Chapter 2

Materials and Methods

2.1 Animals

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

2.2 Procedure for application of substances

Substances were applied to the brain with a volume of 4 μl using a Hamilton microliter syringe. Physiological saline containing (mM): 140 NaCl, 5 KCl, 7 $CaCl_2$, 1 $MgCl_2$, 4 $NaHCO_3$, 5 Trehalose and 5 N-tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) and 100 sucrose (pH 7.3) was applied as the control. The application of saline as a wash followed the drug application. Two hours separated the control (performed at 11h00 $\pm$ 1h) 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, I checked that the 2 hours interval between the control and the drug did not have any effect

on the moths' sensitivity to pheromone.

2.2.1 Serotonin and its antagonists

24 hours before the beginning of the experiments, the head capsule was opened, the tracheas were gently removed and the antennal lobes were desheathed using fine forceps in order to allow the substances to reach the brain. The insects were stored at 26 °C (16:8 light: dark photoperiod) until the experiment. Serotonin (5HT, creatine sulfate, Sigma, St. Louis, MO, USA), and two of its antagonists ketanserin and mianserin (Sigma) were applied at the following concentrations : 10^{-5}M, 10^{-4}M, 10^{-3}M for serotonin, 10^{-4}M and 10^{-3}M for ketanserin and 10^{-4}M for mianserin. Serotonin, mianserin and ketanserin were dissolved in physiological saline.

2.2.2 Nitric oxide cascade

One hour before the beginning of the experiments, a small opening was performed in the head capsule by using the injection syringe. All chemicals were maintained in the dark and, when possible, only dissolved to the required concentration immediately prior (2-5 min) to bath application. L-NAME (Nω-nitro-L-arginine methyl ester, Sigma) and D-NAME (Nω-nitro-D-arginine methyl ester, Sigma) were dissolved in physiological saline whereas NOR3 ((+/-)-(E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide, Wako, Osaka, Japan) and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, Sigma) were first dissolved in DMSO (dimethylsuphoxide, Dojindo, Kumamoto, Japan) and then diluted in physiological saline.

Behavioral experiments

In order to test the effects of the nitric oxide synthase inhibitor, control application (N=66) was followed at a 2 hours interval by a random sequence of L-NAME at

a concentration of 10^{-4}M or 10^{-5}M and D-NAME at a concentration of 10^{-4}M. The effects of a nitric oxide donor on the pheromone sensitivity were measured by applying NOR3 5 × 10^{-4}M (N=65) on *B. mori*'s brain. The sensitivity test was performed 5 minutes after the drug injection. Two hours after NOR3 application, saline was applied as a wash.

Biogenic amines experiments

To investigate a possible relationship between biogenic amines and nitric oxide, I co-injected NOR3 (10^{-4} to 10^{-2}M) with L-NAME (10^{-3}M) or with ODQ (10^{-4}M). As controls I also injected saline, NOR3 (10^{-3}M) and L-NAME (10^{-3}M). The insects were frozen in liquid nitrogen 3 minutes after drug injection.

2.3 Behavioral experiments

2.3.1 Effects of serotonin and nitric oxide

Three minutes after injection, 4 moths were placed in a translucent acrylic closed box (29.5 × 22 × 5cm). The insects were placed 7.3 cm from the pheromone source (Fig. 2.1 on the next page). Air-puff stimulus was used to spread odors into the box through a 2 mm diameter hole in the middle of the lid with a Pasteur pipette containing a piece of filter paper bearing pheromone. 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 3-way solenoid valve controlled by an electronic stimulator. The following series of bombykol concentrations were applied to the moth: n-hexane as a control, (0.01 ng and 0.03 ng when the moths were very sensitive) 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

