Chapter 4

Discussion

4.1 Major findings

The results are summarized in Tables 4.1 on the following page and 4.2 on page 65. Variations of the sensitivity to pheromone was observed, higher during the day and lower at night. Moreover, I modified the behavior by applying neuroactive substances (depending on the concentration, serotonin increased or decreased the sensitivity to pheromone while nitric oxide increased the sensitivity to pheromone and induced wing fluttering). Application of a high concentration (1000 ng) of synthetic pheromone (bombykol) induced short and long term habituation. Dishabituation could be induced in short but not in long term habituated moths (Table 4.1 on the following page).

The role of nitric oxide on modifying the levels of biogenic amines was clarified: nitric oxide increased the levels of Nac-5HT, dopamine, NADA and tyramine.

The levels of biogenic amines were related to different behaviors: serotonin was related to the circadian variation of the behavior, serotonin and 5-HTP increased with pheromone exposure and tyramine increased after mating (Table 4.2 on page 65).

Table 4.1: Summary of the modifications induced in the pheromone-searching behavior

		Behavior modification
Circadian variation		+/-
Drug application	serotonin	+/-
	nitric oxide	+, flutter
Habituation	short term	-
	long term	-
Dishabituation	short term	-
	long term	↔

+ : increase in pheromone sensitivity; - : decrease in pheromone sensitivity; flutter: wing fluttering with pheromone exposure; ↔: no change observed

4.2 Variation of internal cues

In order to synthesize the results, the role of each neuromodulator will be first discussed separately.

4.2.1 Serotonin

Serotonin and circadian rhythm

My results showed a high correlation between the male *Bombyx mori*'s sensitivity to bombykol and brain serotonin levels (Figs. 3.2 on page 27 and 3.1 on page 26). Serotonin levels in the brain displayed a circadian variation with a peak at noon (Fig. 3.2 on page 27) 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 ng, 0.5 ng and 1 ng). At higher concentrations (5 ng, 10 ng

Table 4.2: Summary of the relationship between the behavior and neuroactive substances in the brain of the male moth

	Nitric oxide	Habituation	Exposure to pheromone	Mating	Circadian variation
Serotonin	↔	↓*	↑	↔	↑↓
5-HTP	↔	↔	↑	↔	Not tested
Nac-5HT	↑	↔	↔	↔	Not tested
Dopamine	↑	↔	↔	↔	Not tested
NADA	↑	↔	↔	↔	Not tested
Tyramine	↑	Not tested	↔	↑	Not tested
Octopamine	↔	↔	↔	↔	Not tested
Nitric oxide	Not tested	Not tested	Not tested	Not tested	Not tested

↑: increase in neuroactive substance levels; ↓ : decrease in neuroactive substance levels; ↔ : no change observed; + : increase in behavioral response
∗ : Effect during long term habituation, located in the protocerebrum

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. 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., 1994b; Kloppenburg et al., 1999). In the cockroach *Periplaneta americana* and the cricket *Gryllus bimaculatus*, serotonin is known to be involved in the regulation of the optic lobe circadian clock (Page, 1987; Tomioka, 1999; Saifullah and Tomioka, 2003). 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 antennal lobe and the endogenous clock, which could be partly located in the central brain of moths (Truman, 1974; Helfrich-Forster et al., 1998). 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 light-dark cycle, and in turn, act on the sensitivity to pheromone in the antennal lobe. In both cases, the mechanisms underlying serotonin synthesis and release are still unclear. The moth *Trichoplusia 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., 1994b). In contrast with that study, in *Manduca sexta*, also a nocturnal moth (Lingren et al., 1977), a circadian variation of serotonin in the antennal lobe 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 the scotophase (Kamimura and Tatsuki, 1994). The release of *B. mori* female's pheromone also shows a circadian rhythm: the release of pheromone increases at the beginning of photophase to reach a peak 6 hours later; this peak lasts for 2

hours 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 for *B. mori* that has been selected through evolution.

Serotonin and sensitivity to pheromone

In order to confirm the role of serotonin on the male *B. mori*'s sensitivity to pheromone, I applied serotonin and its antagonists at different concentrations. Serotonin (10^{-4}M) applied to the antennal lobes increased the male silkmoth's sensitivity to pheromone (Fig. 3.10 on page 36). Serotonin's effect was dose-dependent (Fig. 3.11 on page 38): a lower concentration (10^{-5}M) did not affect the behavior whereas a higher concentration (10^{-3}M) decreased the sensitivity. In order to quantify the efficiency of serotonin diffusion into the brain, I also performed HPLC measurements of brains 3 minutes and 24 hours (wash) after application of serotonin 10^{-5}M, 10^{-4}M and 10^{-3}M. My results showed that application of serotonin at 10^{-4}M and 10^{-3}M increased brain serotonin levels by 241 % and 424 % respectively (Fig. 3.14 on page 41). These levels correspond to an efficiency rate of 0.1 % and 0.05 % respectively, a rate comparable to other studies: Linn et al. (1994a) showed that the accumulation of serotonin which was expressed in the moth *T. ni*'s brain after injection was in the range of 0.1 to 0.6 % of the amount injected in the head without desheathing. The distribution of injected solutions was monitored by a 3 minute Lucifer Yellow application on the desheathed brain (data not shown). Local staining of the 2 antennal lobes, 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 antennal lobes subsequent to the application of serotonin may be underestimated due to the fact that the antennal lobe is about 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 serotonin 10^{-4}M to the desheathed brain of *B. mori* increased the peak spike frequency of bombykol responses recorded from the ventral nerve cord, while 10^{-3}M 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 depends on the 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 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 antennal lobe could also affect differently the 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. 3.12 on page 39 and 3.13 on page 40). Mianserin is known to be a 5-HT (serotonin) antagonist (Baines and Downer, 1991; Tierney, 2001), most probably a 5-HT_{1-2} receptor blocker (Dringenberg, 2000) and may act as well 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_{1-2} blocker induced a stronger decrease in sensitivity than the 5-HT_2 blocker, suggesting either that different receptors are activated by serotonin in the antennal lobes and/or a combined action of serotonin and octopamine. Ketanserin at 10^{-3}M unlike at 10^{-4}M 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 besides application of serotonin 10^{-4}M, I did not observe a complete reversion of the wash (mianserin 10^{-4}M, ketanserin 10^{-3}M; Fig. 3.12 on page 39), even in moths not affected by the drug treatment (serotonin 10^{-5}M and ketanserin 10^{-4}M, 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 hour 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 antennal lobes by a single pair of serotonin-immunoreactive neurons with branches in every glomerulus of the antennal lobe 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 serotonin-immunoreactive neuron spikes spontaneously and responds to mechanosensory stimuli to the antennae. Due to the fact that, in *M. sexta*, its branchings in the antennal lobe contain mostly output synapses (Sun et al., 1993), the serotonin-immunoreactive neuron may be involved in a feedback system from the protocerebrum to the antennal lobe. Serotonin application here may therefore mimic the serotonin-immunoreactive neuron's release of serotonin in the antennal lobe. My pharmacological method, similar to the one used by Hill et al. (2003), allows a direct comparison of serotonin's enhancing effects on neuronal populations in specific antennal lobe glomeruli and on pheromone sensitivity at the behavioral level. My results suggest that the neuronal responses to pheromone are modulated by serotonin in the antennal lobes 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 macroglomerular complex of *M. sexta* (Kloppenburg and Heinbockel, 2000). In *B. mori*, application of serotonin to the antennal lobe enhances both the maximum amplitude and duration of antennal lobe optical responses to electrical stimulation of the antennal nerve. In the macroglomerular complex, these effects are stronger in the toroid, the 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 antennal lobe and that these effects should in turn have some behavioral significance. Until now, no such behavioral effects resulting from serotonin application to the antennal lobe have been reported.

