4. Material and Method

4.1 Animals

Male silkmoths, *Bombyx mori* (Lepidoptera: Bombycidae) were reared in the laboratory on artificial diet at 27 °C and 50 - 60% relative humidity. Adult male moths were used within 2 - 4 days after eclosion (see Fig. 4). All males used in this study demonstrated the pheromone-triggered zigzag behavior prior to their use in electrophysiological experiments.

4.2 Olfactory and visual stimuli

Olfactory stimuli were delivered using a puff stimulation system (Kanzaki *et al.*, 2003). An air delivery glass tube (Pasteur pipette, 1mm tip diameter; air flow of 2 m/s) containing a piece of filter paper (1 x 2 cm) bearing major pheromone component bombykol [(E,Z)-10,12-hexadecadienol, 100 ng] (sufficient to elicit the zigzagging behavior) was positioned about 1cm from the same side of the antenna as the LAL inserted microelectrodes. Odorants were removed by gentle suction into an exhaust tube positioned behind the preparation (see Fig. 5).

"Light ON" stimuli were applied to both compound eyes using flashes of ambient
white light (see Fig. 5). The intensity of the light was 500 – 1050 lx under the light ON condition and was below 20 lx under the light OFF condition. Light stimuli were controlled with trigger pulses generated by an electric stimulator (SEN-7203; Nihon Kohden, Tokyo, Japan).

4.3 Physiology

Intracellular recording and staining methods have been described in previous studies (Mishima and Kanzaki, 1999; Kanzaki et al., 2003). The intracellular recording systems were shown in Fig. 5. After cooling (4 °C, approximately 30 min) to achieve anesthesia, all legs were removed, and then the moth was placed in an experimental chamber. The head was immobilized by a plastic plate, and the brain was exposed by opening the head capsule and removing large tracheae. The brain was superfused with saline solution (in mM: 140 NaCl, 5 KCl, 7 CaCl₂, 1 MgCl₂, 4 NaHCO₃, 5 trehalose and 5 N-TRIS [hydroxymethyl]-2-aminoethanesulfonic acid, pH 6.8) and surgically desheathed in the region of the lateral accessory lobe (LAL) or the posterior optic foci (POF). Glass microelectrodes filled with Lucifer yellow CH (LY) solution (Sigma, St Louis, MO, 4 % in distilled water) were inserted into the LAL in order to record physiological responses of DNs. These microelectrodes had resistances of 100 – 200 MΩ. After recording
physiological responses of DNIs, the dye was iontophoresed into the cell with constant hyperpolarizing current of 1 - 2 nA passed for 1 - 5 min. The brain was fixed with 10% formaldehyde and 0.2% saturated picric acid for 1 - 2 h at 4 °C. Then the tissue was dehydrated with an ethanol series and cleared in methyl salicylate.

4.4 Double-labeling a single GI or GII DN with the cv1-NMN

4.4.1 Backfilling of the cv1-NMN

After cooling (4 °C, approximately 30 min) a male moth to achieve anesthesia, all legs and wings were removed. The preparation was mounted ventral-side up on a wax chamber. The ventral part of the neck was dissected to expose the cv1 nerve (see Fig. 6). The nerve was stained by filling with saturated LY dissolved in distilled water from the cut end of the cv1 nerve for 1.5 - 3 h at room temperature. In some preparations, 80 - 90 nA constant hyperpolarizing current was applied for reducing the time of backfilling. After the backfilling the head was immediately removed with the VNC and the cv1 nerve. The brain was dissected from the head capsule and the cell cluster region of the GI and GII DNIs was surgically desheathed carefully with fine forceps in saline solution.
4.4.2 Intracellular staining of a single GI or GII DN

The cell bodies were visualized with a fixed-stage upright microscope (BX50WI or BX51WI; Olympus, Tokyo, Japan) equipped with differential interference contrast (DIC) optics and long-working distance objectives (x 40 LUM Plan FL/IR or x60 LUM Plan FL/IR water immersion). The contrast was enhanced by a CCD camera (C2741-79; Hamamatsu Photonics, Shizuoka, Japan) enhanced contrast. Cell clusters on the surface of the brain can be clearly visualized with this system, allowing us to select the cells for inserting the microelectrode (see Figs. 7, 8). The tips of glass microelectrodes were filled with the solution containing 4% neurobiotin (Vector Laboratories, Burlingame, CA) in 1M KCl. These electrodes had resistances of 50 MΩ. Cell bodies in the GI and GII DNs were visualized and impaled with microelectrodes (see Figs. 7, 8). After stabilization of the impaled neuron, the dye was iontophoresed into the cell with a constant depolarizing current of 30 - 50 nA passed for 10 - 15 min.

The brain was fixed with 10% formaldehyde containing 0.2% saturated picric acid for 1 - 2 h at 4 °C. After being washed twice for 10 min in 0.1 M PBS, the tissue was incubated in Cy3-conjugated streptavidin solution (Jackson Immunologicals, West Grove, PA; 0.5 μg/ml of 0.1 M PBS containing 0.02% Triton X100) overnight at 4 °C. Then the tissue was washed twice for 10 min in 0.1 M PBS and dehydrated with an ethanol series
and cleared in methyl salicylate.

4.5 Confocal microscopy

Each stained neuron was imaged posteriorly using a confocal imaging system (LSM-510; Carl Zeiss, Jena, Germany) with Plan Apochromat x 40 (n.a. = 1.0) objectives. The LY-stained neurons were examined with a 458 nm excitation and a band-pass emission filter (505 - 550 nm). The Cy3-stained neurons were examined with a 543 nm excitation and a long-pass emission filter (> 560 nm). Serial optical sections were acquired at 0.7 μm intervals throughout the entire depth of a neuron. Three-dimensional reconstructions of the labeled neurons were made from these sections. Images of stained neurons were assembled and labeled in Adobe Photoshop version 5.5.

We carefully examined overlapping regions in the SOG between varicose processes in each type of DNs and smooth dendritic arborizations of the cv1 NMN. The number of regions with an overlap between each type of DN and a cv1-NMN in the SOG was counted by examining all the optical slices obtained every 0.7 μm step (Table 2). The overlapping regions were also clarified by examining from different directions (e.g., Fig. 16 Aa). In some preparations two DNs were simultaneously stained. In such cases, when the overlapping pattern with the cv1-NMN was clearly different between these two DNs
and when it was possible to distinguish these two DN s, overlapping regions were counted separately with care (e.g., Figs. 16, 19).