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Coordination of flipflopping neural signals and head turning during pheromone-mediated walking in a male silkworm moth *Bombyx mori*

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Abstract Male silkworm moths, *Bombyx mori*, move their heads side-to-side during zigzag walking toward a source of sex pheromone. High-speed video analysis revealed that changes in walking direction were synchronized with this head turning. Thus the direction of the walking is indicated by the direction of the head turning. Head turning was regulated by neck motor neurons which innervate the cervical ventral muscles and the ventral muscles through the second cervical nerve. To determine the role of the 'flipflop' state transition in spike activity carried by descending interneurons from the brain to the thoracic ganglion, we recorded pheromonal responses simultaneously from flipflop descending interneurons and a single cervical ventral 1 neck motor neuron. The activity of the cervical ventral 1 neck motor neuron was synchronized to that of the flipflop descending interneurons. The cervical ventral 1 neck motor neuron was morphologically identified using confocal imaging. Our results demonstrate that the flipflop signals play an important role in instructing turning signals during the pheromone-mediated behavior in a male *B. mori*.

Key words Moth · Pheromone · Zigzag walking · Flipflop · Neck motor neuron

Abbreviations *cv* cervical ventral muscle · *CN* cervical nerve · *DN* descending interneuron · *NMN* neck motor neuron · *SOG* suboesophageal ganglion · *v* ventral muscle

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Introduction

Male silkworm moths, *Bombyx mori*, exhibit a characteristic zigzagging pattern as they walk upwind to pheromones released by conspecific females. Wing vibrations, head-turning movements and abdominal ruddering accompany pheromone-mediated walking (Kramer 1975, 1986, 1996; Obara 1979; Kanzaki and Shibuya 1992; Kanzaki and Mishima 1996). This coordinated suite of behaviors, released by pheromone, has been termed 'zigzag behavior' (Kanzaki et al. 1994). Males exhibit continuous left and right turning even when both wings and/or the abdomen is removed (Kanzaki 1998).

In response to pheromonal stimulation, characteristic flipflopping neural activity is recorded from descending interneurons (DNs) in the cervical connectives which link the brain and the prothoracic ganglion (Olberg 1983; Kanzaki et al. 1994; Kanzaki and Mishima 1996). This flipflop activity is a state-dependent response; i.e., it has two distinct firing frequencies, high and low, and switches back and forth between the two states in response to pheromonal stimulation. Since their discovery (Olberg 1983), it has been proposed that the state-dependent activity of these flipflopping DNs might underlie the zigzag turning that characterizes the pheromone-mediated behavior of *B. mori* males. The data presented here, together with previous work from this laboratory (Kanzaki et al. 1994; Kanzaki and Mishima 1996) strongly support this earlier proposal.

Recently flipflopping activity has been classified into two types (i.e., 'FF' and 'ff'; Kanzaki et al. 1994; Kanzaki and Mishima 1996). When two suction electrodes are applied to a cut end of the cervical connectives, one to the whole right and the other to the whole left connective, some of the largest units in each connective show a characteristic flipflop activity pattern. The flipflopping activity patterns in each connective consistently show an antiphasic relationship. The flipflop activity shown by some of the largest units in each connective is classified as an 'FF'. In contrast, when a recording is

made from a small bundle split from one connective, some units show a flipflop activity pattern synchronized to the 'FF' recorded from the contralateral connective. This type of flipflop interneuron is classified as 'ff'. Each connective contains both 'FF' and 'ff' DNs and the phase transitions of the 'FF' and the 'ff' occur simultaneously (Kanzaki et al. 1994). Since the amplitude of the 'ff' is smaller than that of the 'FF', it is difficult to detect unless the recording is made from a small bundle split from the connective (Kanzaki et al. 1994).

In a previous paper, we reported the correlation between the timing of change in the direction of walking and head turning during pheromone-mediated walking. The timing for alternating the turning direction is synchronized to that of head turning (Kanzaki and Mishima 1996). We also showed that the activity pattern of some neck motor neurons (NMNs) in the second cervicale nerve (2nd CN) appeared to be synchronized to that of the flipflop DNs (Kanzaki and Mishima 1996).

In the present study we simultaneously recorded responses to pheromone from flipflop DNs and from a single NMN which innervates neck muscles regulating side-to-side head turning. The NMN was also morphologically identified using a confocal imaging technique. Our results demonstrate here that the flipflop signals descending from the brain to the thoracic ganglion play an important role in initiating turning signals during pheromone-triggered behavior in male *B. mori*.

Materials and methods

Animals

Silkworm moths, *B. mori* (Lepidoptera: Bombycidae) were purchased as pupae from Katakura Industries, Japan. Adult male moths were used within 2–4 days after eclosion. They showed zigzag walking in response to pheromonal stimulation applied to the antennae.