Based on these facts, the main expected effect of serotonin application to the antennal lobes would be an enhancement of the male sensitivity to pheromone, as I observed when serotonin 10^{-4}M was applied. In contrast, I also obtained an inhibitory effect with serotonin 10^{-3}M. My results are not irreconcilable with previous studies, in which the main serotonin concentration used was 10^{-4}M. I cannot exclude the possibility that such a high concentration of serotonin (10^{-3}M) 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 twofold increase during the daily variation in the moth's brain (Fig. 3.2 on page 27). This range of increase was obtained when applying serotonin 10^{-4}M, while when applying a concentration of 10^{-3}M, serotonin levels rose of more than 4 times the control (Fig. 3.14 on page 41), an increase much larger than what was observed in the circadian variation of serotonin. A concentration similar to ours could provoke inhibitory effects on antennal lobe neuronal responses, leading to a pheromone

sensitivity decrease. Another possibility, compatible with previous reports, could be that the excitation of antennal lobe neurons due to serotonin 10^{-3}M 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 serotonin-immunoreactive 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 antennal lobes, optic lobes, mushroom bodies have been performed in the honeybee in order to assess the effects of amines on olfactory conditioning (for review see Bicker and Menzel, 1989; Erber et al., 1993). A recent study showed that octopamine increases the responsiveness of honeybees to brood pheromone (an activator of foraging) (Barron et al., 2002). In a noctuid moth, *T. 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 my experiments, I did not observe an enhancement of general locomotor activity; instead I 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 to 8 hour delay of the injection, whereas I 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 antennal lobes, avoiding as much as possible the effects of serotonin receptor desensitization (Hanley and Hensler, 2002). Furthermore I can combine my results and suggest that the ranges of circadian variation of serotonin in the brain match with the excitatory effects of serotonin in the antennal lobes leading to a higher sensitivity to pheromone.

The difference between the serotonin daily concentration in the brain (Fig. 3.2 on page 27) and the serotonin levels in the brain following a 4 μl application of external serotonin (Fig. 3.14 on page 41) could be explained by the difference in brain dissection (levels of serotonin in Fig. 3.2 on page 27 were obtained from antennal lobes, protocerebrum and optic lobes, while Fig. 3.14 on page 41 levels concern mainly the antennal lobes and protocerebrum) as well as a seasonal variation of brain serotonin concentration. My results suggest a significant role of serotonin in enhancing the male moth's sensitivity to pheromone.

4.2.2 Serotonin immunoreactive neuron

The neuron from which a Lucifer Yellow fill is shown in Fig. 3.3 on page 29 seems to be very important in the neuromodulation of the signals in the antennal lobe. An identical neuron has been confirmed to be serotonin immunoreactive through double labeling with serotonin immunocytochemistry (Hill et al., 2002). This neuron, examined with a confocal microscope, revealed arborizations in all the glomeruli of the contralateral antennal lobe, including the 3 compartments of the macroglomerular complex which is known to contain neurons showing response to pheromone and also each ordinary glomerulus, relaying information from normal odors (Fig. 3.4 on page 30). The branchings in the glomeruli in the antennal lobe (Fig. 3.4 on page 30) were thick and varicose in comparison with smooth branchings the mushroom bodies (Fig. 3.6 on page 32) and the central body (Fig. 3.7 on page 33). The branchings into the lateral accessory lobe (LAL) (Fig. 3.5 on page 31) also showed varicosity. The varicose appearance of the antennal lobe and the LAL leads one to speculate that these may represent output synapses (Kondoh and Hisada, 1986; Mishima and Kanzaki, 1999; Lei et al., 2001), and that information is carried from higher centers to a lower center, the antennal lobe, and to the LAL which has been mentioned above to be important for olfactory processing and involved in the flip-flop activity observed in descending neurons and downstream leading to the zigzag pattern (Kanzaki and Shibuya, 1992; Kanzaki

et al., 1994; Kanzaki and Mishima, 1996). This neuron is the unique serotonin-immunoreactive neuron having extensions into the antennal lobe (shown in an immunocytochemical study, Hill et al., 2002). Moreover, serotonin application affects both pheromone-evoked local field potentials and potential oscillations in the macroglomerular complex of *M. sexta* (Kloppenburg and Heinbockel, 2000) and also enhances the maximum amplitude and the duration of the optical response (to electrical stimulation of the antennal nerve) in both the macroglomerular complex and the ordinary glomeruli of *B. mori* (Hill et al., 2003). These effects on the physiological level could be related to an increase of the male pheromone-sensitivity obtained in my behavioral study, as this behavioral response to pheromone is the fastest behavioral change that occurs when pheromone is applied. From physiological recording of this neuron in response to bombykol, bombykal and hexane one can postulate that this neuron is mechanosensory (Figs. 3.8 on page 34 and 3.9 on page 35). This would suggest that the release of serotonin in the antennal lobe and the LAL would occur when the antennae are confronted to any mechanosensory stimulation. One can speculate that this release of serotonin in the antennal lobe would "prepare" the neurons in the antennal lobe to respond faster to a further stimulus which could be olfactory (pheromone or ordinary odor) or mechanosensory. This neuron is the only serotonin-immunoreactive neuron in each antennal lobe of the male silkmoth (Iwano and Kanzaki, 2005). This single neuron may therefore play a key role in the moth's sensitivity to pheromone, a role that can be compared to the VUM_{mx1} neuron in the honeybee *Apis mellifera*, responsible for reinforcement during olfactory conditioning (Hammer, 1993).

