2. Materials and Methods

2.1. Preparation

The moths *Bombyx mori* (Lepidoptera: Bombycidae) (Fig. 1A) were reared on an artificial diet at 26°C and 60% relative humidity under a long-day photoperiod regimen (16 hours light / 8 hours dark). Adult male animals were used 2-6 days following eclosion. After cooling (4°C, ca. 30 minutes) to achieve anesthesia, the head was isolated from the thorax and placed on a dissecting chamber. The brain was exposed by opening the head capsule and some muscles and trachea over the brain were removed. I carefully desheathed parts of the AL using fine forceps. The brain was dissected from the head capsule and transferred to a small chamber. The brain was superfused with physiological saline containing 140 mM NaCl, 5 mM KCl, 7 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$, 5 mM trehalose, 5 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid and 100 mM sucrose (pH 6.8). To prevent the brain from floating, I held it down with two thin glass electrodes placed over the optic lobes (Fig. 2A).

2.2. Intracellular staining with newly developed methods

I adopted a newly developed staining method, the intracellular staining under visual control (Seki et al. 2005). Cell bodies were visualized with a fixed-stage upright microscope (BX50WI or BX51WI; Olympus, Tokyo, Japan) equipped with DIC optics and long-working distance objectives (x40 LUMPlanFL/IR or x60 LUMPlanFL/IR water immersion) (Fig. 3). A CCD camera (C2741-79; Hamamatsu photonics, Shizuoka, Japan) enhanced contrast (Fig. 3). Cell clusters on the surface of the brain can be clearly visualized with this system, allowing me to select the cells for inserting the microelectrode (Fig. 2). In this study, cell bodies in the lateral cell cluster (LC) of the AL were visualized and impaled with a microelectrode filled with Lucifer yellow CH (LY; 4% in distilled H_2O; Sigma, St. Louis, MO) or Alexa flour 568 (2% in distilled H_2O; Molecular Probes, Eugene, OR) to stain antennal lobe LNs (Fig. 2B,C) and cell bodies in the medial cell cluster (MC) of the AL were visualized and impaled to stain antennal lobe PNs. After impaling, I stained neurons by iontophoretic injection of LY and Alexa flour 568 with 1~10 nA constant hyperpolarizing or depolarizing current respectively for 3~10 minutes. This method allows me to stain neurons efficiently.

2.3. Anti-cGMP immunohistochemistry

NO induced anti-cGMP immunohistochemistry has revealed the candidate of the

target neurons of NO in the nervous systems (Fig. 4; De Vente et al. 1987; Scholz et al. 1998; Aonuma, 2002; Aonuma and Niwa, 2004) and staining methods with wholemount preparations were modified from previous works (Aonuma, 2002; Aonuma and Niwa, 2004). The moth brains were preincubated in 1 mM 3-isobutyl-1-methyxanthine (IBMX; Sigma) in saline for 30 minutes at 4°C to block endogenous phosphodiesterase activity. Soluble guanylyl cyclase was stimulated by a NO donor, sodium nitroprusside (SNP; Sigma) or S-nitroso-N-acetylpenicillamine (SNAP; Sigma) (Fig. 4). The tissue was exposed to 10 mM SNP or 200 μM SNAP in saline containing 1mM IBMX for 15 minutes at 25°C after incubation of 1 mM IBMX saline to detect NO-related increase in cGMP (Fig. 4). Incubation in SNP or SNAP saline was followed by fixation in 4% paraformaldehyde overnight at 4°C. Then the preparations were washed in PBS containing 0.2% Triton X-100 (PBST) several times and preincubated with 5% normal donkey serum in PBST (PBST-NDS) for 3 hours at 25°C with agitation. They were incubated with an anti-cGMP antibody (1:20000 in PBST-NDS) at 4°C for 3 days. They were then washed in PBST and incubated in Cy3-conjugated secondary anti-sheep IgG antibody (diluted 1:200 in PBST-NDS; Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C overnight. As a control, some preparations were incubated in IBMX without stimulating NO-sensitive soluble guanylyl cyclase using SNP or SNAP saline

and some preparations were processed without the anti-cGMP antibody. In some preparations (n = 4), moth brains were sliced using vibratome (DTK-1000; Dosaka EM, Kyoto, Japan) at 100 μm after preparations were embedded in 4% agarose. For section preparations the procedures were similar except for incubation times, which were shorter (overnight with an anti-cGMP antibody, 3 hours in Cy3-conjugated secondary anti-sheep IgG antibody). The antiserum was a gift from Dr. Jan De Vente (Rijsuniversiteit Limburg, The Netherlands; see Tanaka et al., 1997 for its specificity and characterization). This Anti-cGMP immunostaining stained the specific deltoid region in the LPC. This deltoid region is observed in every individual consistently (Fig. 5).

2.4. Double labeling of LY staining of a single PN with anti-cGMP immunostaining

To identify NO-induced anti-cGMP immunoreactive neurons, combination of intracellular staining of single PNs with LY and anti-cGMP immunostaining were performed. After a single PN was stained with LY, the brain was incubated in IBMX saline for 30 minutes at 4°C and then processed NO-induced anti-cGMP immunostaining as described above.

2.5. Labeling of antennal sensory axons

To compare arborization area within single glomerulus, I labeled both antennal sensory axons with tetramethylrhodamine-dextran (TMR; Molecular Probes) and a single LN with LY. The anterograde staining was modified from Ai and Kanzaki (2004). I dissected and exposed the antennal nerve of *B. mori*. The cut end of an antenna was immersed in saturated TMR in DW for 24 hours at 4°C and then a single LN was labeled with LY.

2.6. Whole staining of the antennal lobe

I stained whole AL with LY as described in Kanzaki et al. (2003) to observe and define the AL structure. The dissected brains were prefixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) for 2 minutes and immersed in 0.1% LY solution for 4 hours at room temperature. The brains were then fixed in 4% formaldehyde for 1 hours at room temperature and dehydrated with an ethanol series and cleared in methyl salicylate.

2.7. 3-D reconstructions of the labeling neurons

The brains were then dehydrated with an ethanol series and cleared in methyl

salicylate. Each stained neuron was imaged frontally using a confocal imaging system (LSM-510; Carl Zeiss, Jena, Germany) with Plan Apochromat ×20 (n.a. = 0.75) and ×40 (n.a. = 1.0) objectives. The LY-stained neurons were examined with 458 nm excitation and a band-pass emission filter (505-550 nm). The Alexa flour 568-stained, TMR-stained and Cy3-stained neurons were examined with 543 nm excitation and a long-pass emission filter (>560 nm). Serial optical sections were acquired at 0.7 - 2.0 μm intervals throughout the entire depth of a neuron. 3-D reconstruction of the labeled neurons was made from these sections. Then to make closer observations of some preparations, the stained neurons were 3-D reconstructed with the software AMIRA (TGS, Berlin, Germany) from sequential series of confocal slice images. To extract neurons for 3-D reconstructions, I set the appropriate threshold value that left clearly stained parts, and for each section I manually traced parts of neurons that were under the threshold value but surely stained. Projection images made from the traced neurons are shown in Figures 8-12 (e.g., Fig. 8A, D, E).