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Serotonin modifies the sensitivity of the male silkmoth to pheromone

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Summary

Serotonin is known to modulate the response of neuronal populations in the primary olfactory center of the moth olfactory system, the antennal lobe. Here, we analyzed the effects of serotonin on the behavior related to the restricted pheromone olfactory pathway of the male silkmoth, *Bombyx mori*. In order to understand the effects of serotonin at the behavioral level, we applied serotonin (10^{-5} mol l^{-1}, 10^{-4} mol l^{-1} and 10^{-3} mol l^{-1}) to the brain and found that 10^{-4} mol l^{-1} serotonin increases the sensitivity to female pheromone whereas 10^{-3} mol l^{-1} serotonin had the opposite effect. Levels of serotonin in the brain were determined using HPLC with electrochemical detection. Inhibitory effects were observed after applying the serotonin antagonists mianserin (10^{-4} mol l^{-1}) and ketanserin (10^{-3} mol l^{-1}). Additionally, we quantified the circadian variation of serotonin in the brain using HPLC with electrochemical detection. Further, this variation correlated well with a circadian variation of the male sensitivity to pheromone. These results show that the serotonin-related enhancement of neuronal responses at the antennal lobe level is expressed at the behavioral level as a modulation of pheromone sensitivity and that the circadian variation of serotonin levels in the brain correlates with changes in the moth's pheromone sensitivity.

Key words: insect, HPLC, *Bombyx mori*, olfaction, serotonin, pheromone, circadian rhythm.

Introduction

In the insect nervous system, the biogenic amine serotonin acts as a neurotransmitter, neuromodulator and neurohormone. Serotonin affects the central nervous system as well as the sensory periphery and the neuromuscular junction (Evans, 1980; Mercer and Menzel, 1982; Claassen and Kammer, 1986; Nässel, 1988; Casagrand and Ritzmann, 1992; Erber et al., 1993; Menzel et al., 1999).

Serotonin is responsible for the modulation of various behaviors in insects: for example, short-term memory, sensitivity to olfactory stimuli (Mercer and Menzel, 1982; Menzel et al., 1999) and foraging behavior in honeybees (Schulz et al., 2002). Serotonin is also involved in the regulation of the optic lobe circadian clock in the cricket (Tomioka et al., 1993; Tomioka, 1999) and the cockroach (Page, 1987). Moreover, serotonin increases the duration of random activity in the cabbage looper (*Trichoplusia ni*) and the gypsy moth (*Lymantria dispar*; Linn and Roelofs, 1986; Linn et al., 1992).

Investigations of the effects of serotonin in the moth olfactory system have shown that, in the hawkmoth *Manduca sexta*, serotonin enhances the responses of some neurons in the first olfactory center, the antennal lobe (AL), to electrical and pheromonal stimuli (Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999). Furthermore, in cultured AL neurons, serotonin increases the spike number and induces a broadening of action potentials (Mercer et al., 1996). Serotonin application also affects both pheromone-evoked local field potentials and potential oscillations in the macroglomerular complex (MGC) of male *M. sexta* AL (Kloppenburg and Heinbockel, 2000). In the silkworm moth, *Bombyx mori*, high-speed optical imaging with a voltage-sensitive dye has shown that serotonin increases the maximum amplitude and duration of the optical responses in the AL (both the MGC and the ordinary glomeruli), suggesting that serotonin enhances neuronal responses in the AL (Hill et al., 2003).

The effects of serotonin on the response to pheromone in moths may be related to the presence of a pair of unique serotonin-immunoreactive neurons that innervate both ALs and have been identified in *B. mori* (Hill et al., 2002) as well as in several other insects (Schurmann and Klemm, 1984; Kent et al., 1987; Rehder et al., 1987; Breidbach, 1990; Salecker and Distler, 1990).

Insect olfactory systems are useful models for comprehending neural processing since olfactory information is processed through similar mechanisms in vertebrates and insects. The insect AL, while anatomically similar to the vertebrate olfactory bulb, contains far fewer neurons (Hildebrand, 1996). Insect systems are of great interest given

that they can be studied from the single neuron to the neural network and finally at the behavioral level. Moth olfactory systems, such as the pheromone-related olfactory pathway, have been studied extensively, from the ALs to the pre-motor centers (Kanzaki, 1997; De Belle and Kanzaki, 1999; Kanzaki et al., 2003).

In the present study, we therefore focus on the role of serotonin in the olfactory processing pathway of *B. mori*, which shows a stereotypical behavior called the 'mating dance' when encountering female pheromone (Kramer, 1975; Kanzaki et al., 1992; Kanzaki, 1998).

Here, we examine at the behavioral level the modulatory effects of serotonin on the enhancement of neural activity in *B. mori*'s AL. We applied serotonin (10^{-5} mol l^{-1}, 10^{-4} mol l^{-1} and 10^{-3} mol l^{-1}) and two of its antagonists, mianserin (10^{-4} mol l^{-1}) and ketanserin (10^{-4} mol l^{-1} and 10^{-3} mol l^{-1}), to the desheathed ALs of the male silkmoth and measured the pheromone sensitivity. 10^{-4} mol l^{-1} serotonin increased the sensitivity to pheromone while 10^{-3} mol l^{-1} serotonin, 10^{-4} mol l^{-1} mianserin and 10^{-3} mol l^{-1} ketanserin had the opposite effect. We measured the levels of serotonin in the brain after application using HPLC with electrochemical detection: 10^{-3} mol l^{-1} and 10^{-4} mol l^{-1} applications increased the serotonin concentration in the brain, while 10^{-5} mol l^{-1} application did not differ from the control. Furthermore, a circadian variation of serotonin, quantified with HPLC, showed a strong relationship with a circadian variation of the male moth sensitivity to pheromone. Our results demonstrate that the enhancing effects of serotonin in the olfactory neural pathway contribute to an arousal mechanism at the behavioral level, leading to a higher sensitivity to pheromone. Additionally, the moth's sensitivity to pheromone seems to be related to a circadian variation of serotonin in the brain.

Materials and methods

Animals

Bombyx mori L. (Lepidoptera: Bombycidae) were reared from eggs in the laboratory on an artificial diet under a 16 h:8 h light:dark photoperiod at 26°C and 50–60% relative humidity. Adult male moths were used within 3–7 days after eclosion.