4.2.3 Nitric oxide

Nitric oxide has been known as a neuroactive substance in the nervous system since the end of the 1980's (Garthwaite et al., 1988). Nitric oxide role in neurotransmission and neuromodulation seems to be a general mechanism in vertebrates and insects (reviewed by Müller, 1997). In contrast to other neurotransmitters, nitric

oxide is a gas that diffuses from its site of production to the target sites. Its action is limited by its half-life and diffusion barriers and does not depend on synaptic structures, a particularity that distinguishes it from other neuroactive substances. Formation of nitric oxide with a nitric oxide synthase (nitric oxide synthase) is a Ca^{2+}-dependent process (see Fig. 1.1 on page 7 in the Introduction).

As well as in vertebrates, the major target of nitric oxide is the soluble form of guanylate cyclase (sGC) in insects (Müller, 1997), which leads to elevations of cGMP levels (Elphick et al., 1993; Liu et al., 1995). cGMP, in turn, modulates cGMP-dependent protein kinases responsible of the phosphorylation of downstream target protein evoking cellular responses (Bicker and Menzel, 1989; Garthwaite and Boulton, 1995). In insect, nitric oxide seems to have a role in neural plasticity, given that nitric oxide has been related to habituation and long-term memory in the honeybee (Müller, 1996; Müller and Hildebrandt, 2002). Furthermore, nitric oxide production in the antennal lobe of *M. sexta* depends on odorants (including pheromone) stimulation (Collmann et al., 2004).

Nitric oxide and pheromone sensitivity

The application of a nitric oxide synthase blocker, L-NAME at the concentration of 10^{-4}M significantly decreased the male moth's sensitivity to pheromone, while its enantiomer, D-NAME at the same concentration did not significantly differ from the control (Fig. 3.15 on page 42). L-NAME at a lower concentration (10^{-5}M) showed an intermediate response (Fig. 3.16 on page 43). Furthermore, the application of a nitric oxide donor, NOR, at the concentration of 5×10^{-4}M, increased the male moth's sensitivity to pheromone (Fig. 3.17 on page 44). These results suggest an active enhancing role of nitric oxide on the pheromone searching behavior. The nitric oxide donor cells can be located by the use of NADPH-diaphorase histochemistry and anti-cGMP immunohistochemistry allows the location of the target cells in both vertebrates and insects (Matsumoto et al., 1993; Müller, 1997). Existence and localization of nitric oxide synthase is highly diver-

gent among insect species. In *B. mori*, NADPH-diaphorase reactivity was observed widely in the brain, including the antennal lobes, the mushroom bodies and central body (H. Aonuma, unpublished data). Interestingly, anti-cGMP reactivity was specifically observed in projection neurons from the toroid (a specific area in the macroglomerular complex, specialized in bombykol processing) leading to a specific area in the protocerebrum, the Δ ILPC (Seki et al., 2005). This recent finding suggested a new role of nitric oxide/cGMP pathway, closely related to the pheromone processing pathway. In *M. sexta*, nitric oxide donor cells in the antennal lobe are mainly olfactory receptor neurons while nitric oxide target cells are projection neurons (Nighorn et al., 1998). Nighorn et al. (1998) suggested that nitric oxide could be a means of communication within the glomeruli of the antennal lobe, between olfactory receptor neurons and projection neurons, bypassing the inhibitory local interneurons. Furthermore, Collmann et al. (2004) showed that odorant stimulation causes nitric oxide production in the olfactory system of the moth *M. sexta*. My results highlighted one role of nitric oxide in enhancing the response of projection neurons in the antennal lobe. Although I cannot rule out the possibility that nitric oxide diffused deeper in the brain, and acted in higher olfactory center, the short delay between the drug application and the measurement would suggest an action in the antennal lobes, similar to the serotonin bath application. The response of macroglomerular complex neurons to electrical stimulation of the antennal nerve of *B. mori* increased after L-NAME (10^{-4}M) application and decreased after NOR3 (5×10^{-4}M) application (K. Okada, personal communication). The role of nitric oxide could therefore be more complex than a simple enhancement of projection neurons. In the locust an interesting action of nitric oxide has been investigated: nitric oxide was suggested to have a role as intracellular messenger. Indeed, local interneurons in the locust antennal lobe showed both NADPH and c-GMP immunoreactivity (Bicker et al., 1996, 1997). Knowing that these local neurons are known to use GABA as conventional neurotransmitter, Bicker et al. (1996) suggested that nitric oxide plays a role in

synchronizing neural activity in parallel with the odor-evoked synchronization role of GABA (MacLeod and Laurent, 1996). This synchronizing action of nitric oxide is a common feature of olfactory information processing in insects, mollusca and vertebrates (Breer and Shepherd, 1993; Gelperin, 1994). In *B. mori*, c-GMP and NADPH immunoreactivity are both present in local neurons and could play such a synchronizing role (H. Aonuma, unpublished data). This would explain that NOR3 application induced a weaker response to an electrical stimulation of the antennal nerve of *B. mori* while the sensitivity of the moth to pheromone increased with the application of a same concentration of NOR. The level of neuronal response in the antennal lobe may not be related to the sensitivity levels to pheromone if the critical aspect is synchronization of neurons. As presented here, the roles of serotonin and nitric oxide on the male moth's pheromone searching behavior are similar, that is an increase in pheromone sensitivity. However, the means of action of both neuromodulators are different due to their specific properties. Application of nitric oxide donor induced spontaneous wing fluttering of male moths without exposure to pheromone (Fig. 3.18 on page 45). This example illustrates the different action of neuromodulators: serotonin is released in the antennal lobe through a single serotonin-immunoreactive neuron that responds to mechanosensory stimuli to the antennae. In contrast, donor and target neurons of nitric oxide are present widely in the antennal lobe, including in the area specific for bombykol processing, the toroid. The role of nitric oxide in this pathway may therefore be multiple, depending on the zone of action in the antennal lobe. One cannot rule out the possibility that nitric oxide plays a role in triggering some behavior in the male silkmoth, in this case wing fluttering.

