3. Result

3.1 Neuroactive substance in the antennal lobe neural pathways

3.1.1 Antennal lobe structure

The AL was located in the most anterior part of each hemisphere in the moth brain. Fig.1B shows the frontal (left) and dorsal (right) diagram of the neural structure and the cell cluster location. The AL had three cell clusters; 1) the medial cell cluster (MC) was located in the anterior dorso-medial part of the AL (Fig. 1A, B) and comprised about 250 cell bodies (Kanzaki and Shibuya, 1986a; Koontz and Schneider, 1987), 2) the lateral cell cluster (LC) was located in the ventro-lateral side of the AL (Fig. 1A, B) and comprised about 860 cell bodies (Kanzaki and Shibuya, 1986a; Koontz and Schneider, 1987), and 3) the anterior cell cluster (AC) was located most anterior and in the ventro lateral part of the AL. The anterior cell cluster was not an agglomerative cell cluster. Therefore, I could not count the number of the cell bodies in the anterior cell cluster. As shown in Fig. 3C the anterior cell cluster was well observed in FMRFamide immunostaining. The AL consisted of the center fiber core (CFC) and surrounding glomerular structures. Dendritic arborizations of the LNs, PNs and centrifugal neurons distributed from CFC to glomerular structures. In the male moth, the glomerular structures were divided into ordinary glomeruli (OGs) and the male specific macroglomerular complex (MGC). The MGC had three particular compartments called cumulus, toroid and horseshoe (Kanzaki *et al.*, 2003). In the male *B. mori*, the OGs were located ventro-medially and the MGC was located

dorso-laterally in the AL (Fig. 1B, C).

3.1.2 Projection pathways connecting the antennal lobe with the protocerebrum

In the silkworm moth, it has been reported that the AL was connected to the protocerebrum by three tracts, i.e., the inner, middle and outer antenno-cerebral tracts (IACT, MACT, OACT: Kanzaki and Shibuya, 1986a; Kanzaki et al., 2003; Seki et al., 2004). In the present study, I found that the IACT was divided into IACT-a and IACT-b due to the originating positions (Fig. 1C, D). Fig. 1D shows the frontal (left) and dorsal (right) diagram of the antenno-cerebral tracts (ACT) to the protocerebrum. I could not find the dorsal and dorso-medial antenno-cerebral tracts (DACT and DMACT), which were reported in M. sexta (Homberg et al., 1988; Kanzaki et al., 1989). The ACT identified in this study showed the following morphological properties.

1) The IACT-a originated from a dorso-medial bundle located in the posterior ventral side of the medial cell cluster. The IACT-a intersected the lateral accessory lobe commissure (LALC) and ran along the posterior-lateral edge of the central body (CB). At the front of the ventro-lateral edge of the protocerebral bridge, IACT-a bent laterally and ran along the ventral-anterior surface of the calyx of the MB and projected to the LPC (Fig. 1C, D).

2) The IACT-b originated from a ventro-lateral bundle located in the posterior medial side of the lateral cell cluster. The IACT-b ran along the ventro-medial frontal edge of the

lateral accessory lobe (LAL) and merged with the IACT-a and intersected the LALC. The

IACT-b occupied the lateral side of the IACT and followed the same pathway as IACT-a to

the protocerebrum (Fig. 1C, D).

3) The MACT originated from a ventro-lateral bundle located in the posterior medial

side of the lateral cell cluster. The MACT usually combined with the IACT-b in the CFC

region. The MACT occupied the posterior side of the combined fibers and ran along the

IACT-b, but ascended into the protocerebrum separated from the IACT-b. In the lateral

region of the central body (CB), MACT bent laterally and ran along the ventral side of the

peduncle of the MB and projected to the LPC (Fig. 1C, D).

4) The OACT originated from a ventro-lateral bundle located in the posterior medial side

of the lateral cell cluster. In the CFC, the OACT gradually separated from the IACT-b and

MACT. The OACT took a posterior medial direction to the protocerebrum and immediately

bent to a posterior dorso-lateral direction. The OACT ran along the ventro-lateral posterior

edge of the LAL and projected to the LPC (Fig. 1C, D).