Behavioral experiments

Procedure for application of substances

24 h before the beginning of the experiments, the head capsule was opened, the tracheae were gently removed and the ALs were desheathed in order to allow the substances to reach the brain. The insects were stored at 26°C (16 h:8 h light:dark photoperiod) until the experiment. Substances were applied to the brain with a volume of 4 µl using a Hamilton microliter syringe. Physiological saline, containing (in mmol l^{-1}) 140 NaCl, 5 KCl, 7 $CaCl_2$, 1 $MgCl_2$, 4 $NaHCO_3$, 5 trehalose, 5 *N*-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid (TES) and 100 sucrose (pH 7.3), was applied as the control. Serotonin (5-HT creatine sulfate; Sigma, St Louis, MO, USA) and two of its antagonists, ketanserin and mianserin (Sigma), were applied at the following concentrations: 10^{-5} mol l^{-1}, 10^{-4} mol l^{-1}, 10^{-3} mol l^{-1} for serotonin, 10^{-4} mol l^{-1} and 10^{-3} mol l^{-1} for ketanserin and 10^{-4} mol l^{-1} for mianserin. The application of saline as a wash followed the drug application. Two hours separated the control (performed at 11.00±1 h) and the drug in order to avoid the side effect of adaptation or habituation, and one day separated applications of drug and wash so as to match the time of day. As a preliminary experiment, we checked that the 2 h interval between the control and the drug did not have any effect on the moths' sensitivity to pheromone.

Experimental set-up and sensitivity to pheromone experiments

3 min after injection, four moths were placed in a translucent acrylic closed box (29.5×22×5 cm). A 2 mm-diameter hole in the middle of the lid allowed the insertion of a Pasteur pipette tip. The insects were placed 7.3 cm from the pheromone source (see Fig. 1). Air-puff stimulus was used to spread odors into the box. The pheromonal stimulus was the principal pheromone component of *B. mori*: synthetic (E,Z)-10,12-hexadecadien-1-ol (bombykol) dissolved in n-hexane. The olfactory stimulant was applied to a piece of filter paper (1×2 cm) and then inserted into the Pasteur pipette. Pulsed olfactory stimulation was produced with a three-way solenoid valve controlled by an electronic stimulator. The following series of bombykol concentrations were applied to the moth: n-hexane as a control, 0.1 ng, 0.3 ng, 1 ng, 2.5 ng, 5 ng, 10 ng, 30 ng and 100 ng, always one pulse, from the lowest to the highest concentration, at intervals of 30 s. The duration of the pulse was 200 ms and the flux rate was 1.4 l min^{-1}. A smoke test was performed using $TiCl_4$ in order to simulate the shape and position of the pheromone plume. The smoke reached the moths' position within 2 s. The air and odorant were removed after each set of experiments through an exhaust tube placed behind the box. Boxes were changed after each series of bombykol, and contamination by pheromone in the experimental area was constantly checked for by placing moths around the setup. Wing fluttering within 30 s of the puff was the criterion of whether the moth responded to pheromone. The behavioral response of the moths and the pheromone stimulation were simultaneously recorded on a digital video camera (Handycam; Sony, Tokyo, Japan; 30 frames s^{-1}) with an LED lamp connected to the electrical stimulator flashing at each pheromone puff. The light level was kept constant at 480 lux during the day and 2 lux at night.

In the experiments concerning circadian variation of male sensitivity to pheromone, the sensitivity to pheromone was measured in a 24 h time period in intact moths, and the responses were divided into 12 durations of 2 h. The series of pheromone concentrations was, in this case, 0.05 ng, 0.1 ng, 0.5 ng, 1 ng, 5 ng, 10 ng and 50 ng.

Biogenic amines analysis

Animals

When measuring serotonin circadian variation, three-day-old adult males were frozen in liquid nitrogen at the following times during the photoperiod: 0.00 h, 04.00 h, 08.00 h, 12.00 h,

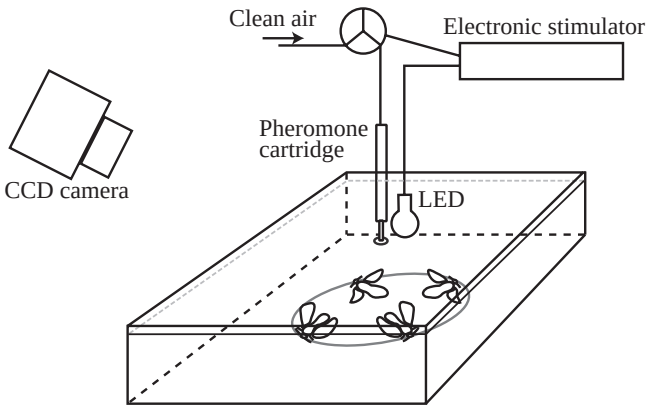

Fig. 1. Experimental set-up. Four moths were placed in a closed box. Pheromone was applied through a 2 mm-diameter hole with a Pasteur pipette containing a piece of filter paper bearing pheromone. The pheromone stimulus was controlled by an electronic stimulator through a three-way solenoid valve, and an LED connected to the electronic stimulator flashed for the duration of the stimulus. The moths' response to pheromone and the LED flash were recorded with a CCD camera.

16.00 h and 20.00 h. In the case of serotonin application, the procedure was similar to the behavioral experiments (control, 11.00 h; drugs, 13.00 h; wash, next day 11.00 h). The heads were cut off and immediately stored at –80°C and lyophilized until dissection and analysis.