Nitric oxide and biogenic amines

Indolealkylamines Fig. 4.1 on the following page shows the metabolic pathway of indolealkylamines (adapted from Evans, 1980; Brown and Nestler, 1985). Indolealkylamines include 5-HTP, serotonin and Nac-5HT. Serotonin, and more re-

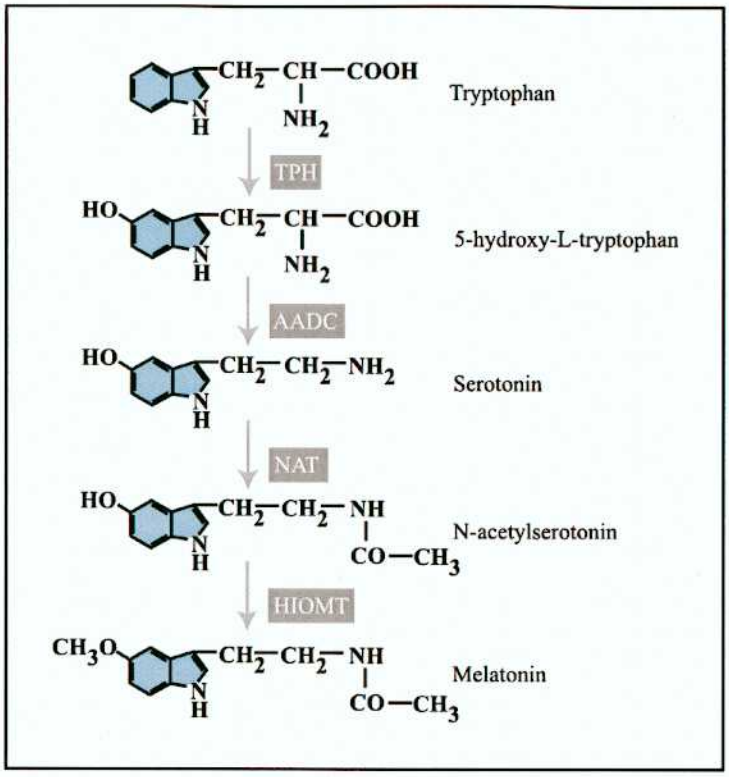

Figure 4.1: Possible metabolic pathway of indolealkylamines in insects
The letters with arrows indicate the enzymes mediating the metabolic pathway. Adapted from Evans (1980); Brown and Nestler (1985). TPH, tryptophan hydroxylase; AADC, L-aromatic amino acid decarboxylase; NAT, N-acetyltransferase; HIOMT, hydroxyindole-O-methyltransferase.

cently melatonin have been described as neuroactive substances in insects (Evans, 1980; Richter et al., 2000; Roeder, 2004). 5-HTP and Nac-5HT are not supposed to play any neuroactive role in insect brains, even though in vertebrates, Nac-5HT is known as a neurohormone (Lotufo et al., 2001).

I evaluated the role of nitric oxide on indolealkylamines. Levels of tryptophan and melatonin could not be measured. Different drugs related to nitric oxide were applied on the moth brain. Similarly to the behavioral experiments, a nitric oxide synthase inhibitor, L-NAME was applied alone at the concentration of 10^{-4}M.

L-NAME at a similar concentration was also applied with different concentrations of a nitric oxide donor, NOR3 (10^{-4}, 10^{-3}, 10^{-2}M). At last, NOR3 was applied at a concentration of 10^{-3}M in combination with a sGC blocker, ODQ (10^{-4}M). Biogenic amine levels in the moth's brain were measured within 3 minutes of the drug application. Fig. 3.19 on page 46 shows the levels of serotonin, 5-HTP and Nac-5HT in the brain of male moths. Serotonin and its precursor were not related to the different drug application. However the levels of Nac-5HT were lower when L-NAME was applied alone or with the lowest concentration of NOR3 (10^{-4}M), which suggests that absence or low levels of nitric oxide are related to low levels of Nac-5HT. This discrepancy in results between serotonin and its metabolite suggests a independent roles of both chemical substances, a fact that has already been established in insects for a neuromodulator (tyramine) and its metabolite (octopamine) (Roeder et al., 2003). Nitric oxide could increase or facilitate the catabolism of Nac-5HT into melatonin, which would not affect the levels of serotonin. Unfortunately, no mapping studies have been performed with Nac-5HT or melatonin. Melatonin has been suggested to depend on nitric oxide in the vertebrate retina (Wellard and Morgan, 2004). My results also showed that the levels of Nac-5HT were not affected by the application of the sGC inhibitor, ODQ (with NOR3) in comparison with application of NOR3 alone. This suggests that the role of nitric oxide on Nac-5HT does not use the nitric oxide/cGMP pathway. It has been reported that nitric oxide can have different targets, olfactory cyclic nucleotide gated channels (Broillet and Firestein, 1996) and GABA receptors (Fukami et al., 1998), both of which are likely to be expressed in the antennal lobe. However, whether these mechanisms apply in insects remains to be shown and molecular research should be performed in that direction to clarify my results.

Catecholamines Fig. 4.2 on the following page shows the metabolic pathway of catecholamines (adapted from Brown and Nestler, 1985). Besides serotonin, two other biogenic amines have been known for decades to play a role as neuromodula-

