4. Discussion

4.1. Newly developed intracellular staining methods

Insect brains have smaller number of neurons than vertebrate brains. The neurons of the insect brains are identifiable neurons. Each neuron has a characteristic feature and multi functions. So, it is important to investigate nervous system from single cell level in the insect brains.

In this study, I developed a novel intracellular staining method using an infrared DIC microscope. With the method I inserted a microelectrode to one of target neurons under visual control (Fig. 2A-C). As a characteristic of insect brains, cell bodies of neurons cluster on the surface of the brain. So I could access and stain most of the neurons from the cell body clusters. In the conventional intracellular staining in insect brains, one has to decide the position of an electrode under stereoscopic microscopy. The success rate to insert the electrode to a target neuron is low except when the neuron has a very thick axon or a large cell body. It is difficult to impale the particular neurons repeatedly. Previous research using morphological and histological approaches have provided information about constituents in the particular cell cluster. For example in *B. mori*, the MC of the AL contained uniglomerular PNs and the LC of the AL contained LNs and

multiglomerular and uniglomerular PNs (Kanzaki and Shibuya, 1986). To insert an electrode to the target neurons, it is effective to aim at the cell body in the cell cluster whose constituent neurons are already known to be a target one. Therefore, I established the intracellular staining under visual control method. This method enabled me to stain target neurons efficiently.

For the mapping the termination of the single PNs comparing with the ΔILPC region, it is necessary to stain single PNs in combination with NO-induced anti-cGMP immunostaining in the same preparation. I stained neurons in the MC of the AL and surely stained single PNs efficiently. In the first work, I also used this method for double labeling of antero-grade staining of ORNs and single neuron staining of a LN or double labeling of single neuron staining of a LN and PN. These staining combinations were useful but extremely difficult with the conventional intracellular staining.

For the morphological analysis of AL LNs, I used this method to sample neurons comprehensively from a target cell cluster. I sampled neurons from the wide region of the LC of the AL (Fig. 18). This way of comprehensive morphological analysis proposes a new way of investigation of a particular network.

4.2. Projection map in the protocerebrum from subdivisions of the AL

4.2.1. ΔILPC: landmark in the LPC

Recent studies have revealed NO-cGMP signaling systems play important roles in olfactory processing in several kinds of insects (Elphick et al., 1995; Müller, 1997; Nighorn et al., 1998; Bicker, 2001; Stengl et al., 2001; Aonuma and Niwa, 2004). In *B. mori*, NO-cGMP signaling system is expressed in the pheromone processing pathway and regulates the threshold of pheromone mediated searching behavior (L.Gatellier, pers. commun.). In this study, I performed NO-induced anti-cGMP immunohistochemistry to identify the target cells of NO in *B. mori* after stimulating soluble guanylyl cyclase by incubating preparations in the saline containing NO donor. Strong anti-cGMP immunoreactivity was expressed in the AL and LPC (Fig. 8). I unexpectedly found that the specific deltoid area was consistently observed in the ILPC with anti-cGMP immunostaining (Fig. 8B). In a previous study, Kanzaki et al. (2003) showed that the MGC-PNs project to the ILPC but not into the LH. They also found that the toroid-PNs project more medially to the ILPC than the cumulus- and horseshoe-PNs. However, they could not identify precise and absolute projection sites among those PNs, because the LPC (including the ILPC) is an indistinct area without any clear neuropile structures, a characteristic that has been a problem for many researchers investigating projection

areas of AL PNs in the LPC. The ΔILPC was observed consistently in every individual with NO-induced anti-cGMP immunostaining, so it became a reference landmark for comparison of terminal arborizations of PNs. Taking advantage of this consistent immunoreactivity as a landmark in the LPC, I could provide further analysis about terminal arborization areas of each type of PNs (Figs. 9-12).

