Chapter 1

Introduction

Insect olfactory systems are useful models for comprehending neural processing since olfactory information is processed through similar mechanisms in vertebrates and insects. The insect antennal lobe, while anatomically similar to the vertebrate olfactory bulb, contains far fewer neurons (Hildebrand, 1996). Insect systems are of great interest given that they can be studied from the single neuron to the neural network, and finally at the behavioral level. Olfaction is very important in many insect species, decisive for the control of behavior (orientation towards food sources, potential mates, spots for oviposition) and essential for maintaining the species.

Olfaction towards the odor of conspecific has been widely studied in invertebrates. Behaviors triggered by reproductive pheromone show a wide range of plasticity: behavioral responsiveness in rodents can be modified by experience (suggesting a complex central mechanism) while in moths, pheromonal responsiveness appears highly stereotypical and symptomatic of a relatively simple 'labeled line' (Kanzaki and Mishima, 1996; Sorensen, 1996). In comparison to vertebrates, the neural pathways underlying behaviors in insects are simpler, and have been suggested to be preprogrammed genetically (Tully, 1996).

Neuroactive substances play an essential role in modulating behaviors of invertebrates and vertebrates. Within neuroactive substances, biogenic amines appear to modulate synaptic activity simultaneously in different areas of the central nervous system, given that they are present in relatively small number of neurons in the brain and that these neurons form synapses with presynaptic and postsynaptic terminals of target neurons.

In insects, biogenic amines are known to modulate specific behaviors. Serotonin phase-shifts the circadian clock in the cricket optic lobe (Tomioka, 1999). Dopamine and octopamine are related to reproductive dominance in the bumble bee, *Bombus terrestris*, to foraging behavior in the honeybee, *Apis mellifera*, and to appetitive olfactory memories in the fruitfly, *Drosophila melanogaster* (Bloch et al., 2000; Schulz et al., 2002; Schwaerzel et al., 2003). Tyramine is related to the olfactory behavior in *D. melanogaster* (Kutsukake et al., 2000).

As the pheromone-searching behavior of the male moth is highly specific, suggesting little flexibility (Sorensen, 1996), the role of biogenic amines in the pheromone-searching behavior would be less significant than that in other behaviors. However, recent immunocytochemical studies (Iwano and Kanzaki, 2005; Seki et al., 2005) suggested that neuromodulators and neurotransmitters play a considerable role in pheromone olfactory processing based on their location in specific areas in the antennal lobe (the macroglomerular complex, which is the pheromone-related processing area in the antennal lobe) and the protocerebrum. The role of these neuroactive substances, mainly serotonin, nitric oxide and tyramine remained to be clarified.

The male silkmoth, *Bombyx mori*, is an appropriate model for understanding potential modulations that may occur in the central nervous system because the neural network underlying the pheromone searching behavior has been studied in detail, from the antennal lobes to the pre-motor centers (Kanzaki, 1997; De Belle and Kanzaki, 1999; Kanzaki et al., 2003). *B. mori*'s behavior comes down to walk towards the female sex pheromone source with a typical behavior called

the "mating dance" (Kramer, 1975; Obara, 1979; Kanzaki and Shibuya, 1992; Kanzaki et al., 1992; Kanzaki and Mishima, 1996; Mishima and Kanzaki, 1998). Males *B. mori* execute a zigzag-walking track towards the plume of female sex pheromone. A single pulse of pheromonal stimulation leads to the following sequence: surge represented by straight-line walking, followed by a zigzagging walk and by a looping behavior (Kanzaki et al., 1992). Olfactory information leading to this behavior first passes through the antennal lobe (AL), which is the first-order olfactory center (Olberg, 1983; Kanzaki and Shibuya, 1983; Kanzaki et al., 1994). The second stage is the protocerebrum (PC) including a specific area called the Δ ILPC (Seki et al., 2005). In the PC, neurons have connections to an important neuropil in the olfactory pathway, the lateral accessory lobe (LAL) (Kanzaki et al., 1992, 1994; Kanzaki and Mishima, 1996; Mishima and Kanzaki, 1999). The olfactory information is processed as well in the mushroom bodies, and also the central body. Multimodal information is then relayed by protocerebral neurons with axons descending in the ventral nerve cord. These neurons are called descending neurons and transfer the information to the thoracic motor circuitry involving the generation of the behavior (Kanzaki and Shibuya, 1983; Kanzaki et al., 1994). A small number of these descending neurons show a characteristic pheromone-triggered state-dependent neural activity, which has been called the flip-flop activity and which may carry information for controlling the pheromone-mediated zigzag walking, because the high and low states correspond to two different positions of the antennae (Olberg, 1983) and also to the neck and wing motor systems (Kanzaki and Mishima, 1996; Mishima and Kanzaki, 1998, 1999).