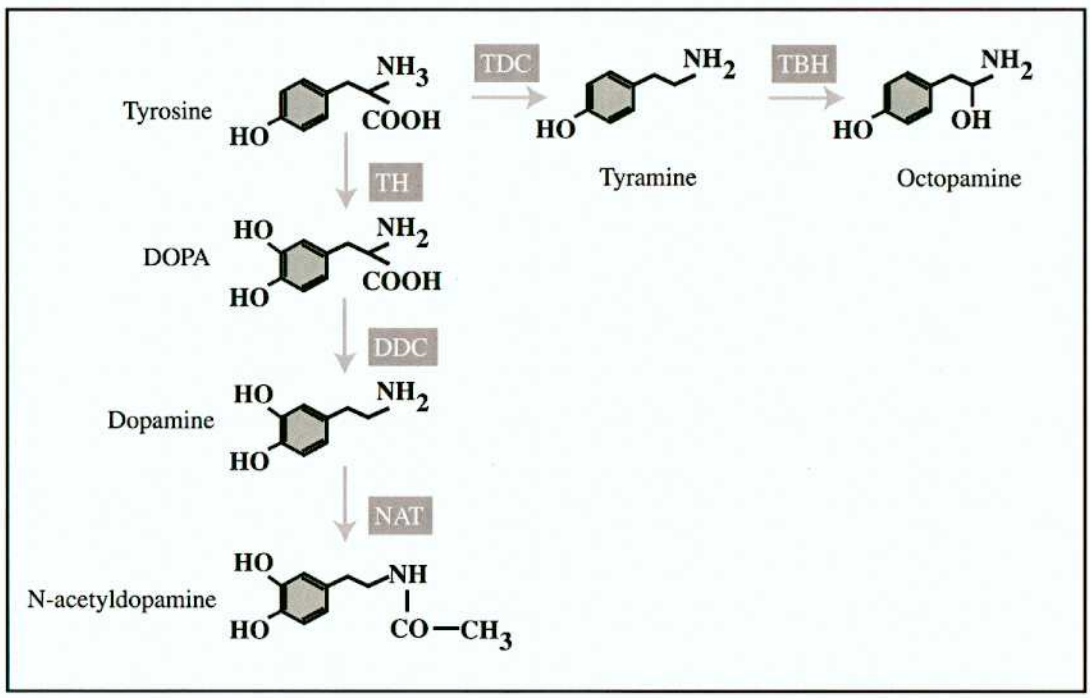

Figure 4.2: Possible metabolic pathway of catecholamines in insects
The letters with arrows indicate the enzymes mediating the metabolic pathway. Adapted from Brown and Nestler (1985). TDC, tyrosine decarboxylase; TBH, tyramine β-hydroxylase; TH, tyrosine hydroxylase; DDC, DOPA decarboxylase; ; NAT, N-acetyltransferase.

tors in insects: octopamine and dopamine (Evans, 1980; Roeder, 1994). Dopamine is related with aggressive behavior in crickets and in ants (Kostowski et al., 1975; Stevenson et al., 2000) and also to olfactory memory retrieval in the honeybee *A. mellifera* (Bicker and Menzel, 1989) and to aversive memory formation in the fruitfly *Drosophila melanogaster* (Schwaerzel et al., 2003). Octopamine is known in insects to be the equivalent of adrenaline in vertebrates and is known to have several physiological actions including appetitive memory formation in the fruitfly (Schwaerzel et al., 2003; for review, see Roeder, 1999). Until recently, tyramine was supposed to play mainly the role of the precursor of octopamine. Tyramine's role as a neuromodulator in its own right (as the equivalent of noradrenaline) has been highlighted more recently by the discovery of a receptor showing a high affinity for tyramine (Saudou et al., 1990; for review, see Roeder et al., 2003; Roeder, 2004). Immunocytochemical studies have provided evidence for the presence of tyramine immunoreactivity in the male *B. mori*'s brain (Iwano and Kanzaki, 2005). Using high performance liquid chromatography with electrochemical detection, I succeeded in quantifying the levels of tyramine, dopamine and *N*-acetyldopamine (NADA). Peculiarly, even though the system allowed octopamine detection, octopamine peak could not be observed, which suggests the absence of octopamine in the moth's brain or, more likely, a presence of octopamine under detection levels (< 0.02 pmol/brain). In contrast I could detect the presence of tyramine, the precursor of octopamine at levels of 0.05 to 2 pmol/brain (Fig. 3.20 on page 47). In the brain of the diurnal moth *T. ni*, the levels of tyramine were approximately 1.7 pmol/brain while levels of octopamine were approximately 4 to 5 pmol/brain (Linn et al., 1994b). In *T. ni*, the levels of octopamine were therefore 3 times the levels of tyramine. Immunocytochemical studies have shed light on the disparity in location and presence of serotonergic, dopaminergic and octopaminergic neurons including identified neurons (for review, see Homberg and Müller, 1999). My results suggest therefore that octopamine could play a minor role in the male *B. mori* in comparison with other moths and in extenso other insects.

This hypothesis still needs to be tested by using immunostaining of octopaminergic neurons in *B. mori*. The role of nitric oxide was therefore evaluated on tyramine, dopamine and NADA. As shown on Fig. 3.20 on page 47, the levels of tyramine after application of L-NAME were significantly lower than when NOR3(10^{-2}M) was added to L-NAME. The levels of tyramine also showed a tendency of decrease after application of NOR3 with ODQ. The levels of dopamine and NADA (Fig. 3.21 on page 48) showed a similar trend, that is, the levels of the neuroactive substance were lowest when L-NAME had been applied, but also when L-NAME had been applied with the low concentration of NOR3(10^{-4}M). It appears therefore that the levels of NOR3 inferior to 10^{-2}M are not sufficient to trigger the increase of the neuroactive substance levels (tyramine, dopamine or NADA). Furthermore, the levels of dopamine and NADA after application of NOR3 with ODQ were low in comparison with application of NOR3 alone, a response interestingly similar to tyramine. Taken together, these results suggest that nitric oxide presence in the brain increases the levels of tyramine, dopamine and NADA, and this increase could be related to the nitric oxide/c-GMP pathway, even though no clear evidence could be provided.

Indolealkylamines and catecholamines Nitric oxide has been suggested to regulate neurotransmission in vertebrates (for review, see Kiss, 2000). In short, nitric oxide has facilitatory actions on serotonin and noradrenaline and both excitatory and inhibitory effects on dopaminergic neurotransmission. Nitric oxide seems to inhibit the function of monoamine uptake systems. The nitric oxide way of action is still unclear and studies involving the sGC-cGMP system showed contradictory results. Nitric oxide affects dopamine transporters using an interneuronal communication, a nonsynaptic interaction without receptors (Kiss et al., 2004). In the invertebrate nervous system, much less is known between a possible role of nitric as a regulator of neurotransmission by biogenic amines (Stefano et al., 1997; D'yakonova, 2002). In the leech and the mussel, nitric oxide modulates

catecholamine release (Stefano et al., 1997) and in the common snail, nitric oxide functions as a second messenger and as a co-transmitter of serotonin (D'yakonova, 2002). The means of action of nitric oxide in invertebrates has not been investigated successfully so far. My study therefore gained a glimpse into the role of nitric oxide on biogenic amines regulation in insects. Nitric oxide could play a similar role of neurotransmitter regulation as in vertebrates (Kiss, 2000), and the molecular systems involved in nitric oxide action could be more diverse than expected, not based mainly on the sGC-cGMP system (Müller, 1997).