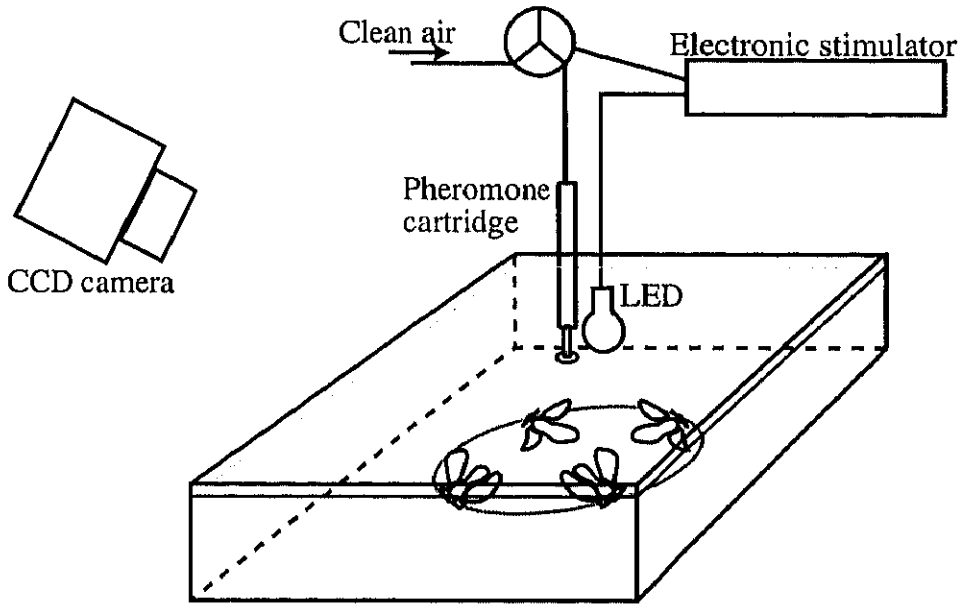


Figure 2.1: Experimental setup
Four moths were placed in a closed box. Pheromone was applied through a 2 mm diameter hole with a Pasteur pipette containing a piece of filter paper bearing pheromone. The pheromone stimulus was controlled by an electronic stimulator through a 3-way solenoid valve and an 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.

30 seconds. The duration of the pulse was 200 ms and the flux-rate was 1.4 l/min. 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 seconds. 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 seconds 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, frame rate = 30 frames/sec) with an LED lamp connected to the electrical stimulator and flashing at each pheromone puff. The light level was kept constant at 480 lx during the day and 2 lx at night.

2.3.2 Circadian variation

Ten moths at a time were placed in an acrylic translucent box. The experimental setup was similar to the previous section (Fig. 2.1 on the preceding page). The sensitivity to pheromone was measured in a 24 hour time period in intact moths, and the responses were divided into 12 durations of 2 hours. 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.

2.3.3 Habituation

Short term habituation

Two hours before the training, all moths were exposed to low levels of bombykol (up to 30 ng) as a pre-test in order to control their response to pheromone (Fig. 2.2 on the next page). The habituation group was exposed to 3 × 500 ms of bombykol (1000 ng; interstimulus interval = 500 ms) 30 minutes before the sensitivity test. The dishabituation group was similarly exposed to 3 × 500 ms of bom-