Chromatography

When measuring serotonin circadian variation, an improved version of the method originally designed by Nagao and Tanimura (1988, 1989) was used for serotonin detection. Each brain (including protocerebrum, ALs and optic lobes) was dissected and homogenized in a micro-glass homogenizer (Wheaton, Millville, NJ, USA) in 50 µl of ice-cold 0.1 mol l^{-1} perchloric acid containing 12.5 ng ml^{-1} 3,4-dihydroxybenzylamine (DHBA) as the internal standard. After stirring for 3 min on ice, the homogenate was centrifuged at 17 400 ***g*** for 30 min at 0°C. The supernatant was transferred to a micro-vial for immediate injection onto an HPLC column for analysis. The HPLC system was composed of a pump (501; Waters, Milford, MA, USA), a refrigerated automatic injector (231-401; Gilson, Middleton, WI, USA) and a C_{18} reversed-phase column (250 mm×4.6 mm i.d.; 5 µm average particle size; Capcell Pak C_{18} MG, Shiseido, Tokyo, Japan). A glassy carbon electrode (WE-GC; Eicom, Kyoto, Japan) was used for electrochemical detection. The detector potential was usually set at 0.85 V against an Ag/AgCl reference electrode, which was maintained at 30°C. Signals from the electrochemical detector were recorded and integrated using data analysis software (Millennium; Waters). The mobile phase contained 0.18 mol l^{-1} monochloroacetic acid and 6 mg l^{-1} of EDTA disodium salt and was adjusted to pH 3.6 with sodium hydroxide. 1.6 mmol l^{-1} of sodium-1-octanesulfonate and 9% (v/v) acetonitrile were added to the solution. The mobile phase buffer was filtered through a 0.22 µm filter (GVWP 04700; Millipore, Bedford, MA, USA) and degassed under vacuum. The flow rate was kept constant at 0.7 ml min^{-1}.

In the case of serotonin application, each brain (including protocerebrum and ALs) was dissected and transferred into an Eppendorf tube containing 50 µl of 10 ng ml^{-1} isoproterenol (as the internal standard), 100 µmol l^{-1} EDTA disodium salt and 0.1 mol l^{-1} perchloric acid. The samples were sonicated for 3 min and centrifuged at 17 400 ***g*** for 30 min at 4°C. The supernatant was injected directly onto the column. The method consisted of an HPLC system (HTEC-500; Eicom), a refrigerated automatic injector (234; Gilson), a temperature regulator (832; Gilson), a C_{18} column (Eicompak SC-5ODS; 3×150 mm; Eicom) and a graphite electrode (WE-PG; Eicom). The mobile phase contained 100 mmol l^{-1} citrate acetate buffer (pH 3.6). 0.58 mmol l^{-1} sodium-1-octanesulfonate and 12% (v/v) methanol were added to the solution. The flow rate was kept constant at 0.5 ml min^{-1}. The detector was set at a working potential of 950 mV against an Ag/AgCl reference electrode and kept at 23°C. External standards were run at the beginning and at intervals throughout the runs.

In both cases, measurements based on the peak height of the chromatogram were obtained by calculating the ratio of the peak height of a substance to the peak height of the internal standard. Concentrations were obtained by comparison of the ratios between the sample and standard chromatograms. Chemicals were obtained from Sigma Chemical Co., except for SOS, acetonitrile (Nacalai Tesque, Kyoto, Japan), monochloroacetic acid and sodium hydroxide (Wako, Osaka, Japan).

Data analysis

Sensitivity to different pheromone concentrations was analyzed with the General Linear Model (GLM) Univariate (Edwards, 1993), followed by the Bonferroni adjustment for multiple comparisons among groups. The dependent variable was the number of moths fluttering their wings; the independent variables were pheromone concentration (0.3, 1, 2.5, 5, 10, 30, 100 ng) and treatment (control, drug and wash) in the case of drug application, and pheromone concentration (0.05, 0.1, 0.5, 1, 5, 10, 50 ng) and time of day (0.00, 02.00, 04.00, 06.00, 08.00, 10.00, 12.00, 14.00, 16.00, 18.00, 20.00, 22.00 h) in the case of circadian behavioral variation. Figs 2, 5, 8 show the detailed behavioral responses for each pheromone concentration while Figs 3, 6 represent the difference of mean of behavioral responses obtained with the GLM between the drug and the control for the set of pheromone concentrations. In both cases, the percentage of responses (Figs 2, 5, 8) and of difference of responses between drug and control (Figs 3, 6) was presented on the *y*-axis in order to allow a comparison between treatments. The efficiency of serotonin application was measured with the Kruskal–Wallis test. Serotonin circadian variation was evaluated with one-way ANOVA followed by Tukey's pairwise comparison. The relationship between serotonin levels in the brain and the pheromone sensitivity within 24 h was analyzed with the Pearson correlation coefficient.

In all cases, significant difference was set at $P<0.05$. All

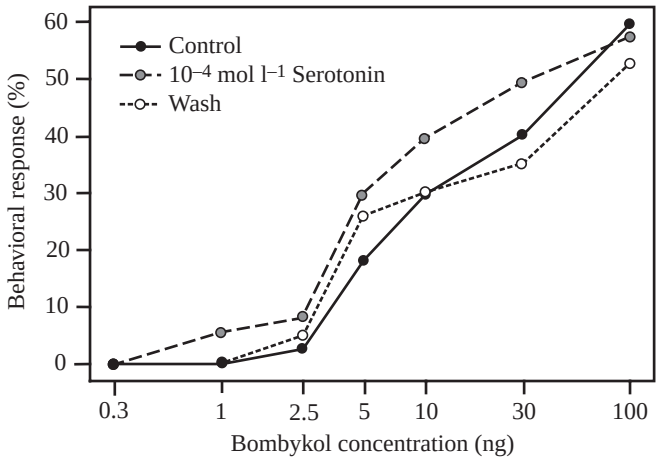

Fig. 2. Effects of serotonin on the behavioral response of the male moth to different concentrations of artificial pheromone. 4 µl of 10^{-4} mol l^{-1} serotonin were applied to the desheathed brain 3 min before pheromone exposure. Sample size: control (*N*=76), serotonin (*N*=73), wash (*N*=75). Serotonin shifted the curve to the left; its effect was higher than the control and the wash (GLM, *P*<0.05).

