3. Results

3.1. Newly developed intracellular staining methods

I developed a new intracellular staining method. With the method I inserted a microelectrode to a target cell body under visual control (Fig. 2). Infrared DIC microscopy enabled me to visualize cell bodies about 50 μm in depth (data not shown) from the surface of the brain. The size of the most of the cell bodies in the MC and LC of the AL was 10-20 μm (Fig. 2B, C), so I could visualize cell bodies at the third to fourth layer (data not shown). I evaluated this method by trying to stain neurons from the various region of *B. mori* brain primarily in the olfactory processing pathway (Figs. 6, 7). I confirmed that various kinds of neurons can be stained with this method. For instance, AL LNs, AL PNs, Kenyon cells (intrinsic neurons of the MB), Group I, II, III descending neurons, neurosecretory cells, efferent optic lobe neurons, bilateral neurons in the protocerebrum and other unknown neurons were stained with this method (Figs. 6, 7). Numerous cell clusters not stained in this study were still left, so this method can be used to explore unknown neurons. I hold the electrode into the cells for more than 10 minutes, so I injected fluorescent dyes to the cells enough to be fully stained. I could confirm the state of dye injection by use of fluorescent light microscopic view. The

smallest step of movement of the electrode controlled with the micromanipulator was 0.1 μm. I could insert the electrode to the smallest cell bodies (7-10 μm) in the *B. mori* brain, the Kenyon cells (Fig. 6).

Due to high efficiency of staining with this method, I achieved double labeling of single neuron staining with immunostaining (Figs. 9-12), anterograde staining (Fig. 19A-C) and of two single neurons (each neuron stained with different dyes) (Fig. 19D, E). I also used this method to comprehensively sample neurons in a particular cell cluster (Fig. 18).

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

3.2.1. Anti-cGMP immunostaining

I performed NO-induced anti-cGMP immunostaining in male *B. mori* brains (n = 31). Strong anti-cGMP immunoreactivity was observed in the AL and the LPC (Fig. 8A, B, F-H). I analyzed immunoreactivity using 3-D reconstruction of the stained neurons (Fig. 8D, E).

In the lateral protocerebrum (LPC), NO-induced anti-cGMP immunostaining revealed the specific delta area (Fig. 8B). This region was situated about 150μm from

the surface of the AL and about 150 μm from the surface of the calyces of the mushroom body (Ca). This region showed a triangular shape from the frontal view with a base and height of respectively about 200 μm and 80 μm (Fig. 8B). This region also showed a triangular shape from the dorsal view with a respective base and height of about 200 μm and 50 μm (Fig. 8E). The three dimensional view of this region represents therefore a triangular pyramid (Fig. 8D). I named this area the delta area in the inferior lateral protocerebrum (ΔILPC).

Remarkably the ΔILPC was innervated by neurons from three AL output tracts (inner, middle and outer antenno-cerebral tracts; IACT, MACT and OACT respectively) (Fig. 8A- D).

Neurons of the IACT showed the strongest NO-induced anti-cGMP immunoreactivity (Fig. 8A-D). In the AL of *B. mori*, there are two cell clusters that comprise local interneurons (LNs) and projection neurons (PNs). One is the medial cell cluster (MC) and the other is the lateral cell cluster (LC) (Fig. 8A). The MC resides medio-dorsally and comprises about 250 neurons (Kanzaki and Shibuya, 1986; Koontz and Schneider, 1987), most of which are thought to be uniglomerular projection neurons (Kanzaki and Shibuya, 1986). In the MC, 39 ± 4 (mean ± S.D. n = 10) neurons showed NO-induced anti-cGMP immunoreactivity (Fig. 8A, F). Those neurons innervated the

toroid of the MGC (Fig. 8F, G) and connected to the IACT (Fig. 8A, G). The IACT exits the AL medio-dorsally and ascends into the protocerebrum, bypassing the postero-lateral edges of the central body. Most fibers then bend laterally and frontally and run along the antero-ventral face of the Ca to the ΔILPC (Fig. 8A-D). From the Ca to the ΔILPC two pathways were observed (Fig. 8C-E). One was a pathway from the Ca along with the pedunculus of the MB to the ΔILPC. Most of the neurons from the IACT passed through this pathway. The other was a pathway extending more laterally through the lateral horn (LH) to the ΔILPC. A few neurons passed through this pathway (Fig. 8C-E). Neurons passing through the IACT were presumed to be a group of toroid-PNs due to morphological similarities. This was confirmed later by the double-labeling experiments (see below).