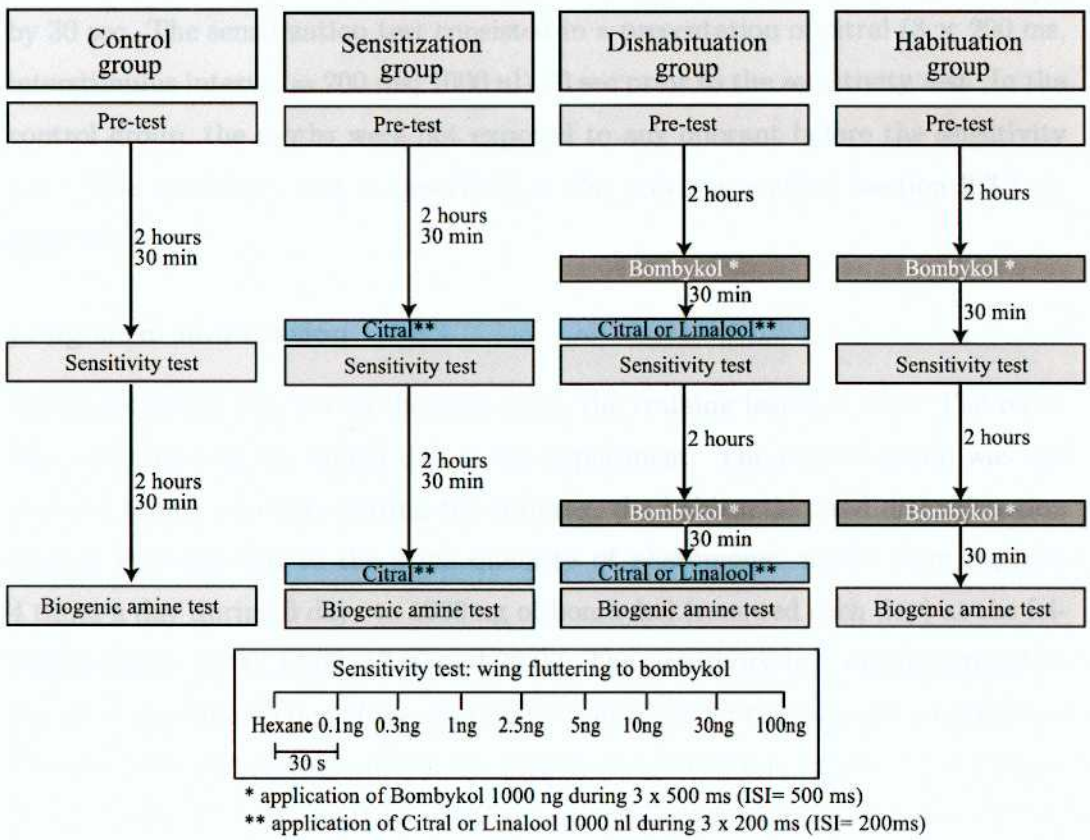


Figure 2.2: Short term habituation protocol

The pre-test consisted of a sensitivity test prior to habituation experiments. 30 minutes before the sensitivity test, the habituation and dishabituation groups were exposed to high levels of pheromone (3 × 500 ms of bombykol, 1000 ng; interstimulus interval = 500 ms). Shortly prior to the sensitivity test, the dishabituation and sensitization groups were exposed to a plant odorant (linalool or citral, 3 × 200 ms, 1000 nl, interstimulus interval = 200 ms). The control group was not exposed to any odorant between the pre-test and the sensitivity test. The four groups were exposed to the same treatments before being frozen in liquid nitrogen for measurement of biogenic amines in a high performance liquid chromatography system.

bykol (1000 ng) but a short presentation of a different odorant (linalool or citral, 3 × 200 ms, interstimulus interval = 200 ms, 1000 nl) preceded the sensitivity test by 30 sec. The sensitization test consisted in a presentation of citral (3 × 200 ms, interstimulus interval = 200 ms, 1000 nl) 30 sec prior to the sensitivity test. In the control group, the moths were not exposed to any odorant before the sensitivity test. The sensitivity test is described in the previous section (section 2.3.1 on page 13).

