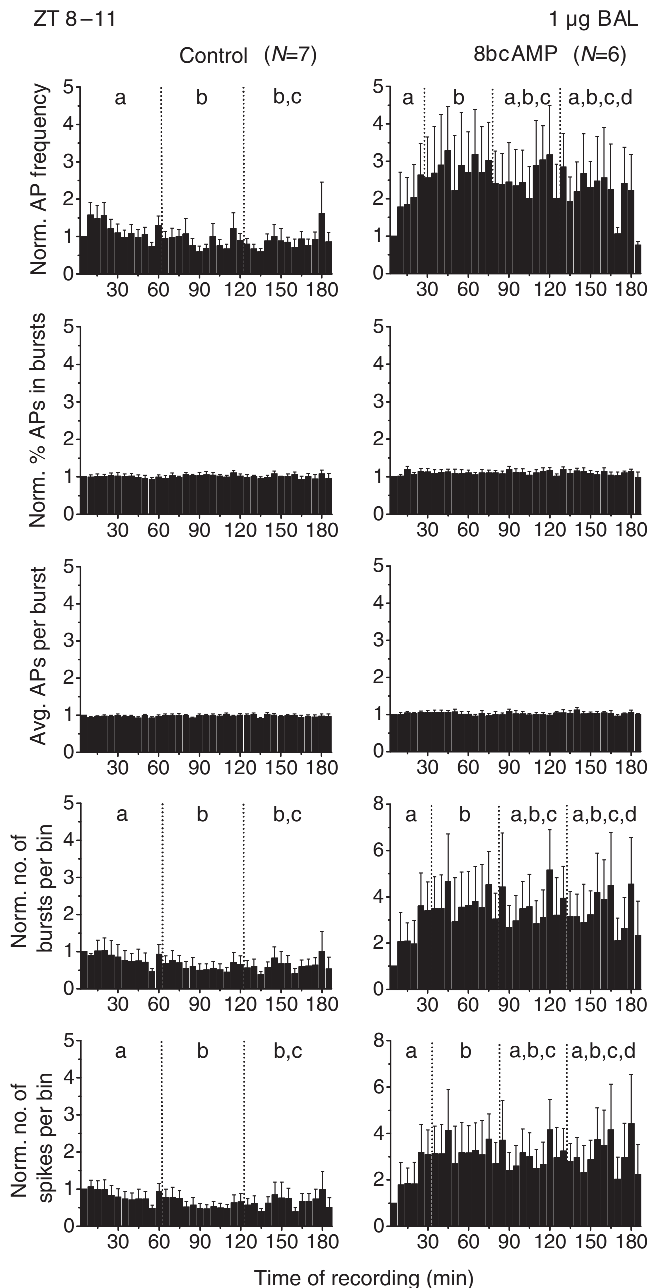

Fig. 6. In recordings at ZT 8–11 with 1 μg BAL stimulation, the perfusion with 8bcAMP significantly increased the normalized spontaneous AP frequency, the number of bursts per bin and the number of spikes per bin with very similar time courses (Mann–Whitney test, $P<0.01$, or ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). In contrast, in the associated controls slight but significant decreases were found for the normalized spontaneous AP frequency, the number of bursts per bin and the number of spikes per bin (Mann–Whitney test, $P<0.01$ or ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). The normalized percentage of APs occurring in bursts and the normalized average APs per burst were not affected. Values are means + s.e.m. Same lower case letters denote no significant differences between tested groups of mean values.

ineffective, the upregulation of cAMP *via* a pathway involving serotonin receptors is unlikely (C.F. and M.S., unpublished results). Thus, it is assumed that the effects are OA specific. The majority of insect OA receptors were shown to be positively coupled to adenylyl cyclases (Evans and Maqueira, 2005; Farooqui, 2007). The antennal OA receptor cloned from *M. sexta* has not yet been pharmacologically characterized, but shares high sequence similarity with other insect α-adrenergic-like OA receptors (Dacks et al., 2006). This receptor type increases both intracellular cAMP and Ca^{2+} concentration. Therefore, it is likely that the OA receptor involved activates an adenylyl cyclase and also affects Ca^{2+} concentration. Possibly, the pheromone dose dependency, the time dependency, and the lack of direct correlations between the 8bcAMP effects on the normalized SP and AP response indicate the involvement of synergistic or antagonistic Ca^{2+} concentration changes. Interestingly, the 8bcAMP effects differed at different BAL doses. At ZT 8–11, 8bcAMP increased the SP amplitude at the higher BAL dose but it decreased it at the lower BAL dose. Given BAL elicits Ca^{2+} rises in ORNs dose dependently (Stengl et al., 1999), it is possible that there is an interaction between the intracellular Ca^{2+} levels and cyclic nucleotide concentrations. Experiments are underway to test this hypothesis.

