2. Materials and methods

2.1 Animals

Experiments were performed on an adult male silkwom moth, *Bombyx mori* (Lepidoptera: Bombycidae). Adult male moths were reared from eggs in the laboratory on an artificial diet under a 16h : 8h light : dark photoperiod at 26 °C and 50 - 60% relative humidity. Adult male moths within 2 – 4 days after eclosion were used.

2.2 Histochemistry

2.2.1 Immunocytochemistry

GABA, FMRFamide, serotonin and histamine immunolabelling

The brain was fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer solution (PBS, pH 7.4) for 2 - 18 hours at room temperature. Preparations for histamine immunolabelling were fixed in 4% 1-ethyl-3-carbodiimide in 0.1 M sodium phosphate buffer (pH 7.4) for 14 hours. All dissections were performed during daytime. The brain was permeabilized overnight at 4 °C in 0.1 M phosphate buffered saline solution containing 0.5% Triton X-100 (PBSX, pH 7.4). The brain was embedded in 3 - 5% agarose and 200 μm thick sections were made with vibrating microtome (Microslicer, Dosaka EM, Kyoto, Japan). The plane of sectioning was frontal in all preparations. Microtome sections were collected and washed in PBSX for 30 - 60 min at 4 °C. Subsequently, sections were incubated in PBSX containing 5% normal goat serum (NGS, Sigma G9023) for 2 - 4 hours

at room temperature in order to block non-specific staining. The sections were incubated with primary antiserum for 18 - 72 hours at 4 °C: rabbit anti-GABA (Sigma A2052) diluted 1:1000 - 1:2000; rabbit anti FMRFamide (Diasorin 20091) diluted 1:500 - 1:1000; rabbit anti serotonin (Diasorin 20080) diluted 1:2000; rabbit anti histamine (Diasorin 22939) diluted 1:1000. Primary antisera were diluted in PBSX containing 5% NGS. The sections were next rinsed with PBSX and then incubated in anti-rabbit secondary antibody for 18 - 48 hours at 4 °C: Cy3 conjugated goat anti-rabbit (Chemicon AP132C) diluted 1:200 - 400. Secondary antiserum was diluted in PBSX containing 5% NGS. The sections were then washed at room temperature with PBS. Finally sections were dehydrated through an ethanol series and cleared in methyl salicylate.

Tyramine immunolabelling

The brain was fixed in 2.5% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with sodium metabisulfite (SMB, Sigma S1516) for 14 hours at room temperature. After fixation, the brains were treated with 0.5% of sodium borohydride ($NaBH_4$, Sigma 45, 288-2 Aldrich) in 0.05 Tris-HCl buffer containing 0.45% SMB (Tris HCl SMB, pH 7.4) for 20 minutes to saturate double bonds. After washing with Tris-HCl SMB, the brain was embedded in 5% agarose and 200 μm thick sections were made with vibrating microtome. The plane of sectioning was frontal. Microtome sections were collected and washed in Tris-HCl SMB containing 0.5% Triton X-100 (Tris-HCl SMBX)

for 80 minutes at room temperature. Subsequently, sections were incubated with Tris-HCl SMBX containing 5% NGS for 3 hours at room temperature in order to block non-specific staining. The sections were incubated with primary antiserum for 72 hours at 4 °C: rabbit anti-tyramine (Chemicon AB124) diluted 1:1000. Primary antiserum was diluted in Tris-HCl SMBX containing 5% NGS. The sections were next washed with Tris-HCl SMBX and then incubated in anti-rabbit secondary antibody for 48 hours at 4°C: Cy3 conjugated goat anti-rabbit (Chemicon AP132C) diluted 1:200. Secondary antiserum was diluted in Tris-HCl SMBX containing 5% NGS. The sections were then washed at room temperature with Tris-HCl SMB. Finally sections were dehydrated through an ethanol series and cleared in methyl salicylate.

Immunocytochemical double labeling

Lucifer yellow CH (LY, Sigma L-0259) stained brains, which were preserved in the Neuron Database freezer, were returned to 100% ethanol, and then rehydrated through an ethanol series. Immunocytochemical double labeling was then performed on the LY-stained brain. After rehydration, the method of immunocytochemical double labeling was performed similarly to normal preceding immunolabelling methods. However, I had special attention about shading not to fade away the LY-staining.

Specificity controls

The following specificity control experiments were performed. First, I confirmed that omission of the primary antiserum prevented immunolabelling on the sections. Second, I determined the specificity of the antiserum by liquid-phase preadsorption. Immunolabellings disappeared after preadsorption of the diluted antiserum with various concentrations of target substances.