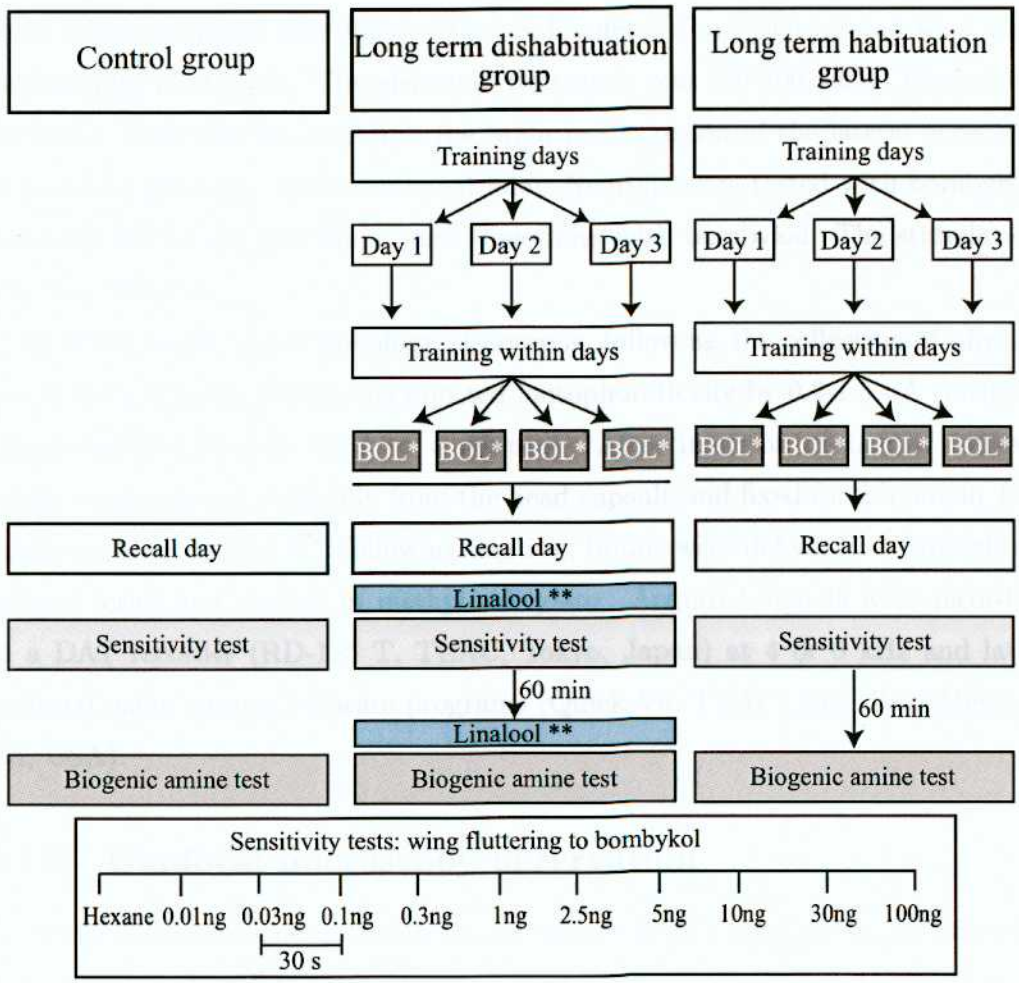
Long term habituation

As presented in Fig. 2.3 on the next page, the training lasted 3 days. The recall was performed on the fourth day of the experiment. The control group was not exposed to any odorant. During the training, the habituation and dishabituation groups were exposed to the same quantity of pheromone: moths were exposed 4 times a day during 3 days to 1000 ng of bombykol (renewed each day) at the following times: 11:00, 14:00, 17:00 and 20:00. The sensitivity test was performed on the recall day (fourth day of the experiment), at 13:00 for the 3 groups (control, habituation and dishabituation). In the case of dishabituation, linalool (3 × 200 ms, interstimulus interval = 200 ms, 1000 nl) was applied 30 sec prior to the sensitivity test described in a previous section (section 2.3.1 on page 13).

2.4 Intracellular recordings

2.4.1 Physiology

The male was placed ventral-side-up on a fixed chamber made of acrylic plastic after the legs were removed. The head was immobilized with a notched plastic yoke slipped between the head and the thorax. The brain was exposed by opening the head capsule and removing large tracheae; intracranial muscles were removed to eliminate brain movements. The brain was superfused with saline solution.



* application of bombykol 1000 ng during 500 ms
** application of linalool 1000 nl during 3 x 200 ms (ISI= 200ms)
Trainings were performed at 11:00, 14:00, 17:00 and 20:00.
Sensitivity tests were performed at 13:00.
Biogenic amine tests were performed at 14:00

Figure 2.3: Long term habituation protocol
During long term habituation and dishabituation, the moths were exposed 4 times a day for 3 days to high levels of pheromone (500 ms of 1000 ng bombykol, BOL). On the next day, the sensitivity test was performed at 13:00. The dishabituation group was furthermore exposed to linalool (3 × 200 ms, 1000 nl, interstimulus interval = 200 ms) shortly prior to the sensitivity test. The control group was not exposed to any odorant before the sensitivity test. One hour after the sensitivity test, the dishabituation group was exposed to linalool (3 × 200 ms, 1000 nl, interstimulus interval = 200 ms) and the 3 groups were frozen in liquid nitrogen for measurement of biogenic amines in a high performance liquid chromatography system.