Potential antennal targets of cAMP

Several directly or indirectly cAMP-dependent ion channels are known in ORNs of *M. sexta* (Stengl et al., 1999; Dolzer, 2002; Krannich and Stengl, 2008). An apparently directly cAMP-dependent non-specific cation channel and a cAMP-dependent Ca^{2+} channel were identified in *M. sexta* (Krannich and Stengl, 2008), next to other cyclic nucleotide-sensitive cation and potassium channels (Dolzer, 2002; Dolzer et al., 2008). While these channels were specifically sensitive to 8bcAMP at concentrations lower than $1\ \mu mol\ l^{-1}$ cyclic nucleotide, at considerably higher concentrations of cAMP cGMP-dependent cation channels were also activated. Because 8bcAMP and 8-bromo 3′,5′-cyclic guanosine monophosphate perfusions *via* the tip recording electrode revealed significantly different effects (Flecke et al., 2006), apparently, despite the rather high cyclic nucleotide concentrations in the recording pipette, only physiological levels reached the inside of the ORNs.

Another potential target for cAMP, which might increase SP amplitude, could be the ubiquitous OR83b analogue MsextaOR2 (Patch et al., 2009; Wicher et al., 2008). It remains to be investigated whether MsextaOR2 is cAMP sensitive and involved in pheromone transduction of *M. sexta*. Alternatively the 8bcAMP-dependent effects could be explained by an action on V-ATPases located in the accessory cells of trichoid sensilla of moth species (Klein and Zimmermann, 1991; Klein, 1992), thereby increasing the driving force of the SP. Analogous to the larval midgut, the TEP in trichoid sensilla is supposed to be generated by the activity of a V-ATPase-powered K^+/nH^+ antiporter which builds up a high K^+ gradient between the sensillum lymph space and the haemolymph (Thurm, 1972; Thurm, 1974; Wieczorek et al., 2009). This potential difference is added to the membrane potential of the dendrites. It was shown that the application of cAMP and forskolin on salivary glands of the blowfly significantly increased V-ATPase activity (Dames et al., 2006) and altered the TEP (Prince and Berridge, 1972; Berridge and Prince, 1972). Whether V-ATPases in accessory cells of *M. sexta* are in fact cAMP sensitive, and whether changes in the TEP could indirectly regulate the SP amplitude remains to be studied.

Increase in SP amplitude without strong effects on AP frequency

While 8bcAMP strongly affected the normalized SP amplitude at ZT 8–11 and ZT 1–4 it had only minor effects on the generation of BAL-dependent APs. Only at ZT 1–4 in recordings with both BAL doses were increases in the mean SP amplitude followed by shifts to higher