3.1.3 Distribution pattern of immunoreactivity in the antennal lobe

GABA immunoreactivity

A large number of GABA immunoreactive cell bodies were observed in the lateral

cell cluster (Fig. 2A). In the anterior cell cluster and medial cell cluster, I could not find

any cell bodies (arrow in Fig. 2A). The cell bodies were distributed widely in the whole

area of the lateral cell cluster. 80% to 90% (n = 6) of the cell bodies in the lateral cell

cluster showed GABA immunoreactivity (Fig. 2A). Immunoreactive fibers were observed

in the CFC, the OGs and each compartment of the MGC (Fig. 2A). High intensity of

immunoreactivity was identified in some axons in the CFC (Fig. 2A). Immunoreactive

arborization was distributed in the whole region of each OG (surrounded by the arrow

heads in Fig. 2C). However, in the MGC, the immunoreactive branchings were restricted to

some small partitions in their compartments (arrows in Fig. 2B). Some axons ran through

the MACT (arrow in Fig. 2D) from the lateral cell cluster. I could not find any GABA

immunoreactivity in the IACT-a, IACT-b, OACT and in the AN.

FMRFamide immunoreactivity

Cell bodies showing FMRFamide immunoreactivity were located in the lateral cell

cluster and anterior cell cluster (Fig. 3A, C), but not in the medial cell cluster (Fig. 3B).

Although cell bodies were distributed through the whole area of the lateral cell cluster, a

bunch of cell bodies were found in a dorsal part of the lateral cell cluster (Fig. 3A). The

number of immunoreactive cell bodies in the lateral cell cluster was 24 ± 2 (mean ± S.D. n

= 4). In the anterior cell cluster 2 ± 1 (mean ± S.D. n = 4, max = 4) immunoreactive cell

bodies were observed (Fig. 3C). Immunoreactive fibers were observed in the CFC, all the

OGs and the MGC (Fig. 3A, D). I could observe weak immunoreactive axons in the CFC

(Fig. 3D). In the whole region of each OG, varicose arborizations were stained with

high-density (surrounded by the arrow heads in Fig. 3D). In each compartment of the MGC, immunoreactive arborizations were observed with dense and varicose patterns (Fig. 3D). However, these patterns were not as dense as in the OGs (surrounded by the arrow heads in Fig. 3D). I could find a few immunoreactive axons passing through the OACT (arrow in Fig. 3E) and MACT (arrow in Fig. 3F) from the lateral cell cluster and anterior cell cluster. I could not find any immunoreactive axons in the IACT-a, IACT-b and in the AN.

Serotonin immunoreactivity

A single serotonin immunoreactive cell body was observed in the boundary region between the lateral cell cluster and the protocerebrum (Fig. 4B). The diameter of the cell body was about 20 μm (Fig. 4B), which was larger than the cell bodies (ca. 10 μm) in the lateral cell cluster. Immunoreactive fibers were observed in the CFC, the OGs and each compartment of the MGC (Fig. 4A, C). I found some immunoreactive fibers in the CFC (Fig. 4C). The immunoreactive arborization pattern in the OG was restricted to the interior side (arrow 1 in Fig. 4C). Especially in the MGC, the immunoreactive branchings were restricted to some small partitions within the compartment (arrow 2 in Fig. 4C). I could find two axons passing through the IACT-b (arrow in Fig. 4D) but not in the IACT-a, OACT, MACT and in the AN.

Immunocytochemical double labeling with single lucifer yellow staining neuron, which was registered on Neuron Database, identified serotonin immunoreactive fibers in

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the AL originated from a pair of serotonin immunoreactive neurons (Fig. 5). The cell body

of this neuron was located in the boundary region between the lateral cell cluster and the

protocerebrum and branched throughout the contralateral AL (Fig. 6A). This neuron also

had processes in both the ipsilateral and contralateral superior protocerebrum, the

ipsilateral LAL, the calyces of both mushroom bodies, and in the central body.