Glass microelectrodes were filled with 4 % Lucifer Yellow (Sigma) and were used as recording electrodes. The electrode resistance was 150-200 MΩ. The microelectrodes were then inserted into the brain in the region of the lateral accessory lobe (LAL), posterior to the antennal lobe. Neurons were tested with bombykol, n-hexane and/or air as a blank, and sometimes with bombykal. The stimulation time was 500 ms.

In order to get the morphology of neurons, following the collection of physiological data, Lucifer Yellow was injected iontophoretically by 0.2-1.5 nA constant hyperpolarizing current for 1 to 4 minutes. After injection of Lucifer Yellow, brains were removed surgically from the head capsule and fixed for 2 hours in 4 % paraformaldehyde at 4 °C. Following fixation, brains were dehydrated through an ethanol series and cleared in methyl salicylate. Acquired signals were recorded on a DAT recorder (RD-125 T, TEAC, Tokyo, Japan) at 4 or 6 kHz and later analyzed using various software programs (Quick Vu, TEAC; Igor, WaveMetrics, OR, USA).

2.4.2 Confocal microscopy observation

Lucifer Yellow-stained neurons were examined as whole mounts and imaged frontally and sometimes dorsally using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany). Serial optical sections were acquired at 1-2 μm interval throughout the entire depth of the neuron.

2.5 Biogenic amine analysis with HPLC

In all cases, heads were cut off in liquid nitrogen and immediately stored at -80 °C. In serotonin, serotonin antagonists and circadian variation experiments, the heads were lyophilized within 3 days after isolation. The heads were dissected at room temperature just before being analyzed with the HPLC. In habituation and nitric oxide experiments, the heads were stored at -80 °C until dissection; dissection

was performed on ice shortly before HPLC analysis. In habituation experiments, antennal lobes were carefully isolated from the protocerebrum using a sharpened tungsten wire (Nilaco, Tokyo, Japan) which tip had been previously bent oval-shaped. Protocerebra were analyzed per unit while 4 antennal lobes (from 2 brains) were gathered in one Eppendorf tube before analysis.

2.5.1 Serotonin circadian variation

Three days old adult males were frozen in liquid nitrogen at the following times during the photoperiod : 0h00, 4h00, 8h00, 12h00, 16h00 and 20h00. The HPLC with electrochemical (amperometric) detection system is presented on Fig. 2.4 on the next page. An improved version of the method originally designed by Nagao and Tanimura (1988, 1989) was used for serotonin detection. Each brain (including protocerebrum, antennal lobes and optic lobes) was dissected and homogenized in a micro-glass homogenizer (Wheaton, Millville, NJ, USA) in 50 μl of ice-cold 0.1 M perchloric acid containing 12.5 ng/ml 3,4-dihydroxybenzylamine (DHBA) as the internal standard. After stirring for 3 minutes on ice, the homogenate was centrifuged at 15000 rpm for 30 min at 0 °C. The supernatant was transferred to a micro-vial for immediate injection onto a 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 $\times$ 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 850 mV against a Ag/AgCl reference electrode which was maintained at 30 °C. Signals from the electrochemical detector were recorded and integrated by using data analysis software (Millennium, Waters). The mobile phase contained 0.18 M monochloroacetic acid and 6 mg/l of EDTA disodium salt and was adjusted to pH 3.6 with sodium hydroxide. 1.6 mM of sodium-1-octanesulfonate and 9 % (v/v) acetonitrile were added to the solution. The mobile phase buffer was filtered