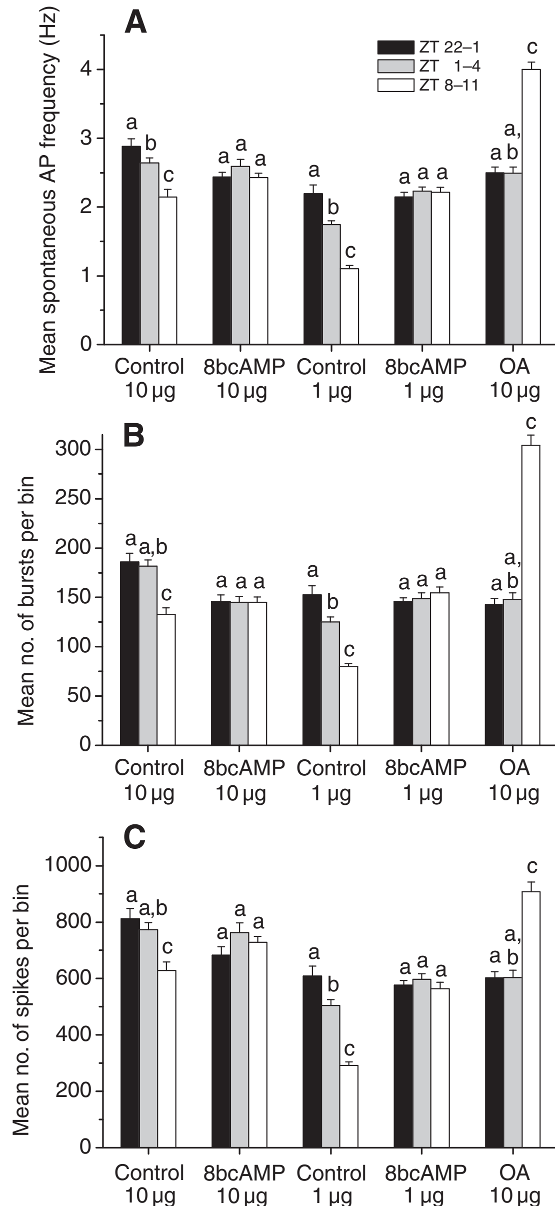

Fig. 7. Both 8bcAMP and OA antagonized endogenous shifts to a lower spontaneous AP activity during the photophase. The mean spontaneous AP frequency (A), the mean number of bursts per bin (B) and the mean number of spikes per bin (C) calculated from the absolute values for ZT 22–1, ZT 1–4 and ZT 8–11. All values are means + s.e.m. (A) In control recordings with both 10 and 1 μg BAL stimulation the mean spontaneous AP frequency decreased gradually at ZT 1–4 and ZT 8–11 (Mann–Whitney test, P<0.001). In contrast, application of 8bcAMP in recordings with both BAL doses antagonized this shift at ZT 1–4 and ZT 8–11. The application of 1 mmol l^{-1} OA antagonized the decrease at ZT 1–4 and increased the mean spontaneous AP frequency significantly at ZT 8–11 (Mann–Whitney test, P<0.001). Significant decreases in the mean number of bursts per bin (B) and the mean number of spikes per bin (C) were found at ZT 8–11 for controls with 10 μg BAL stimulation and at ZT 1–4 and ZT 8–11 for controls employing 1 μg BAL (Mann–Whitney test, P<0.001). 8bcAMP antagonized the shift to a lower mean number of bursts and a lower mean number of spikes per bin. In addition, OA increased the mean number of bursts and spikes per bin significantly at ZT 8–11 (Mann–Whitney test, P<0.001). The OA-dependent increase in the mean number of bursts per bin was 1.4-fold stronger than the increase in the mean number of spikes per bin. Same lower case letters denote no significant differences between tested groups of mean values.

mean AP frequencies. Remarkably, the increase in the normalized SP amplitude, which is apparently due to a stronger depolarization of the dendrite, was not transformed into an elevation of the normalized AP frequency. Thus, either the 8bcAMP-dependent augmentation of the receptor current did not reach the AP triggering zone or an additional adaptation mechanism at the spike trigger zone prevented the increase in the normalized AP frequency. Previous investigations of adaptation processes in trichoid sensilla of *M. sexta* indicated the existence of additional adaptation mechanisms at the level of the AP response (Dolzer et al., 2003). This is further supported by experiments in which the application of 8-bromo 3′,5′-cyclic guanosine monophosphate only affected the AP response but not the SP (Flecke et al., 2006). It remains to be examined whether the SP amplitude additionally is attenuated during propagation towards the AP generator, e.g. *via* 8bcAMP-dependent activation of an I_h current or other cyclic nucleotide-sensitive channels which could decrease the input resistance locally as in ORNs of the vomeronasal organ (Dibattista et al., 2008). In contrast to 8bcAMP, OA and tyramine increased both the SP amplitude and the AP frequency in tip recordings of trichoid sensilla (Flecke and Stengl, 2009). Alternatively, it is likely that OA and tyramine activate more than one signalling pathway (Han et al., 1998; Bischof and Enan, 2004; Balfanz et al., 2005; Ohtani et al., 2006; Farooqui, 2007), possibly involving a Ca^{2+}-dependent modulation of the spike generator.

Effects on the spontaneous AP frequency

Perfusion with 8bcAMP counteracted the decline of the absolute values of different parameters in the spontaneous AP activity at both pheromone doses at ZT 1–4 and 8–11 (Fig. 7). This was not observed to the same extent at the higher BAL dose in the normalized time courses at ZT 8–11. Possibly, with the higher BAL dose the 8bcAMP-dependent increases were very rapid and occurred immediately after the beginning of the recording and thus could not be resolved with a bin width of 5 min. While OA appears to predominantly affect the burst behaviour of the ORNs by increasing the percentage of APs occurring in bursts, 8bcAMP appeared to influence the generation of APs occurring as single spikes or in bursts to the same extent (Flecke and Stengl, 2009). In addition, OA effects were much stronger than 8bcAMP effects. These findings again suggest that OA activates more than one second messenger pathway, possibly changing both cAMP and Ca^{2+} concentrations.