Olfactory stimulation

A continuous air flow system (Kanzaki et al. 1989), was used to deliver odors to the antennae. Pure air was passed into an air delivery tube (glass syringe barrel, 2.5 cm diameter, 5 l min^{-1}, 23 cm s^{-1}) positioned about 1 cm from each antenna. The air stream from this tube was aimed at both antennae. Synthetic (*E*,*Z*)-10,12-hexadecadien-1-ol (bombykol), the principal pheromone component of *B. mori*, was solved in *n*-hexane and used as the olfactory stimulant in these experiments. The olfactory stimulant was applied to a piece of filter paper (1 cm × 2 cm) as a 50 µl solution; it was then inserted into a glass stimulant cartridge (Pasteur pipette, 1 mm tip diameter). Concentrations of odorant are expressed as the amount of stimulant on the filter paper in the stimulus cartridge; 0.3–0.6 µg of bombykol were applied to the filter paper. Odor stimuli were delivered from one of two injection cartridges. Switching the air stream from one injection cartridge to the other cartridge (control) was controlled by a solenoid driven valve (MTV-31-M6, Takasago Electric). After passing over the preparation, odorants were removed by gentle suction into an exhaust tube positioned nearby.

Behavior

The methods for behavioral experiments were similar to those described by Kanzaki and Mishima (1996). The head movements during walking were recorded by a high-speed video camera system (Kodak, Ektapro EM) at 125 frames/s. The video camera was positioned above the male moth. The moth was tethered to a steel bar on the dorsal part of the prothorax. The moth walked on the Styrofoam ball. The ball was not attached to the moth, but the moth kept the ball suspended by leg contact. The diameter of the ball was 3 cm and the weight was 0.45 g, which was approximately the same as that of the moth (Fig. 1). The surface of the ball was painted randomly with ca. 120 black dots (0.5 mm in diameter). The direction of walking was analyzed by computing the movement of the dots caused by the male's walking.

Analysis of the behavior

The direction of walking was determined by computing the changes in x–y coordinates of the positions of dots marked on the Styrofoam ball. The coordinate changes were represented as changes of the rotational angles of the ball (Fig. 1 inset). The turn angle (ϕ) was calculated by a computer (NEC, PC-9801VX) frame by frame (every 8 ms). The cumulative turn angles ($\sum\phi$) were plotted by summing the turn angles in successive time periods. As a convention, left turning rotation of the ball was defined as increasing the cumulative angle, whereas rotation to the right was defined as decreasing the cumulative angle.

Since even when the head was moving, the moth hardly moved its antennae while walking on the ball, the distal parts of the antennae were marked as measuring points for later analysis of the head's sideways movements. The head angle (θ) was determined as the angle between the body axis and the line connecting the measuring points on the antennae (Fig. 1 inset). The head angle was calculated frame by frame (every 8 ms) by the computer.

Physiology

After cooling (4 °C, approximately 30 min) to achieve anesthesia, the abdomen and all legs of the moth were removed and the dorsal part of the thorax was dissected with wings. The male was mounted ventral-side-up on a wax chamber. The head was immobilized with a notched plastic plate slipped between the head and thorax. The ventral part of the neck was opened to expose the cervical nerves (CNs) and the ventral nerve cord that consists of two fused connectives. Using two suction electrodes recordings were made simultaneously from a cut end of the connective and the CN which contains NMNs. The electrodes were filled with saline solution containing (in mmol · l^{-1}): 140 NaCl, 5 KCl, 7 $CaCl_2$, 1 $MgCl_2$, 4 $NaHCO_3$, 5 trehalose and 5 *N*-TRIS[hydroxymethyl]-2-aminoethanesulfonic acid, pH 6.8.

Acquired signals recorded with an FM-tape recorder (TEAC, R-60) were digitized and stored on a computer (DELL, 4100/LV) through an A/D converter (Axon Instruments, TL-1-125 interface). The voltage level of the baseline was set above the noise voltage level. The number of impulses above a baseline was measured and processed by the computer and shown as impulse-frequency histograms (0.5 s bins, Kanzaki et al. 1994).

Anatomy

A male was mounted ventral-side-up on a wax chamber by methods similar to the physiological methods described above. An NMN contained in the CN was stained by filling with 5% lucifer yellow CH (LY, Sigma) from the cut end of the nerve. The nerve was exposed to LY for 12–24 h at 4 °C. The CNS was then fixed in 4% formaldehyde in 0.2 mol · l^{-1} phosphate buffer (pH 7.4) for 1 h and dehydrated with an ethanol series and cleared in methyl salicylate. The LY-stained neck motor neuron in the brain was imaged with a Confocal Imaging System (Carl Zeiss LSM-510) using Plan Apochromat 20× (n.a. = 0.75) and ×40 (n.a. = 1.0) objectives. The stained neuron was examined with 458-nm excitation and a long-pass emission filter at 457 nm in a whole mount. Serial optical