4.2.2. Identification of the pheromone processing center in the LPC

Homberg et al. (1988) showed that the PNs from the MGC and the PNs from the ordinary glomeruli project to different areas in the LPC in the brain of the male moth *M. sexta*. They showed that the projection area of the MGC PNs is the ILPC and the projection area from the ordinary glomerulus PNs (G-PNs) is the LH, which is posterior lateral to the ILPC. Hence they suggested that pheromone information processing pathways might be distinguished from general odor processing pathways; it is what we call labeled line processing. This separation of the pheromone and general odor pathway is analogous to the 'main' and 'accessory' olfactory systems of vertebrates (Hildebrand and Shepherd, 1997).

In the *B. mori* AL, 39 ± 4 (mean ± S.D. n = 10) neurons in the MC showed anti-cGMP immunoreactivity. These neurons were presumed to be a group of the

toroid-PNs (Fig. 8). This was confirmed clearly by double labeling of single toroid-PNs with NO-induced anti-cGMP immunolabeling (Figs. 9, 20). I also confirmed by double labeling experiments that cumulus-PNs and horseshoe-PNs projected to the lateral half of the ΔILPC (Figs. 10, 11, 20). I also demonstrated that not only the PNs passing through the IACT but also the PNs passing through the OACT and MACT projected to the ΔILPC (Fig. 8). Considering the fact that the PNs passing through the OACT had cell bodies in the LC and dendrites in the MGC (Fig. 8H), these neurons could be the c+t-PNs that were identified in the previous work (Kanzaki et al., 2003; Table 1). Similarly the PNs with their axon passing through the MACT and not characterized in this study may be the other type of the c+t-PNs that were identified in the previous work (Kanzaki et al., 2003; Table 1). I therefore presume that all the MGC-PNs project to the ΔILPC. That is, all types of MGC-PNs carrying pheromone information is transmitted exclusively to the ΔILPC, which gives strong evidence that this ΔILPC is the pheromone processing center in the LPC.

In this study, I also double labeled 16 G-PNs in comparison to the ΔILPC (Table 2). These neurons showed a variety of arborization patterns in density and location in the LH but not in the ΔILPC (Figs. 12, 20). This result supports the idea that pheromone and non-pheromone information are independently processed in the higher brain

centers.

4.2.3. Integration and segregation of pheromone information

In this study, I revealed that toroid-PNs projected to the whole area of the ΔILPC and that cumulus and horseshoe-PNs projected to the lateral half of the ΔILPC (Figs. 9-11, 20). Our previous intracellular recording work revealed that MGC-PNs' responses to pheromonal stimulation correlate with their dendritic arborizations in the subdivisions of the MGC (Kanzaki et al., 2003; Table 1). The toroid-PNs show excitatory response to bombykol and the cumulus- or horseshoe-PNs show excitatory response to bombykal (Kanzaki et al., 2003; Table 1). Therefore the toroid-PNs transmit the information of the major pheromone component (bombykol) to the whole area of the ΔILPC and the cumulus- and horseshoe-PNs transmit the information of the minor pheromone component (bombykal) to the lateral half of the ΔILPC (Fig. 20).

In this study, I was not able to label c+t-PNs with anti-cGMP immunostaining. Since I revealed the approximate position of the ΔILPC, it partly allowed me to reproduce the putative region of the ΔILPC without anti-cGMP immunostaining. So I reexamined the terminal arborization area of the c+t-PNs characterized in the previous study (Kanzaki et al., 2003, Fig. 20) comparing with the putative ΔILPC region. I found that their

terminal arborization area may correspond to the lateral part of the ΔILPC (dotted lines in Fig. 20). These results suggest that the bombykol and bombykal information is transmitted to the lateral half of the ΔILPC and only the bombykol information is transmitted independently to the medial part of the ΔILPC. Therefore the integration and segregation of the pheromone-component information may be processed in this ΔILPC. It has been shown that only bombykol can sufficiently trigger the whole mating behavior (Kaissling and Kasang, 1978; Kanzaki 1998). It is possible that protocerebral neurons which have dendritic arborizations in the medial part of the ΔILPC may play an important role as a trigger for the mating behavior.

