Chapter 3

Results

3.1 Variation of internal cues

3.1.1 Circadian variation

Behavior and circadian variation

Males *Bombyx mori*'s sensitivity to pheromone was measured during a period of 24 hours, by confronting the moths with a concentration gradient of synthetic pheromone (Fig. 3.1 on the following page). The N value for each group varied between 41 and 88. 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 6:00) rose until noon (the percentage increased to 63 %) before decreasing until the scotophase (the number dropped to 16 % at 22:00). Furthermore, pheromone concentration had a significant effect on the behavioral response ($P<0.001$): at critical concentrations, a two-fold increase in concentration (from 0.5 ng to 1 ng) led to a drastic change of behavioral response to pheromone over 24 hours ($P<0.001$).

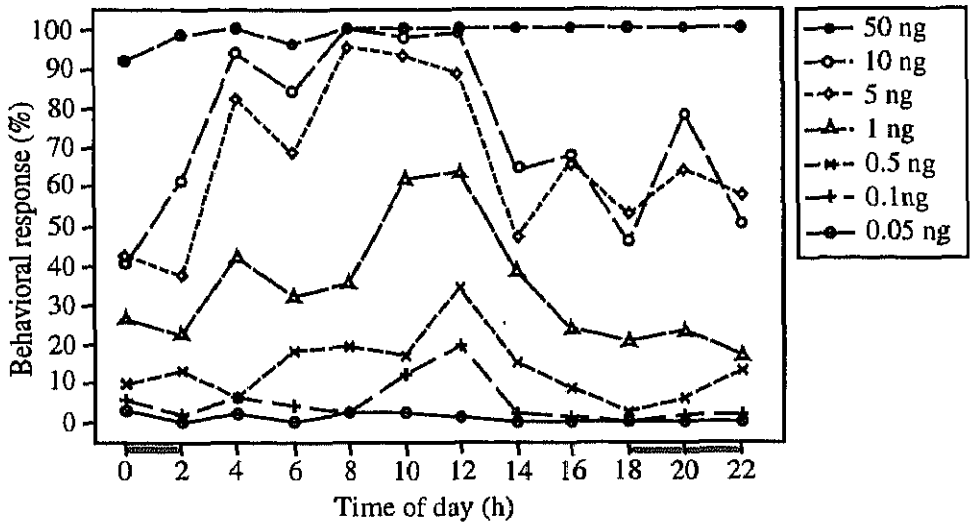

Figure 3.1: Daily variation of the male moth's behavior
The moth was exposed to increasing concentrations of pheromone during 24 hours. Period during which the light was turned off is indicated by a gray bar along the x-axis.

Serotonin and circadian variation

The content of serotonin in the brain was evaluated every 4 hours over 24 hours (Fig. 3.2 on the next page). The N values for each group varied between 6 and 9. From the beginning of the photophase until the noon peak, the concentration remained fairly constant (0.57 pmol/brain). The levels of serotonin in the brain were highest (0.72 pmol/brain) at noon and decreased progressively until 2 hours after the beginning of the scotophase (0.41 pmol/brain). 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). Circadian variation of pheromone sensitivity (Fig. 3.1) strongly correlated with the circadian variation of serotonin in the brain (Fig. 3.2 on the next page) (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).

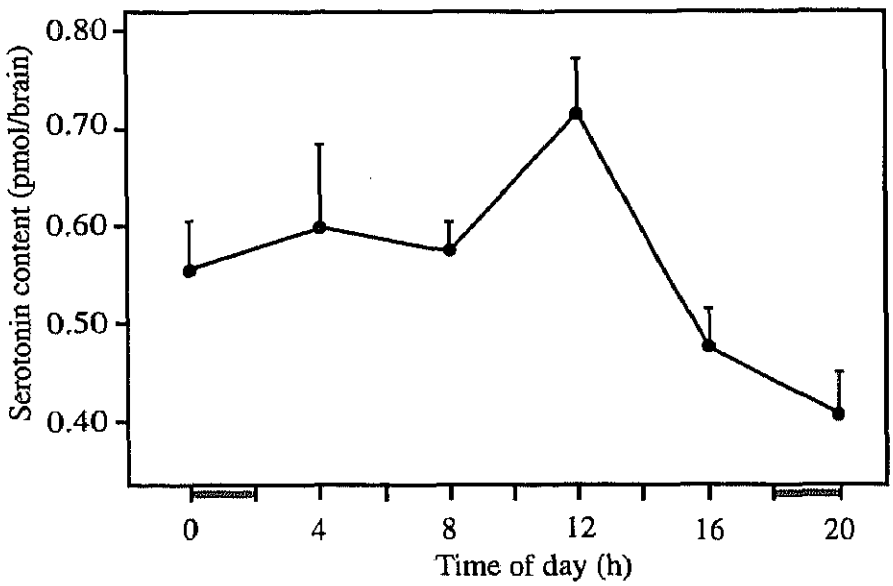

Figure 3.2: Daily variation of serotonin in *B. mori*'s brain
Values are means + S.E.M). The period during which the light was turned off is indicated by a gray bar along the x-axis.

3.1.2 Serotonin immunoreactive neuron

Morphology

A confocal microscope was used to examine in detail the morphology of the only serotonin-immunoreactive neuron in the antennal lobe. Fig. 3.3 on the following page shows the entire brain obtained from confocal stacks of optical section fitted together and the schematic diagram of the brain regions innervated by this neuron. The soma was in the posterior portion of the lateral cell cluster of the left antennal lobe and the neuron had branches in the contralateral side in every ordinary glomerulus and also in each compartment (cumulus, toroid and horsehoe 1 and 2) of the macroglomerular complex. Fig. 3.4 on page 30 shows a thin section of the antennal lobe in which the branchings to all the glomeruli can be seen. The branchings were thick and varicose. This neuron also had processes in both the ipsi- and contralateral superior protocerebrum (PC). Thick branchings were present in the dorsal part of the ipsilateral lateral accessory lobe (LAL) (Fig. 3.5 on page 31). Smooth branchings could be observed in the whole volume of the calyces of both mushroom bodies (Fig. 3.6 on page 32). In both mushroom bodies, a thick process was seen running along the dorsal edge of the calyx. Fine processes were observed in the central body (Fig. 3.7 on page 33) and in both anterior and posterior stacks, thick processes were seen entering the dorsal part of the central body, with finer processes extending through the neuropil.

Physiology

Figs. 3.8 on page 34 and 3.9 on page 35 represent the physiological responses and the instantaneous spike frequency of this neuron respectively to the major component of pheromone, bombykol, the minor component, bombykal and the control (hexane), recorded from the axon, with a spike frequency of $\simeq$ 10 Hz. The background frequency level was $\simeq$ 2.5 Hz. The response of this neuron to these 3 stimuli was clearly an excitation (Fig. 3.9 on page 35) which allows to postulate

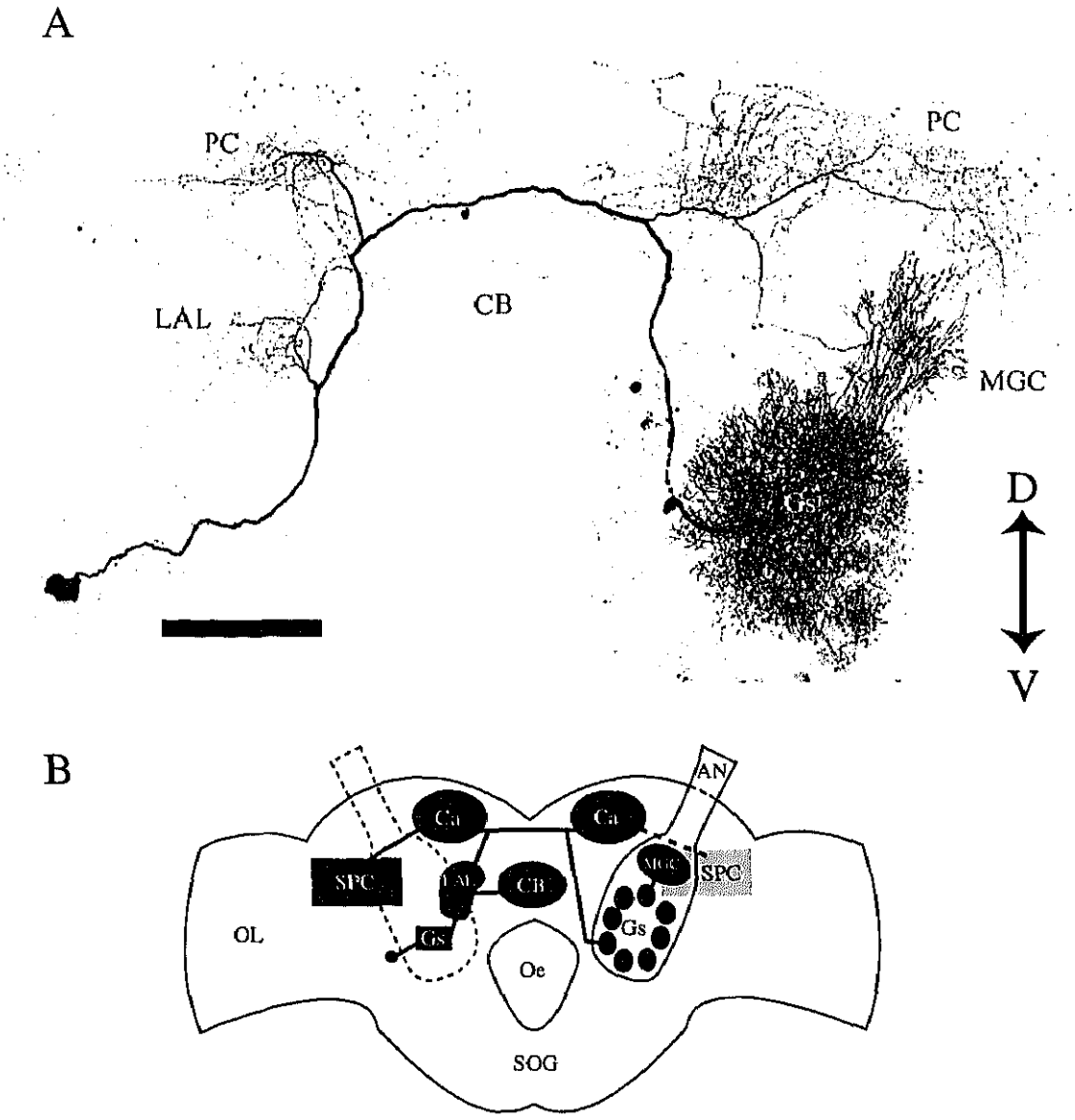

Figure 3.3: Serotonin immunoreactive neuron in the antennal lobe
A. Confocal image of the serotonin-immunoreactive neuron (frontal view): branchings in most of the neuropils in the brain. B. Schematic diagram of the brain regions innervated with this neuron. Scale bar = 100 μm. AN, antennal nerve; Ca, calyx of the mushroom body; CB, central body; Gs, ordinary glomeruli; LAL, lateral accessory lobe; MGC, macroglomerular complex; Oe, oesophagus; OL, optic lobe; SOG, suboesophagal ganglion; SPC, superior protocerebrum.