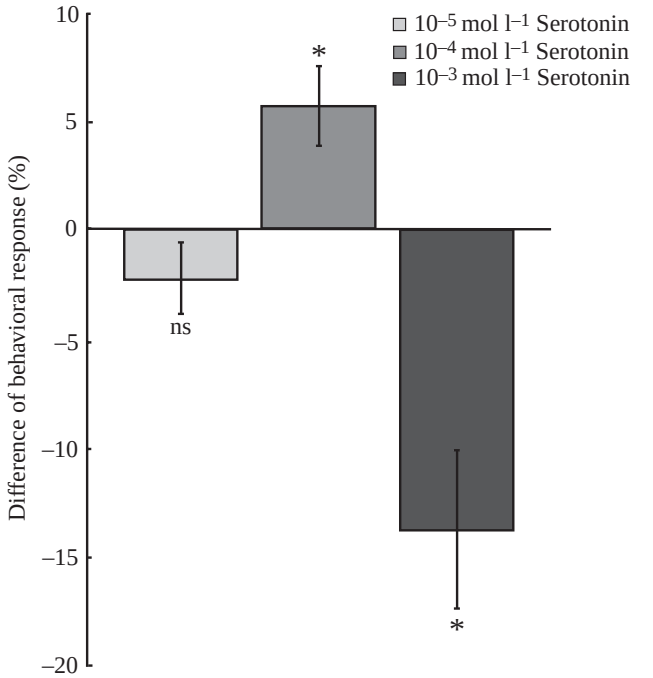

Fig. 3. Difference of means between the percentage of moths that responded to pheromone before and after three different concentrations of serotonin application. Values are means ± S.E.M. Sample size: 10^{-5} mol l^{-1} (*N*=129), 10^{-4} mol l^{-1} (*N*=73), 10^{-3} mol l^{-1} (*N*=60). The asterisk indicates significant difference (GLM, *P*<0.05); ns, non-significant.

statistical analyses were performed using commercially available software (SPSS, Chicago, IL, USA).

Results

Effects of serotonin on the male sensitivity to pheromone

Serotonin (10^{-5}, 10^{-4} and 10^{-3} mol l^{-1}) was applied to the desheathed ALs 3 min before testing the moths' sensitivity with increasing concentrations of the synthetic pheromone bombykol. The moths' sensitivity to pheromone was also tested with application of saline 2 h previous to and 24 h following the serotonin injection, as a control and a wash, respectively. Fig. 2 presents the percentage of control, serotonin-applied (10^{-4} mol l^{-1}) and wash moths responding to different concentrations of pheromone. Drug application to the brain had a significant effect on the moth's sensitivity to pheromone (*P*<0.02). We found that serotonin shifted the behavioral curve of response to pheromone to the left: the moths became more sensitive to pheromone than controls (control–drug, *P*<0.03) and reverted after 24 h (drug–wash, *P*<0.03; control–wash, non-significant). In the control, where saline had been applied to the moths' ALs, 18% of the subjects responded to a 5 ng pheromone concentration, while in the serotonin-applied moths, 30% responded to the same concentration. This increasing tendency in behavioral response due to serotonin was observed at all the pheromone concentrations with the exception of the lowest (0.3 ng), to which no moths were sensitive to pheromone, and the highest (100 ng), at which a large proportion of control moths responded to the pheromone. The effects of serotonin were dose dependent (Fig. 3): application of a lower concentration (10^{-5} mol l^{-1}) did not lead to a significant variation of the behavioral sensitivity to pheromone compared with controls (mean difference=–2.27±1.61; non-significant), application of an intermediate concentration (10^{-4} mol l^{-1}) increased the behavioral response (mean difference=5.68±1.85; *P*<0.03) while application of a higher concentration (10^{-3} mol l^{-1}) provoked an inhibition of the behavioral sensitivity (mean difference=–13.81±3.65; *P*<0.01).

Concentration of serotonin in the brain after application of drugs

Serotonin levels in the brain were measured 3 min after application of saline (the control) and serotonin (10^{-5}, 10^{-4} and 10^{-3} mol l^{-1}) and 24 h after washing with saline (10^{-4} and 10^{-3} mol l^{-1} serotonin) (Fig. 4). Application of 10^{-5} mol l^{-1} serotonin produced no change in levels in the brain compared with control levels, but there was a significant difference (*P*<0.001) when comparing 10^{-4} and 10^{-3} mol l^{-1} serotonin with the control [increasing the control levels (0.18 pmol $brain^{-1}$) to 0.44 and 0.80 pmol $brain^{-1}$, resulting in an increase of 241 and 424%, respectively]. After 24 h, the levels of serotonin decreased to the control levels.

Effects of the serotonin antagonists mianserin and ketanserin on the male sensitivity to pheromone

The effects of application of serotonin antagonists on the behavioral sensitivity were also evaluated. Mianserin (10^{-4} mol l^{-1}), a 5-$HT_{1\text{-}2}$ blocker (Dringenberg, 2000; Tierney, 2001), shifted the response to pheromone to the right, as shown

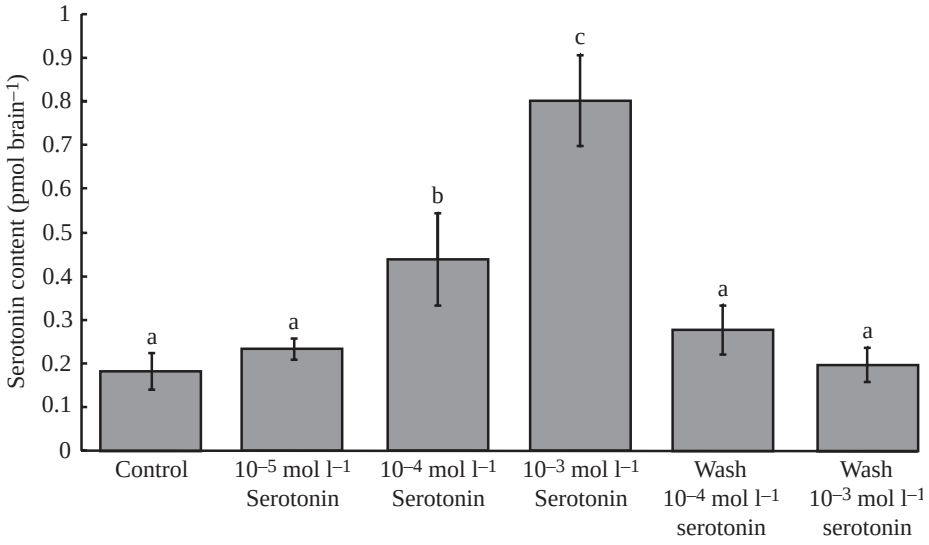

Fig. 4. Serotonin levels in the silkmoth's protocerebrum and antennal lobe (AL) after application of different concentrations of serotonin (10^{-5} mol l^{-1}, 10^{-4} mol l^{-1} and 10^{-3} mol l^{-1}). 4 µl of serotonin were applied to the desheathed brain 3 min before measurement with HPLC. Values are means ± S.E.M. Sample size: control (*N*=18), 10^{-5} mol l^{-1} serotonin (*N*=18), 10^{-4} mol l^{-1} serotonin (*N*=25), 10^{-3} mol l^{-1} serotonin (*N*=26), wash for 10^{-4} mol l^{-1} serotonin (*N*=9), wash for 10^{-3} mol l^{-1} serotonin (*N*=9). Differences between bars marked with the same letters were not significant (Kruskal–Wallis test, $P<0.05$).

in Fig. 5A. In response to 5 ng of pheromone, the behavioral response decreased from 46% with the control to 33% with the serotonin antagonist. The subjects did not completely revert to control behavioral sensitivity after 24 h.