2.2.2 Confocal microscopic study

Immunolabeled neurons were imaged frontally using a confocal imaging system (LSM-510, Carl Zeiss, Jena, Germany) with plan apochromat x 20 (n.a. = 0.75) and x 40 (n.a. = 1.0) objectives. The Cy3 immunolabeled neurons were examined with 543 nm excitation and a long-pass emission filter (> 560 nm) in serial sections. The Lucifer Yellow CH stained neural structures were examined with 458 nm excitation and a band-pass emission filter (505 - 550 nm) in serial sections. Serial optical sections were acquired at 0.8 μm intervals throughout the entire depth of the agarose section. Three-dimensional reconstructions of the immunolabeled neurons were made from these sections.

2.2.3 Three-dimensional reconstruction of the antenno-cerebral tracts and surrounding neural structures

In order to know fine distribution patterns of the neural structure, I reconstructed the ACT and surrounding neural structures of the AL and LAL-VPC neural structure from

serotonin immunostaining sections, which were imaged using a confocal imaging system. The ACT and neural structures were reconstructed with the software AMIRA (TGS, Berlin, Germany) from sequential series of confocal slice images.

2.3 Neuron Database analysis

Neuron Database is developed by the Kanzaki laboratory (Department of Mechano-Informatics, Graduate School of Information Science and Technology, The University of Tokyo, Japan) for collecting morphological and physiological properties of single brain neurons of the male *B. mori*. The Database contains three-dimensional structures and physiological responses to odors including pheromones of more than 800 single neurons. All the data are obtained by using intracellular recording and staining from individual brains. Since all the single-cell stained brains are preserved in a refrigerator, each single-cell stained brain is available for another experiments such as immunocytochemistry.

2.3.1 Selection of immunocytochemical double labeling candidate from Neuron Database

I applied double labeling by immunocytochemical staining and intracellular staining to identify the immunoreactivity of single neurons. I selected brains, which were already stained by intracellular staining and registered in the Neuron Database, as

candidates for immunocytochemical staining.

As the first step of immunocytochemical double labeling, I distilled the morphological properties of immunoreactive neurons from normal immunocytochemical methods. Then, I extracted their characteristic morphological properties. For example, 1) Immunoreactive cell body or cell clusters position, 2) Their arborization pattern in the neural structures, 3) The pathway of immunoreactive axons in their neural tract. As the second step, I compared the morphological properties of immunoreactive neurons with morphological properties of single neurons, which were registered on the Neuron Database. If there was a strong resemblance between morphological properties of immunoreactive neurons and single neuron, I could consider strong possibility of their identity. Therefore, I applied immunocytochemical double labeling methods to the candidate brain, which showed strong resemblance.

2.3.2 Extraction of neurons for cellular organization analysis

To investigate the cellular organization around the antennal lobe, I extracted olfactory neurons from the Neuron Database. In this study, I extracted AL organized neurons (LNs, PNs and centrifugal neurons) using the following criterion; about their cell body position and projection pathways (antenno-cerebral tract) to the protocerebrum. For investigation of the LAL-VPC, I extracted and collected unclassified LAL-VPC intrinsic neurons from their arborization patterns and regions.

2.3.3 Representation of morphological properties of LAL-VPC intrinsic neurons

The arborization patterns of LAL-VPC intrinsic neurons were usually restricted to the individual subregion, LAL subregions (ventro-medial LAL / dorso-lateral LAL) and VPC subregions (medial VPC / lateral VPC) or combinations of these subregions. Even in the histochemical study, LAL-VPC neural structure was divided into four particular regions by the pathways of LALC and OACT (Fig. 9C). Therefore, I defined four particular subregions (vmLAL, dlLAL, mVPC and lVPC) in one side of the LAL-VPC. Furthermore, the LAL-VPC intrinsic neurons showed characteristic distribution patterns of smooth and varicose terminals, which corresponded to these subregions. A previous electron microscopic study suggested smooth arborizations of the neurite interpreted as postsynaptic neurites (input) and varicose arborizations of the neurite as presynaptic connections (output) (Waston and Hardt, 1996). Therefore in this study, I registered these morphological properties of the LAL-VPC intrinsic neurons corresponding to their representation. By these methods, I represented morphological properties of LAL-VPC intrinsic neurons in the schematic diagram (Figs. 21, 22).