The pheromone-related behavior of the male *B. mori* is therefore a suitable model to highlight the role of biogenic amines on rigid behaviors. Is there a circadian effect on the behavior? Is there a relationship between neurotransmitters, circadian effects and the behavior? In the present study, the role of internal and external cues related to the neural network underlying the pheromone-searching

behavior have been analyzed.

The role of internal cues on the pheromone searching behavior included the circadian variation of serotonin and pheromone sensitivity behavior, the role of an identified specific serotonin-immunoreactive neuron, the role of internal variation of neuromodulators (mainly serotonin and nitric oxide) and the role of nitric oxide on biogenic amines.

External cues referred to the variation of biogenic amines due to exposure to pheromone and habituation mechanisms (short and long term) in relation with biogenic amine variation.

1.1 Internal cues

I considered as internal cues the variations occurring inside the brain and responsible for modulations of the neural network underlying behavioral modifications. By applying neuromodulators in the brain and measuring the behavioral changes, I evaluated their role on the neural network. The relationship between two neuromodulators, serotonin and nitric oxide, and insects' behaviors are introduced here.

1.1.1 Role of serotonin

In the insect nervous system, the biogenic amine serotonin acts as neurotransmitter, neuromodulator and neurohormone. Serotonin affects the central nervous system as well as the sensory periphery and the neuromuscular junction (Evans, 1980; Mercer and Menzel, 1982; Claassen and Kammer, 1986; Nässel, 1988; Casagrand and Ritzmann, 1992; Erber et al., 1993; Menzel et al., 1999). Serotonin is responsible for the modulation of various behaviors in insects such as the short-term memory, sensitivity to olfactory stimuli and foraging behavior in the honeybee *A. mellifera* (Mercer and Menzel, 1982; Menzel et al., 1999; Schulz et al., 2002). Serotonin is also involved in the regulation of the circadian clock in the optic

lobe of the cricket (Tomioka et al., 1993; Tomioka, 1999) and the cockroach *Leucophaea maderae* (Page, 1987). Moreover, serotonin increases the duration of random activity in the cabbage looper moth, *Trichoplusia ni*, and the gypsy moth, *Lymantria dispar* (Linn and Roelofs, 1986; Linn et al., 1992). Investigations of the effects of serotonin in the moth olfactory system have shown that, in the hawkmoth *Manduca sexta*, serotonin enhances the responses of some neurons in the first olfactory center, the antennal lobe to electrical and pheromonal stimuli (Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999). Furthermore, in cultured antennal lobe neurons, serotonin increases the spike number and induces a broadening of action potentials (Mercer et al., 1996). Serotonin application also affects both pheromone-evoked local field potentials and potential oscillations in the macroglomerular complex of the male *M. sexta* antennal lobe (Kloppenburg and Heinbockel, 2000). In the moth *B. mori*, high-speed optical imaging with a voltage-sensitive dye has shown that serotonin increases the maximum amplitude and the duration of the optical responses in the antennal lobe (both the macroglomerular complex and the ordinary glomeruli), suggesting that serotonin enhances neuronal responses in the antennal lobe (Hill et al., 2003).

In several insects, a unique serotonin-immunoreactive neuron innervates both antennal lobes and has been identified using immunocytochemical studies (Schurmann and Klemm, 1984; Kent et al., 1987; Rehder et al., 1987; Breidbach, 1990; Salecker and Distler, 1990). The physiological properties of this neuron had never been described. Furthermore, whether such neuron was present in *B. mori* was still unknown.