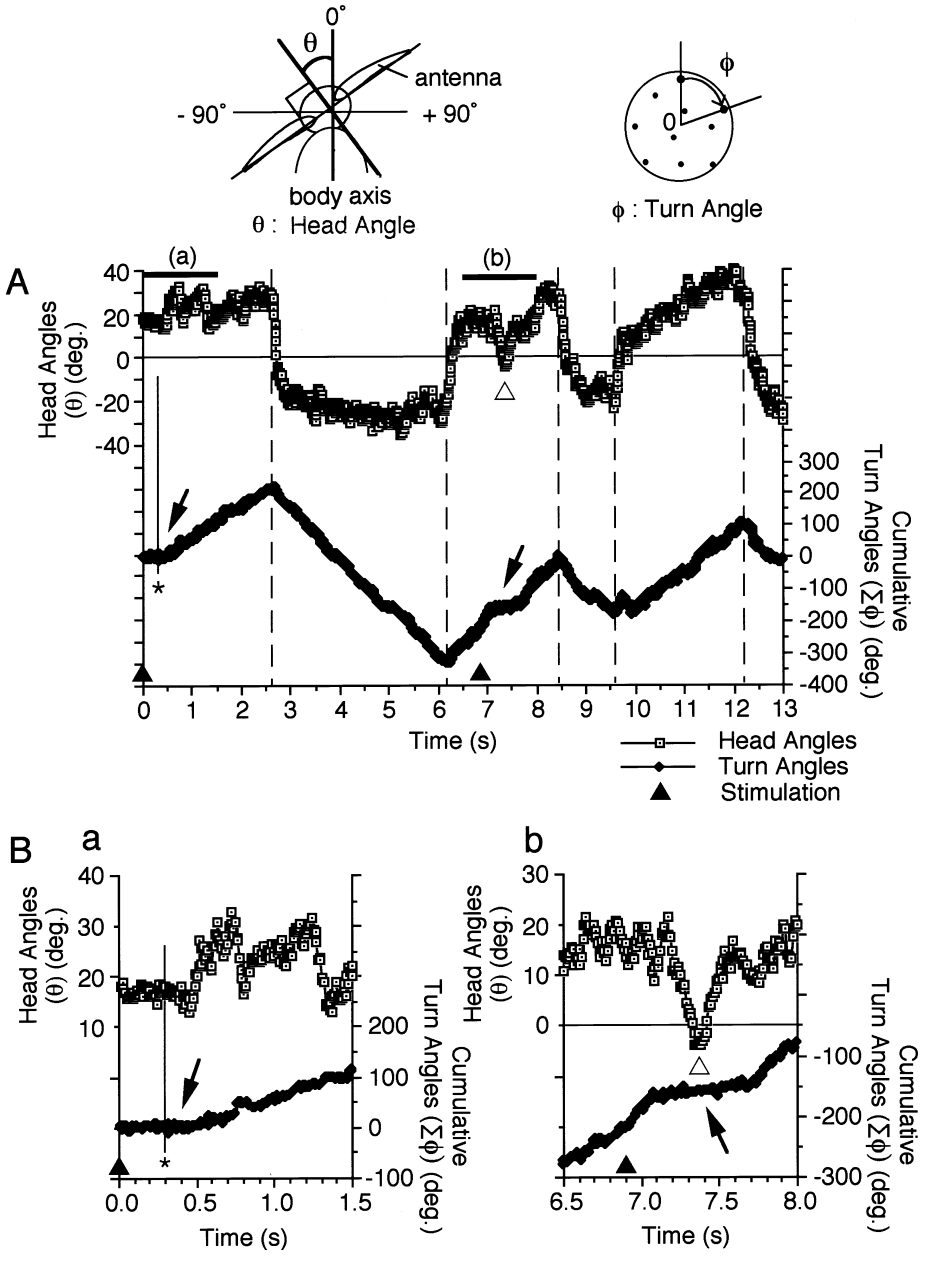

Fig. 1A, B Correlation between head turning and walking during zigzag behavior. **A** The plots indicate the correlation between the head angles (θ inset) and whole body turn angles (ϕ inset) of a male moth. The turn angles were plotted as a running sum of each angle frame by frame (every 8 ms). The value of turn angle increased during the right turn, whereas it decreased during the left turn. During the straight-line walking, the plot of the running sum of turn angles were maintained at a constant value. The male moth began to walk ca. 0.3 s after the onset of the stimulation (*asterisk*). *Dashed lines* indicate the timing to change the turning direction. **B** The plots (**a**, **b**) expand the segments corresponding to the *solid lines* (**a**, **b**) above the plots in **A**. *Filled triangles* indicate pheromonal stimulation to both antennae (200 ms duration). During straight-line walking (*arrows*) the male moth's head angle is kept at 0 degree (*open triangle*)

sections were acquired at 1- to 2-μm intervals throughout the entire depth of the neuron. Three-dimensional reconstruction of the labeled neuron was made from these sections.

The terminology used to describe the neuron, muscles and skeleton is the same as that used by Nüesch (1953, 1957) and Eaton (Eaton 1971, 1974).