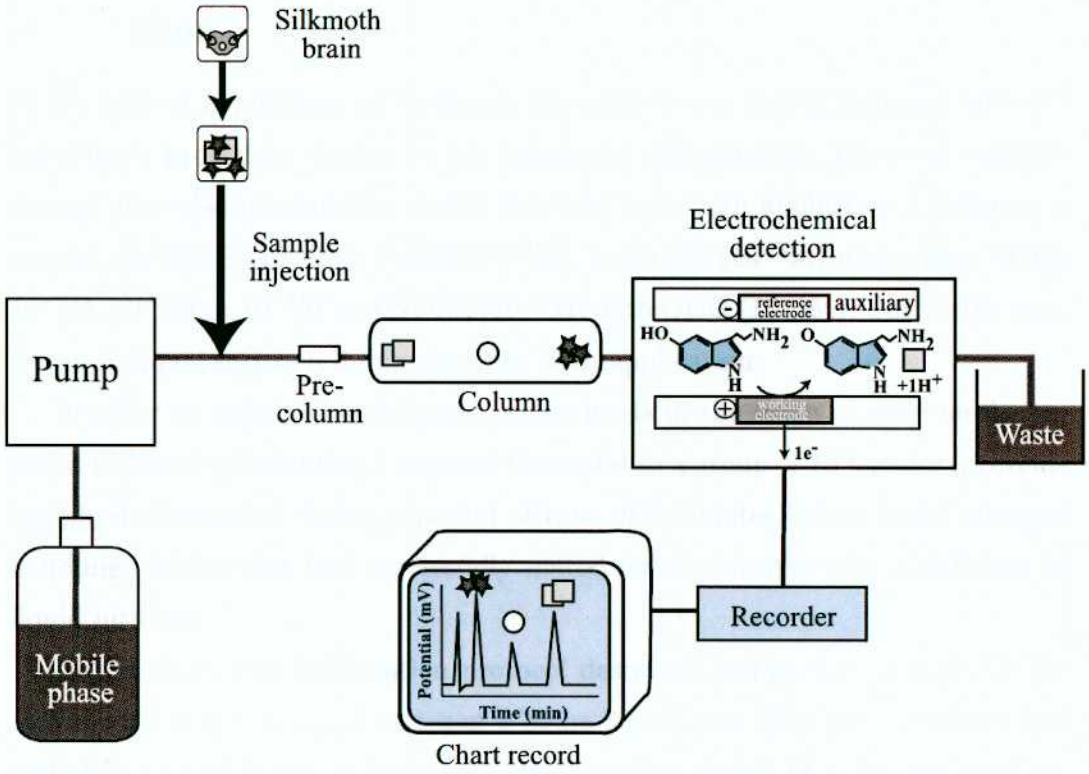


Figure 2.4: High performance liquid chromatography (HPLC) system with electrochemical detection
After dissection, the brains were sonicated and injected in the HPLC system. In the HPLC column, the biogenic amines were separated according to their physical properties such as their size, shape, charge, hydrophobicity, and affinity for the static molecules in the column. Each substance was quantified through amperometric detection, where the potential detector was usually set at 850 or 950 mV.

through a 0.22 μl filter (GVWP 04700, Millipore, Bedford, MA) and degassed under vacuum. The flow rate was kept constant at 0.7 ml/min.

2.5.2 Drug application, pheromone exposure and habituation

In the case of application of serotonin, the moths were frozen in liquid nitrogen following a procedure similar to the behavioral experiments. Levels of biogenic amines were also measured in moths that had been bath applied with saline as a control, L-NAME (10^{-3}M), NOR3 (10^{-3}M), L-NAME (10^{-3}M) with either NOR3 10^{-2}M, 10^{-3}M or 10^{-4}M and NOR3 (10^{-3}M) with ODQ (10^{-4}M). The moths were frozen in liquid nitrogen 3 minutes after bath application.

In order to measure the biogenic amine levels in the brain of male moths exposed to female pheromone, I exposed the males to a group of 10 females (preventing physical contact) during a period of time of 5 minutes before liquid nitrogen handling. Males that had successfully mated for 10 minutes were also frozen in liquid nitrogen.