In order to understand the role of serotonin on the pheromone-searching behavior, I performed three series of experiments. First, at the behavioral level, I examined the modulatory effects of serotonin on the enhancement of neural activity by applying different concentrations of serotonin to the moth's desheathed antennal lobes. Second, I measured the levels of serotonin after application in the brain using high performance liquid chromatography (HPLC) with electrochemi-

cal detection. Third, I also performed intracellular recordings and stainings of one serotonergic neuron in the moth's antennal lobe.

1.1.2 Nitric oxide

Nitric oxide (NO) has been known as a neuroactive substance in the nervous system since the end of the 1980's (Garthwaite et al., 1988) and is thought to be a part of a general mechanism in vertebrates and insects (reviewed by Müller, 1997). It is suggested to play a considerable role in olfactory processing due to the organization of the primary olfactory center composed of glomeruli in both vertebrates and invertebrates (Hildebrand, 1996; Collmann et al., 2004).

Nitric oxide is mainly formed when a nitric oxide synthase (NOS) is activated (by Ca^{2+}) and the target of nitric oxide is a soluble guanylate cyclase (sGC), which in turn triggers the production of cGMP (see Fig. 1.1 on the following page). This nitric oxide/cGMP pathway is supposed to be the main pathway involving nitric oxide in insects (Müller, 1997; Müller and Hildebrandt, 2002). Using nitric oxide induced cGMP immunohistochemistry, Seki et al. (2005) identified the target neurons of nitric oxide, mainly located in the macroglomerular complex in the antennal lobe of *B. mori*.

However, the role of nitric oxide in the olfactory pathway is still unclear. Nitric oxide is thought to be produced after pheromone stimulation and in *M. sexta*, the single serotonin-immunoreactive neuron appears to be a target cell of nitric oxide (Collmann et al., 2004). Even though a similar result was not obtained in *B. mori*, nitric oxide has been suggested to affect levels of biogenic amines.

To understand the role of nitric oxide in the pheromone-searching behavior, I measured the variation in pheromone sensitivity after application of drugs related to nitric oxide. Furthermore, as no clear evidence had been presented yet, I evaluated the variation of biogenic amines in the brain after application of drugs related to nitric oxide.

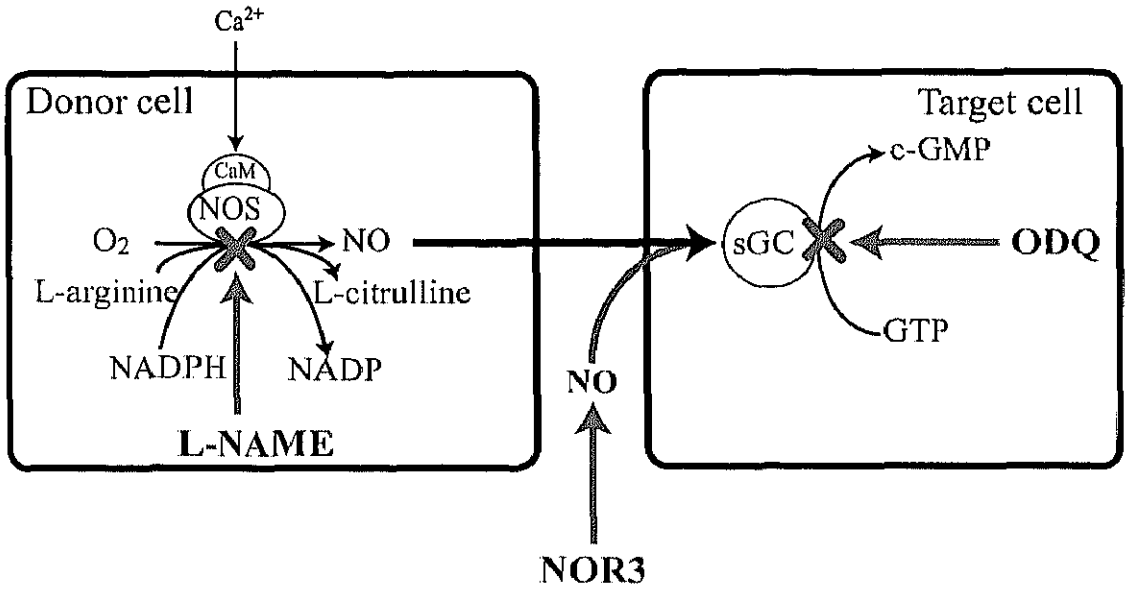

Figure 1.1: Nitric oxide pathway and drugs related with nitric oxide
CaM, calmoduline; NO, nitric oxide; NOS, nitric oxide synthase; NADP, Nicotinamide adenine dinucleotide phosphate; sGC, soluble guanylate cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate. Drugs hindering the nitric oxide pathway: L-NAME, Nω-nitro-L-arginine methyl ester; NOR3, (+/-)-(E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