Results

Head movements and walking during pheromone-triggered behavior

Single pulses of pheromonal stimuli (200 ms) were applied to a tethered male moth to examine the relationship between head turning and the walking direction during pheromone-triggered behavior. Figure 1 shows an example of the time-dependent changes of the walking direction and those of the head turning. In this experiment, pheromonal stimulation was applied twice to both antennae equally at 0.0 s and 6.9 s (filled triangles). The head angle of the male was ca. 20 degrees before the first stimulation because the male moth was resting at a state of turning its head to right. The male began to walk with wing vibrations ca. 0.3 s after the onset of the stimulation (asterisk). The moth immediately showed straight-line walking for 0.2 s (arrow); this behavior was followed by series of constant left and right turning. Straight-line walking recurred for ca. 0.4 s (arrow) just after the second stimulation. During the straight-line walking the head angle was maintained at 0 degree (open

triangle). In other experiments ($n = 8$) the head angle was usually maintained at that of the body axis (0 degree) during the straight-line walking, which was only observed just after pheromonal stimulation. In 15 out of 24 trials (62.5%) using nine *Bombyx* males, single-pulsed stimulation elicited straight-line walking to approximately due upwind. When the moth turned to the right, its head simultaneously showed a sideways movement to its right, and conversely for a left turn (dashed lines). Maximal change of the head angle during the continuous turning was approximately ± 40 degrees. In all nine males tested, the timing to change the turn angle and the head angle was closely related at the behavioral level. Reversal of the direction of the head occurred 0.076 s (± 0.091 SD; $n = 31$) earlier than that of the walking direction. This is because there is a time lag for the moth to begin to turn the ball by extending the foreleg to the ball.

Muscle arrangements and innervation

The head of *B. mori* is supported by two sclerites spanning the soft cuticle between the head and the prothorax called the cervicales. The frontal part of such cervicale articulates with the postocciput (Fig. 2), and the posterior part articulates with the prothoracic episternum. Head movement is controlled by the action of 11 pairs of neck muscles. Based on the anatomy of the neck cuticle and the arrangement of the neck muscles (Fig. 2), sideways movements of the head are caused by the action of the following three neck muscles: the ventral muscles (v) and the cervical ventral muscles 1 and 2 (cv1, cv2). The origin of the v muscle is the median edge of the 1st furca and its insertion is on the ventrolateral end of the postocciput. Thus, contraction of the v muscle retracts the lateral margin of the head capsule causing a sideways movement of the head. The origin of the cv1 muscle is the median edge of the 1st furca and its insertion is on the ventral arm of the cervicale. The origin of the cv2 muscle is the median edge of the 1st furca and its insertion is on the anterior arm of the cervicale. Contraction of the cv1 and cv2 muscles on one side adducts the head.

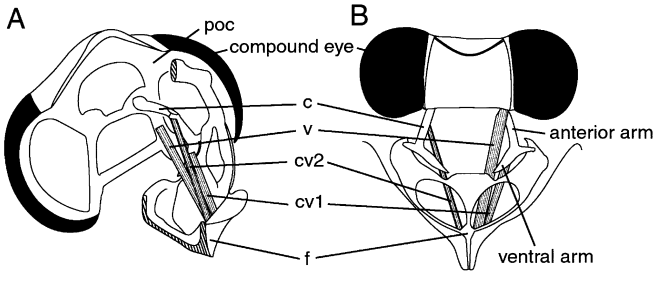

Fig. 2A, B Structure of the neck cuticle and candidate neck muscles which control sideways head movement. **A** View from diagonally behind. **B** Ventral view (*c* cervicale, *f* furca, *poc* postocciput)

The neck muscles were innervated by three nerves, the first cervical nerve (1st CN), the second cervical nerve (2nd CN) and the IN1a (Fig. 3). The 2nd CN arises from the connective between the suboesophageal ganglion (SOG) and prothoracic ganglion. It is divided into dorsal (2nd CNa) and ventral (2nd CNb) branches. The ventral branch (2nd CNb) extends laterally and sends branches to v and cv2. The main branch innervates cv1. We named the nerve that innervates cv1 muscle the cv1 nerve.

Morphology of the neck motor neuron in the cv1 nerve

Lucifer yellow backfilling from a cut end of the cv1 nerve always stained a single NMN (cv1 NMN; Fig. 4A, B). Cross section of the cv1 nerve revealed an axon of 10 μm in diameter (Fig. 4C). Only a single motor neuron was contained in the cv1 nerve and no sensory neuron was observed. The cell body of the cv1 NMN was situated in the ventromedial part of the SOG. The diameter of the cell body is 30–40 μm. The primary neurite (4–6 μm in diameter) runs laterally and posteriorly approximately 80 μm, where it forms several secondary neurites (arrow heads). The secondary neurites run dorsally and spread widely at the posterior part of the SOG. The branching area of the cv1 NMN is restricted to the SOG ipsilateral to the cell body. The