Besides, in many kinds of moths the ratio of each pheromone component is a critical factor for locating conspecific females and/or for discriminating interspecific females (Hansson and Christensen, 1999). In *B. mori*, the minor component, bombykal is known for its inhibitory effects on the mating behavior (Kaissling and Kasang, 1978; L.Gatellier, pers. commun.). Present study indicates that both bombykol and bombykal information individually project to the lateral half of the ΔILPC and there is a possibility that the c+t-PNs also project to this area. This suggests that the inhibitory effect on the mating behavior occurs at the ΔILPC by the interaction between the major and minor pheromone components.

The previous study showed that differences in arborization patterns are observed in the Ca among MGC-PNs and G-PNs (Kanzaki et al. 2003). The toroid-PNs send noticeably smaller branches but the cumulus and horseshoe-PNs send wide field blebby axonal branches into the Ca (Kanzaki et al., 2003; Table 1; Figs. 9-11, 20). This result suggests that only bombykal and general odor information may relate to the processing in the Ca which may include learning, memory or other specific functions. Another possibility is that the MB may play a role in bombykal inhibitory effects on the behavior. To elucidate the functional significance of bombykal, more analysis of behavioral experiments and electrophysiological investigations of protocerebral neurons receiving information in the ΔILPC or the Ca are necessary.

The present study provides the precise projection map of pheromonal information in the protocerebrum from the MGC of *B. mori*. This information serves for further investigation of pheromonal information processing in the protocerebrum. The present investigation elucidates a basic pathway of pheromonal information at the complicated neural circuits in the protocerebrum, and will lead to understand how odor information is processed in the higher brain centers.

4.3. Morphological classification of LNs

4.3.1. Comprehensive sampling of LNs in the lateral cell cluster of the AL

In this study I stained 126 LNs using the newly developed intracellular staining method (Table 3). There was a tendency in distribution of the cell bodies among the types (Fig. 18). The cell bodies of the type I LNs distributed widely from the surface to middle depth (0-60 μm), the cell bodies of the type II LNs situated in the under part of the LCI, the LCII (Fig. 18). The type IVc LNs that extended branches to the AMMC were found at the deep region in the LCI (Fig. 18). These results suggest that to some extent the approximate distribution of cell bodies is retained among individuals. The observation that several PNs in the middle of the cell cluster supports this idea (data not shown). However the possibility of variation in location of the cell bodies within the cell cluster by individuals was also found. The type IIIb LNs had notably large sized cell bodies (Figs. 16D, I, 18). I easily identified the cell bodies when they were observed on the surface of the LCI but they were not observed in every preparation. This suggests one possibility that the cell bodies of the type IIIb move within the cell cluster by individuals. While I obtained neurons in the wide range of regions in the cell cluster using partial cut of the cluster to impale the deeply positioned cells, the number of neurons I stained would not reflect the actual distribution of these LNs. I tended to

select the cells near the surface and edge of the cluster or conspicuous large cell bodies (Fig. 18). However there were many variations of LNs in *B. mori*. This way of comprehensive morphological analysis is efficient to reveal the neuronal elements of the particular neural circuits.

4.3.2. Neural circuits within single glomerulus

Many researchers have investigated synaptic connections in the AL using the electron microscopic techniques (Tolbert and Hildebrand, 1981; Gascuel and Masson, 1991; Malun, 1991a,b; Boeckh and Tolbert, 1993; Distler and Boeckh, 1996, 1997a,b; Sun et al., 1997). These results revealed that many dyadic synapses are observed and ORNs, LNs and PNs are connected mutually within a glomerulus.

I observed differences of dendritic profiles, density and distribution within single glomerulus. The type I LNs had coarse arborization biased to the core region (Fig. 14D), the type II LNs had densely packed arborization all over the glomerulus (Fig. 15E), the type IIIa LNs had sparse and dense arborization surrounding the edge of the glomerulus (Fig. 16B, C), the type IIIb LNs had very fine and dense arborization in the whole glomerulus (Fig. 16E, F).