Examination of individual optical sections revealed that this neuron is branched in every

OGs, and each compartment of the MGC. The primary neurite of this neuron projected

thought the ipsilateral AL where it had a few fine branches in the posterior coarse neuropile

region of the AL. This neuron fired spontaneous with long duration action potentials and

showed very long delay response to mechanosensory stimulation to the antennae (Fig. 6B).

Tyramine immunoreactivity

Tyramine immunoreactive cell bodies were located in the lateral cell cluster (Fig.

7A) but not in the medial cell cluster (arrow in Fig. 7A) and anterior cell cluster. These cell

bodies were restricted to the posterior middle region of the lateral cell cluster (Fig. 7A).

The number of the immunoreactive cell bodies was 28 ± 2 (mean ± S.D. n = 4).

Immunoreactive intensity fluctuated with the preparation (Fig. 7A,B). Immunoreactivity

was usually observed in the CFC, the OGs and each compartment of the MGC (Fig. 7A,B).

In the center region of the CFC, immunoreactive staining usually faded out from primary

neurites (Fig. 7A). Immunoreactive patterns in the OGs were usually restricted to the

medial side (Fig. 7A). In the MGC, the branchings were restricted to some small partitions

within the compartments (arrow in Fig. 7C). I could consistently find two axons in the

IACT-b (arrow 1 in Fig. 7D), which linked to the arborizations in the OGs and each

compartment of the MGC (arrow in Fig. 7B). These two neurites also extended toward the

suboesophageal ganglion (SOG) (arrow 2 in Fig. 7D). However, I could not find the cell

bodies in the AL. Moreover, I observed 4 ± 1 (mean $\pm$ S.D. n = 4) immunoreactive axons in

the AN (arrow in Fig. 7E).

Histamine immunoreactivity

In the protocerebrum and the optic lobe, I could find histamine immunoreactivity

(Fig. 8A, C). There was no histamine immunoreactivity in the cell clusters, the glomeruli,

the ACT of the AL and the AN (Fig. 8B,C).

3.2 Odor-evoked locomotion control mechanism in the protocerebrum

3.2.1 Neural structure of the LAL and VPC

The LAL and VPC in the protocerebrum of the moth brain were shown in the

images of immunocytochemical staining (Fig 9D, E, F). The LAL was located in both sides

of the protocerebrum ventro-lateral to the central body and just posterior to the medial cell

cluster of the AL. The LAL had a spheroid neuronal structure (Fig 9C) (average width: 150

μm x height: 100 μm x depth: 100 μm). Both LALs were linked to each other via the

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commissure of the LALs (LALC). In the middle depth (about 50 µm), it was dominated by

β-lobe of the mushroom body medially, peduncle of the mushroom body dorso-laterally,

superior-lateral lateral protocerebrum (slLPC) medially and the OGs in the AL

ventro-laterally (Fig. 9D). At this depth, IACT-a intersected the LALC in the medial side of

LAL (Fig. 9D). In deep parts (about 70 - 90 µm), the surrounded nearness neural structure

gradually separates, on this altitude it was surrounded by IACT-b and OACT in

ventro-medially, tract of Group-II descending interneurons (Kanzaki *et al.*, 1994) and

other type neurons passed dorsally. At this depth, LALC divided LAL neural structure into

ventro-medial LAL subregion (vmLAL) and dorso-lateral LAL subregion (dlLAL) (Fig.

9E).

The VPC was a distracted neuropil structure and difficult to summarize from the

LAL (Fig 9C, E). In the present study, I found that the distracted neuropil structure was

divided into the LAL and VPC due to the neural structure layout. On my description, the

LAL and VPC were divided by the OACT. This tract traversed LAL-VPC region from

extension of vmLAL to the inferior medial protocerebrum (IMPC) dorso-laterally and

separated continuous LAL-VPC neural structure into LAL region and VPC region (Fig. 9F,

G). Furthermore, morphology of single neurons obtained by intracellular staining revealed

that the LALC divided VPC into medial VPC subregion (mVPC) and lateral VPC

subregion (lVPC) (Fig. 9G). On my observation, VPC occupied a wide area in the ventral

protocerebrum. It was surrounded by inferior medial protocerebrum (IMPC) dorso-lateraly

and by the SOG ventrally.