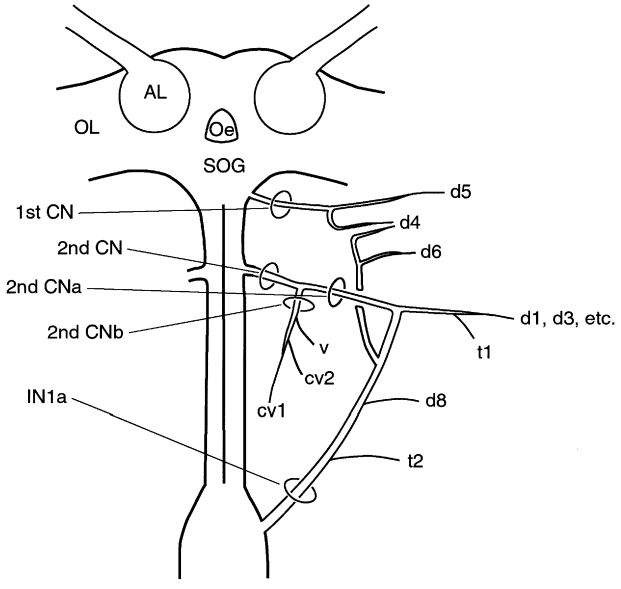

Fig. 3 Schematic drawing of nerve innervation to neck muscles. Dorsal view. Nomenclature is after Nüesch (1953, 1957) and Eaton (1971, 1973). *AL* antennal lobe; *cv* cervical ventral muscle; *d* dorsal muscle; *Oe* oesophageal foramen; *OL* optic lobe; *SOG* suboesophageal ganglion; *t* tentorial muscle; *v* ventral muscle; *1st CN* first cervical nerve; *2nd CN* second cervical nerve; *IN* prothoracic ganglion nerve