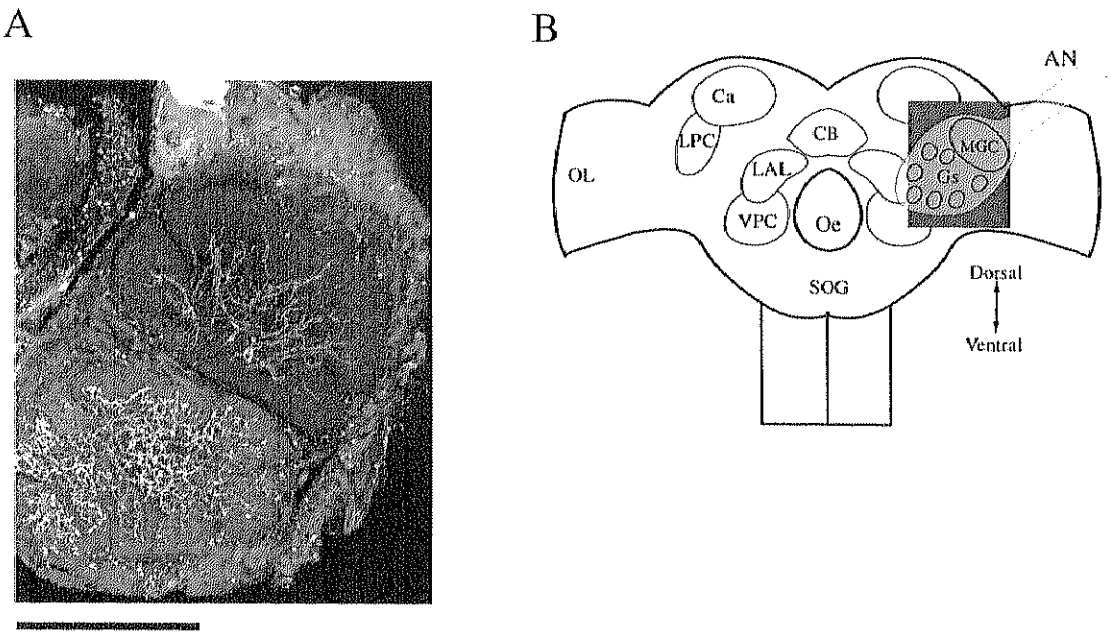

Figure 3.4: Antennal lobes of the serotonin-immunoreactive neuron
A. Confocal image of the contralateral antennal lobe (frontal view): arborization in all the glomeruli. Branchings were thick and varicose. Scale bar = 100 μm. B. Schematic diagram of the brain showing the position of the confocal image in A (dark square). AN, antennal nerve; Ca, calyx of the mushroom body; CB, central body; Gs, ordinary glomeruli; LAL, lateral accessory lobe; LPC, lateral protocerebrum; MGC, macroglomerular complex; Oe, oesophagus; OL, optic lobe; SOG, suboesophagal ganglion; VPC, ventral protocerebrum.

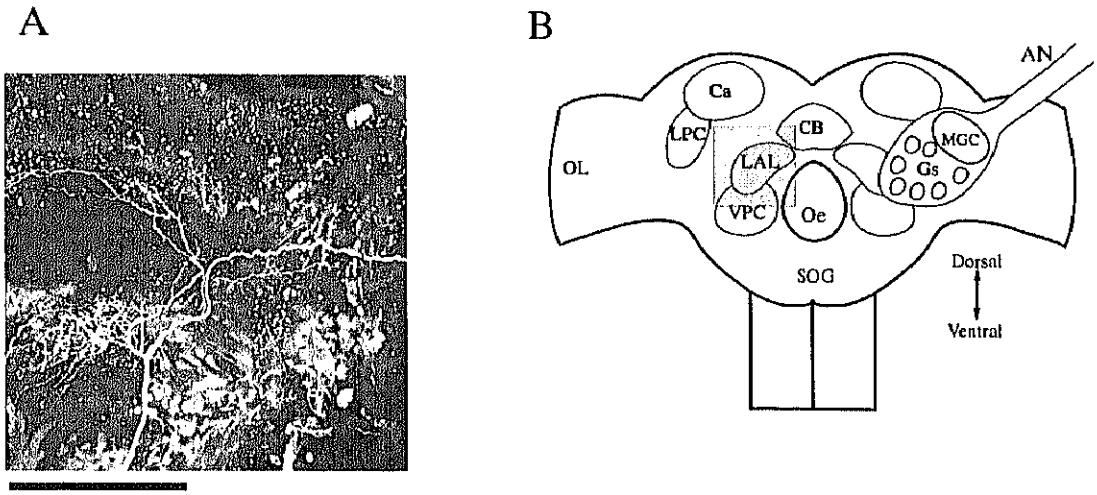

Figure 3.5: Lateral accessory lobe of the serotonin-immunoreactive neuron A. Confocal image of the ipsilateral LAL (frontal view): thick and varicoses branchings are observed in the dorsal part of the LAL. Scale bar = 100 μm. B. Schematic diagram of the brain showing the position of the confocal image in A (dark square). AN, antennal nerve; Ca, calyx of the mushroom body; CB, central body; Gs, ordinary glomeruli; LAL, lateral accessory lobe; LPC, lateral protocerebrum; MGC, macroglomerular complex; Oe, oesophagus; OL, optic lobe; SOG, suboesophagal ganglion; VPC, ventral protocerebrum.

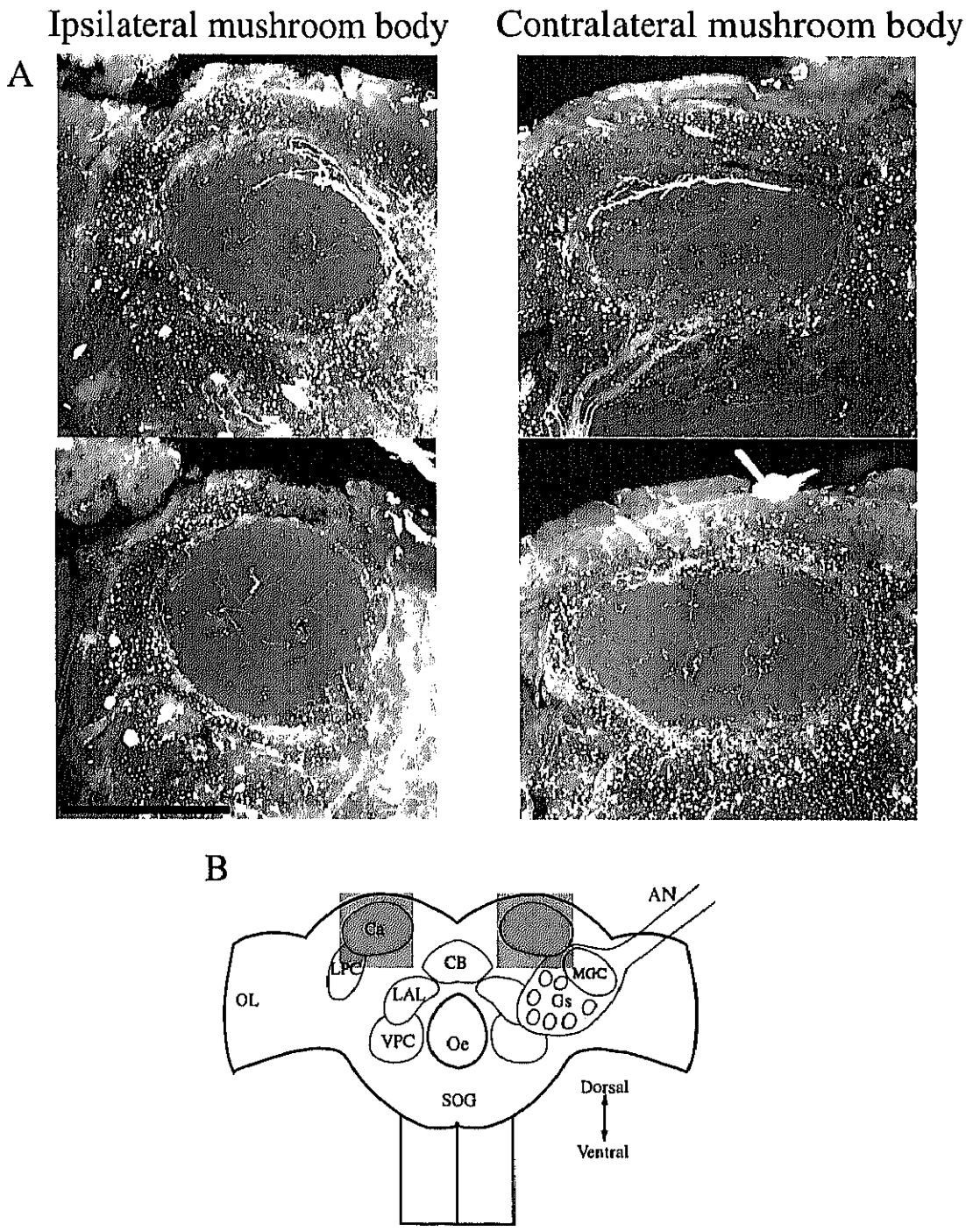

Figure 3.6: Calyces of mushroom bodies of the serotonin-immunoreactive neuron A. Confocal image of the ipsilateral and contralateral calyces (frontal view). Arborizations (very fine processes) were present in the entire volume of both calyces. Upper figures are anterior stacks and lower figures are posterior stacks. Scale bar = 100 μm. B. Schematic diagram of the brain showing the position of the confocal image in A (dark square). AN, antennal nerve; Ca, calyx of the mushroom body; CB, central body; Gs, ordinary glomeruli; LAL, lateral accessory lobe; LPC, lateral protocerebrum; MGC, macroglomerular complex; Oe, oesophagus; OL, optic lobe; SOG, suboesophagal ganglion; VPC, ventral protocerebrum.

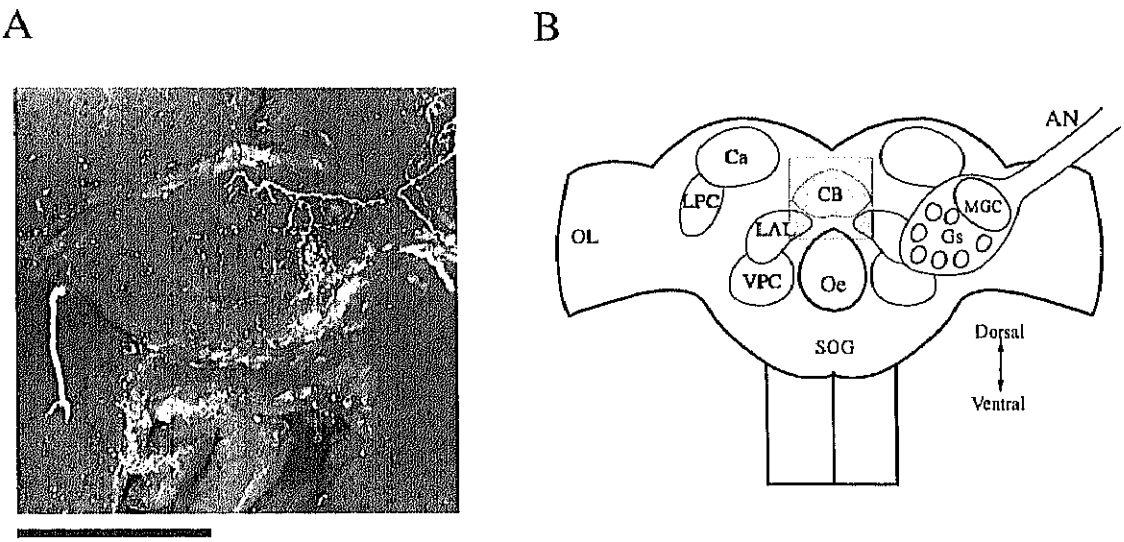

Figure 3.7: Central body of the serotonin-immunoreactive neuron
A. Confocal image of the central body (frontal view). Fine processes were observed throughout the entire central body and a thick process was seen running along the dorsal part of the central body. The image is a stack of 30 individual confocal sections. Scale bar = 100 μm. B. Schematic diagram of the brain showing the position of the confocal image in A (dark square). AN, antennal nerve; Ca, calyx of the mushroom body; CB, central body; Gs, ordinary glomeruli; LAL, lateral accessory lobe; LPC, lateral protocerebrum; MGC, macroglomerular complex; Oe, oesophagus; OL, optic lobe; SOG, suboesophagal ganglion; VPC, ventral protocerebrum.