The second component was a group of neurons extending into the ΔILPC through the OACT (Fig. 8A-D). In the AL of *B. mori*, the LC resides ventro-laterally and comprises about 860 neurons (Kanzaki and Shibuya, 1986; Koontz and Schneider, 1987), which contain local interneurons and multiglomerular and uniglomerular projection neurons (Kanzaki and Shibuya, 1986). In the LC, ca. 150 neurons showed NO-induced anti-cGMP immunoreactivity but the number of cells labeled with anti-cGMP immunostaining showed more variations than in the MC (a variation

between 100 and 200 depending on the individual; data not shown). Most of them were considered as local interneurons judging from projection patterns of primary neurites that entered the coarse neuropile of the AL (Fig. 8G). Others innervated the MGC and left the AL through the OACT (Fig. 8H). The OACT passes through the AL more ventrally than the IACT and bends laterally right out of the AL. Fibers of the OACT innervated the ΔILPC bypassing the Ca (Fig. 8C, D).

The third component was a group of neurons extending into the ΔILPC through the MACT (Fig. 8A-D). The origin of neurons in the MACT could not be detected in the AL because those fibers fused in the AL. The MACT runs parallel to the IACT, but it bends laterally at the level of the central body. Fibers of the MACT innervated the ΔILPC bypassing the Ca (Fig. 8C, D).

3.2.2. Analysis of projection sites in the LPC from subdivisions of the AL

NO-induced anti-cGMP immunostaining revealed the ΔILPC, a specific area in the LPC. It allowed me to use the ΔILPC as a structural reference marker and to compare projection sites of each type of PNs with the ΔILPC. I performed double labeling experiments which enable single neuron staining of PNs combined with anti-cGMP immunostaining (Figs. 9-12). I stained 25 PNs (9 MGC-PNs and 16 ordinary

glomerulus PNs (G-PNs) in the MC combined with NO-induced anti-cGMP immunostaining (Table 2). The nomenclature of the MGC-PNs follows that of Kanzaki et al. (2003).

Toroid-PNs

I stained 4 toroid-PNs, combined with NO-induced anti-cGMP immunostaining (Table 2). The toroid-PNs innervated the toroid of the MGC and sent axons through the IACT (Fig. 9A, E). Their cell bodies were located in the MC (Fig. 9A). They projected to the Ca with a few short blebby branches and to the ILPC with thick blebs (Fig. 9A-C). After the intracellular staining of the toroid-PN with LY, NO-induced anti-cGMP immunostaining was applied to the same brain. The intracellularly stained toroid-PN was double labeled with NO-induced anti-cGMP immunostaining (Fig. 9D, E). The 3-D reconstruction of a toroid-PN with anti-cGMP immunostaining showed that the toroid-PN sent branches to all over the ΔILPC (Fig. 9F, G). Another 3 toroid-PNs showed similar projection patterns (Table 2). This type of PNs has been characterized morphologically and physiologically (Kanzaki et al., 2003; Table 1). They show excitatory responses selectively to the major pheromone component, bombykol (Kanzaki et al. 2003; Table 1).

Cumulus-PNs

I stained 4 cumulus-PNs, combined with NO-induced anti-cGMP immunostaining (Table 2). The cumulus-PNs had dendrites in the cumulus of the MGC and exit the AL via the IACT (Fig. 10A, E). Their cell bodies were located in the MC (Fig. 10A). They extended branches broadly in the Ca with thick blebs and terminate in the ILPC (Fig. 10A-E). The 3-D reconstruction of a cumulus-PN with anti-cGMP immunostaining showed that the cumulus-PN extended its branches to the lateral half of the ΔILPC (Fig. 10F, G). Another 3 cumulus-PNs showed similar projection patterns (Table 2). This type of PNs has been characterized morphologically and physiologically (Kanzaki et al., 2003; Table 1). They show excitatory responses selectively to the minor pheromone component, bombykal (Kanzaki et al., 2003; Table 1).