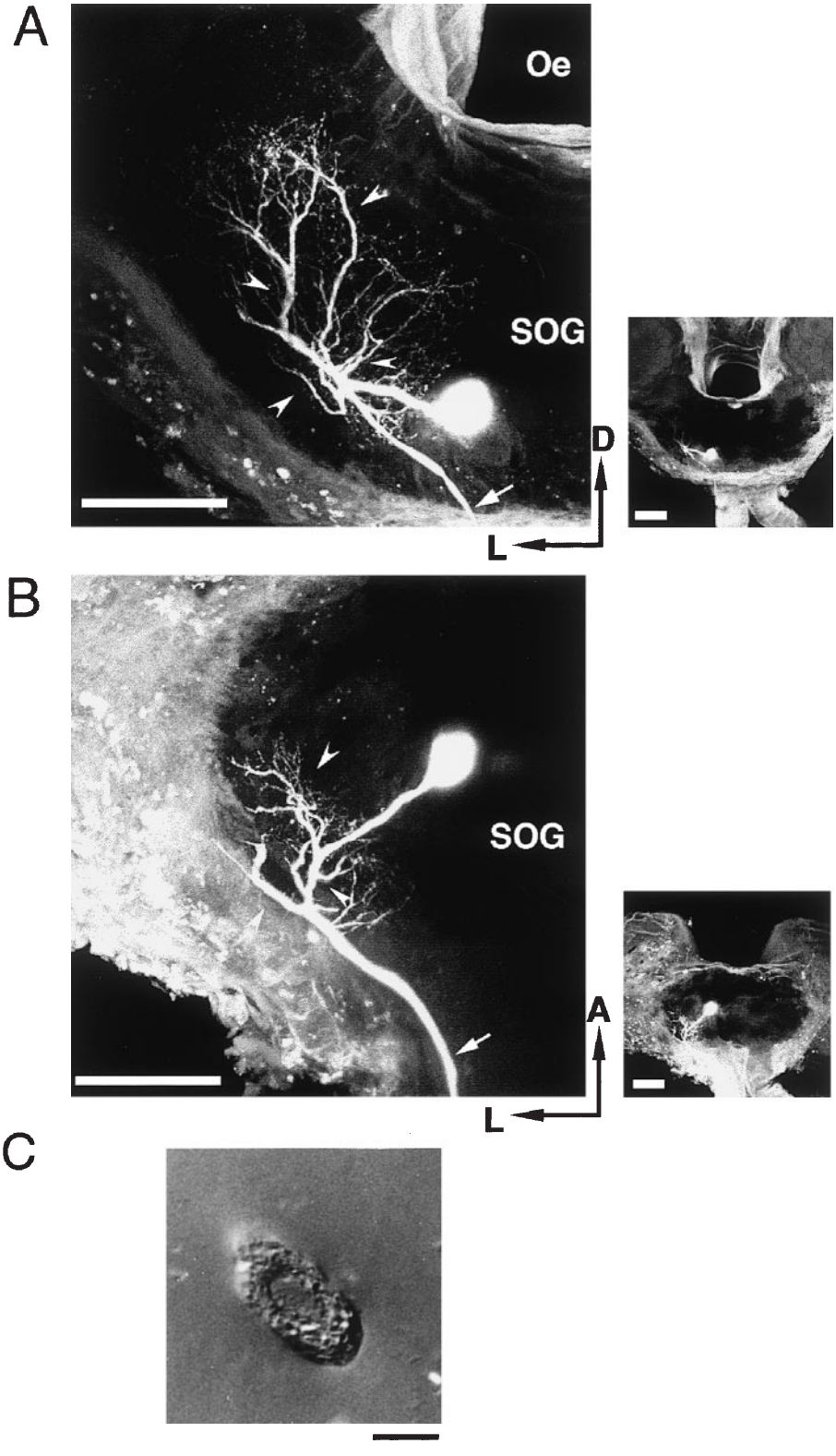

Fig. 4A–C Confocal images of a lucifer yellow-stained neck motor neuron (NMN) in the cv1 nerve. **A** Frontal view. **B** Ventral view. Fine processes of the neuron are restricted to the posterior part of the SOG ipsilateral to the cell body. The axon descends the connective ipsilateral to the cell body (*arrows*). *Insets* show confocal images of the cv1 nerve in a whole SOG. *Oe* oesophageal foramen; *SOG* suboesophageal ganglion. **C** Cross-section of the cv1 nerve. The nerve contains only a single NMN. Scale bars: 100 μm in **A** and **B**. 10 μm in **C**

axon (6–8 μm in diameter) enters the ventral nerve cord (arrows) ipsilateral to the cell body and runs at the most lateral part of the connective. The axon of cv1 NMN then extends to 2nd CNb and innervates the cv1 muscle (Fig. 3).

Correlation in activity pattern between flipflop DNs and a cv1 nerve

In order to examine the relationship in activity pattern between the flipflopping DNs and the cv1 NMN,

recordings were made from the cv1 NMN and, for convenience, from the cervical connective contralateral to the cv1 NMN. Flipflop activity patterns were recorded with a suction electrode applied to a small bundle split from the cervical connective (Fig. 5A). In all the preparations tested ($n = 43$), single unit activity was observed by recording from the cv1 nerve (Figs. 5A, 6, 7A). This is because the cv1 nerve contains only a single motor neuron (Fig. 4C). In the absence of pheromone, the cv1 NMN was silent in most of the cases. The cv1 NMN alternated its activity pattern between a high-spike-frequency state (15–40 Hz) and a low-spike-frequency state (0–20 Hz) in response to single pulses of pheromonal stimulation (Figs. 5B, 7B). The activity pattern of the cv1 NMN had an antiphasic relationship to that of flipflop ('FF') DNs recorded from the connective contralateral to the cv1 NMN (Fig. 5B). Even when the recordings were from the cv1 nerve and the whole connective contralateral to the cv1 nerve, the activity pattern of some of the largest units was antiphasic to that of the cv1 nerve (Fig. 5C). In some cases both 'FF' and 'ff' activity patterns were simultaneously recorded by a single suction electrode (Fig. 6). As shown in our previous study, the amplitude of 'ff' activity is smaller than that of 'FF' activity because of the differences in the axon diameter (Kanzaki et al. 1994; Kanzaki and Mishima 1996). In this preparation, activity of the left cv1 NMN showed an antiphasic relationship to 'FF' and synchronized to 'ff' in the right connective (Fig. 6). Thus in all the experiments ($n = 32$) the cv1 NMN was activated at the same times that the 'FF' DNs contralateral to the cv1 NMN were in a low state (or the 'FF' DNs ipsilateral to the cv1 NMN were in a high state) (Figs. 5, 6).

Activity patterns from left and right cv1 NMNs in response to pheromonal stimulation were antiphasic (Fig. 7A). In all the preparations tested ($n = 7$), similar results were obtained. In some cases, the stimulation failed to shift the activity state. In these cases, only phasic excitation was elicited (Fig. 7A, B*).

In cv1 NMNs the delay of the state transition from a low-spike-frequency state to a high-spike-frequency state was 0.8–1.4 s after the onset of the stimulation, while a longer delay (1.8–2.4 s) was usually observed in the transition from high to low state ($n = 13$; Fig. 8A). State transition from high to low state or low to high state was observed 6–29 times from one animal. Thus, the cv1 NMN in each cv1 nerve was simultaneously excited in a high state for a brief period (ca. 0.8 s) just after the pheromonal stimulation.

