1. Introduction

An animal can determine adaptive behavior for survival based on environmental information that they receive through the sensory systems and process it in their neural pathways (Nicholls *et al.*, 2001). For animals, detecting chemical substances is important for individual's subsistence (Nicholls *et al.*, 2001). For detecting chemical substance, olfaction is regarded as an essential sense. The subtle odor substance emitted from food source will inform animals the location of the food. Various pheromones, kairomones and allomones emitted from the same or other kind of animals will induce them beneficial behavior (Brown *et al.*, 1970). Without exception, the insect usefully exploits olfactory information for struggle for existence in nature (Stengl *et al.*, 1999).

In this study, in order to investigate olfactory neural pathways in the insect brain, I applied histochemical techniques with the support of Neuron Database. Neuron Database is developed in our laboratory for collecting morphological and physiological properties of single brain neurons of the male silkworm moth *Bombyx mori*. The aim of this study is to make a comprehensive research of the olfactory neural pathways in the insect brain with the support of Neuron Database. First, I discuss olfactory processing in the antennal lobe (AL) and projection pathways from the AL to the protocerebrum. Second, I discuss the control systems of odor-evoked locomotion in the protocerebrum.

1.1 Neuroactive substance in the antennal lobe neural pathways

In insects, olfactory information received by the antennae is first processed in the antennal lobe (AL), the primary olfactory center in the brain. The AL of insects has a similar neural architecture and basic aspects of olfactory processing to the vertebrate olfactory bulb (Hildebrand and Sheperd, 1997). Therefore, neural processing in the AL of insects has been a popular model for understanding olfactory processing. In *B. mori*, the AL is composed of local interneurons (LNs), projection neurons (PNs) and centrifugal neurons (Homberg *et al.*, 1988; Anton and Homberg, 1999; Kanzaki *et al.*, 2003). The odor information is received by olfactory receptor neurons (ORNs) and transmitted to glomerular structures in the AL. In the glomeruli, neural information is processed by synaptic connections of the LNs terminating only in the AL, and PNs. The processed neural information is transferred by the PNs to higher olfactory centers, the protocerebrum (PC). The PNs, passing through antenno-cerebral tracts (ACT), ascend into the PC including the mushroom body (MB) and the lateral protocerebum (LPC) (Schildberger, 1983; Kanzaki *et al.*, 1989; Malun *et al*, 1993; Hildebrand, 1996; Anton and Homberg, 1999; Kanzaki *et al.*, 2003; Seki *et al.*, 2004) . In addition, centrifugal neurons are involved in modulation of this processing (Kent *et al*, 1987; Salecker and Distler, 1990; Hammer, 1993; Hill *et al.*, 2003).

Since neural processing in the AL is based on chemical neurotransmission and neuromodulation (Waldrop *et al.*, 1987; Anton and Homberg, 1999; Christensen *et al.*,

1993, 1996, 1998; Okada *et al.*, 1996; Ai *et al.*, 1998; Kloppenburg *et al.*, 1999; Kloppenburg and Heinbockel, 2000; Ai and Kanzaki, 2004), it is important to identify neurotransmitter and/or neuromodulator as signaling molecules for understanding olfactory processing in the AL. In insect species, a variety of neuroactive substances in the AL neurons have been clarified using immunocytochemical techniques (Homberg *et al.*, 1990; 1995; Homberg and Muller, 1999). Those studies showed a high interspecific variation in the distribution patterns of neuroactive substances in the AL. For example, histamine and dopamine immunoreactive neurons in the AL neurons showed a high interspecific variation. Histamine immunoreactivity in the AL is found in bees, moths, crickets, locusts and cockroaches but not in flies (Pirvola *et al.*, 1988; Homberg and Hildebrand, 1991; Beat *et al.*, 1997; Bornhauser and Meyer 1997; Ignell, 2001). Dopamine immunoreactivity in the AL is found in bees, moths and crickets but not in flies and locusts (Klemm, 1974, 1976; Schürmann *et al.*, 1989; Distler, 1990). It is necessary to identify interspecific variations of neuroactive substances in order to understand olfactory processing in a particular insect.

Of all neuroactive substances, serotonin has been vigorously investigated with respect to its neural functions in the olfactory neural processing in some insect species. In the hawkmoth *Manduca sexta*, bath application of serotonin enhances the olfactory response of neurons in the AL (Kloppenburg *et al.*, 1999). Additionally, serotonin application increases the amplitude and duration of pheromone-evoked local field potentials, as well as the amplitude of potential oscillations in the male specific neural

compartments of the AL in *M. sexta* (Kloppenburg and Heinbockel, 2000). In the male *B. mori*, behavioral studies and electrochemical detection using high-performance liquid chromatography (HPLC) demonstrated that serotonin modifies olfactory sensitivity to pheromone stimulation (Gatellier *et al.*, 2004). Moreover, high-speed optical imaging with a voltage-sensitive dye revealed the modulatory effect of serotonin in a particular neural compartment of the AL in the male *B. mori* (Hill *et al.*, 2003). Those studies demonstrate that olfactory processing in the AL is dynamically modified by serotonin. Therefore, dynamic neural modulations by neuroactive substances must be an important factor for olfactory processing in the AL.