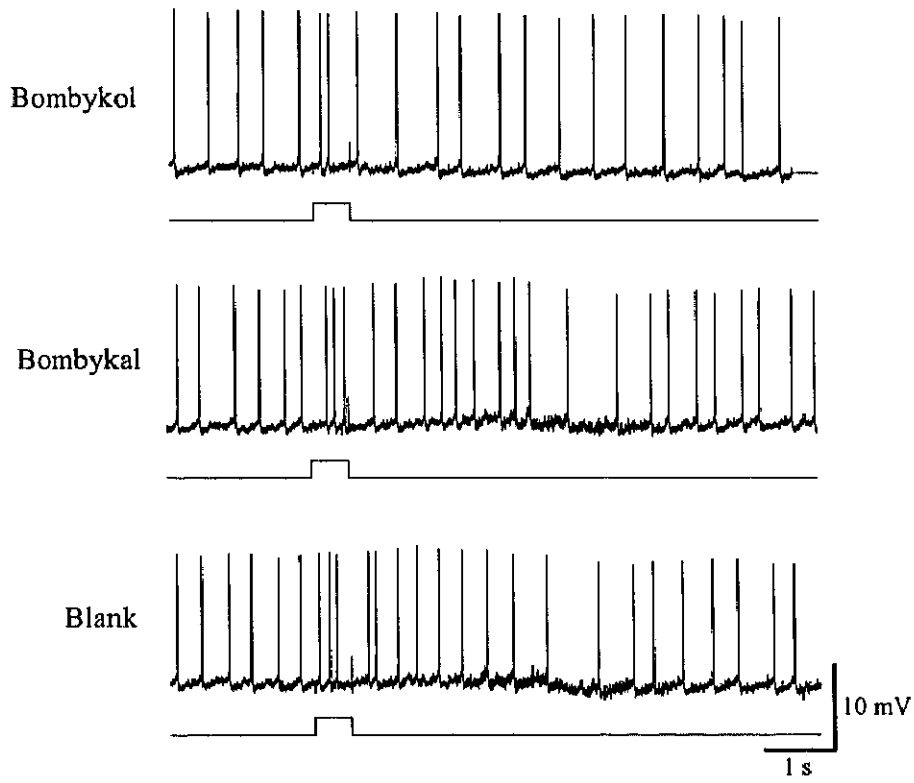

Figure 3.8: Physiological responses of the serotonin-immunoreactive neuron A. Response of the neuron to bombykol. B. Response of the neuron to bombykal. C. Response of the neuron to the blank. The background spike frequency was regular and the neuron showed a very weak response to the 3 stimuli. The lower trace indicates the stimulus (500 ms).

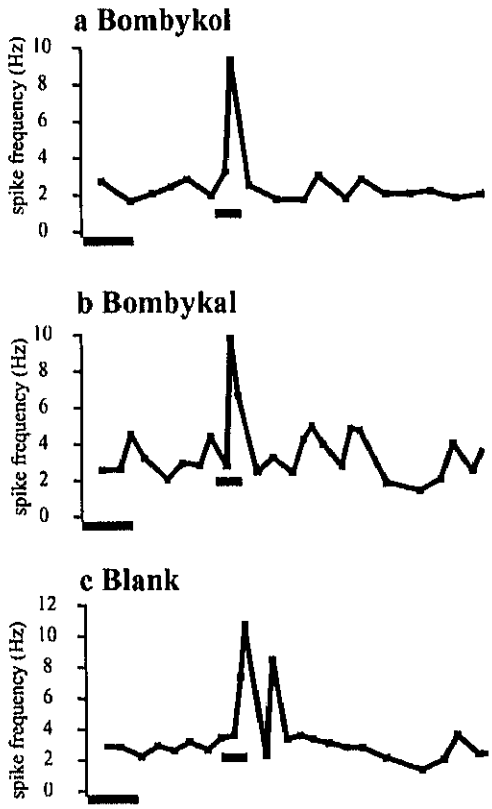

Figure 3.9: Instantaneous spike frequency of the serotonin-immunoreactive neuron A. Response of the neuron to bombykol. B. Response of the neuron to bombykal. C. Response of the neuron to the blank. The neuron responded to the 3 stimuli with a brief increase in spike frequency, reaching a peak spike frequency of $\simeq$10 Hz. Scale bar = 1 s. Stimulus = 500 ms.

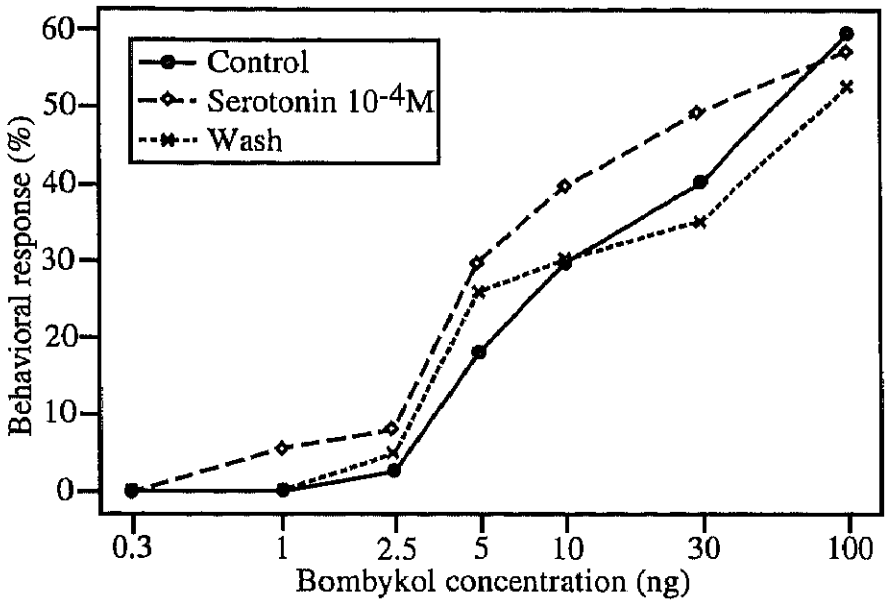

Figure 3.10: Effect of serotonin (10^{-4}M) on the moth's sensitivity to pheromone

that this neuron is mechanosensory rather than specifically pheromone-sensitive. The neuron did not respond to light stimulation (data not shown).

3.1.3 Serotonin and sensitivity to pheromone

Behavior

Serotonin Serotonin [10^{-5} (N=129), 10^{-4} (N=73) and 10^{-3}M (N=60)] was applied to the desheathed antennal lobes 3 minutes before testing the moths' sensitivity with increasing concentrations of synthetic pheromone, bombykol. The moths' sensitivity to pheromone was also tested by application of saline 2 hours previous to and 24 hours following the serotonin injection, as a control and a wash. Fig. 3.10 presents the percentage of control, serotonin applied (10^{-4}M) 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). I found that serotonin shifted the behavioral curve of response to pheromone to

the left: the moths became more sensitive to pheromone (control-drug: $P<0.03$) and reverted after 24 h (drug-wash: $P<0.03$; control-wash: P=NS). In the control, where the moths had been applied with saline, 18 % of the subjects responded to a 5 ng pheromone concentration, while with 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 besides 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. 3.11 on the next page): application of a lower concentration (10^{-5}M) did not lead to a significant variation of the behavioral sensitivity to pheromone (Mean difference = -2.27 ± 1.61, P=NS), application of an intermediate concentration (10^{-4}M) increased the behavioral response (Mean difference = +5.68 ± 1.85, $P<0.03$) while application of a higher concentration (10^{-3}M) provoked an inhibition of the behavioral sensitivity (mean difference = -13.81 ± 3.65, $P<0.01$). The time taken by the moths to react to pheromone was also measured when serotonin 10^{-4}M was applied. Serotonin was not related to the reaction time to pheromone (data not shown).

Serotonin antagonists The effects of application of 2 serotonin antagonists on the behavioral sensitivity were also evaluated. **Mianserin** (10^{-4}M, N=65), a $5HT_{1-2}$ blocker (Dringenberg, 2000; Tierney, 2001) shifted the response to pheromone to the right in comparison with the control (N=65), as shown in Fig. 3.12 on page 39. 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 after 24h. **Ketanserin** is a highly selective $5HT_2$ antagonist (Chen et al., 1999; Dringenberg, 2000; Saifullah and Tomioka, 2003). At 10^{-3}M (N=82), ketanserin decreased the behavioral sensitivity in a significant manner ($P<0.01$, Fig. 3.12 on page 39) in comparison with the control (N=84): over the whole pheromone concentration gradient, the response was lower

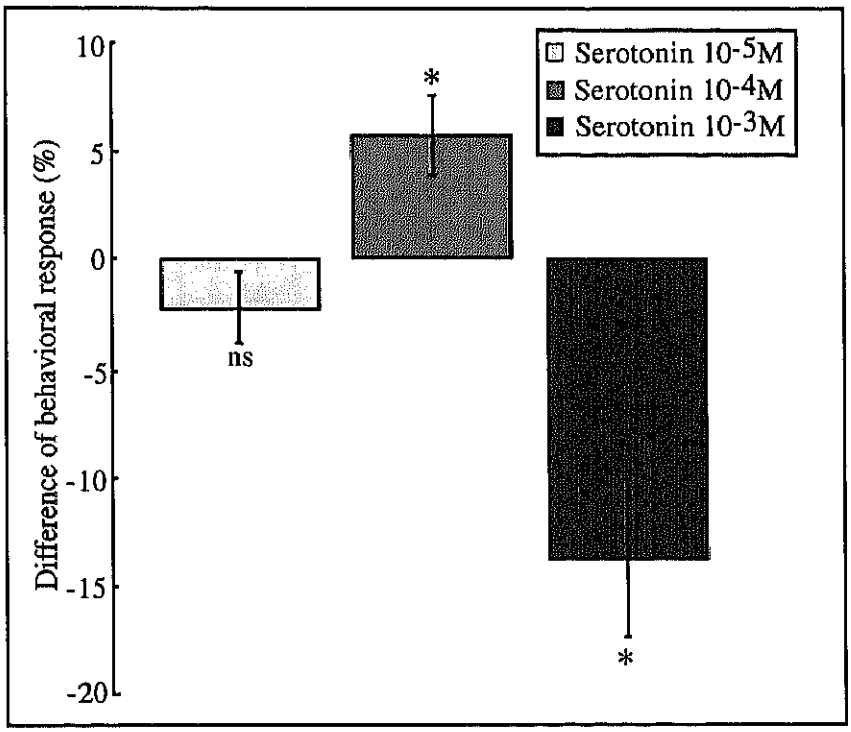

Figure 3.11: Effect of different concentrations of serotonin on the moth's sensivity to pheromone
Difference of means between the percentage of moths that responded to pheromone before and after serotonin application (10^{-3}, 10^{-4} and 10^{-5}M). Values are means ± S.E.M. The asterisks indicate significant difference; ns, non-significant (GLM test followed by the Bonferroni pairwise comparison; $P<0.05$).

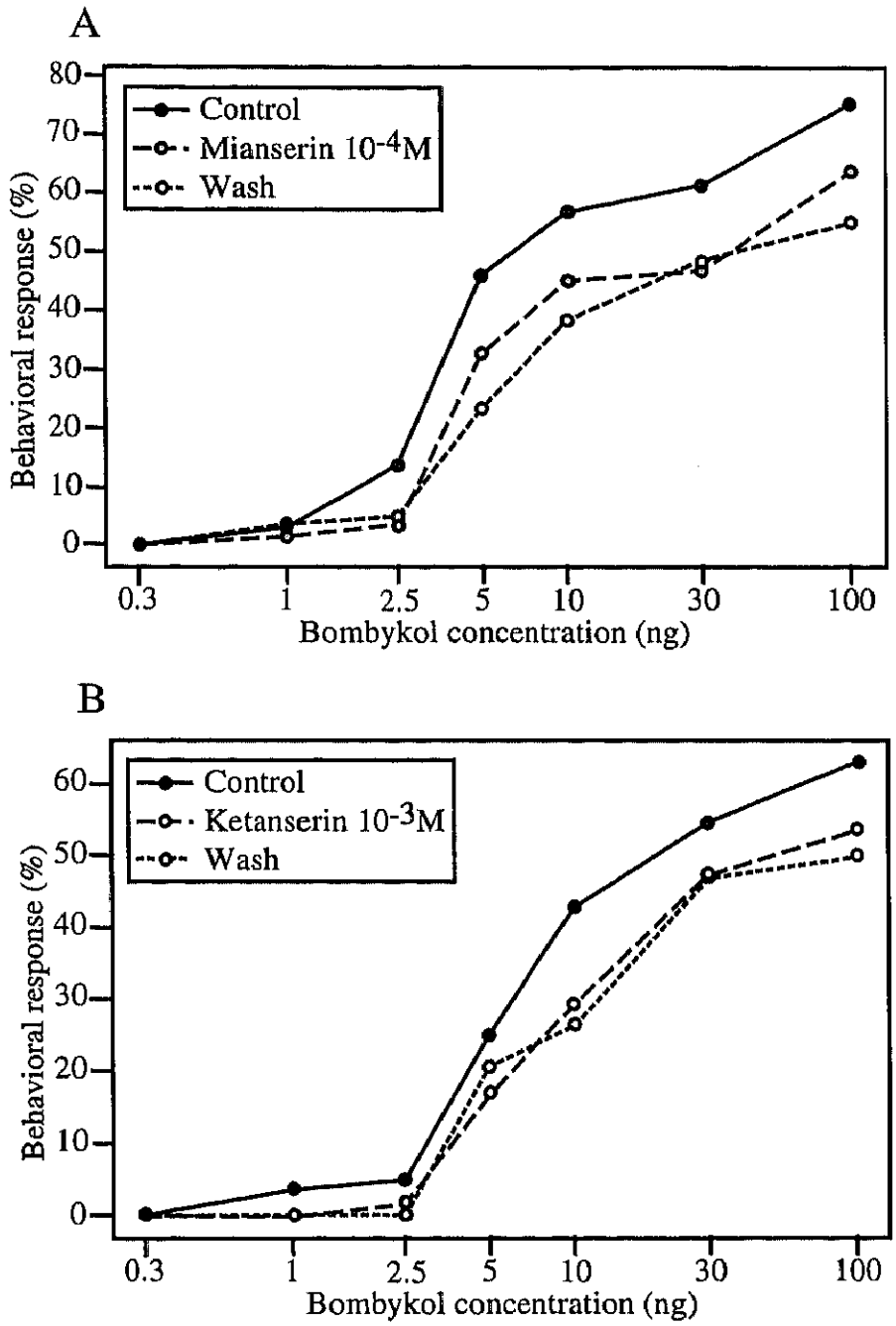

Figure 3.12: Effects of serotonin antagonists on the moth's sensitivity to pheromone
A. Application of mianserin 10^{-4}M. B. Application of ketanserin 10^{-3}M.