4.3 Variation of external cues

4.3.1 Exposure to pheromone

The role of biogenic amines has been studied extensively in insects, mainly by the use of pharmacological experiments (for review, see Bicker and Menzel, 1989; Bicker, 1999). Such types of experiments are effective to understand the role of neuroactive substances but their considerable disadvantage consists in an exposure to artificial conditions. Biogenic amine variation has also been measured in relationship with circadian variation (Muszynska-Pytel and Cymborowski, 1978; Tomioka et al., 1993; Linn et al., 1994b; Kloppenburg et al., 1999, see also our discussion, on page 64). However the variation of biogenic amine levels related directly to a specific behavior has not been studied much in insects. Measurement of biogenic amines in the brain of an animal performing a precise action leads to evidences of relationship between amines and behavior: levels of biogenic amines can be measured in natural conditions. A HPLC study showed that the levels of biogenic amines vary depending on the state of the honeybee (worker vs. forager) (Schulz et al., 2002). I therefore aimed to assess a relationship between biogenic amines and exposure to pheromone in natural conditions. Exposure to pheromone increased significantly the levels of serotonin and its precursor 5-HTP in the male moth's brain, but not in the female's brain (Figs. 3.22 on page 49 and 3.24 on page 51). Levels of Nac-5HT in males' brain also tended to increase with pheromone exposure. The mating activity slightly decreased the levels of serotonin, 5-HTP and Nac-5HT but significantly decreased the levels of tyramine, which were not significantly affected by pheromone exposure in males (Fig. 3.23 on page 50). Again there was a discrepancy between males and females, given that the levels of tyramine did not vary in females after mating. Mating was not related to a variation of biogenic amines (5-HTP, serotonin, Nac-5HT, tyramine, dopamine or NADA) in the brain of females. These results confirm my pharmacological results (see discussion, on page 64) and also other physiological studies

(Kloppenburg and Hildebrand, 1995; Mercer et al., 1996; Kloppenburg et al., 1999; Kloppenburg and Heinbockel, 2000; Hill et al., 2003), implying serotonin as a main neuromodulator in pheromone searching behavior. Serotonin circadian variation allows the increase of serotonin at noon, when females are most active (Kamimura and Tatsuki, 1994). In my experiments, the male moths exposed to pheromone for $\simeq$ 5 minutes were very actively looking for females when the levels of biogenic amines were measured. It is however difficult to clarify the biological significance of serotonin increase on the behavior. Such exposure to pheromone could lead to sensitization, meaning a higher sensitivity to pheromone, mediated by an increase of serotonin levels. In the honeybee, biogenic amines did not seem to be related to the sensitization of an appetitive conditioning (Menzel et al., 1999), but in other invertebrates such as the leech and the common snail, serotonin is known to be related to sensitization to specific behaviors (Ehrlich et al., 1992; Barbas et al., 2003). The role of serotonin as increasing pheromone sensitivity would therefore not be restricted to the behavioral response to a single exposure of pheromone, but also to a more long term phenomenon, which leads to the same behavioral results (increase of pheromone sensitivity). Further behavioral experiments, such as measuring the sensitivity to pheromone after a 5 minutes exposure would be necessary to confirm this hypothesis. On the other hand, tyramine was related to the mating behavior (Fig. 3.23 on page 50). The levels of tyramine were lower after mating. Biogenic amines are related to various behaviors including mating in invertebrates (Ureshi et al., 2002; Kiehn et al., 2001). Tyramine decreases the locomotion of the fruitfly *Drosophila melanogaster* larvaes (Saraswati et al., 2004) and also increases *D. melanogaster*'s olfactory behavior (Kutsukake et al., 2000). The levels of tyramine did not vary after mating in the female's brain, suggesting that tyramine plays a specific role in the male's physiology. These findings unravel the complex role of biogenic amines on the silkmoth's behavior. However, their exact role in the neural pathway underlying the pheromone-searching behavior is still unclear and would require further physiological and pharmacological studies

combined with behavioral experiments.

4.3.2 Habituation

Habituation, the simplest form of learning, is considered as a central non-associative learning process characterized by a progressive decrease in the activity of an unconditioned response that may occur with repeated presentation of an unconditioned stimulus (for review, see Thompson and Spencer, 1966; Groves and Thompson, 1970). By habituating to less important signals, an animal can focus its attention on the most important features of its environment. Habituation has been suggested to take place in higher centers (Krasne and Teshiba, 1995; Stopfer and Carew, 1996). Habituation can be produced in a wide range of animals and has been studied extensively with the gill withdrawal of the sea snail, *Aplysia californica* (for review, see Kandel et al., 2000). The mechanism of memory, which includes habituation, can be divided into short term memory, which lasts minutes or hours, and long term memory, which can last hours or days (Kandel, 1997). Kandel (1997) suggested that the switch to long-term memory requires the induction of genes and proteins not required for short-term memory. In short term processes, the presynaptic action of cAMP-dependent protein kinase (protein kinase A), and also protein kinase C leads to an increase in transmitter release from the presynaptic terminals of the sensory neurons. By contrast, in long term habituation, c-AMP-mediated gene expression and new protein synthesis lead to the growth of new synaptic connections (Kandel, 1997). In *D. melanogaster*, long-term memory is also dependent on cAMP-mediated gene expression (Yin et al., 1994, 1995). Furthermore, biogenic amines are known to be related to habituation in *A. californica* (Ruben and Lukowiak, 1983) and in insects such as the honeybee (Braun and Bicker, 1992). A phenomenon closely related to habituation is dishabituation or sensitization (for review, see Groves and Thompson, 1970). Conversely to habituation, sensitization is an increased response to a stimulus as a result of a different stimulus (sensitizing stimulus). In *A. californica*, the mecha-

nism of presynaptic facilitation includes the activity of serotonergic neurons which enhance the release of neurotransmitters (Brunelli et al., 1976). In the locust, sensitization is related to another biogenic amine, octopamine (Sombati and Hoyle, 1984). As presented here, the mechanisms related to habituation and sensitization appear to be different. One of the aims of this section was to test whether the male silkmoth pheromone searching behavior can be subject to short- and/or long term habituation and also if such neural plasticity, if it occurs, is related to biogenic amine modification in the brain.