In flipflop DNs, the latency from a low state to a high state was 0.6–1.2 s and that from a high state to a low

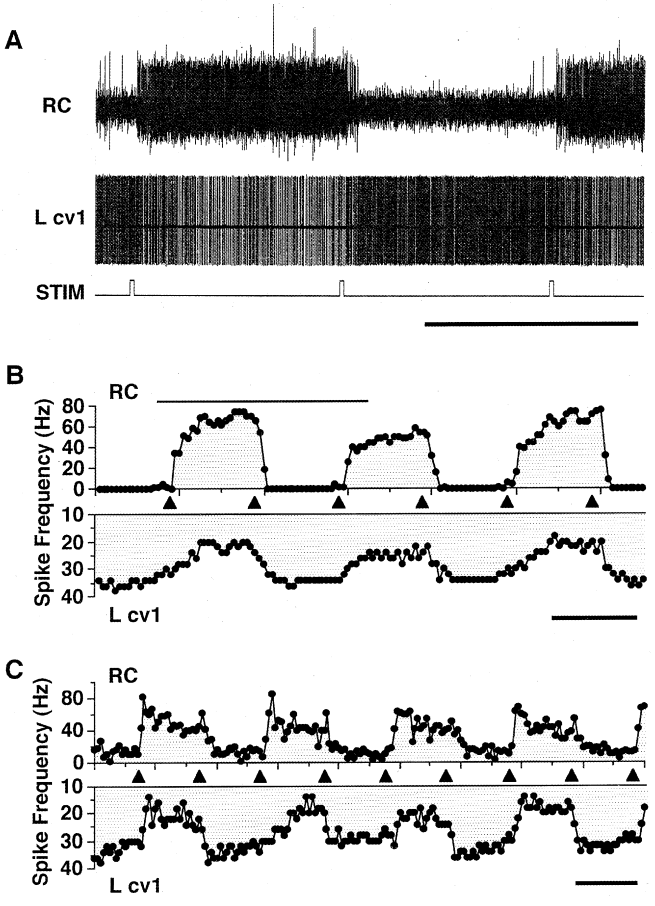

Fig. 5A–C Correlation in activity pattern between flipflop descending interneurons (DNs) and a cv1 neck motor neuron (NMN). **A** Simultaneous recordings from flipflop DNs and a cv1 NMN. Recordings were from a small bundle split from a whole right connective (*RC*) and a left cv1 NMN (*L cv1*). Pheromonal stimulation (200 ms) was applied to a left antenna (*STIM*). **B** Spike-frequency histogram (0.5-s bin) of **A** is illustrated. The frequencies are indicated on the y-axis with opposite direction between *RC* and *L cv1*. *Solid bar* above the histogram indicates the segment corresponding to the traces in **A**. **C** Simultaneous recordings were from a cv1 NMN and a whole connective contralateral to the cv1 NMN. The cv1 NMN is activated when some of the largest units in the connective are in the low firing frequency state. *Triangles* indicate the stimulation. Scale bars in **A**, **B**, **C**: 10 s

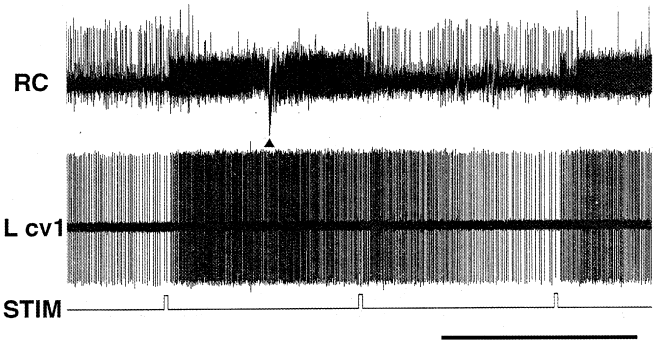

Fig. 6 The cv1 NMN is activated when the 'FF' DN contralateral to the cv1 NMN is in a low state. The cv1 NMN is activated when the ipsilateral 'FF' type is in a high state. Recordings were made from a small bundle split from a right whole connective (*RC*) and left cv1 NMN (*L cv1*). The amplitude of 'ff' activity is reported to be smaller than that of 'FF' activity because of the differences in axon diameter (Kanzaki et al. 1994; Kanzaki and Mishima 1996). The signal was disturbed by sudden movement of the moth (*triangle*). Scale bar: 10 s