Ketanserin is a highly selective 5-HT_2 antagonist (Chen et al., 1999; Dringenberg, 2000; Saifullah and Tomioka, 2003). At 10^{-3} mol l^{-1}, ketanserin decreased the behavioral sensitivity in a significant manner ($P<0.01$; Fig. 5B): over the whole pheromone concentration gradient, the response was lower with the drug, leading to a drop from 25% to 17% with a 5 ng pheromone exposure. In this case too, the reversion to control behavioral sensitivity was not totally accomplished after 24 h.

The inhibitory effects of serotonin antagonists were dependent on the antagonist type (mianserin *versus* ketanserin) and on the antagonist concentration (for ketanserin, 10^{-4} mol l^{-1} *versus* 10^{-3} mol l^{-1}) (Fig. 6). 10^{-4} mol l^{-1} ketanserin did not have any effect on the moth's sensitivity to pheromone compared with the control (mean difference=–0.94±2.20, non-significant) whereas a concentration of 10^{-3} mol l^{-1} decreased the behavioral response (mean difference=–6.47±1.67; $P<0.01$). 10^{-4} mol l^{-1} mianserin (mean difference=–9.02±2.69; $P<0.02$) had a stronger effect than both 10^{-3} and 10^{-4} mol l^{-1} ketanserin.

Circadian variation of serotonin in the brain

Fig. 7 shows the content of serotonin in the brain every 4 h over 24 h. From the beginning of the photophase until the noon peak, the concentration remained fairly constant (0.57 pmol $brain^{-1}$). The levels of serotonin in the brain were highest at noon (0.72 pmol $brain^{-1}$) and decreased progressively until 2 h after the beginning of the scotophase (0.41 pmol $brain^{-1}$). The variation of serotonin levels in the brain was statistically significant, with higher levels of serotonin at noon than at the beginning of the scotophase ($P<0.01$; family error rate <0.05).

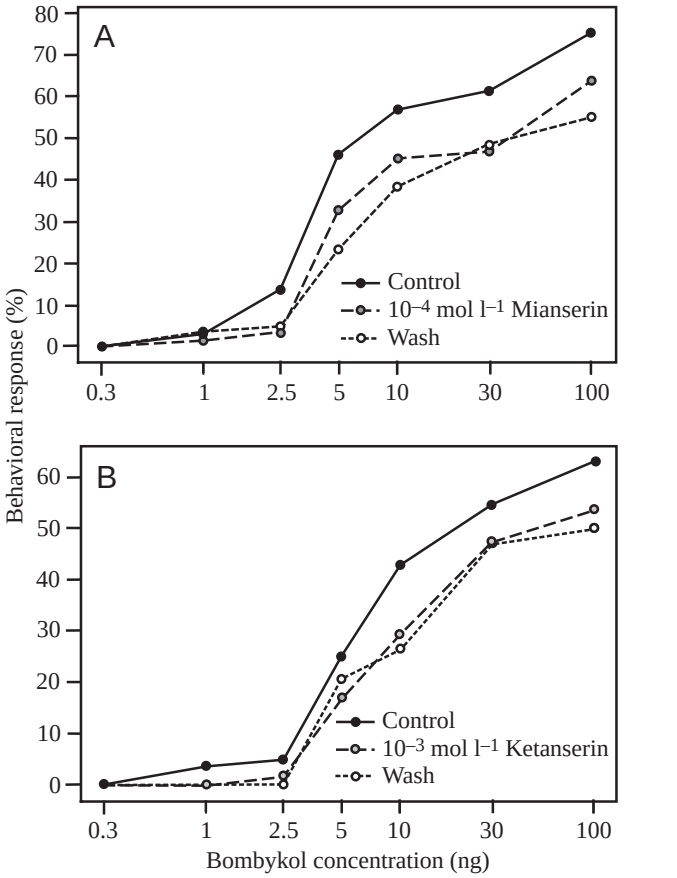

Fig. 5. Effects of serotonin antagonists on the behavioral response of the male moth to different concentrations of artificial pheromone. 4 µl of the drug were applied to the desheathed brain 3 min before pheromone exposure. Both drugs shifted the curve to the right (GLM, $P<0.05$), decreasing significantly the behavioral response in comparison with the control. (A) Mianserin at 10^{-4} mol l^{-1}. Sample size: control (*N*=65), mianserin (*N*=64), wash (*N*=60). (B) Ketanserin at 10^{-3} mol l^{-1}. Sample size: control (*N*=84), ketanserin (*N*=82), wash (*N*=34).

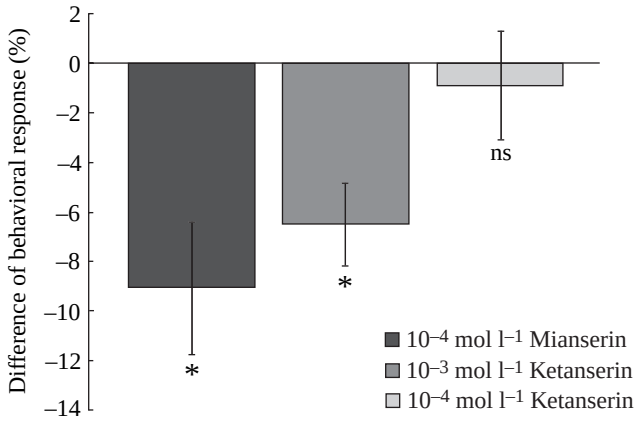

Fig. 6. Difference of means between the percentage of moths responding to pheromone before and after serotonin antagonist application. Values are means ± S.E.M. Sample size: 10^{-4} mol l^{-1} mianserin (N=64), 10^{-3} mol l^{-1} ketanserin (N=82), 10^{-4} mol l^{-1} ketanserin (N=84). Asterisks indicate significant difference (GLM, P<0.05); ns, non-significant.