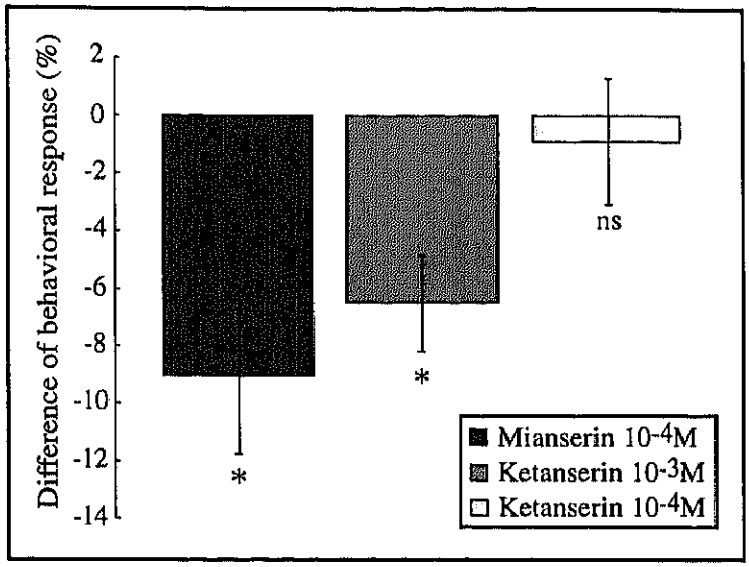

Figure 3.13: Difference of means between the percentage of moths responding to pheromone before and after serotonin antagonist application
Values are means ± S.E.M. Asterisks indicate significant difference; ns, non-significant (GLM test followed by the Bonferroni pairwise comparison; $P<0.05$).

with the drug leading to a drop from 25 % to 17 % with a 5 ng of pheromone exposure. In this case too, the reversion was not totally accomplished after 24h (Mianserin wash, $N=60$; ketanserin wash, $N=34$). The inhibitory effects of serotonin antagonists were dependent on the antagonist type (mianserin versus ketanserin) and on the antagonist concentration (ketanserin 10^{-4}M versus 10^{-3}M) (Fig. 3.13). Ketanserin 10^{-4}M did not have any effect on the moth's sensitivity to pheromone (Mean difference = -0.94 ± 2.20, P=NS) whereas a concentration of 10^{-3}M decreased the behavioral response (Mean difference = -6.47 ± 1.67, $P<0.01$). Mianserin (10^{-4}M) (Mean difference = -9.02 ± 2.69, $P<0.02$) had a stronger effect than both ketanserin 10^{-3}M and 10^{-4}M. The time taken by the moths to react to pheromone was also measured after ketanserin 10^{-4}M application. Ketanserin was not related to the reaction time to pheromone (data not shown).

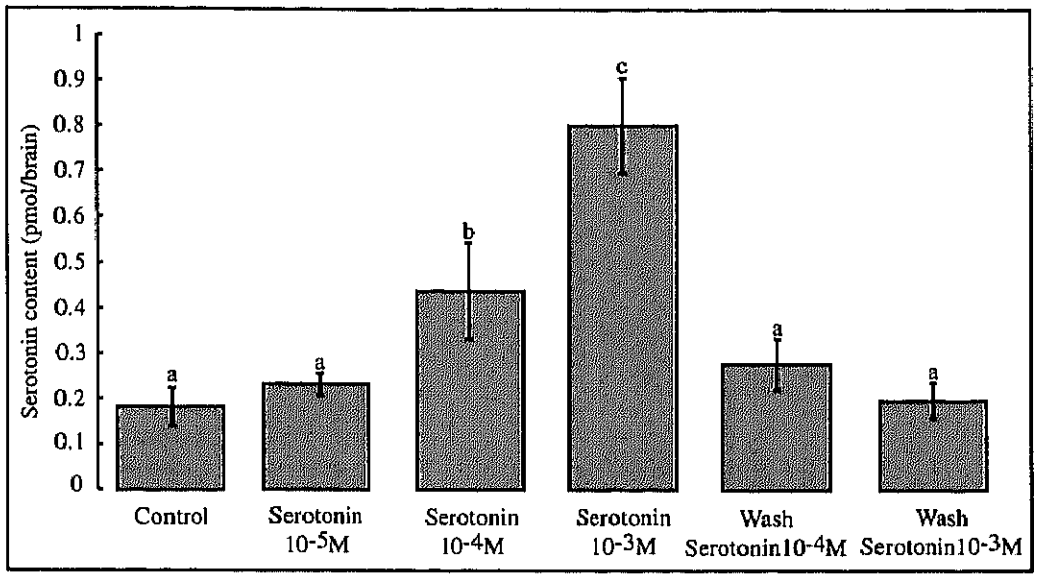

Figure 3.14: Levels of serotonin in the moth's protocerebrum and antennal lobe after application of different concentrations of serotonin
Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (Kruskal-Wallis test; $P<0.05$).

Levels of serotonin in the brain after bath application

Serotonin levels in the brain were measured 3 minutes after application of saline (the control, $N=18$), serotonin 10^{-5} ($N=18$), 10^{-4} ($N=25$) and 10^{-3}M ($N=26$) and 24 hours after serotonin 10^{-4} ($N=9$) and 10^{-3}M ($N=9$) application (the wash) (Fig. 3.14). Application of serotonin 10^{-5}M produced no change in levels in the brain compared with control levels, but there was a significant difference ($P<0.001$) while comparing serotonin 10^{-4} and 10^{-3}M with the control (increasing the control levels, 0.18 pmol/brain, respectively to 0.44 and 0.80 pmol/brain, resulting in an increase of 241 and 424 %). After 24 hours, the levels of serotonin decreased to the control levels.

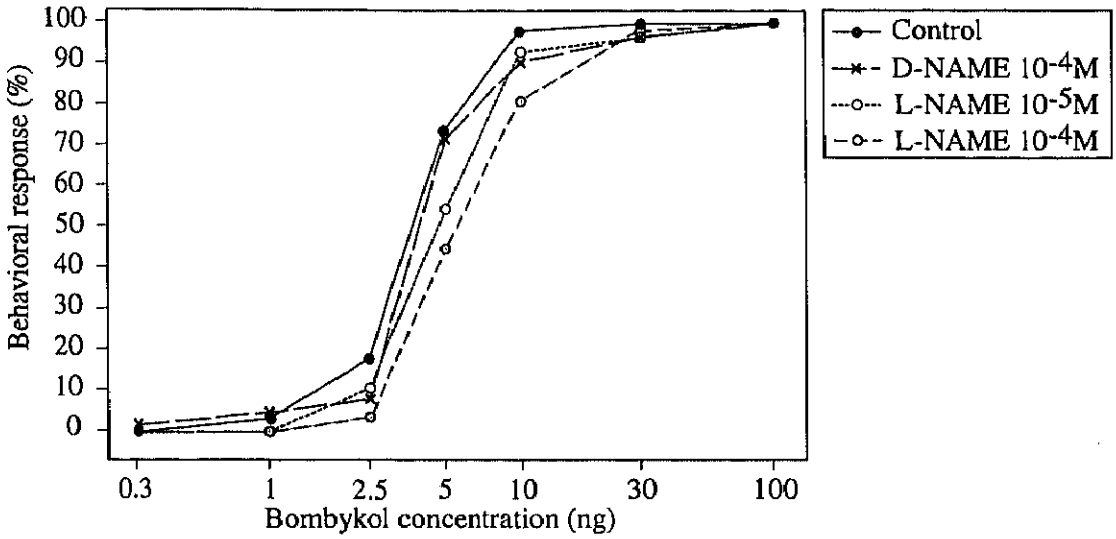

Figure 3.15: Effects of L-NAME (10^{-4}M and 10^{-5}M) and D-NAME (10^{-4}M) on the moth's sensitivity to pheromone

3.1.4 Nitric oxide and sensitivity to pheromone

Sensitivity to pheromone and nitric oxide

Behavioral and pharmacological experiments were performed to analyze the effects of NO-signaling in the phenomenal controls in male silkmoth. As shown in Fig. 3.15 and Fig. 3.16 on the next page, application of L-NAME 10^{-4}M (N=65) decreased the moth's sensitivity to pheromone of more than 8 % (P=0.002) in comparison to the control and of 5.7 % (P=0.042) in comparison with D-NAME 10^{-4}M. Application of D-NAME (the L-NAME enantiomer, N=65) at the same concentration did not significantly affect the behavioral sensitivity even though a slight decrease of response (2.5 %) was observed. The nitric oxide synthase inhibitor may present a dose-dependent effect on the behavioral sensitivity to pheromone, given that L-NAME at a lower concentration (10^{-5}M, N=66) showed a weaker tendency (5.7 %) to shift the sensitivity curve to the right although there was no significant difference between the response due to L-NAME 10^{-5}M and either

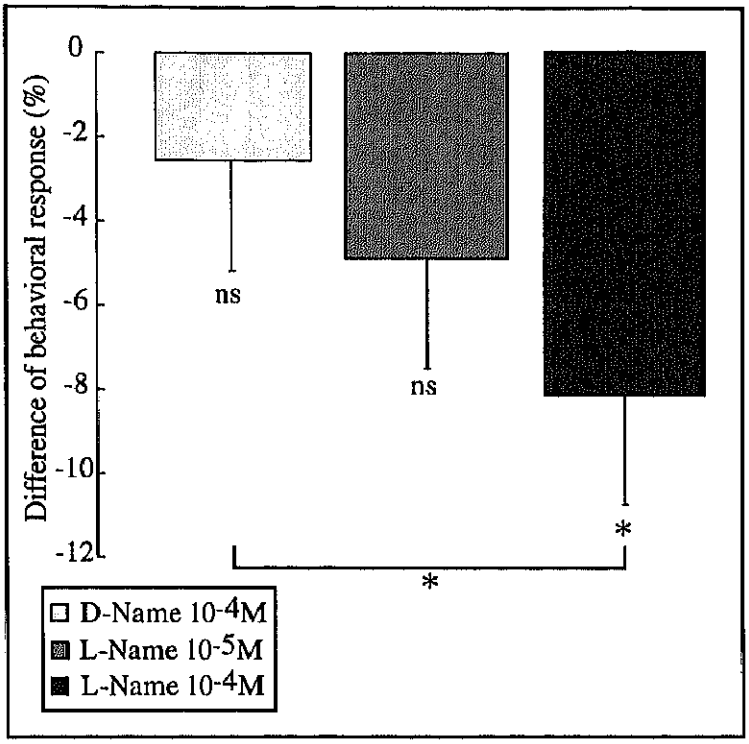

Figure 3.16: Difference of means between the percentage of moths responding to pheromone before and after D-NAME 10^{-4}M, L-NAME 10^{-5}M and L-NAME 10^{-4}M application
Values are means ± S.E.M. Asterisks indicate significant difference; ns, non-significant (GLM test followed by the Least Significant Different pairwise comparison; $P<0.05$).