Short term habituation

Behavior Short term habituation (30 minutes) could be induced in males moths in response to pheromone (Figs. 3.25 on page 52 and 3.26 on page 53). The response of the moths decreased significantly after 3 exposures to high concentration of pheromone (1000 ng, the equivalent of the contents in the gland of one female). Dishabituation could also be induced after habituation, by the use of an unrelated odorant (in this case, I successfully tested two odorants: citral and linalool): the response of the moths first habituated and dishabituated was higher than the response of moths habituated to pheromone. A single exposure to an odorant did not have any effect on the sensitivity to pheromone (Fig. 3.26 on page 53). This last result suggests that sensitization does not occur in the short term (with the odorant stimulus), but (with the same odorant), dishabituation does occur. Sensitization and dishabituation seem therefore to be two different mechanisms in this case. In *A. californica*, Antonov et al. (1999) suggested that a similar mechanism, the heterosynaptic facilitation of the monosynaptic PSPs, contributes to learning and memory for dishabituation and sensitization of the withdrawal reflex and that other sites and mechanisms of plasticity also contribute. This hypothesis is controversial given that other studies concluded that different mechanisms were responsible for dishabituation and sensitization (Marcus et al., 1988; Wright et al., 1991). My results tend to confirm the second hypothesis, suggesting that the simple

dual process proposed by Groves and Thompson (1970), where dishabituation would be the result of habituation and sensitization, is the main mechanism. More complex multiprocesses could be involved, with specific mechanisms underlying the dishabituated behavior.

Sensory adaptation and muscle fatigue are peripheral mechanisms reducing responsiveness (Thompson and Spencer, 1966). The occurrence of dishabituation suggests that the reduction in responsiveness to pheromone is habituation rather than sensory adaptation or muscle fatigue. Moreover, electroantennogram studies showed that the responses from olfactory receptors were not affected during short term habituation, ruling out an eventual role of sensory adaptation (Kuenen and Baker, 1981, Ryota Fukushima, personal communication).

Bred in captivity for thousands of years, the male silkmoth is fully domesticated and lost the functional mouth parts and therefore the capacity of feeding from plant nectar like other moths. As a result, exposure to plant odorants such as citral and linalool, does not induce any specific behavior; the moth stays still (personal observations). However, plant odorants induce dishabituation to pheromone (Fig. 3.26 on page 53). Given that the pheromone searching behavior is the only behavior performed by the adult male silkmoth, dishabituation produced by an odorant could therefore be the remains of an old behavior.

Biogenic amines Insects' brain can be divided into the first olfactory center (the antennal lobes) and the higher olfactory center (the protocerebrum). The location of synaptic plasticity is an important aspect of learning. In the honeybee *Apis mellifera*, the antennal lobe seems to be the site of early processing of reinforcement pathway, and octopamine is a key neuromodulator for this early olfactory processing (Farooqui et al., 2003). Also in *A. mellifera* and in *D. melanogaster*, the mushroom bodies, in the protocerebrum, are known to be related to olfactory conditioning (for details, see Heisenberg, 1988). In order to separate the role of the antennal lobes and the mushroom bodies, I separated the antennal lobes from

the protocerebrum before measuring levels of biogenic amines in the brain. As shown on Figs. 3.27 on page 54, 3.28 on page 55 and 3.29 on page 57, there was no significant variation of the levels of biogenic amines (serotonin, 5-HTP, Nac-5HT, dopamine, NADA) after short term habituation.

Sensitization led to a significant decrease of NADA, and a tendency of decrease of dopamine in both the antennal lobes and the protocerebrum. The parallel level changes of dopamine and NADA could be explained by the metabolic pathway of dopamine in the brain (see pathway 4.2 on page 79). The positive correlation between levels of dopamine and NADA suggests that individuals with low dopamine levels in their brains might have respectively lower enzymatic activity for metabolism of dopamine, and consequently have low levels of NADA, as NADA is the principal metabolite of dopamine (Sasaki and Nagao, 2001). NADA is also known as a dopamine agonist in the hawkmoth *Manduca sexta* (Granger et al., 2000). NADA is also postulated to be a sclerotizing agent in many insect species (Karlson and Sekeris, 1962; Mills et al., 1967). Furthermore, NADA is also related with dopamine to the synthesis of the juvenile hormone (Hentschel, 1981; Granger et al., 1999, 2000). Nevertheless, the role of the dopamine and NADA decrease in the male moth after exposure to an odorant is still unclear. Dopamine is not known to be related to olfaction in insects, besides to aversive olfactory memories in *D. melanogaster* (Schwaerzel et al., 2003). In invertebrates, dopamine modulates the neural dynamics of oscillations of the local field potential in the protocerebrum (Rhines et al., 1993). Dopamine could play a similar role in the male silkmoth as oscillations are known to occur in both the protocerebrum of insects (Laurent and Naraghi, 1994) and the antennal lobes (Heinbockel et al., 1998; Okada and Kanzaki, 2001). However, the neurotransmitter GABA seems to be responsible for neural synchronization in the olfactory pathway (MacLeod and Laurent, 1996). Further studies are therefore necessary to assess the role of dopamine and NADA in olfaction of general odorants.

Long term habituation

Behavior Long term habituation to pheromone has been described in several flying moths (Figueredo and Baker, 1992; Daly and Figueredo, 2000) even though all responses by male moths to pheromone have been considered genetically pre-programmed and relatively inflexible (Sorensen, 1996). In contrast with these flying and feeding moths, the adult *Bombyx mori* does not show any behavior besides the zigzag walking loop towards pheromone. Its response to pheromone could therefore be less flexible than other moths species. Long term habituation to pheromone (24 hours) was induced in the male moth's sensitivity to pheromone. In contrast with short term habituation, the habituated moths could not be dishabituated with a single puff of a different odorant shortly before the test (Figs. 3.30 on page 58 and 3.31 on page 59). As explained on page 85, the molecular mechanisms related to short and long term plasticity are different: gene expression and new protein synthesis are responsible for the growth of new synaptic connections in long term memory, which is not the case for short term memory (Kandel, 1997). In my experiments, synaptic connection changes could occur after long term habituation, which would prevent dishabituation or recovery. A lack of recovery after long term habituation was also observed with the oriental fruit moth, *Grapholita molesta*, (Figueredo and Baker, 1992) and the tobacco budworm moth, *Heliothis virescens*, which did not recover for 96 hours, a substantial portion of the adult life span (Daly and Figueredo, 2000). The biological significance of long term habituation to pheromone could be related to the efficiency of mating rate of the male moth. If the male moth is exposed to pheromone a few times a day, but does not find the female and therefore does not succeed the mating behavior, it will "learn" to respond only to higher pheromone concentrations. Low concentrations of pheromone implies covering long distances. By the time the male reaches the pheromone source, the likeliness another male mated the female is therefore greater. As the lifespan of *B. mori* is relatively long considering the insect does not feed, the male moth probably "learns" to be more effective and to move towards

pheromone only with substantial chances to reach it in time.