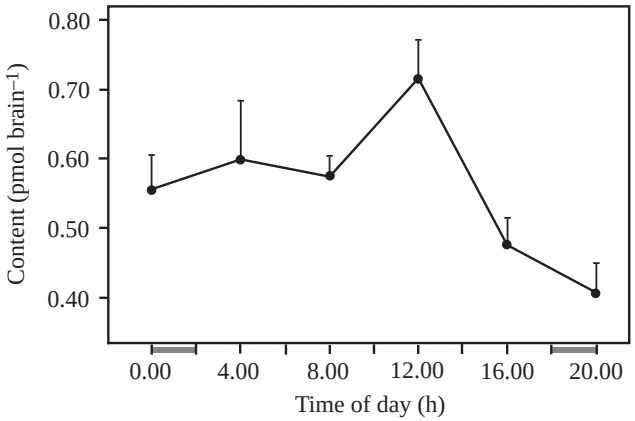

Fig. 7. Serotonin levels (+S.E.M.) in the brain (protocerebrum, antennal lobe and optic lobe) of *B. mori* exposed to a 16 h:8 h light:dark cycle, at different time points over 24 h. The period during which the light was turned off is indicated by a gray bar along the *x*-axis. The *N* values for each group varied between 6 and 9. There was a circadian variation of serotonin in the brain (one-way ANOVA, P<0.05).

Circadian variation of the pheromone-related behavior

The male sensitivity to pheromone was measured over a period of 24 h by confronting the moths with a concentration gradient of synthetic pheromone (Fig. 8). The GLM set a significant circadian variation (P<0.001): the activity at the beginning of the photophase (32% of the moths responded to 1 ng pheromone at 06.00 h) rose until noon (the percentage increased to 63%) before decreasing until the scotophase (the number dropped to 16% at 22.00 h). Furthermore, pheromone concentration had a significant effect on the behavioral response (P<0.001): at critical concentrations, a twofold increase of the concentration level (from 0.5 ng to 1 ng) led to a drastic change of behavioral response to pheromone over 24 h (P<0.001).

Circadian variation of serotonin in the brain (Fig. 7) strongly correlated with the circadian variation of pheromone sensitivity (Fig. 8) (Pearson correlation coefficient >0.91 between the serotonin variation in the brain and the circadian behavioral response to 0.1, 0.5 and 1 ng of pheromone).

Discussion

Serotonin modulates sensitivity to bombykol

This study demonstrates that serotonin plays an important role at the behavioral level of the male silkmoth's sex-pheromone olfactory pathway: 10^{-4} mol l^{-1} serotonin applied to the ALs increased the male silkmoth's sensitivity to pheromone (Fig. 2). Serotonin's effect was dose dependent (Fig. 3): a weaker concentration (10^{-5} mol l^{-1}) did not affect the behavior whereas a higher concentration (10^{-3} mol l^{-1}) decreased the sensitivity.

In order to quantify serotonin diffusion efficiency into the brain, we also performed HPLC measurements of brains 3 min and 24 h (wash) after application of 10^{-5} mol l^{-1}, 10^{-4} mol l^{-1} and 10^{-3} mol l^{-1} serotonin. Our results showed that application of serotonin at 10^{-4} mol l^{-1} and 10^{-3} mol l^{-1} increased brain serotonin levels by 241% and 424%, respectively (Fig. 4). These levels correspond to an efficiency rate of 0.1% and 0.05% respectively, a rate comparable with other studies; Linn et al. (1994) showed that the accumulation of serotonin that was expressed in the moth *T. ni*'s brain after injection was in the range of 0.1–0.6% of the amount injected in the head without desheathing.

The distribution of injected solutions was monitored by a 3 min Lucifer Yellow application on the desheathed brain (data not shown). Local staining of the two ALs, excluding the protocerebrum, suggested that the effects of serotonin and serotonin antagonists are mainly restricted to the ALs. Furthermore, the increase of serotonin in the ALs subsequent to the application of serotonin may be underestimated due to the fact that the AL is ~15% of the whole brain volume.

Contradictory effects of different serotonin concentrations have been reported in vertebrates and invertebrates. Serotonin has an excitatory effect on the chick biventer cervicis muscle at suitable concentrations and an inhibitory dose-dependent effect at high concentrations. An irreversible toxic effect was observed with repeated exposures to serotonin (Teerapong and Harvey, 1977). Application of 10^{-4} mol l^{-1} serotonin to the desheathed brain of *B. mori* increased the peak spike frequency of bombykol responses recorded from the ventral nerve cord (VNC), while 10^{-3} mol l^{-1} serotonin decreased the peak spike frequency of bombykol responses (E. S. Hill, unpublished observations). In the crayfish, the neuromodulatory effect of serotonin on the lateral giant neurons is dependent on its dose, rate and duration: inhibitory effects are obtained when high concentrations are reached rapidly whereas excitatory effects occur when low or high concentrations are reached gradually (Teshiba et al., 2001). Serotonin could activate two parallel intracellular signaling pathways through either different

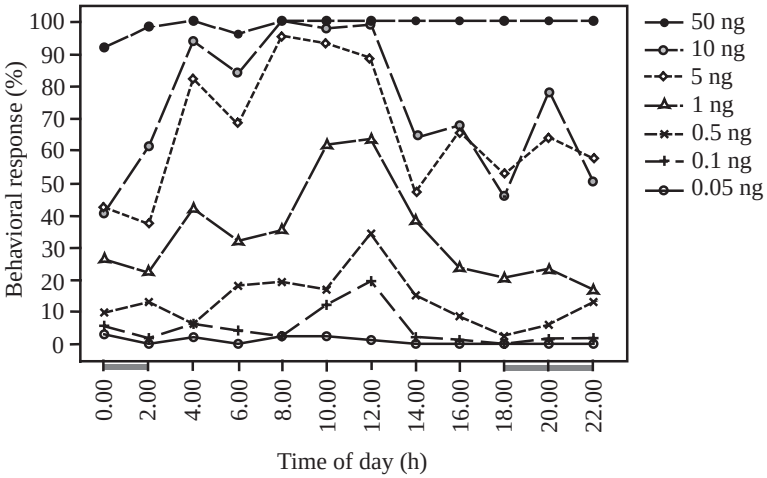

Fig. 8. Daily variation of the male *B. mori*'s behavior to different concentrations of artificial pheromone. Period during which the light was turned off is indicated by a gray bar along the *x*-axis. The *N* values for each group varied between 41 and 88. Around noon (10.00 h and 12.00 h), moths showed highest sensitivity to pheromone, significantly higher than during scotophase (GLM, $P<0.05$). The daily behavioral response to pheromone was strongly correlated to the daily variation of serotonin in the moth's brain (Fig. 7) (Pearson correlation coefficient: >0.90 for 0.1 ng, 0.5 ng and 1 ng).