3.2.2 LAL-VPC intrinsic neurons extracted from Neuron Database

I could extract 50 LAL-VPC intrinsic neurons from the Neuron Database. Arborization of individual neurons did not often penetrate the whole area of the LAL and VPC, but was restricted to certain parts of the LAL-VPC subregions. The extracted LAL-VPC intrinsic neurons were classified by morphological properties into two categories, 1) bilateral neurons which linked LALs and VPCs in both sides of the hemispheres (LAL-bilateral neurons; LAL-BLs), 2) interneurons which linked the LAL and VPC (LAL-VPC interneurons; LAL-VPC LNs). Pheromonal stimulation was applied to 34 out of 50 preparations. 85.3% of these neurons (n = 29) had a definite response to pheromonal stimulation (bombykol or bombykol-bombykal mixture).

3.2.3 General morphology and physiology of LAL-BLs

Thirty-three LAL-BLs were registered in the Neuron Database. Particularly well-stained morphology was important for solving its arborization subregions in the LAL and VPC. Therefore in this study, I adopted 22 neurons because of good morphological and physiological qualities. These 22 neurons had branching in both sides of LALs and VPCs (Fig. 21). All of the LAL-BLs had smooth dendritic arborizations in the LAL and VPC ipsilateral to the cell body. Moreover, their branching in the LAL and VPC contralateral to

the cell body was blebby. A mixed pattern between smooth and blebby arborization in one side in the LAL and VPC was not observed in the confocal optical imaging observations (Figs. 10, 11). The main arborizations linked both sides by a single major neurite, which crossed the midline of the brain through the LALC or the tract located on the dorsal side of the central body. In both sides of the LAL and VPC, the arborizations pattern of smooth or varicose was often restricted to the LAL-VPC subregion (vmLAL, dlLAL, mVPC and lVPC) or combinations of these subregions (Fig. 21). The size of the cell body was 20 - 30 μm in diameter. These LAL-BLs exhibited the following response patterns to bombykol (Fig. 21); long-lasting excitation (LLE, n=6), brief excitation (BE, n=7), brief inhibition (BI, n=2) and long-lasting inhibition (LLI, n=2). One cell did not show any response.

3.2.4 Analysis of LAL-BLs

Statistics of input region of LAL-BLs

The detailed input area of the LAL-BLs was analysed because the input information is one of the most important factor for analysing the roles of single neurons. I analysed input subregions of the LAL-BLs on the base of two LAL subregions (vmLAL and dlLAL), because I could not find any LAL-BLs with dendritic arborization only in the VPC subregions. The LAL-BLs had dendritic arborization in both the LAL subregions with VPC subregions. Fig. 16 shows the distribution of input subregions of the LAL-BLs. From this analysis, I could find that vmLAL subregion accompanied with other regions equality.

However, dlLAL subregion accompanied with lVPC mainly. Therefore, I roughly

classified the input area of the LAL-BLs into vmLAL group and dlLAL + lVPC group (Fig.

16). From these result, I defined the LAL-BLs into two types of neurons, Type-A (major

input received from vmLAL) and Type-B (major input recived from dlLAL and lVPC).

Relationship between input region classification and output regional property

To describe the general morphological character of Type-A and B LAL-BLs, I

analysed the relationship in properties between input region and output region (Fig. 17).

In this study, I defined a proportion of regular criterion for simplifying their

miscellaneous morphological property. In Fig. 16, the ratio of the lVPC subregion to the

dlLAL input area neuron was 63.2%, but it was clear that the input of most Type-B

LAL-BLs was located in dlLAL and IVPC (Fig. 21). So I defined 63.2% as a regular

criterion proportion of strong linkage relationship between subregions. In this study, I

multiplied a regular criterion proportion (63.2%) and the most high appearance ratio output

subregion in the Type A and B LAL-BLs for calculating the theoretical strong linkage ratio.

If the proportion of the secondary subregion to the most high appearance subregion exceeds

the theoretical strong linkage ratio, I defined these subregion has strong linkage each other.