However, in *B. mori* brain, the localization of putative neuroactive substances is still unclear. To investigate the localization of neuroactive substances in the AL, I applied immunocytochemical techniques to the brain of the male *B. mori*. I revealed the distribution patterns of GABA, which is regarded as the principal inhibitory neurotransmitter in the AL (Waldrop *et al.*, 1987; Anton and Homberg, 1999; Christensen *et al.*, 1993, 1996, 1998; Okada *et al.*, 1996; Ai *et al.*, 1998; Ai and Kanzaki, 2004) and FMRFamide, which is not specifically involved in the olfactory system but exists as a subpopulation of the GABA immunoreactive neurons in the AL (Homberg *et al.*, 1990). Furthermore, I investigated serotonin, tyramine and histamine immunoreactive neurons in the AL. All these biogenic amines have been implicated as transmitters or modulators in the insect brain (Gatellier *et al.*, 2004; Hill *et al.*, 2003; Homberg and Muller, 1999; Saraswati

et al., 2003). Besides, I applied cellular organization classification to these immunoreactive AL neurons. This classification revealed basic distribution patterns of neuroactive substances in the cellular organization of the AL showing that each projection pathway from the AL to the protocerebrum contains a specific combination of neuroactive substances.

1.2 Odor-evoked locomotion control system in the protocerebrum

Neural information transmitted from the primary olfactory center to the protocerebrum (PC) has multiple neural processing stages and finally evokes various behaviors. Zigzag behavior is often observed in the odor orientation behavior of various animals. For example in insects, the ladybeetle shows characteristic straight walking and zigzag behavior after their preyed upon a woolly aphid (Nakamura, 1982). In the ant, the tracing orbit, which ushered by the guide pheromone, is a zigzagging pattern (Hangartner, 1967) . In addition, the desert ant foragers show zigzag-like orientation paths for approaching to the upwind food odor sources (Wolf and Wehner, 2000).

Male *B. mori* evoked a characteristic zigzag behavior similar to other insect species. In male *B. mori*, this zigzag behavior was induced by a key chemical substance, called sex-pheromone "bombykol" and was a sequential orientation behavior. At the first stage of a sequential orientation behavior, male moths make a straight-line walking just after the stimulation, at the following second stage they show left and right turning (as so

called a zigzagging walking). The inter turn interval time is gradually prolonged. Then, they show a lopping behavior (turns of more than 360 degrees). This sequential pheromone orientation behavior is reset by pheromone stimulation and is repeated from the beginning in response to each pheromone stimulation (Kanzaki *et al.*, 1992; Kanzaki, 1998).

This zigzag behavior is elicited by neural activity transmitted from the protocerebrum to descending interneurons (DNs). The passage of pheromonal information from the protocerebrum to a thoracic motor center has been well studied in *M. sexta* and *B. mori* (Olberg, 1983; Kanzaki and Shibuya, 1986b; Kanzaki *et al.*, 1991b; Kanzaki *et al.*, 1994; Mishima and Kanzaki, 1999). In both moth species, some descending interneurons show a characteristic long-lasting activity in response to the pulsed pheromone stimulation (Kanzaki *et al.*, 1991b; Mishima and Kanzaki, 1999). Especially a set of *B. mori* descending interneurons showed a characteristic "flip-flop activity pattern" (Olberg, 1983; Kanzaki *et al.*, 1994; Kanzaki and Mishima, 1996; Mishima and Kanzaki, 1999). While, the activity states of both sides of flip-flopping descending interneurons show anti-phasic relationship (Kanzaki *et al.*, 1994; Kanzaki and Mishima, 1996). Further, intracellular recording and staining studies demonstrate the morphology of these flip-flopping descending interneurons (Mishima and Kanzaki, 1999). These flip-flopping descending interneurons have major dendritic arborization in one side of the lateral accessory lobe (LAL) and ventrolateral protocerebrum (VPC), which have bilaterally symmetrical spheroidal neuropil structure in the protocerebrum. These morphological and physiological

properties of flip-flopping descending interneurons suggest the existence of "alternating activity pattern" between both sides of the LAL-VPC.

Additionally, numerous LAL-VPC intrinsic bilateral neurons show a characteristic long-lasting activity in response to pheromone stimulation (Kanzaki *et al.*, 1991b; Kanzaki and Shibuya, 1992). Further, it is reported that many olfactory responsive protocerebral extrinsic neurons innervate the LAL in *M. sexta* and *Agrotis segetum* (Kanzaki *et al.*, 1991a; Lei *et al.*, 2001). From these evidences, both sides of the LAL-VPC are considered to be an important neuropil area through the olfactory neural pathways for generating "long-lasting activity" and "alternating activity pattern" for "flip-flop activity" in descending interneurons.

In previous studies, it has already been demonstrated that the function of "flip-flop activity" is a signal associated with the initiation of turns during pheromone searching zigzag behavior (Kanzaki and Mishima 1996; Mishima and Kanzaki 1998). However, the neural mechanisms for generating the "long-lasting activity" and "alternating activity pattern" in both sides of the LAL-VPC are not yet well known. In this study, at the first setout I demonstrated an intimate neural structure of the LAL-VPC and showed that this neural structure is formed by four neural subregions, which are divided by neural tracts. In the second, I examined the morphological, physiological and neurochemical properties of LAL-VPC intrinsic neurons, which were extracted from the Neuron Database. My purpose is to construct neural circuit models for generating the "long-lasting activity" and

"alternating activity pattern" in both sides of the LAL-VPC, which is triggered by pulse of odor information and transferred to descending interneurons as "flip-flop activity" for releasing characteristic zigzag behavior.