serotonin receptors (Bermudez et al., 1992; Tierney, 2001) or different levels of a common initial second messenger (Teshiba et al., 2001). At high concentrations, serotonin could also be activating other biogenic amine receptors (Herman et al., 2003). Different concentrations of serotonin in the AL could also differently affect synapses of the three types of neurons in the AL: olfactory receptor neurons, local interneurons and projection neurons.

Mianserin and ketanserin, serotonin receptor antagonists, showed an inhibitory effect on the behavioral sensitivity to pheromone (Figs 5, 6). Mianserin is known to be a 5-HT (serotonin) antagonist (Baines and Downer, 1991; Tierney, 2001), most probably a 5-$HT_{1\text{-}2}$ receptor blocker (Dringenberg, 2000), and may also act on octopamine receptors (von Nickisch-Rosenegk et al., 1996). Ketanserin is a highly selective 5-HT_2 antagonist (Chen et al., 1999; Dringenberg, 2000; Saifullah and Tomioka, 2003). The 5-$HT_{1\text{-}2}$ blocker induced a stronger decrease in sensitivity than the 5-HT_2 blocker, suggesting that different receptors are activated by serotonin in the ALs and/or a combined action of serotonin and octopamine. Ketanserin at 10^{-3} mol l^{-1} unlike at 10^{-4} mol l^{-1} had an effect on the pheromone sensitivity, which suggests that ketanserin action is dose dependent. Both serotonin antagonists showed an opposite effect to the excitatory effect of serotonin. Application of a wider range of serotonin antagonists at a wider concentration range would help to understand the role of serotonin receptors in excitation and inhibition of the pheromone searching behavior.

In all pharmacological experiments apart from application of 10^{-4} mol l^{-1} serotonin, we did not observe a complete reversion of behavioral response after the wash (10^{-4} mol l^{-1} mianserin, 10^{-3} mol l^{-1} ketanserin; Fig. 5), even in moths not affected by the drug treatment (10^{-5} mol l^{-1} serotonin and 10^{-4} mol l^{-1} ketanserin; data not shown). The behavioral measurement of the wash was performed one day after the drug treatment in order to avoid a circadian effect and allow a natural chemical wash-out; this 24 h delay, in addition to the brain dissection performed previous to the experiments, could partly explain why the sensitivity did not reverse to 'control' levels.

In moths, serotonin may be released in the ALs by a single pair of serotonin-immunoreactive (SI) neurons with branches in every glomerulus of the AL as well as in higher order neuropil regions of the brain (Kent et al., 1987; Hill et al., 2002). Furthermore, Hill et al. (2002) showed that this SI neuron spikes spontaneously and responds to mechanosensory stimuli to the antennae. Due to the fact that, in *M. sexta*, its branchings in the AL contain mostly output synapses (Sun et al., 1993), the SI neuron may be involved in a feedback system from the protocerebrum to the AL. Our serotonin application may therefore mimic the SI neuron's release of serotonin in the AL.

Our pharmacological method, similar to the one used by Hill et al. (2003), allows for a direct comparison of serotonin's enhancing effects on neuronal populations in specific AL glomeruli and on pheromone sensitivity at the behavioral level. Our results suggest that the neuronal responses to pheromone are modulated by serotonin in the ALs and transferred *via* higher information processing centers in the protocerebrum to descending neurons related to the pheromone-searching behavior (Kanzaki et al., 1991, 1994).

In the AL, serotonin enhances central olfactory neuron responses to electrical stimulation of the antennal nerve and female sex pheromone in *M. sexta* (Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999). Furthermore, serotonin application increases the amplitude and duration of pheromone-evoked local field potentials and the magnitude of potential oscillations in the MGC of *M. sexta* (Kloppenburg and Heinbockel, 2000). In *B. mori*, application of serotonin to the AL enhances both the maximum amplitude and duration of AL optical responses to electrical stimulation of the antennal nerve. In the MGC, these effects are stronger in the toroid (neuropil specialized in processing bombykol information) than in the cumulus, the neuropil processing mainly the minor pheromone component, bombykal (Hill et al., 2003; Kanzaki et al., 2003). All these findings suggest that the male moth is strongly affected by serotonin in the AL and that these effects should in turn have some behavioral significance. Until now, no such behavioral effects resulting from serotonin application to the AL have been reported.

Based on these facts, the main expected effect of serotonin application to the ALs would be an enhancement of the male sensitivity to pheromone, as we observed when 10^{-4} mol l^{-1} serotonin was applied. Surprisingly, we also obtained an inhibitory effect with 10^{-3} mol l^{-1} serotonin. Our results are not irreconcilable with previous studies, in which the main serotonin concentration used was 10^{-4} mol l^{-1}. We cannot exclude that such a high concentration of serotonin (10^{-3} mol l^{-1}) does not occur in nature and that the behavioral decrease of response to pheromone is artificial. Supporting this idea, serotonin levels show a maximum of a twofold increase during the daily variation in the moth's brain (Fig. 7). This range of increase was obtained when applying 10^{-4} mol l^{-1} serotonin; when applying a concentration of 10^{-3} mol l^{-1}, serotonin levels rose more than four times the control (Fig. 4), an increase much larger than that observed in the circadian variation of serotonin. A concentration similar to ours could provoke inhibitory effects on AL neuronal responses, leading to a pheromone sensitivity decrease. Another possibility, compatible with previous reports, could be that excitation of AL neurons due to 10^{-3} mol l^{-1} serotonin application could cause an inhibition of neurons in higher centers in the moth brain. A low rise in serotonin concentration would lead to an increase in sensitivity to pheromone, while a higher serotonin concentration would prevent the moth from responding further to pheromone. This hypothesis would give a new insight into the functional significance of the feedback role of the SI neuron in the moth brain.