From this criterion, I decided to admit the other secondary subregions for basic simplify

morphology or not.

Relationship between morphological classification and physiological properties

The physiological characteristics of all the LAL-BLs were classifed into five categories; i.e., LLE (33.3%), BE (38.9%), BI (11.1%), LLI (11.1%) and no response (5.6%). Physiological and morphological correlations were observed in the Type-A and B neurons. Type-A LAL-BLs had two physiological categories; i.e., LLE (62.5 %) and BE (37.5 %). Type-B LAL-BLs had five physiological categories, i.e., LLE (10.0%), BE (40.0%), BI (20.0%), LLI (20.0%) and no response (10.0%).

In these physiological properties of LAL-BLs, I measured the appearance ratio of BE. The appearance ratio of the BE was always very stable and regular in all types of LAL-BLs. In the all LAL-BLs (38.9%), TypeA LAL-BLs (37.5%) and TypeB LAL-BLs (40.0%) showed an approximate appearance ratio (Fig. 17). This consequence probably showed that the BE physiological response was very common in all the types of LAL-BLs. From this analysis, I could estimate the characteristic physiological properties of Type-A and B LAL-BLs. Type-A LAL-BLs distinguished itself by LLE (long-lasting excitation). Type B LAL-BLs distinguished itself by inhibitoryy responces (Fig. 17).

Classification of LAL-BLs

From the preceding analysis, I classified the LAL-BLs into two groups. The Type-A included LAL-BLs with inputs in the vmLAL and outputs in the vmLAL judging from the basic morphology. They showed characteristic long-lasting excitation (LLE) in

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response to a pulsed olfactory stimulation (Figs. 17, 19). In contrast, the Type-B included

LAL-BLs with inputs in the dlLAL and lVPC, and output in the vmLAL and dlLAL.

They typically showed inhibitory responses (Figs. 17, 19).

Type-A LAL-BLs

The branchings of the Type-A LAL-BLs were restricted in the LALs in both sides

of the PC (Fig. 10). Their cell bodies were usually located anterio-dorsally in the brains,

posterior of the medial cell cluster and lateral side of γ-lobe of the mushroom body.

The primary neurite ran ventrally to the ipsilateral vmLAL, where it ramified into smooth

branches. The main neurite crossed the brain via the LAL commissure and mainly

arborized profusely in the contralateral vmLAL. These arbors exhibited remarkable

varicose characteristics. Type-A LAL-BLs usually responded with LLE (62.5 %) and BE

(37.5 %) to antennal stimulation with bombykol (Fig.17).

Type-B LAL-BLs

Type-B LAL-BLs (Fig. 11) showed a wide range of morphological properties by

comparison with the Type-A LAL-BLs. Their cell bodies were located posterio-dorsally,

just at the ventro-lateral edge in the median line of the calyx. The primary neurite ran

forwardly to ipsilateral LAL, where it formed smooth branches in the dlLAL and lVPC

subregions. The main neurite crossed the midline of the brain via the LAL commissure

through the contralateral LAL, where it usually formed wide varicose terminals that were distributed in the vmLAL and dlLAL. Type-B LAL-BLs usually exhibited LLE (10.0%), BE (40.0%), BI (20.0%), LLI (20.0%) and no response (10.0%) by bombykol stimulation to the antenna (Fig.17).

3.2.5 General morphology and physiology of LAL-VPC LNs

Seventeen LAL-VPC LNs were registered in the Neuron Database. I extracted 12 neurons, which showed complete staining. Twelve LAL-VPC LNs neurons innervated only in one side of the brain (Fig. 22). Eleven neurons out of 12 showed remarkable olfactory responses.

All of the 12 neurons sent major branches into the LAL and VPC (Fig. 22). They were unilateral interneurons connecting the LAL to the VPC. These neurons exhibited a characteristic brief excitation and/or recurrent brief excitation, which repeated brief excitation at regular intervals, activity (n=7), long-lasting excitation (LLE, n=4). No LAL-VPC LNs showed inhibitory response (Fig. 22).