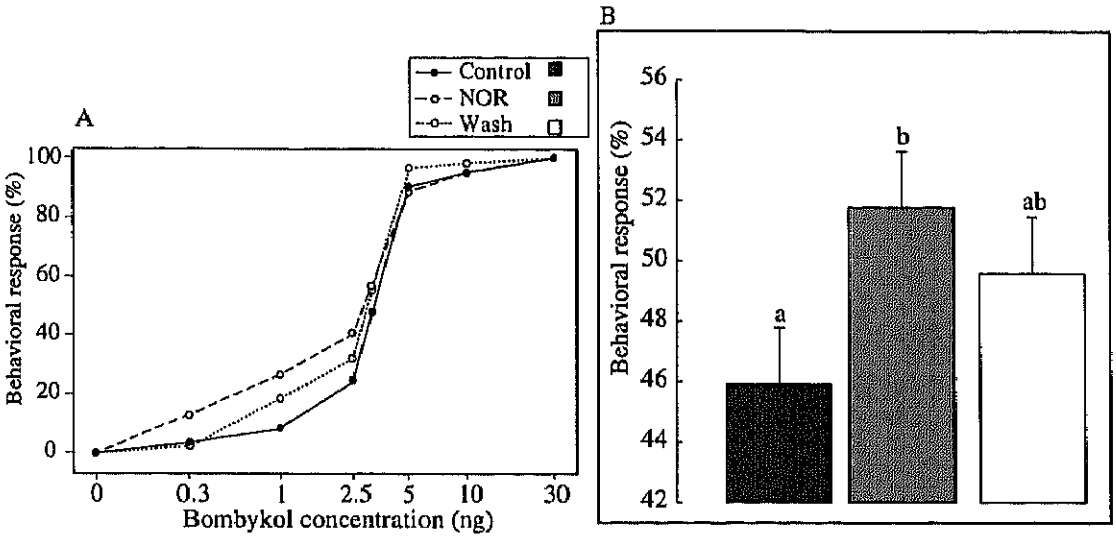

Figure 3.17: Effects of NOR3 (5×10^{-4}M) on the moth's sensitivity to pheromone A. Effect of NOR3 on the behavioral response of the male moth to increasing concentrations of pheromone. B. Means of behavioral response after NOR3 application. Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

L-NAME 10^{-4}M or the two controls D-NAME and saline (N=66). The time taken by the moth to react to pheromone after application of the drugs (D-NAME 10^{-4}M, L-NAME 10^{-4} and 10^{-5}M and control) was also statistically evaluated. There was no significant variation of the moths' reaction time to pheromone (data not shown).

Application of NOR3 (nitric oxide donor, 5×10^{-4}M, N=65) on the brain increased considerably (above 5 %) the response to low pheromone concentrations (0.3, 1 and 2.5 ng; P=0.014, Fig. 3.17) in comparison with the control while the wash showed a partial recovery (3 % above the control) (P=NS). The effects of NOR3 were most visible at low pheromone concentrations. Furthermore, application of NOR3 led to a strong spontaneous wing fluttering as shown on Fig. 3.18 on the next page. More than 47 % of the moths (N=59) fluttered their wings

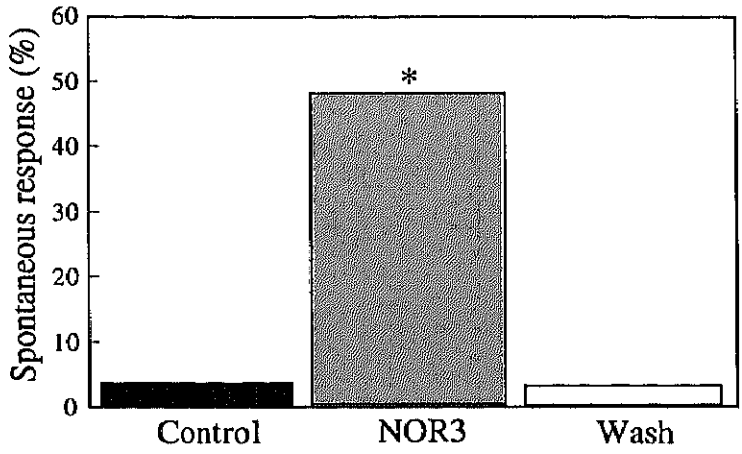

Figure 3.18: Spontaneous wing fluttering following NOR3 (5×10^{-4}M), control and wash
Asterisk indicates significant difference (Fisher's Exact Test; $P<0.05$).

within 30 seconds following NOR3 application while less than 4 % of them reacted to saline prior (control, N=61) or after (wash, N=64) the NOR3 application, which corresponded to a significant difference of $P<0.0001$ in both cases (NOR3 vs control and NOR3 vs wash).

These results suggest that the NO-cGMP signalling pathway mediates olfactory and pheromone processing in the male silkmoth and might have other roles in the behavior such as spontaneous wing fluttering.

Biogenic amines and nitric oxide

The levels of biogenic amines were measured after application of several substances related to nitric oxide in order to assess a potential relationship between nitric oxide and biogenic amines. The role of these substances has been described in the introduction (for details, see Fig. 1.1 on page 7). L-NAME (10^{-3}M) was applied alone or simultaneously with different concentrations of NOR3 (10^{-2}, 10^{-3} and 10^{-4}M). NOR3 (10^{-3}M) was also applied concurrently with ODQ (10^{-4}M). Fig. 3.19 on the next page shows the effects of these drugs on serotonin and

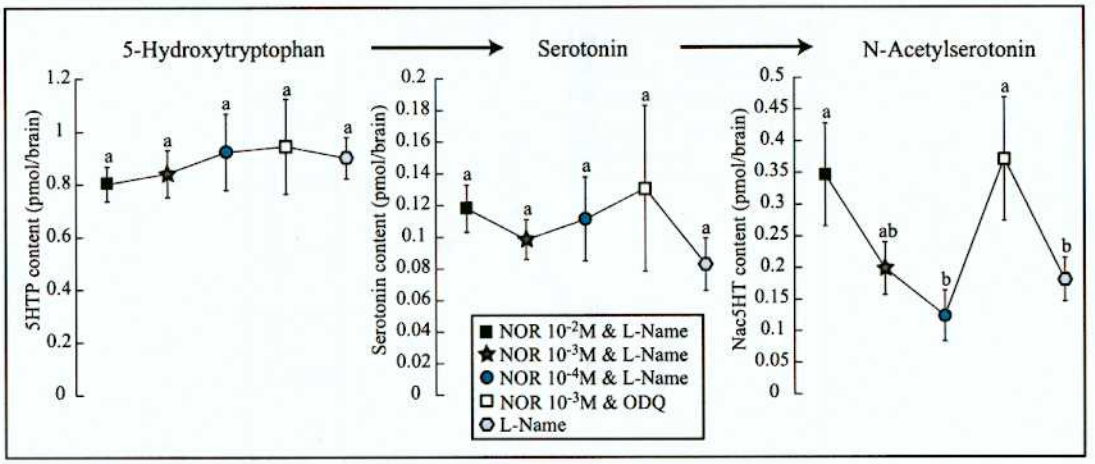

Figure 3.19: Levels of 5-HTP, serotonin and Nac-5HT in brains exposed to different combinations of nitric oxide related drugs
The combinations were: different concentrations of NOR3 (10^{-2}, 10^{-3} and 10^{-4}M) with L-NAME (10^{-3}M), L-NAME (10^{-3}M) and NOR3 (10^{-3}M) with ODQ (10^{-4}M). Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

its precursor (5-hydroxytryptophan, 5-HTP) and metabolite (*N*-acetylserotonin, Nac-5HT). Levels of 5-HTP (N=12 to 23) on the left were steady, varying slightly between 0.8 and 0.95 pmol/brain, unrelated to drug application. Levels of serotonin (N=11 to 30) did not show any significant difference in relationship to drug application. However, levels of serotonin seemed to be lower after L-NAME application than after other drugs application. Levels of Nac-5HT (N=4 to 7), on the right, were significantly lower after L-NAME alone or applied simultaneously with NOR3 10^{-4}M (respectively 0.181 ± 0.035 and 0.124 ± 0.041 pmol/brain) than after L-NAME with NOR3 10^{-2}M and ODQ with NOR3 (respectively 0.347 ± 0.081 and 0.372 ± 0.097 pmol/brain). This suggests that levels of Nac-5HT were higher in presence of NO-donor than in presence of nitric oxide synthase inhibitors.

The levels of tyramine (N=2 to 11) were also related to drug application (Fig. 3.20 on the following page). In this case, levels of tyramine were signifi-

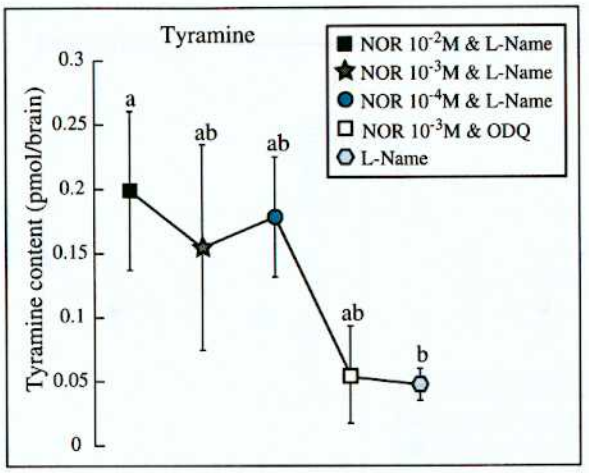

Figure 3.20: Levels of tyramine in brains exposed to different combinations of nitric oxide related drugs
The combinations were different concentrations of NOR3 (10^{-2}, 10^{-3} and 10^{-4}M) with L-NAME (10^{-3}M), -Name (10^{-3}M) and NOR3 (10^{-3}M) with ODQ (10^{-4}M). Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

cantly higher (0.199 ± 0.062 pmol/brain) after application of L-NAME with NOR3 10^{-2}M than after L-NAME alone (0.047 ± 0.013 pmol/brain). Lower concentration of NOR3 (10^{-3} and 10^{-4}M) did not have any significant effect when applied with L-NAME, even though a similar tendency could be observed. ODQ applied with NOR3 was related to low levels of tyramine in the brain, although no significant difference could be observed. Similarly to Nac-5HT, tyramine levels appear to vary depending on NO-donor and nitric oxide synthase-inhibitor presence.