The 8bcAMP-dependent increase in the spontaneous AP frequency alone could be explained by the activation of hyperpolarization-activated cyclic nucleotide-gated (HCN, I_h) cation channels, also observed in ORNs of moths (Krieger et al., 1999; Krannich, 2008). Dibattista and colleagues recently showed that the activation of adenylyl cyclases by forskolin increased the resting potential of ORNs in the vomeronasal organ (Dibattista et al., 2008), and in somatosensory neurons the elevation of cAMP increased the AP frequency after depolarizing steps due to a I_h-dependent increase in the membrane potential (Momin et al., 2008). In contrast, the blocking of I_h channels or of adenylyl cyclases in ORNs of the vomeronasal organ decreased the resting potential, increased the current threshold to elicit APs (Dibattista et al., 2008), and decreased the spontaneous AP activity in ORNs of *Panulirus argus* (Gisselmann et al., 2005). The 8bcAMP-dependent opening of I_h channels might increase the resting potential of the ORNs, thus enabling more subthreshold depolarisations to trigger spike generation. It remains to be studied whether I_h channels are indeed responsible for the setting of the resting potential and whether they are involved in the control of the spontaneous activity in *M. sexta* ORNs.

Furthermore, 8bcAMP might affect the spontaneous activity *via* direct or indirect interaction with the putative cAMP-sensitive OR83b analogue MsextaOR2 (Patch et al., 2009; Wicher et al., 2008), because olfactory sensilla lacking OR83b receptors showed very little or no spontaneous AP activity (Larsson et al., 2004). However, the physiological relevance of the observed changes in the spontaneous AP frequency remains to be determined.

Time dependency of 8bcAMP effects

Here we have shown for the first time that cAMP affects the time-dependent modulation of insect odour transduction and the generation of spontaneous APs. These time-dependent differences in cAMP effects were probably caused by circadian release of OA. Biogenic amines were suggested to control circadian rhythms in pheromone sensitivity in several moth species. In *M. sexta* the level of OA in the haemolymph is regulated in a circadian rhythm with a maximum during the scotophase when the nocturnal moths showed maximal mating behaviour and with low levels during rest in the photophase (Lehman, 1990; Lingren et al., 1977). Also, for *Trichoplusia ni* time-dependent changes in the OA levels in the haemolymph and brain were found which correlated with behavioural rhythms (Linn et al., 1994; Linn et al., 1996). In *M. sexta*, both OA and 8bcAMP perfusion increased the BAL-dependent normalized SP amplitude and antagonized an endogenous decrease in the spontaneous AP activity during the photophase, while both substances were ineffective during the scotophase, when ORNs were maximally sensitized (Flecke and Stengl, 2009). While the 8bcAMP-dependent increases with 1 μg BAL stimulation were almost equal in amplitude between ZT 1–4 and ZT 8–11, OA strongly increased the SP amplitude only at ZT 8–11, suggesting a time-dependent desensitization of OA receptors or adenylyl cyclases. Therefore, we are currently investigating whether cAMP concentrations express circadian rhythms and whether OA stimulates adenylyl cyclases time dependently in the antennae of *M. sexta*.

In addition, our results suggest that circadian changes in odour sensitivity in moths are also regulated at the periphery as they are in *D. melanogaster* (Tanoue et al., 2004; Krishnan et al., 2008), cockroaches (Saifullah and Page, 2009) and other moths (Merlin et al., 2007). The endogenous decline in the mean SP amplitude, AP frequency, and the spontaneous AP activity during the photophase suggests that the observed circadian rhythms in pheromone sensitivity are at least partly due to time-dependent regulation of single ORNs. Because the circadian clock protein PERIOD was located in ORNs and accessory cells of *M. sexta* (Schuckel et al., 2007) we assume that ORNs in *M. sexta* are independent peripheral circadian pacemakers which are synchronized in their sensitivity *via* OA release from centrifugal OA neurons projecting into the antenna.