In many kinds of insects ORNs terminate at the rim or hemisphere of a glomerulus

and most frequently reported type of LNs (like type I LNs found in this study) arborized core region of a glomerulus (Anton and Homberg, 1999). So it has been speculated that synaptic regions are separated into the rim, core and base region of a glomerulus. Some previous studies speculated that the LNs with arborization in the core region of a glomerulus have no connections with the ORNs. In this study using double labeling of ORNs and a type I LN which has core type arborization within a single glomerulus, I demonstrated that several overlaps at the rim region of a glomerulus were observed (Fig. 19A-C). Although it did not surely demonstrate the existence of synaptic connections between them but the possibilities are remained. I also double labeled a type I LN and uniglomerular PNs. I found the PNs' dendrites extending the whole glomerulus (Fig. 19D, E). The type I LN and the PN overlapped at the core region of the glomerulus (Fig. 19D, E). Sun et al. (2003) demonstrated that many output synapses of PNs are observed at the core of a single glomerulus. The type I LN may receive information from the uniglomerular PNs. Electron microscopic study is necessary to reveal the synaptic relationship among ORNs, PNs and various LNs. However the difference of dendritic shapes may reflect the combination of synaptic contacts. In vertebrate, at least two layers of lateral interactions are observed; periglomerular neurons at the glomerular layer and granule cells at the external plexiform layer out of the glomerulus

(Christensen and White, 2000). In insects, most of synapses are confined within a glomerulus. The results of the present study suggest that multiple synaptic layers may be constructed within a glomerulus.

4.3.3. Relationship between LNs and functional representation of the AL glomeruli

In the AL, each glomerulus is considered as a functional representation of a particular olfactory receptor (Clyne et al., 1999; Vosshall et al., 1999, 2000). So it is considered that an odor is represented by a combination of several glomeruli. The MGC is projected by pheromone receptor neurons and specialized for the pheromone processing (Kanzaki and Shibuya, 1986; Christensen and Hildebrand, 1987; Kanzaki et al., 1989; Hansson et al., 1991, 1992; Hildebrand, 1996; Christensen et al., 1996; Hansson and Christensen, 1999). The type I, II, IV LNs had arborization in the MGC so they might relate pheromone information processing. Of those types, the type IVa,b LNs had dense arborization in the MGC, whereas the type I, II LNs had relatively sparse arborization (Figs. 14F, 15B, 17A, B, C, D). Further more, the type IVa LNs connected the MGC and the selected pluri-Gs (Fig. 17A, B). Koontz and Schneider (1987) demonstrated that the anterograde staining from the trichodeal sensilla stains the MGC and the LLG1, LLG2, and MSG. Type IVa connected the MGC with some of Gs right

under the MGC including the LLG1, LLG2 and MSG specifically. So the pheromone related information may be processed and some functional modulation might be added by these neurons.

There is the PV region under the LLG1, LLG2 and MSG (Fig. 13C, D). The function of the PV region has not been known. The PNs arborizing in the PV showed inhibitory responses to mechanosensory stimuli, so the PV might be a region related to the mechanosensory (K. Soo, pers. commun.). The type IVc LNs extended branches in the AMMC and had thick arborization in the PV (Fig. 17E, F). This type of neurons might mediate the interaction between the PV and AMMC. The type II and III LNs densely arborized in the Gs and PV regions while sparse or no arborization in the MGC, LLG1, LLG2 and MSG (Figs. 15D, 16). These two types of LNs connected the Gs on the surface of the AL and the PV strongly so there appear to be some strong connection between these two regions.