Fig. 3.21 on the next page shows the levels of dopamine and its metabolite *N*-acetyldopamine (NADA) (respectively N=9 to 25 and N=11 to 28). Both graphs show a noticeable similarity. Levels of dopamine on the left were higher in presence of L-NAME with NOR3 10^{-2}M (10.40 ± 1.24 pmol/brain) than after L-NAME alone (5.35 ± 1.14 pmol/brain, P=0.002) or after L-NAME with NOR3 10^{-4}M (5.75 ± 0.80 pmol/brain, P=0.012). When an intermediate concentration

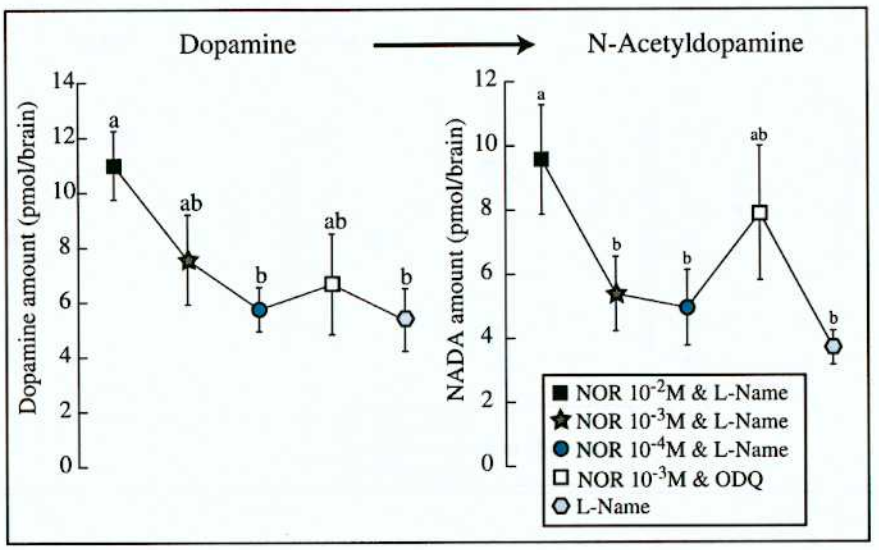

Figure 3.21: Levels of dopamine and *N*-acetyldopamine in brains exposed to different combinations of nitric oxide related drugs
The combinations were different concentrations of NOR3 (10^{-2}, 10^{-3} and 10^{-4}M) with L-NAME (10^{-3}M), L-NAME (10^{-3}M) and NOR3 (10^{-3}M) with ODQ (10^{-4}M). Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

of NOR3 (10^{-3}M) was applied with L-NAME, dopamine was present at an intermediate concentration (7.56 ± 1.63 pmol/brain). Levels of dopamine were also intermediate when ODQ was applied with NOR3 10^{-3}M (6.66 ± 1.84 pmol/brain). Levels of NADA were also significantly higher when L-NAME was applied with NOR3 10^{-2}M (9.58 ± 1.70 pmol/brain) than when L-NAME was applied alone (3.69 ± 0.54 pmol/brain, P=0.002) or with NOR3 10^{-4}M (4.96 ± 1.18 pmol/brain, P=0.018) but also with NOR3 10^{-3}M (5.39 ± 1.16 pmol/brain, P=0.014). Levels of NADA were intermediate when ODQ was applied with NOR3 10^{-3}M (7.91 ± 2.09 pmol/brain). These results suggest that the concentration of both dopamine and NADA is related to a nitric oxide donor and nitric oxide synthase inhibitor presence in a similar way.

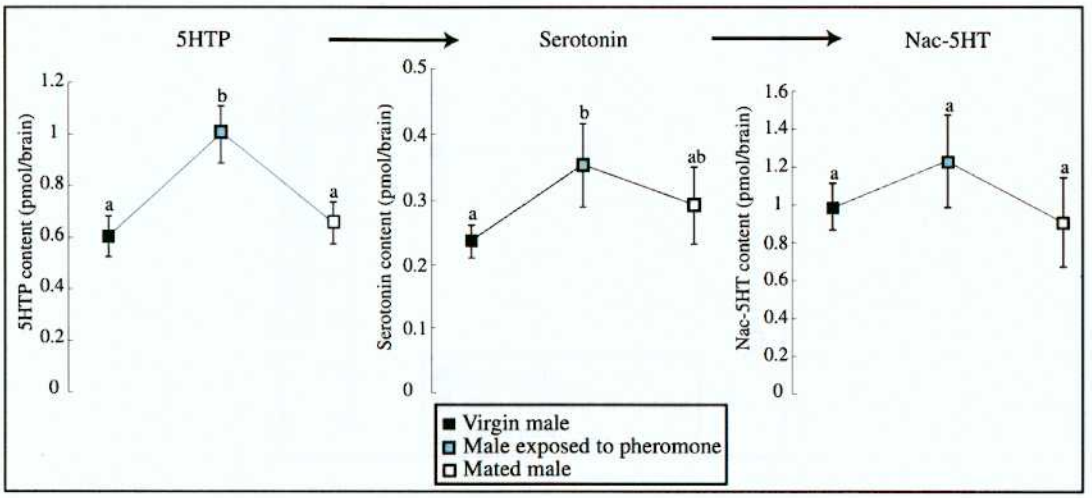

Figure 3.22: Levels of 5-HTP, serotonin and Nac-5HT of virgin males, males exposed to pheromone and mated males
Levels of 5-HTP, serotonin and Nac-5HT were measured in the brain of control moths, moths exposed to pheromone and mated moths. Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

3.2 Variation of external cues

3.2.1 Exposure to pheromone

The levels of biogenic amines were measured in brains of male moths exposed to female pheromone for 5 minutes ("Pheromone exposed males", $N=14$), males having mated for over 5 minutes with a female ("Mated males", $N=9$) and males not having encountered pheromone (the control, "Virgin males", $N=14$). The levels of serotonin, its precursor 5-hydroxytryptophan (5-HTP) and its metabolite N-acetyl-hydroxytryptamine (Nac-5HT) showed the same tendency: the levels increased with exposure to pheromone and decreased after mating (Fig. 3.22). After exposure to pheromone, the levels of serotonin, 5-HTP and Nac-5HT were higher than the control (respectively a 50 %, 46 % and 34 % increase in levels of serotonin 5-HTP and Nac-5HT). A significant difference was obtained with serotonin and

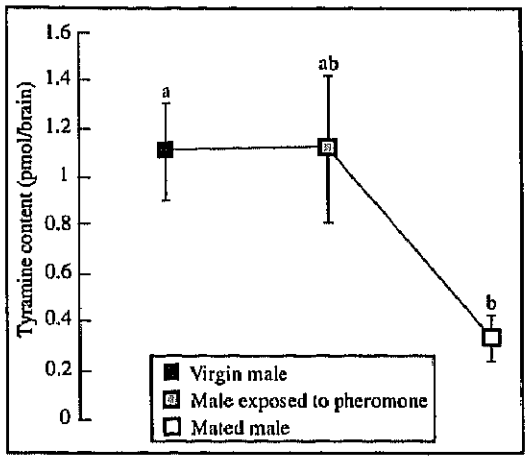

Figure 3.23: Levels of tyramine of virgin males, males exposed to pheromone and mated males
Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

5HTP ($P<0.05$). After mating, the levels of 5-HTP were significantly lower than the levels of males that were exposed to pheromone (39 % decrease, $P<0.02$), while the levels of serotonin and Nac-5HT showed a decreasing tendency (respectively 18 and 32 % decrease). In all cases, the levels of amines in the brains of mated males did not show any significant difference with the levels of control males. Tyramine was related with the mating activity: the levels of tyramine in the brain of moths after performing mating were significantly lower than the control ones (55 % decrease, $P<0.05$)(Fig. 3.23).

Dopamine and its metabolite, *N*-acetyldopamine were also measured in the brains of control, exposed to pheromone and mated male moths, but I could not highlight any relationship between the samples and the drugs (data not shown).

The levels of biogenic amines were also measured in the brain of virgin females ($N=19$)and females after mating ("Mated females", $N=12$) (Fig. 3.24 on the following page). There was no significant difference between the levels of 5-HTP, serotonin, Nac-5HT and tyramine in the brain of virgin females or females that

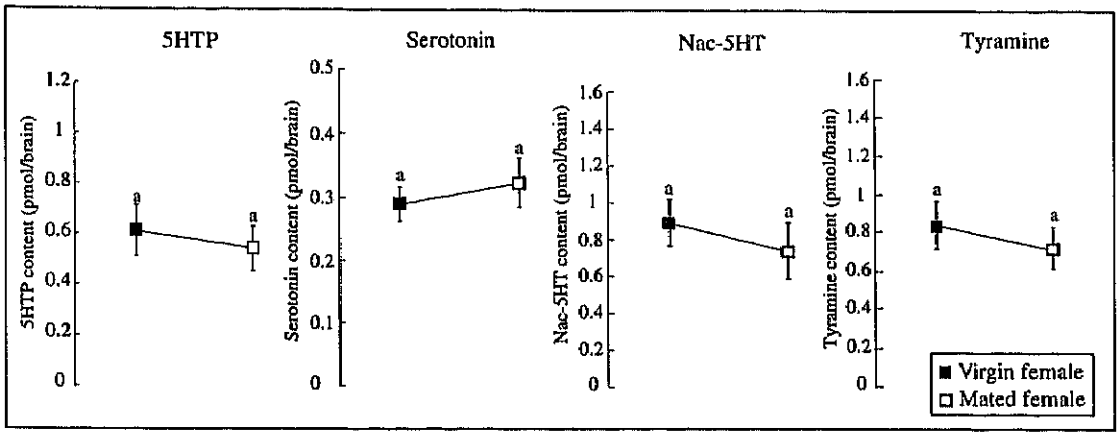

Figure 3.24: Levels of biogenic amines in virgin females and females after mating Levels of 5-HTP, serotonin, Nac-5HT and tyramine were measured in the brain of virgin females and females after mating. The scales are similar to Figs. 3.22 on page 49 and 3.23 on the previous page. Values are means ± S.E.M. No significant difference was observed (GLM test); $P>0.05$).

had mated. The levels of dopamine and NADA showed a similar tendency (data not shown).

3.2.2 Short term habituation

A similar protocol was used in both behavioral and biogenic amines experiments: after the behavioral experiments, the habituation and dishabituation groups were exposed to high level of pheromone (3 × 1000 ng). 30 minutes later, the control and habituation groups were exposed to the sensitivity test. Right before the sensitivity test, sensitization and dishabituation groups were exposed to a brief shock consisting of a short puff of a different odorant (citral or linalool) (for details, see Fig. 2.2 on page 16).

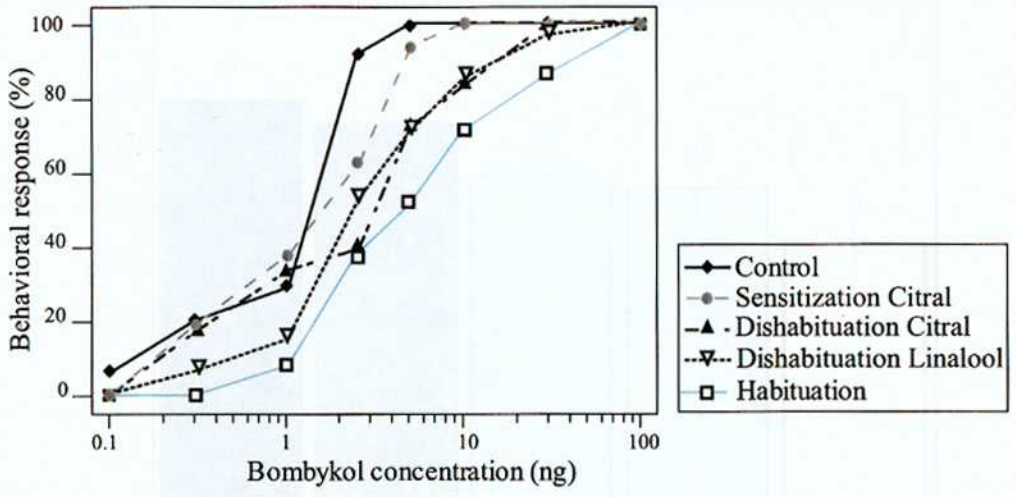

Figure 3.25: Effect of short term habituation, dishabituation and sensitization on the moth's response to pheromone

Short term habituation: behavior

Fig. 3.25 shows the response curve of the 4 groups to different concentrations of bombykol. The habituation curve showed a strong shift to the right at all applied bombykol concentrations (besides the highest) in comparison with all groups (with a small exception at the concentration of 2.5 ng of bombykol in comparison to the "dishabituation due to linalool" group). The difference between the habituation group and the control was superior to 20 % at 5 different concentrations and reached 50 % at 2 bombykol concentrations (2.5 and 5 ng). The 2 dishabituation groups (citral and linalool) showed an intermediate response, between the control and the habituation groups. At low concentrations of bombykol, the response of the "dishabituation due to citral" group was higher than the "dishabituation to linalool" group, but the curves superimposed at higher concentrations. The curve of the sensitization group was intertwined with the control group.