Horseshoe-PN

I stained 1 horseshoe-PN, combined with NO-induced anti-cGMP immunostaining (Table 2). The horseshoe-PN had dendrites in the horseshoe of the MGC and left the AL via the IACT (Fig. 11B). Its cell body was located in the MC (data not shown). It extended branches broadly in the Ca with thick blebs (data not shown) and terminated

in the ILPC (Fig. 11A-C). The 3-D reconstruction of the horseshoe-PN with anti-cGMP immunostaining showed that the horseshoe-PN extended its branches to the lateral half of the ΔILPC (Fig. 11D, E). This projection region was similar but slightly lateral to that of the cumulus-PNs (Figs. 10F, G, 11D, E). This type of PNs has also been characterized morphologically and physiologically (Kanzaki et al., 2003; Table 1). They show excitatory responses selectively to the minor pheromone component, bombykal (Kanzaki et al., 2003; Table 1).

G-PNs

I stained 16 G-PNs, combined with NO-induced anti-cGMP immunostaining (Table 2). The G-PNs had dendritic branches in one or two ordinary glomeruli (Fig. 12A, D). Their cell bodies were located in the MC (Fig. 12A). They ran into the IACT and terminated largely in the Ca showing variety of terminal projection patterns in the LPC (Fig. 12A-D). The 3-D reconstruction of G-PNs with anti-cGMP immunostaining showed that the G-PNs characterized in this study (n=16) did not overlap with the ΔILPC (Fig. 12E, F) but they had variety of projection patterns in the LPC. Their projection areas were mainly in regions posterior and lateral to the ΔILPC, the lateral horn (LH).

3.3. Morphological classification of LNs

3.3.1. Organization of the antennal lobe

I stained 126 local interneurons (LNs) intracellularly and observed them closely with a confocal microscope (Table 3). The AL LNs were defined as their confined arborization within the AL without axons. Cell bodies of LNs resided in the LC. The LC is located at the lateral edge of the AL and is composed of about 860 neurons (Kanzaki and Shibuya, 1986; Koontz and Schneider, 1987), which contain LNs and multi- and uniglomerular projection neurons (Kanzaki and Shibuya, 1986). As described in *M. sexta* (Hoskins et al. 1986; Homberg et al. 1988), the LC of *B. mori* was divided into the large upper part; lateral cell cluster I (LCI) and the small lower portion; lateral cell cluster II (LCII). The boundary between LCI and LCII was not distinct on confocal images, so I defined the LCII is the cluster of neurons sending their main neurite through the ventral small tract (arrow in Fig. 13D). Most of LCI neurons sent their main neurite through the major thick fiber tract (arrow in Fig. 13B) but a few neurites running through the pathway posterior to the major tract were observed (described below). To stain LNs, I inserted an electrode to various regions in the LC under visual control (Fig.

3A-C).

To classify the LNs, I defined several regions in the AL. At the entrance of the antenna, there is a large spheroid macroglomerular complex (MGC; diameter 150-200μm). The MGC is subdivided into three subdivisions, cumulus, toroid and horseshoe (Fig. 13B, C; Kanzaki et al. 2003). The response patterns of MGC projection neurons (PNs) to pheromonal stimuli correlate with their dendritic arborization in the subdivisions of the MGC (Kanzaki et al. 2003). In *B. mori*, ordinary glomeruli (Gs) are composed of 60 ± 2 glomeruli (Terada et al. 2003). From surface to middle depth there are approximately homogeneous sized (diameter 30-50μm) round glomeruli surrounding the central fiber core (Fig. 13A, B). At the bottom of the AL, characteristic glomeruli were arranged. As observed by Koontz and Schneider (1987) there are two large glomeruli called LLG1,LLG2 laterally under the MGC (Fig.13C). Medial to the LLG1,2 there are small 5-6 glomeruli called medial small glomeruli (MSG) (Fig. 13C). The LLG1,2 and MSG were easily identifiable among individuals. Ventral to these glomeruli there was a region fusing some glomeruli without clear demarcations. I named this region as the posterior ventral (PV) region (Fig. 13C, D).