LIST OF SYMBOLS AND ABBREVIATIONS

8bcAMP	8-bromo 3′,5′-cyclic adenosine monophosphate
AP	action potential
BAL	bombykal
cAMP	3′,5′-cyclic adenosine monophosphate
EAG	electroantennogram
HCN	hyperpolarization-activated cyclic nucleotide-gated (I_h)
ISI	interspike interval
OA	octopamine
ORN	olfactory receptor neuron
PSTH	post-stimulus time histogram
SP	sensillar potential
TEP	transepithelial potential
ZT	zeitgeber time

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A

10 µg BAL
1 µg BAL

ZT 1-4
Control
8bcAMP
Control
8bcAMP

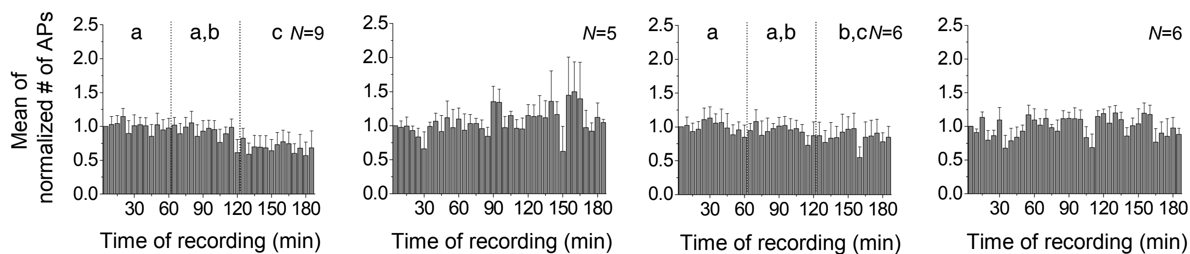
i
0 – 20
N=35
N=12
N=21
N=20

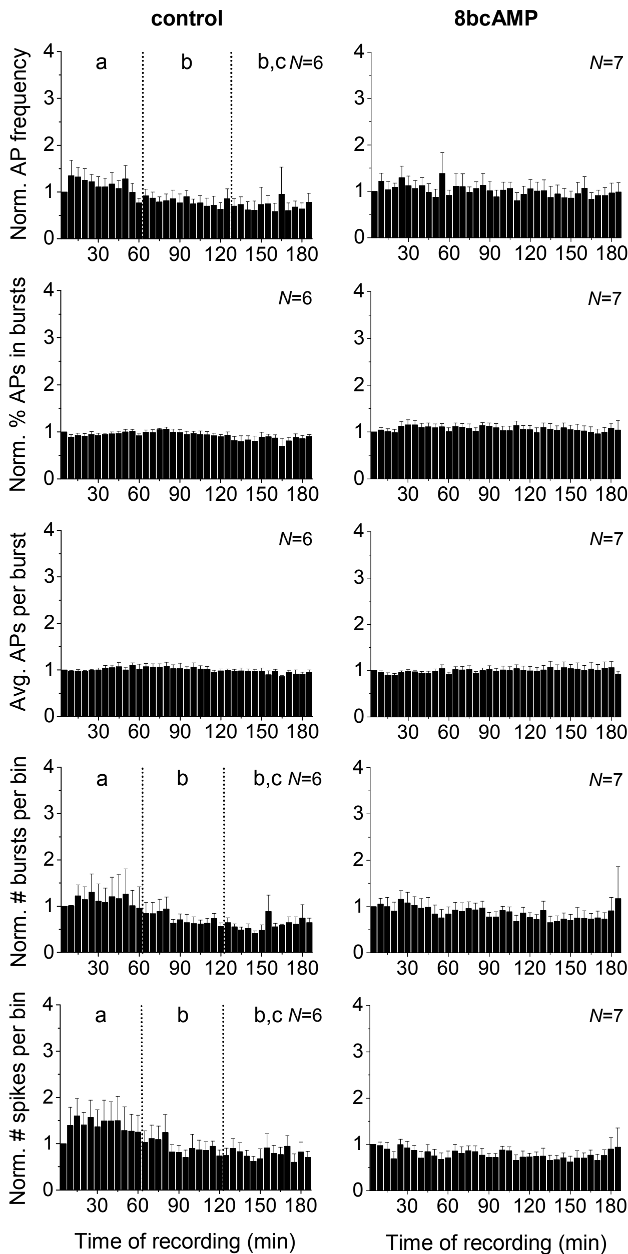
ii
80 – 100
N=30
N=14
N=24
N=25

iii
160 – 180
N=32
N=17
N=23
N=22

spikes
Time (ms)

B





ZT 8 - 11
10 µg BAL
control
8bcAMP
Norm. AP frequency
a
b
b,c N=6
N=7
Norm. % APs in bursts
N=6
N=7
Avg. APs per burst
N=6
N=7
Norm. # bursts per bin
a
b
b,c N=6
N=7
Norm. # spikes per bin
a
b
b,c N=6
N=7
Time of recording (min)
Time of recording (min)