Odor information is represented as a combination of particular Gs (Joerges et al., 1997; Sachse et al., 1999; Galizia and Menzel, 2001; Sachse and Galizia, 2002; Wang et al., 2003). The type III and type IV LNs sent their branches to not all of Gs but heterogeneously to pluri-Gs (Figs. 16, 17), so they might relate the processing of a particular odor. A few studies on insects have showed that other modalities such as

cold/warm, dry/moist, CO_2 sensory neurons project to the AL forming a glomerulus (Anton and Homberg, 1999). The glomerulus receiving other modality was not identified in *B. mori* but some types of LNs may connect these glomeruli and function as an integrator of multimodal information in the AL.

4.4. General Discussion

R. Axel and L. Buck have won the 2004 Nobel Prize in physiology or medicine for their work of identification of the DNA sequences for odorant binding proteins in mouse (Buck and Axel, 1991). They discovered a gene pool of more than 1,000 different genes that encode olfactory receptors. Olfactory processing mechanisms have been investigated rigorously for these three decades (Hildebrand and Shepherd, 1997) and since the work of Axel and Buck in 1991 the understanding of peripheral level especially proceeded (Vosshall, 2000). However olfactory processing mechanisms in central nervous system (CNS) remain conundrum. Olfactory processing in the first order center (AL in insects and OB in vertebrates) has been gradually elucidated that the spatio-temporal coding mechanisms work with glomerulus as a functional unit but several researches provide controversial results (Hildebrand and Shepherd, 1997; Christensen and White, 2000). Furthermore, olfactory processing in the next order

center is more difficult to investigate. It is difficult to understand how the topographic map constructed in the AL is represented in the higher brain centers. Present work aimed at revealing the olfactory processing mechanisms in the CNS. I employed an insect which has many experimental merits such as simple nervous system (Hildebrand, 1996). First, I got the significant result that identify the pheromone processing centers and revealed the integration and segregation sites for pheromone components in the second order centers receiving output from the AL. Secondly I revealed the morphological variation of AL LNs as a first step to reveal the mechanisms of inter- and intra- glomerular computation.

Pheromone processing systems in the moths are one of the most well known fields in olfaction (Kanzaki and Shibuya, 1986; Christensen and Hildebrand, 1987; Kanzaki et al., 1989; Hansson et al., 1991, 1992; Hildebrand, 1996; Christensen et al., 1996; Hansson and Christensen, 1999). In the MGC, correspondence between the subdivisions and pheromone components has been investigated mainly using intracellular recording and staining of the PNs (Hansson and Christensen, 1999). As discussed above, it is important to identify the pheromone processing centers precisely in the LPC from the MGC subdivisions. Here I added one more significance to this work. Pheromone-mediated mating behavior is a very simple and excellent model for tracing

neural pathways from the stimulus to behavior. Particularly in *B mori*, the works of our laboratory have promoted the understanding of where and how the command information controls the mating behavior (Kanzaki et al. 1992; Kanzaki and Mishima, 1996; Kanzaki, 1998). There is a region that generates command information of the mating behavior. It is called the lateral accessory lobe (LAL), a couple of which locating in the protocerebrum. It is not yet confirmed but the previous work suggested that information is projected from the LPC to the LAL (Kanzaki et al. 1991). Identifying the precise region of the pheromone information flow in the LPC will lead to connect the complete pheromone processing pathways in the brain of *B mori*.

On the other hand, the odor information is represented as a spatio-temporal map of glomeruli in the AL. Due to the structural and functional similarity to the vertebrate olfactory bulb, many disciplinary studies have been done to reveal the coding mechanisms based on the glomerular structures in the AL (Christensen and White, 2000). The functions of LNs connecting glomeruli remain conundrum. I got the 3D morphological data of the LNs and revealed the variety of LNs. I provided the basic information about the constituents of the AL neural circuits. My next aim is what function the LNs have in processing odor information. As a trial to investigate them, I plan to extract some morphological parameters to construct compartment models from

these precise morphological data and analyze the electrical properties of LNs on processing. These works will contribute to reveal the puzzle of olfactory coding mechanisms in the AL.