3.3.2. Criteria for classification of LNs

126 LNs were three-dimensionally characterized by intracellular staining and confocal microscopic observation (Table 3). I classified them into 4 types and 7 subtypes (Table 3). I adopted functionally related criteria following that: 1) LNs have arborizations in the MGC or not, 2) LNs have arborizations in all Gs or pluri-Gs, 3) dendritic profiles within a glomerulus and 4) cell body position (Table 3). Type I and II LNs arborized in the MGC and almost all Gs. They differed in cell body position and the dendritic shape within the glomeruli. Type III and IV LNs arborized in pluri-Gs. Type III LNs had no arborization in the MGC (Table 3; described below in detail).

3.3.3. Analysis of each type of LNs

Type I LNs

I stained 91 type I LNs. This was the most frequently observed type of neurons in this study (Table 3). This type of LNs sent branches all over the AL including the MGC and almost all Gs. Their primary neurite entered into the center core of the AL through the thick fiver tract from the LC I and radially branched or bifurcated (Fig. 14A, B, C). The first bifurcation point showed variation among individuals, most of the type I LNs bifurcated around at the center core of the AL (Fig. 14A) but some neurons bifurcated

just at the entrance of the AL (Fig. 14B). The density of arborization in the MGC showed variation. Most of the type I LNs arborized all over the MGC sparsely with no compartmentalization for the subdivisions of the MGC (Fig. 14F) but some neurons showed less branches in the MGC (Fig. 14C). In Gs region, their arborizations within each glomerulus were comparatively coarse and inclined to core regions (Fig. 14D; detailed in Fig. 19A-C). The type I LNs had branches studded with spine-like process (Fig. 14E). Within the same neuron, the dendritic profiles and density were homogeneous all over the AL without any difference in specific region of the AL.

This type of LNs has been reported in many kinds of insects (moth: Matsumoto and Hildebrand, 1981; Christensen et al., 1993. locust: Leitch and Laurent, 1996. honeybee: Flanagan and Mercer, 1989b; Fonta et al., 1993; Sun et al., 1993. fly: Stocker, 1994. cockroach: Ernst and Boeckh, 1983). In *M. sexta,* it is demonstrated with double labeling techniques that this type has GABA as their neurotransmitter (Hoskins et al., 1987)

Type II LNs

This type of neuron arborized in the MGC and almost all Gs. They had cell bodies in the LC II (Fig. 15A). I stained 6 type II LNs (Table 3). They sent their primary neurite

through the deeper and thinner second fiber tract (Fig. 15A, C). Their first bifurcation point was posterior to the central fiber core and showed complicated ramification pattern (Fig. 15A, C). The one branch seemed to extend to the anterior and middle of Gs and the other branch extended to the posterior region of Gs (including LLG1, LLG2, MSG and PV) and the MGC. Most of this type of LNs arborized in the MGC sparsely (Fig. 15A) and the one neuron had no arborization in the MGC (data not shown). The arborization in the MGC appeared fewer in the toroid than the cumulus (Fig. 15B). They had thicker arborization than type I LNs (Figs. 14A-D, 15A, E). They had thick and dense arborization in Gs at the surface to middle layer (Fig. 15E) and in the PV region but sparse arborization in the LLG1, LLG2 and MSG (Fig. 15D). The LNs with same morphological characteristics have been reported in *M. sexta* (Matsumoto and Hildebrand, 1981).

Type III LNs

I stained 19 type III LNs (Table 3). I subdivided this type of LNs into two subtypes, type IIIa and type IIIb. This type of LNs had no arborization in the MGC and arborized in pluri-Gs (Fig. 16). Of 19 type III LNs, 5 neurons were type IIIb LNs (Table 3).

Type IIIa LNs

The numbers of the glomeruli occupied constantly about 50-70% of all Gs (Fig. 16A, G, H). Cell bodies were located in the LCI and they sent their main neurite to the central core of the AL and sent branches to each glomerulus. Their dendritic profiles surrounded the edge of the glomerulus (Fig. 16B, C). The density of arborization in the Gs differed in each glomerulus (Fig. 16B, C). The type IIIa LNs had no arborization in the LLG1, LLG2 and the MSG (only one neuron had little arborization in the LLG1; data not shown).