Biogenic amines The levels of biogenic amines were also measured in the antennal lobes and protocerebrum of moths exposed to long term habituation or dishabituation. The levels of serotonin were lower in the protocerebrum of moths exposed to long term habituation than in control moths. Serotonin levels in the protocerebrum of moths exposed to dishabituation (with a different odorant shortly before the test) were similar to those of long term habituated moths (Fig. 3.32 on page 60). This last result is concordant with our behavioral experiments where the responses to pheromone of habituated and dishabituated moths were similar (see also Fig. 3.31 on page 59). Serotonin seemed therefore to be closely related to long term habituation but not short term habituation (Fig. 3.27 on page 54). This is the first report showing a variation of serotonin during long-term olfactory habituation in insects. Immunocytochemical studies revealed the presence of 40-50 serotonin-immunoreactive neurons innervating all neuropils of the male silkmoth, including the antennal lobes and the mushroom bodies (M. Iwano, personal communication), brain structures known to be involved in olfactory and courtship conditioning in different insects (fruitflies: Cho et al., 2004; honeybees: Menzel, 2001). There is therefore a possibility that the release of serotonin is reduced in the calyces of the mushroom bodies. There was no quantifiable variation of either 5-HTP, Nac-5HT or NADA after long term habituation (data not shown), which suggests that long term habituation is specifically related to the biogenic amine serotonin. Short term habituation of an appetitive reflex in the honeybee is known to be related to both octopamine and tyramine and can be dishabituated (Braun and Bicker, 1992). In the snail *Helix lucorum*, levels of serotonin in the hemolymph are related to long term synaptic facilitation (Malyshev et al., 1997). Furthermore, serotonin has been related for long to long term memory in invertebrates (Barzilai et al., 1989). In *A. californica*, the gill withdrawal reflex is prevented by dopamine (Ruben and Lukowiak, 1983). In the honeybee, protein kinase A (PKA) is in-

volved in the induction of long-term memory during associative learning (Fiala et al., 1999; Müller, 2000) and in the habituation of the proboscis extension response (Müller and Hildebrandt, 2002). Furthermore, the injection of octopamine, which leads to an increase in gustatory responsiveness in the range of minutes to hours (Scheiner et al., 2002), also increases the PKA activity in the antennal lobes of honeybees (Hildebrandt and Müller, 1995), although on a much shorter time scale. These findings suggest that octopamine, by acting on the protein kinase A may affect habituation in the honeybee. However, Müller and Hildebrandt (2002) suggested that the cAMP/PKA pathway responsible for habituation is independent from biogenic amines-mediated processes. By contrast with the honeybee, octopamine does not seem a predominant biogenic amine in the male silkmoth.

In summary, we have introduced here a new model to study neural plasticity in a simple neural system, the pheromone-related olfactory pathway of the male silkmoth. Both short and long term habituation could be induced by simple behavioral experiments (sensitivity test). Variations of serotonin were related to long term habituation, a fact that had not been shown yet in insects. However, the molecular mechanisms underlying the variation of serotonin after long term habituation and the general mechanisms of short term habituation remain to be clarified.

4.4 Concluding remarks

This study demonstrated that the pheromone-searching behavior is highly flexible and can be modulated by applying external cues such as pheromone or by modifying the levels of neuroactive substances in the brain of the male moth *Bombyx mori*. Neuroactive substances were shown to be related to the neural pathway underlying different aspects of the pheromone-searching behavior of *B. mori* (see summary, Tables 4.1 on page 64 and 4.2 on page 65).

Serotonin is presented as a key neuromodulator in the sensitivity to pheromone. Given that a single serotonin-immunoreactive neuron is present in each antennal lobe, this single neuron could modulate the general responsiveness of the moth to pheromone. Furthermore,this neuron responds to mechanosensory stimulation (Figs. 3.8 on page 34 and 3.9 on page 35), which suggests that the state of the moth, "at rest", would be modified by wind exposure, to a more sensitive state. In the light of these new findings, it seems that serotonin allows the male moth to be most fitted to its environment: circadian variation of serotonin in the brain of the moth (Fig. 3.2 on page 27) gives a time window during which the female releases pheromone. Furthermore, more subtle modulations are also controlled by the same neuromodulator serotonin, and seem to prepare the moth to respond maybe faster to pheromone carried by the wind.

Nitric oxide seems also to be a key neuromodulator in the moth's sensitivity to pheromone. According to several immunocytochemical studies, nitric oxide donor and target neurons are both present in the antennal lobes of moths (Nighorn et al., 1998; Collmann et al., 2004; Seki et al., 2005), but the location of the target cells appear to differ across species. Furthermore, I highlighted a new role of nitric oxide in *B. mori*, that is, nitric oxide modulates the levels of biogenic amines. Serotonin levels were not modified but dopamine and tyramine levels were closely related to nitric oxide presence. However, the molecular pathway underlying this modulation remains to be clarified.

The response of male moths to pheromone has been considered for long as a genetically preprogrammed behavior and relatively inflexible. However, in my research, I could modulate this behavior similarly to two previous studies using other moth species that are known to perform several behaviors not observed in *B. mori* such as feeding and flying (Figueredo and Baker, 1992; Daly and Figueredo, 2000).

The measurement of biogenic amines after habituation was performed here for the first time. The variation of serotonin in the protocerebrum unraveled a new potential role of this biogenic amine in long term neural plasticity in the protocerebrum (Fig. 3.32 on page 60), a role that could be once again related to pheromone sensitivity.

This study completes the current knowledge concerning nitric oxide and biogenic amines in relationship with behaviors in insects, throwing light on the essential role of neuromodulators, which act as conductors in the symphony of neuronal activity in brains.