In the short term habituation protocol described in Fig. 2.2 on page 16, the moths were frozen in liquid nitrogen 2 hours 30 minutes after the sensitivity test and after a second series of high concentration of bombykol (3 $\times$ 500 ms, 1000 ng, interstimulus interval = 500 ms) for the habituation and dishabituation groups and linalool or citral (3 $\times$ 200 ms, 1000 nl, interstimulus interval = 200 ms) for the sensitization and dishabituation group. In the long term habituation protocol described in Fig. 2.3 on page 18, the moths were frozen in liquid nitrogen one hour after the sensitivity test on the fourth day of the experiments.

Each brain (including protocerebrum and ALs) was dissected and transferred into an Eppendorf tube containing 50 μl of 10 ng/ml isoproterenol (as the internal standard), 100 μM EDTA disodium salt and 0.1 M perchloric acid. The samples were sonicated for 3 min and centrifuged at 15 000 rpm for 30 min at 4 °C. The

supernatant was injected directly to the column. The method consisted of an HPLC system (HTEC-500, Eicom, Kyoto, Japan), a refrigerated automatic injector (234, Gilson, Middleton, WI), 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 mM citrate-acetate buffer (pH 3.6). 0.58 mM of sodium-1-octanesulfonate and 12 % (v/v) methanol were added to the solution. The flow rate was kept constant at 0.5 ml/min. The detector was set at a working potential of 950 mV against a Ag/AgCl reference electrode and kept at 23 °C. External standards were run at the beginning and at intervals throughout the runs. This system usually allowed the simultaneous measurement of levels of serotonin, *N*-acetylserotonin (Nac-5HT), 5-hydroxytryptophan (5HTP), octopamine, tyramine, dopamine, *N*-acetyldopamine (NADA). Is several samples, some biogenic amine levels could not be measured due to superimposition with unknown peaks.

In both HPLC systems, 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. (St. Louis, MO, USA) except for SOS (Sodium 1-Octanesulfonate), acetonitrile (Nacalai Tesque, Kyoto, Japan), monochloroacetic acid and sodium hydroxide (Wako, Osaka, Japan).

2.6 Data analysis

Sensitivity to different pheromone concentrations was analyzed with the General Linear Model (GLM) Univariate (Edwards, 1993), followed by the Bonferroni or Least Significant Difference adjustment for multiple comparisons among groups. The dependent variable was the number of moths fluttering their wings; the inde-

pendent variables were pheromone concentration (0.01, 0.03, 0.1, 0.3, 1, 2.5, 5, 10, 30, 100) and treatment (control, drug and wash) in the case of drug application, pheromone concentration (0.05, 0.1, 0.5, 1, 5, 10, 50) and time of day (0h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, 18h, 20h, 22h) in the case of circadian behavioral variation and groups (control, odorant, dishabituation with linalool or citral, habituation) in the case of short term and long term habituation. Figs. 3.10 on page 36, 3.12 on page 39 and 3.1 on page 26 show the detailed behavioral responses for each pheromone concentration while Figs. 3.11 on page 38 and 3.13 on page 40 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. 3.10 on page 36, 3.12 on page 39 and 3.1 on page 26) and of difference of responses between drug and control (Figs. 3.11 on page 38 and 3.13 on page 40) 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 a one-way ANOVA followed by Tukey's pairwise comparison. Levels of biogenic amines after exposure to pheromone were evaluated using a one-way ANOVA followed by the Least Significant Difference pairwise comparison.

Similarly, levels of neuromodulators after nitric oxide related drug application as well as levels of neuromodulators in the antennal lobes and protocerebrum in short and long term habituation were evaluated using a one-way ANOVA followed by the Least Significant Difference pairwise comparison.

The spontaneous reaction following NOR3 application was evaluated using the Fisher exact test.

The relationship between serotonin levels in the brain and the pheromone sensitivity within 24 hours was analyzed with the Pearson correlation coefficient.

In all cases, significant difference was set at $P<0.05$. All statistical analysis were performed using commercially available software (SPSS, Chicago, IL, USA).