Type IIIb LNs

The type IIIb LNs had approximately similar arborization pattern with the type IIIa LNs (in number of Gs they had branches and no arborization in the MGC, LLG1, LLG2 and MSG) (Fig. 16D, I). They were characterized by their thick axons running ventro-lateral to medio-dorsally through arch like tract and by their densely fine arborization within single G (Fig. 16D, E, F, I). They had larger cell body (> 20 μm) than the type IIIa LNs (Figs. 16D, I).

Type IV LNs

I stained 10 type IV LNs (Table 3). The type IV LNs arborized in the MGC and pluri-Gs (Fig. 17A-E). The neurons of this type had cell body in the LCI (Fig. 17A-E). They showed variation in arborization pattern in the Gs region, so I further subdivided them into 3 subtypes, type IVa-c.

Type IVa

The type IVa LNs had arborization in a few Gs neighboring to the MGC (Fig. 17A ,B; n = 4). They sent branches to the restricted region in the Gs. The Gs included the LLG1, LLG2 and MSG and a few Gs at the surface and middle depth. The type IVa LNs had dense arborization in the MGC. The dendritic profiles surrounded the edge of each glomerulus similar with the type IIIa LNs (Fig. 17A, B).

Type IVb

The type IVb LNs had pluri-Gs with no regional restriction (Fig. 17C, D; n = 4). The number of Gs innervated by this type of neurons showed variation from 40% of Gs (Fig. 17C) to 10% of Gs (Fig. 17D). The dendritic profiles showed variation which was different in each neuron. One neuron showed dense and sparse density with circumscribed fashion around the edge of the glomerulus similar to the pattern in the

type IIIa, IVa LNs (Fig. 17C). Another one showed profiles studded with spine-like process (Fig. 17D).

Type IVc

The type IVc LNs arborized in pluri-Gs and extended branchings out of the AL to the putative antennal mechanosensory and motor center (AMMC) area (Fig. 17E, F n = 2). This type of neurons also had thicker arborizations in the PV region (Fig. 17E, F).

3.3.4. Distribution of the LN cell bodies in the LC

Using the fixed stage DIC microscope I clearly visualized the LC in the lateral edge of the AL (see Materials and Methods; Fig. 3A, B). I plotted position of the cell bodies of the stained LNs relative to the standard LC. Figure 18 shows the distribution of the cell bodies. The cell bodies of the type I LNs spread widely and shallowly (0-60 μm from the surface of the LC) in the LCI. The cell bodies of the type II LNs were bigger (>17 μm) and resided deep in (60-90μm) and below the LCI, LC II. The cell bodies of the type IIIb LNs were large (>20 μm) and located at the upper surface or deep medially in the LCI. The cell bodies of the type IIIa and type IVa,b LNs resided intermingled with main distribution region of the type I LNs and particular inclination was not

observed. The cell bodies of the type IVc LNs were observed in the deep region of the LC. PNs were also stained and they inclined to reside middle region in the LC (data not shown). I intended to insert the electrode in the wide range of the LC but the inserting points seemed to be biased at the surface and edge of the LC (Fig. 18).

3.3.5. Comparison of the synaptic region among ORNs, PNs and type I LNs

In this study, the most frequently observed type of neurons was the type I LNs (n = 91/126; Table 3). They had sparse arborization biased in the core region within a glomerulus. To confirm the regional segregation of the core and rim region, I employed a double labeling technique on single neuron of a type I LN with anterograde staining of ORNs or with single neuron of PNs. ORNs extended their axons to the rim area of a glomerulus (Fig. 19A-C). The type I LNs showed sparse arborization within the glomerulus and biased at the core region (Figs. 14D, 19A-C). Double labeling of ORNs and type I LNs showed overlap in the rim region in a glomerulus (Fig. 19B, C).The type I LNs had few terminal branches in the rim region but the possibility of existence of the direct connections between ORNs and type I LNs were remained.

I also checked the relation of arborization area within a glomerulus between a type I LN and a PN. The PN had dense arborization within a glomerulus (Fig. 19D, E). Double

labeling of the uniglomerular PNs and the type I LN showed their arborization region overlapped completely at the core region of the glomerulus (Fig. 19E). I could not confirm the existence of connections between them on confocal observation.