1.2 External cues

Even though insects' behaviors are thought to be very rigid, some insects have demonstrated a surprising degree of experience-dependent modification of several other behaviors (De Belle and Kanzaki, 1999). Honeybees are remarkable insects, being able to learn the odor, color and shape of flowers. Hive mates attending a dance performance learn the odor emanating from the dancing bee and seek it at the indicated food site (for review, see Hammer and Menzel, 1995). In the honeybee *A. mellifera* and the fruitfly *D. melanogaster*, olfactory based associative learning (odor paired with food) could be performed in laboratory settings, suggesting that memory formation is located in the antennal lobes (first olfactory center) and in the mushroom body (in the protocerebrum, higher olfactory center) (Hammer and Menzel, 1995; Tanaka et al., 2004). Appetitive olfactory conditioning has also been performed in the moths *Spodoptera littoralis* (Fan et al., 1997) and *M. sexta* (Daly et al., 2001). In *M. sexta*, olfactory-based neural plasticity has been shown to first occur in the antennal lobes, producing a restructuring of spatial and temporal components of network responses to odor in the antennal lobe (Daly et al., 2004). When odor does not predict food, non-associative learning occurs, that is habituation (in moths : Daly and Figueredo, 2000; Daly et al., 2004; in honeybees : Braun and Bicker, 1992; in fruitflies : Cho et al., 2004). In insects, the molecular process underlying non-associative learning has been studied only in *A. mellifera*, using the appetitive proboscis extension reflex, and showed the importance of the nitric oxide/cGMP pathway in the antennal lobe (Müller, 1996).

I therefore tempted to study another olfactory learning, the pheromone-searching behavior and made the challenge of modifying this highly stereotyped behavior. Habituation is a suitable experiment for studying the modification of a pathway using external cues given that the pheromone-related olfactory pathway can be modified by repetitive application of pheromone, excluding the contribution of ex-

ternal pathways (such as the visual pathway). 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). Dishabituation and sensitization are two mechanisms related to habituation (for review, see Groves and Thompson, 1970).

The mechanisms underlying learning and memory (including habituation) can be classified into short (from minutes to hours) and long (from hours to days) term memory (Kandel, 1997). I therefore used two different protocols to induce short term (30 minutes) and long term (24 hours) habituation. I performed a dishabituation protocol in both cases.

Furthermore, biogenic amines are known to be related to habituation in the sea snail *Aplysia californica* (Ruben and Lukowiak, 1983) and in insects such as the honeybee (Braun and Bicker, 1992). I therefore evaluated the role of biogenic amines in habituation. To this aim, I dissected brains of habituated, dishabituated and control moths into first olfactory center (the antennal lobes) and higher olfactory center (the protocerebrum).

1.3 Objective of the present work

Insects adapt to variations of their environments by modifying the neural networks underlying their behavior. Such neural modifications are thought to be controlled by neuromodulators (for review, see Erber et al., 1993; Menzel and Müller, 1996). For instance, neuroactive substances are known to modify olfaction in insects (for review, see Homberg and Müller, 1999). The pheromone-searching behavior of the male moth *B. mori* is however thought to be controlled by a self-generated zigzagging program, in which plasticity plays a minor role (Kanzaki et al., 1992; Kanzaki, 1996). Behavioral plasticity and the role of neuroactive substances have

therefore not been seriously taken in account hitherto.

In order to verify the degree of plasticity of the pheromone-searching behavior of the male moth *B. mori*, and to evaluate the role of neuromodulators in this behavior, I performed behavioral and pharmacological experiments, combined with measurements of biogenic amine levels using a high performance liquid chromatography with electrochemical detection. I also carried out physiological recording and staining of a serotonin-immunoreactive neuron.