The different effects were analyzed statistically as shown on Fig. 3.26 on the next page. The pheromone response of moths exposed to high concentration of pheromone (1000 ng, habituation group, N=37) was significantly lower than the

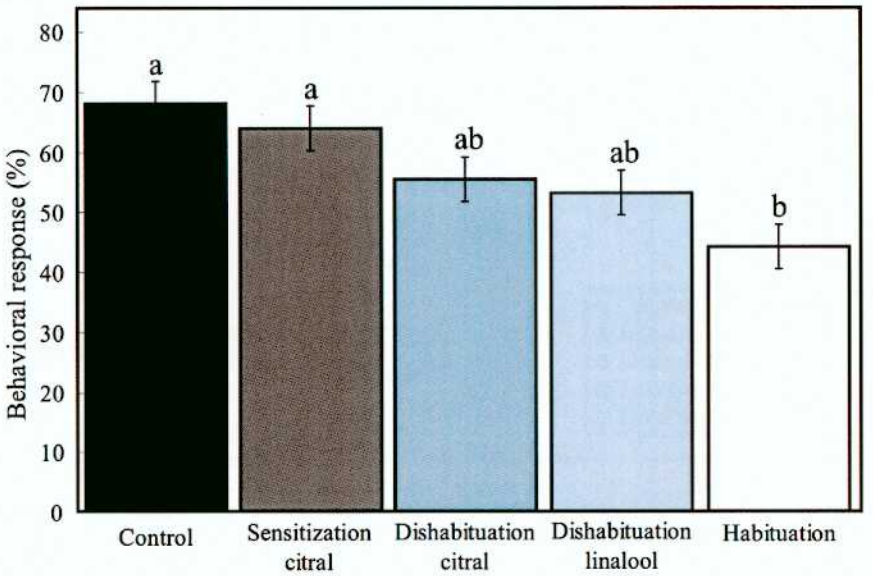

Figure 3.26: Means of behavioral response after short term habituation, dishabituation and sensitization
Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Bonferroni pairwise comparison; $P<0.05$).

control and the sensitization groups (respectively $N=35$, $P=0.001$ and $N=16$, $P=0.009$). The dishabituation groups (citral, $N=18$ and linalool, $N=34$) were not significantly different from the other groups. These results suggest the occurrence of short term habituation and dishabituation. Application of a different stimulus (in this case, short puff of linalool) did not have any effect on the response to pheromone, implying that sensitization cannot be obtained with this protocol.

Short term habituation: biogenic amines

Levels of serotonin, 5-hydroxytryptophan (5-HTP), *N*-acetylserotonin (Nac-5HT), dopamine and *N*-acetyldopamine (NADA) were measured in brains of moths exposed to habituation, dishabituation, sensitization and control moths. Fig. 3.27 on the following page shows the levels of serotonin in the antennal lobes and

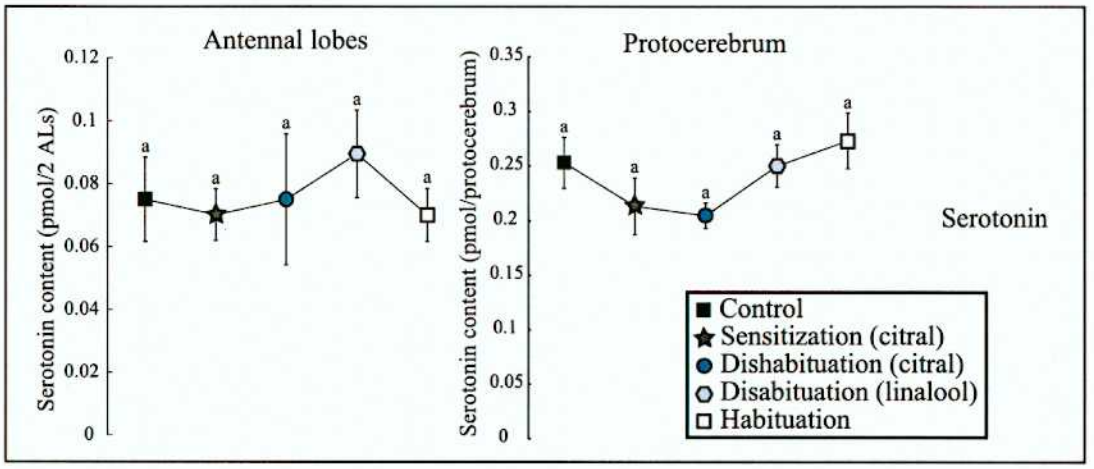

Figure 3.27: Levels of serotonin in the antennal lobes and protocerebrum of male moths after short term habituation, dishabituation and sensitization.
Values are means ± S.E.M. No significant difference was observed (GLM test followed by the Least Significant Difference pairwise comparison); $P>0.05$).

protocerebrum of moths trained to pheromone, exposed to citral (sensitization), dishabituated with citral or linalool, and control moths. I did not observe any significant variation of serotonin in relation to habituation, sensitization or dishabituation, even though the levels of serotonin in the protocerebrum of sensitized moths to citral showed a tendency to be lower than in controls and habituated moths (respectively 28 and 7 % decrease). Similarly to this result, the levels of serotonin in the protocerebrum of moths dishabituated with citral were slightly lower than in that of the controls hand habituated moths (respectively 33 and 14 % decrease), a variation that was not significantly different.

5-HTP (ALs: N=7 to 14 and protocerebrum: N=16 to 31), Nac-5HT (ALs: N=8 to 13 and protocerebrum: N=16 to 32) levels were also measured as shown on Fig. 3.28 on the next page. The 2 graphs (ALs, protocerebrum) of 5-HTP and Nac-5HT show striking similarities with one another and with serotonin.

Levels of 5-HTP and Nac-5HT in the antennal lobes and protocerebrum of moths dishabituated with linalool were slightly higher than when moths were ex-

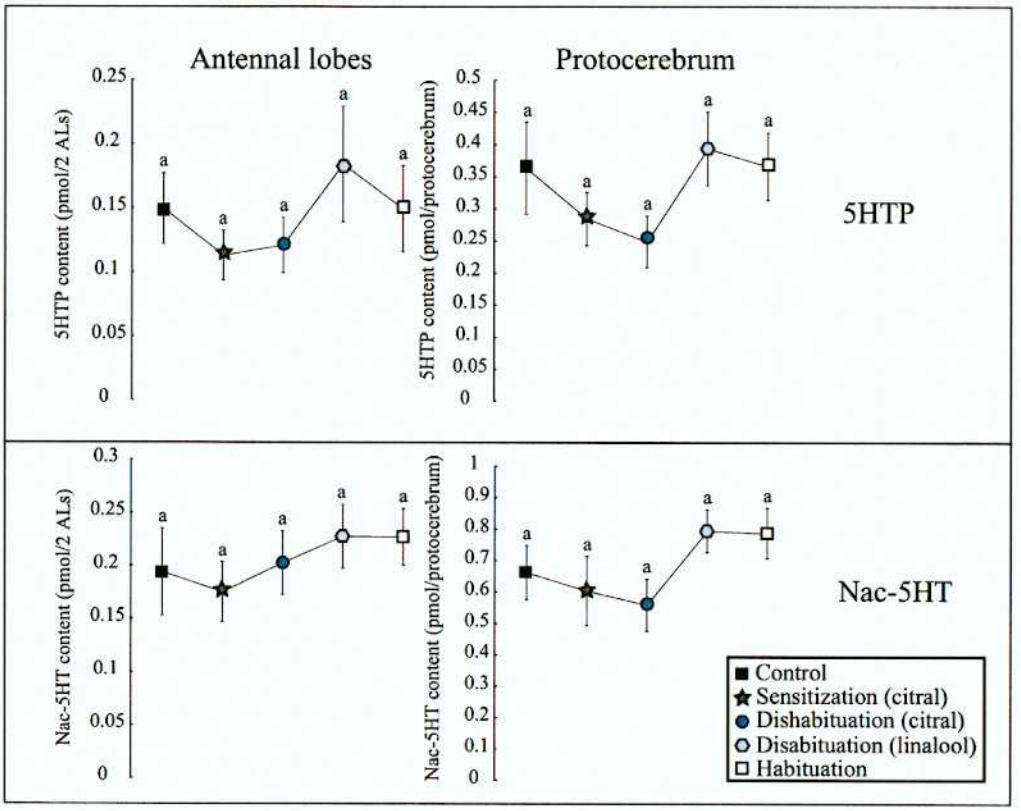

Figure 3.28: Levels of 5-HTP, Nac-5HT in the antennal lobes and protocerebrum of male moths after short term habituation, dishabituation and sensitization. Values are means ± S.E.M. No significant difference was observed (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

posed to citral (exposure to citral alone, sensitization and dishabituation with citral). Even though there was no significant difference for the 3 amines, the similar tendency increases the possibility of a biological meaning of these indolealkylamine variations.

Dopamine (ALs: N=6 to 16 and protocerebrum: N=14 to 31) and NADA (ALs: N=7 to 16 and protocerebrum: N=7 to 27) levels are shown on Fig. 3.29 on the following page. The dopamine levels did not significantly vary in relation to habituation, dishabituation or sensitization even though the levels of dopamine in both the antennal lobes and the protocerebrum of moths exposed to citral seemed to be lower than that in controls and habituated moths. NADA levels were higher in the protocerebrum of moths dishabituated with linalool than exposed to citral (291 %, P=0.042). In the antennal lobe, the control moths showed a higher concentration of NADA than the moths exposed to citral and dishabituated with citral (respectively 218 and 209 %, P=0.018 in both cases).

These results suggest that serotonin, its precursor 5-HTP and its metabolite Nac-5HT are not related to short term habituation. The possibility of a relationship with the exposure to different distinct odorants (in this case, linalool and citral) cannot be excluded. NADA is related to sensitization and dopamine seems to follow a similar tendency.

3.2.3 Long term habituation

During both the behavioral and biogenic amines experiments, the habituation and dishabituation moths were exposed for 3 days to 4 pheromone exposures. On the day following the last exposure, the dishabituation group was exposed to a short puff of linalool shortly before the test. This protocol is exposed in details on Fig. 2.3 on page 18.

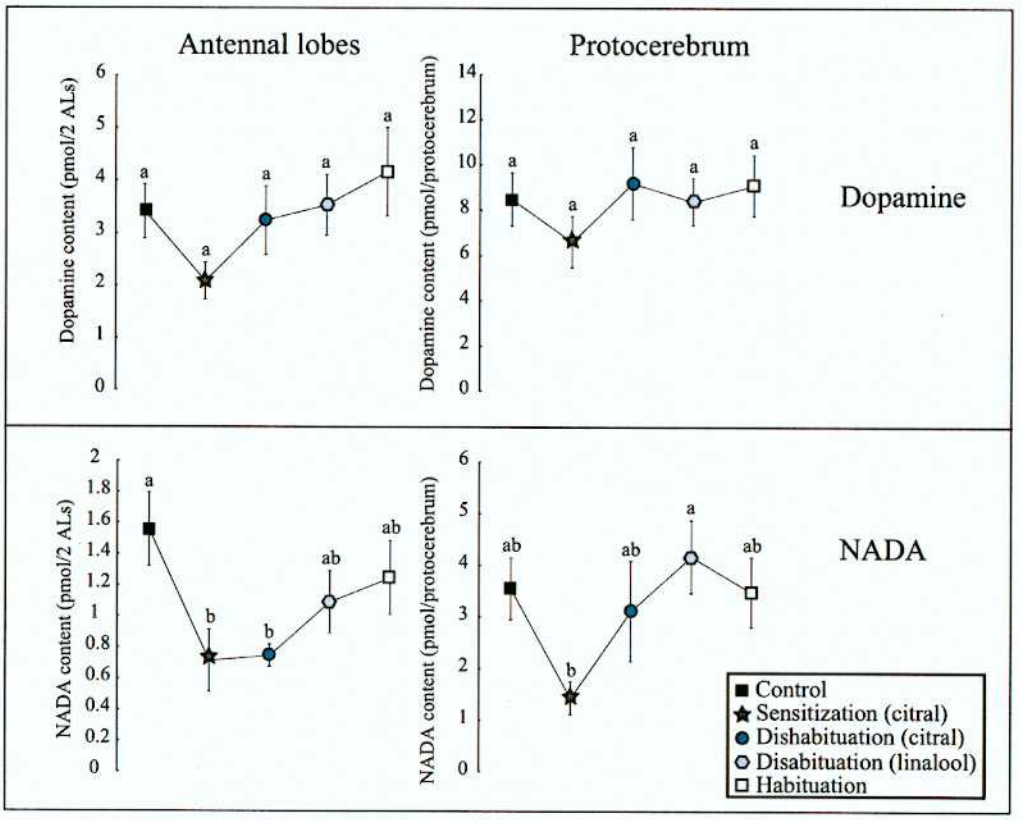

Figure 3.29: Levels of dopamine and NADA in the antennal lobes and protocerebrum of male moths after short term habituation, dishabituation and sensitization. Values are means ± S.E.M. Differences between groups marked with the same letters were not significant (GLM test followed by the Least Significant Difference pairwise comparison; $P<0.05$).