Classification of LAL-VPC LNs

From morphological properties, I could classify the LAL-VPC LNs into two types; i.e., Type-A (input from LAL, output to VPC: n=6) and Type-B (input from VPC, output to LAL: n=6). Type-B LAL-VPC LNs could be subdivided into Type-B-α and

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Type-B-β from their physiological properties (Fig. 22). Similarly to Type-A LAL-VPC LNs, Type-B-α LAL-VPC LNs showed a brief excitatory response to pheromone stimulation. However, the response of Type-B-α LAL-VPC LNs presented a delay beat in comparison with Type-A LAL-VPC LNs. Type-B-β showed a long-lasting excitation in response to pheromen stimulation (Fig. 22).

Type-A LAL-VPC LNs

The cell body of Type-A LAL-VPC LNs (Fig. 12) was usually located anterio-dorsally of the lACT-a and lACT-b (Fig. 9D, E). The primary neurite ran ventro-laterally and along a part of OACT ventro-laterally, where it divided into two axons. One neurite ran anteriorly and then made smooth arbors in the whole area of the LAL (vmLAL and dlLAL). A second neurite ran posteriorly and then made several conspicuous varicose terminals extending widely into the VPC (mVPC and lVPC). Type-A LAL-VPC LNs showed a brief excitation when the antenna was stimulated with bombykol. In response to the stimulation with the mixture of bombykol and bombykal, they usually showed a recurrent brief excitation (Fig. 21).

Type-B-α LAL-VPC LNs

The cell bodies of Type-B-α LAL-VPC LNs (Fig. 13) were located posterio-dorsally in the brain, on the dorsal side of protocerebral bridge. The primary

neurite ran toward the divided point of the LAL and VPC medially, where it innervated to LAL-VPC region from the adjacent winding point of the OACT. On this position, the stout axon parted smooth branches on the whole VPC region (mVPC and lVPC) mainly and varicose terminals on the whole LAL region (vmLAL and dlLAL) mainly. From detailed observation, the axon with varicose terminals on the LAL had secondly neurite on the border region of LAL and VPC. From neural structure layout, I defined this border region as mVPC anterior region (mVPCa). Even on the mVPCa, I could identify petty varicose terminals. In response to bombykol, Type-B-α LAL-VPC LNs usually showed a delayed brief excitatory response. While, the response of Type-B-α LAL-VPC LNs presented a delay in comparison with Type-A LAL-VPC LNs. In Fig. 13 Type-B-α LAL-VPC LNs, the spike number of this neuron increased 500ms after pheromone stimulation. This time course showed a long delay of excitatory response by comparison with the Type-A LAL-VPC LNs, which showed brief excitation or recurrent brief excitation (Fig. 22).

Type-B-β LAL-VPC LNs

The cell body of Typical Type-B-β LAL-VPC LNs (Fig.14) was situated in the same cell cluster of the typical Type-A LAL-VPC LNs. This somata located on slightly posterior comparing with the somata of typical Type-A LAL-VPC LNs. The primary neurite ran down to the same route of the typical Type-A LAL-VPC LNs. Then the neurite arrived at the border region of LAL and VPC. At this area the main neurite made

conspicuously smooth branches in the mVPCa. The neurite ascended toward the vmLAL.

In the vmLAL, this neuron formed distinct varicose terminals. Type-B-β LAL-VPC LNs

always exhibited a long-lasting excitation or flip-flop activity in response to pheromonal

stimulation (Fig. 22).

3.2.6 Distribution pattern of immunoreactivity in the LAL-VPC

GABA immunoreactive in the LAL-VPC region

Some GABA immunoreactive neurites (arrow in Fig. 15A) ran through the LAL

from immunoreactive cell cluster. This GABA immunoreactive cell cluster was observed in

the lateral side of the γ-lobe of the mushroom body (Fig. 15). Immunoreactive fibers were

observed in the whole LAL and VPC (Fig. 15). Some immunoreactive axons ran through

the frontal area of the LALC and connected to each side of the LAL bilaterally (arrow2 in

Fig. 15).