The effects of serotonin and other amines (mainly octopamine and dopamine) have been studied on a few insects' brains with different experimental approaches. Local injections in various parts of the brain such as ALs, optic lobes and mushroom bodies have been performed in the honeybee in order to assess the effects of amines on olfactory conditioning (for a review, see Bicker and Menzel, 1989; Erber et al., 1993). In a noctuid moth, *Trichoplusia ni*, and in a diurnal moth, *Lymantria dispar*, serotonin injection prior to light-off enhanced general locomotor activity at night but not the sensitivity to pheromone (Linn and Roelofs, 1986; Linn et al., 1992). In our experiments, we did not observe an enhancement of general locomotor activity; instead we observed that serotonin modifies the moth's sensitivity to pheromone. This discrepancy in results can be explained by the method used: Linn et al. injected serotonin in the head capsule without desheathing, and the behavior was measured with a 1–8 h delay of the injection, whereas we chose application and desheathing in order to measure the behavior without delay to obtain a fast effect of the drug in a similar way in both ALs, avoiding as much as possible the effects of serotonin receptor desensitization (Hanley and Hensler, 2002).

Circadian variation of serotonin and of bombykol sensitivity

Given that, including the pair of AL-SI neurons, approximately 40–50 SI neurons innervate all the brain neuropils (M. Iwano, personal communication), we predict that serotonin also acts at other information processing levels, such as the modulation of higher processing of the characteristic zigzagging pattern following female pheromone release (Kanzaki, 1998) or the internal clock regulation in the optic lobe, a characteristic known in other insects such as the cockroach (Page, 1987) and the cricket (Tomioka, 1999).

Our results showed a high correlation between the male silkmoth's sensitivity to bombykol and brain serotonin levels (Figs 7, 8). Serotonin levels in the brain displayed a circadian variation with a peak at noon (Fig. 7) and a strong decrease around the beginning of the subjective night (more than 40% decrease). The curve's shape showed a striking similarity to the male moth's sensitivity to pheromone, mainly at intermediate pheromone concentrations (0.1, 0.5 and 1 ng). At higher concentrations (5, 10 and 50 ng), the correlation with serotonin circadian variation appeared to be hidden by a saturation of the behavioral response at noon. This similarity powerfully supports the notion that serotonin is at least partly responsible for pheromone sensitivity and, furthermore, would have a significant role in the circadian regulation of male behavior. Furthermore, we can combine our results and suggest that the ranges of circadian variation of serotonin in the brain match with the excitatory effects of serotonin in the ALs leading to a higher sensitivity to pheromone.

The difference between the serotonin daily concentration in the brain (Fig. 7) and the serotonin levels in the brain following a 4 μl application of external serotonin (Fig. 4) could be explained by the difference in brain dissection (levels of serotonin in Fig. 7 were obtained from ALs, protocerebrum and optic lobes, while Fig. 4 levels concern mainly the ALs and protocerebrum) as well as a seasonal variation of brain serotonin concentration.

Circadian variation of serotonin in the brain has been reported in several insects (Muszynska-Pytel and Cymborowski, 1978; Bult et al., 1991; Tomioka et al., 1993; Linn et al., 1994; Kloppenburg et al., 1999). In the cricket and the cockroach, serotonin is known to be involved in the regulation of the optic lobe circadian clock (Page, 1987; Saifullah and Tomioka, 2003; Tomioka, 1999). In *B. mori*, serotonin circadian variation in the brain could also be related to an endogenous clock and subsequently act simultaneously on the sensitivity to pheromone in the AL and the endogenous clock, which could be partly located in the central brain of moths (Helfrich-Forster et al., 1998; Truman, 1974). However, this dual role of serotonin can only be speculated, given that in moths such involvement has not been clarified yet. As another possibility, serotonin levels in the moth brain could also be regulated predominantly by photic inputs through the light–dark cycle and, in turn, act on the sensitivity to pheromone in the AL. In both cases, the mechanisms underlying serotonin synthesis and release are still unclear. The moth *T. ni* shows a diel fluctuation of serotonin in the brain, with maximal levels in the light period even though the moth is inactive during the day (Lingren et al., 1977; Linn et al., 1994). In contrast to that study, in *M. sexta*, also a nocturnal moth (Lingren et al., 1977), a circadian variation of serotonin in the AL peaks at the beginning of the subjective night

(Kloppenburg et al., 1999). In another nocturnal moth, *Helicoverpa assulta*, the peak of pheromone release occurs during scotophase (Kamimura and Tatsuki, 1994).

B. mori female pheromone release also shows a circadian rhythm: the release of pheromone increases at the beginning of photophase to reach a peak 6 h later; this peak lasts for 2 h before decreasing until the beginning of scotophase (Ichikawa, 1998). The circadian variation of the male's sensitivity to pheromone allows the male to locate more efficiently the female during its pheromone release peak window. This daily correlation between male and female behavior and physiology creates a specific ecological niche of *B. mori* that has been selected through evolution.

Behavioral experiments, such as pheromone sensitivity behavioral response, are important in order to grasp the concrete effect of neural substances on neural systems. However, they provide general concepts, given that the results are means of a great number of individuals. Therefore, in order to unravel the complex mechanisms underlying the excitatory/inhibitory effects of serotonin on sensitivity to pheromone, to confirm our hypothesis that the pheromone-sensitivity regulation is modulated in the AL by serotonin and to separate the effects of serotonin in the AL and in the PC, we are planning to combine behavioral and pharmacological experiments with physiological experiments, such as calcium imaging and extracellular recordings of the VNC, which would allow the use of a great range of concentrations combined with various measurements in single individuals.

In order to clarify the modulatory effects of serotonin in the moth brain, the possible involvement of serotonin in the endogenous clock should be investigated. In addition, so as to validate the hypothesis that serotonin circadian variation modulates the male silkmoth sensitivity to bombykol at the AL level, we are planning to measure the possible circadian variation of serotonin in the AL, as well as in other neuropils of the brain.

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