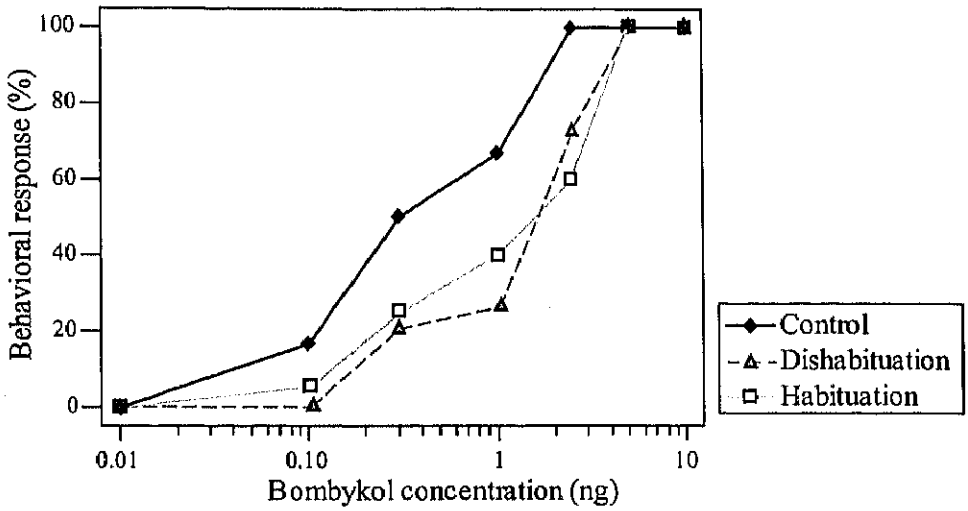

Figure 3.30: Effect of long term habituation and dishabituation on the moth's response to pheromone

Long term habituation: behavior

Moths exposed to 'high' levels of artificial pheromone (bombykol, 1000 ng) 4 times a day for 3 days were subjected to habituation on the following day. The sensitivity curve (Fig. 3.30) of the habituation group showed a strong shift to the left, which implies a general decrease in pheromone sensitivity: at all concentrations besides the lowest and the 2 highest, the response of the habituated moths were lower than the control, showing a decrease in response superior to 25 % at 0.3, 1 and 2.5 ng. The dishabituation curve showed a tendency similar as the habituation curve, with responses much lower than the control at most pheromone concentrations. At low pheromone concentrations, the dishabituation response was slightly lower than the habituation response, a tendency that reverted at higher concentrations.

The means of behavioral response were measured for each group (habituation group, N=20; dishabituation group, N=19 and control group, N=18) as shown on Fig. 3.31 on the next page. The behavioral response of the habituation and dishabituation groups did not exceed 54 %, which corresponded to a response 12 %

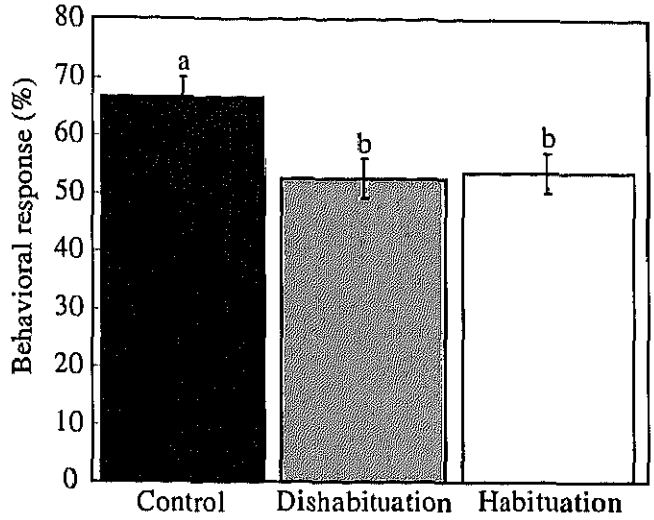

Figure 3.31: Means of behavioral response after long term habituation and dishabituation
Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Bonferroni pairwise comparison; $P<0.05$).

lower than the control, representing a significant different (habituation, $P=0.046$; dishabituation, $P=0.027$). Habituation and dishabituation groups showed a similar response level (no significant difference). These results suggest that habituation occurs after a long term habituation protocol lasting for 3 days, and that this habituation mechanism can last for 24 hours. Furthermore, dishabituation cannot be obtained by exposing the moths to a different odor than pheromone shortly before the test.

Long term habituation: biogenic amines

Levels of biogenic amines were measured in the antennal lobes and protocerebrum of moths exposed to long term habituation. Fig. 3.32 on the following page shows the levels of serotonin in the antennal lobes and protocerebrum of control moths ($N=9$ to 16), dishabituated moths ($N=10$ to 19) and habituated moths ($N=9$

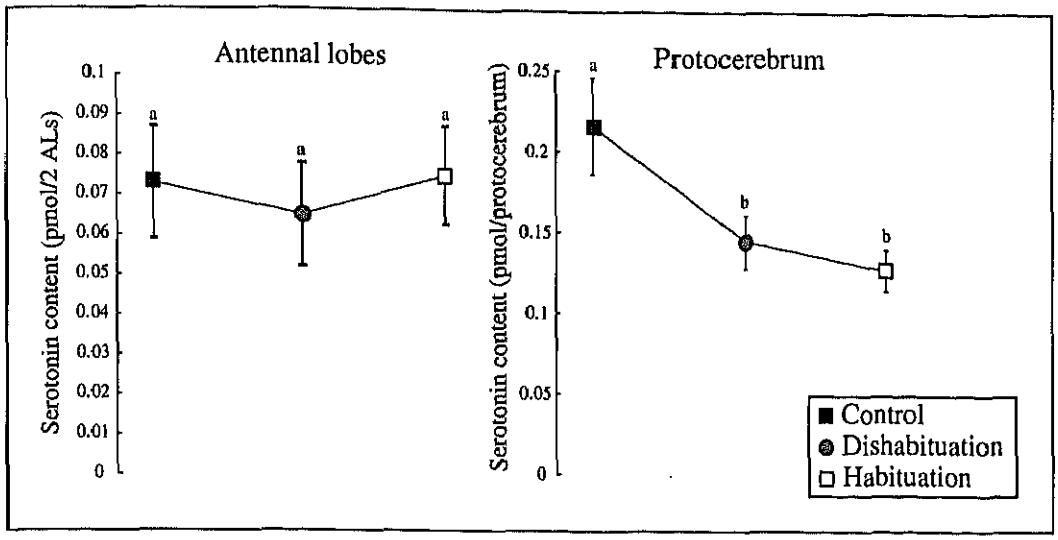

Figure 3.32: Levels of serotonin in the antennal lobes and protocerebrum of male moths after long term habituation and dishabituation
Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Bonferroni pairwise comparison; $P<0.05$).

to 18). Serotonin levels in the antennal lobes did not fluctuate in relation to habituation or dishabituation. In contrast, levels of serotonin were significantly lower in the protocerebrum of dishabituated moths ($P=0.049$) and habituated moths ($P=0.01$) than the control (respectively a drop of 33 and 41 % in comparison with the control).

Fig. 3.33 on page 62 shows the levels of 5-HTP, Nac-5HT and NADA in the antennal lobes and protocerebrum of male moths after long term habituation and dishabituation. Similarly to short term habituation, levels of 5-HTP (ALs: $N=7$ to 9 and protocerebrum: $N=16$ to 18), Nac-5HT (ALs: $N=9$ and protocerebrum: $N=17$ to 18) and NADA (ALs: $N=8$ to 10 and protocerebrum: $N=11$ to 15) did not significantly fluctuate after a long term habituation or dishabituation training in comparison with the control. However, the levels of 5-HTP and Nac-5HT, which are respectively the precursor and metabolite of serotonin, showed a tendency sim-

ilar to serotonin in the protocerebrum, meaning that their levels were slightly lower after long term habituation. Levels of NADA also showed an interesting tendency of slight increase in the antennal lobes and protocerebrum of dishabituated moths in comparison with the control and habituated moths. Unfortunately in long term habituation experiments, levels of dopamine could not be measured. These results suggest that serotonin plays a role in the protocerebrum of moths after long term habituation and that dopamine could play a role in dishabituation.

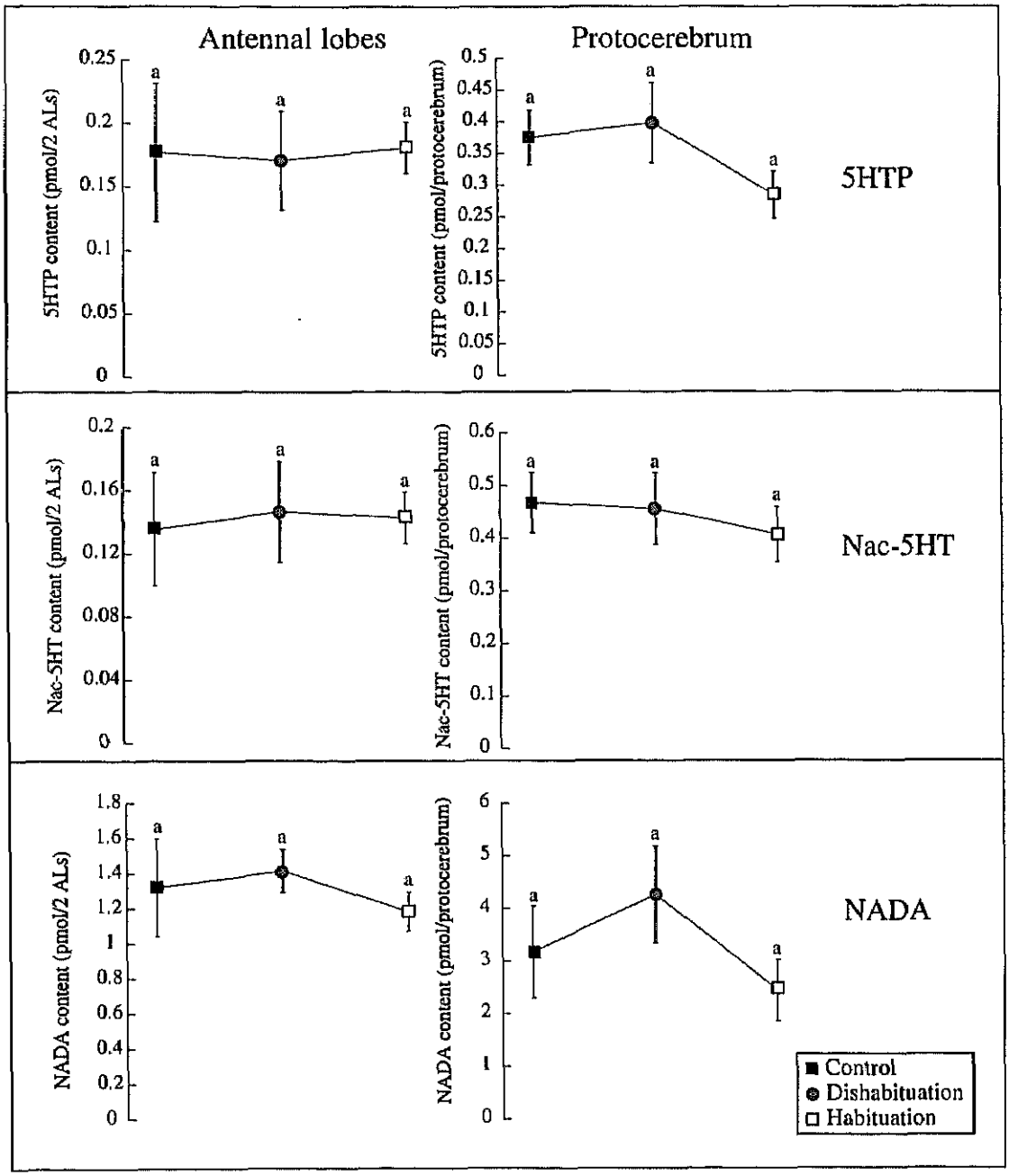

Figure 3.33: Levels of 5-HTP, Nac-5HT, NADA in the antennal lobes and protocerebrum of male moths after long term habituation and dishabituation
Values are means ± S.E.M. Differences between bars marked with the same letters were not significant (GLM test followed by the Bonferroni pairwise comparison; $P<0.05$).