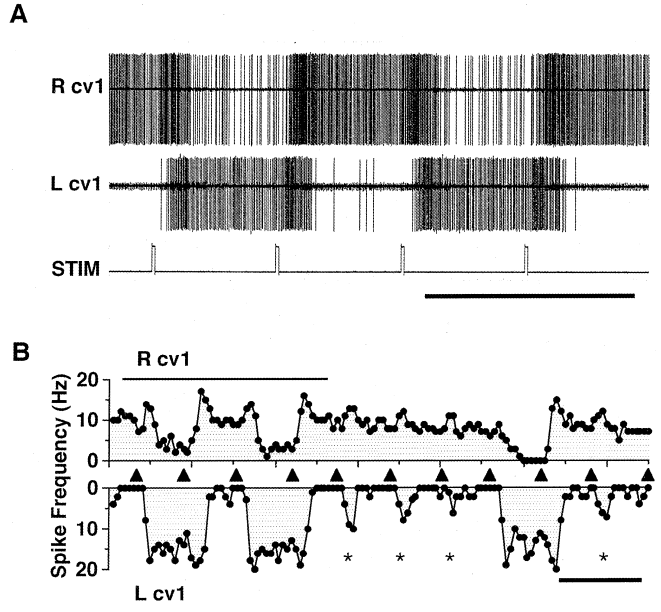

Fig. 7A, B Correlation in activity pattern between a right (*R cv1*) and a left cv1 NMNs (*L cv1*). **A** Activity pattern of the R cv1 and L cv1 is antagonistic. **B** Impulse-frequency histogram (0.5-s bin) of **A** is illustrated. *Triangles* indicate the stimulation applied to the antennae. *Solid bar* above the histogram indicates the segment corresponding to the traces in **A**. Stimulation sometimes failed to alternate the activity state (*), but elicited only phasic activity. Scale bar: 10 s

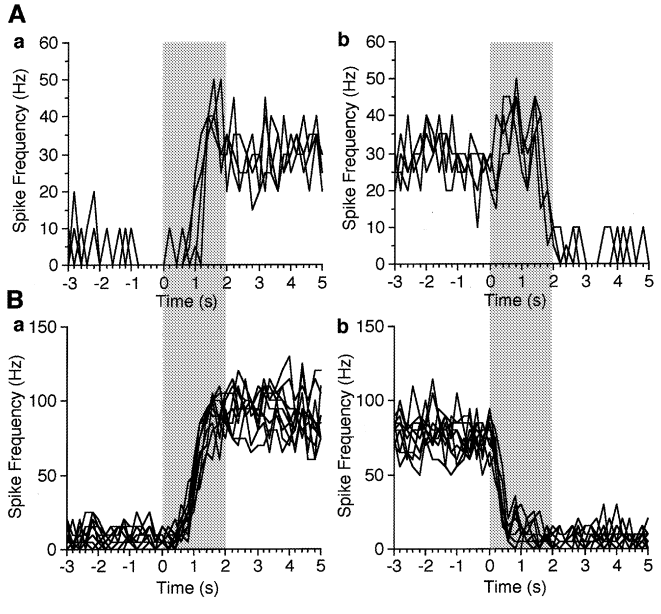

Fig. 8A, B Time-dependent changes of a state transition in the cv1 NMN and 'FF' DNs. **A** State transition in cv1 NMNs. State transition from high state to low state (**a**) and from low state to high state (**b**). Overlay plots of the firing frequency (0.2-s bins) in multiple trials ($n = 4$) from single preparation just before and after the stimulation. **B** State transition in flipflop DNs. State transition from high state to low state (**a**) and from low state to high state (**b**). Overlay plots of the firing frequency (0.2-s bins) in multiple trials ($n = 10$) from a different preparation to **A** just before and after the stimulation. Stimulation is applied at 0 s for 200 ms. *Gray band* indicates onset of pheromonal stimulation and approximate period of state transition of cv1 NMN

state was 0.6–0.8 s after the onset of the pheromonal stimulation ($n = 7$; Fig. 8B). State transition from high to low state or low to high state was observed 6–12 times from one animal. In most cases, flipflop DNs alternated state without any overlapping of the high-spike-frequency state.

Discussion

Relationship between head turning and walking direction

In response to pheromonal stimulation, *B. mori* males display a zigzag behavior that consists of several elements: zigzag turns, wing fluttering, head turning and abdomen curvature (Obara 1979; Kanzaki and Shibuya 1992; Kanzaki 1998). Previous behavioral studies on the walking direction of male *B. mori* revealed that a single puff of stimulation with pheromone triggers a sequential pattern of a walking, which consists of a brief upwind straight-line walk, zigzag turns and looping (Kanzaki et al. 1992; Kanzaki 1998). Both the interturn interval and the turn angle of zigzag walking gradually increase turn by turn after the loss of pheromone stimulation (Kanzaki 1998). This sequence of behavior is reset every time a moth is stimulated with pheromone suggesting an internal program (Kanzaki 1998). In the present study, similar characteristics of the behavior were observed under tethered conditions (Fig. 1). Tethered male moths performed a brief bout of straight walking followed by series of continuous left and right turning in response to pheromonal stimulation (Kanzaki et al. 1992). The continuous turning of more than 360 degrees corresponds to looping in free walking conditions. The sequence of the walking pattern was reset by each pheromonal stimulation and started with an initial straight-line walk (Fig. 1).

In order to understand the function of the flipflop neural signals involved in pheromone-oriented behavior, it is necessary to clarify the relationship between the flipflop signals and some motor activity underlying the behavior. For this purpose, a behavioral element in the zigzag behavior should be selected as an index of zigzag behavior.

As shown in Fig. 1, behavioral analyses using a high-speed video system revealed that the timing for changing the turning direction during series of continuous left and right turning was synchronized with that of head turning. This shows that the turning direction of walking is indicated by the direction of head turning. In *B. mori* males, even when the head was fixed with glue, typical walking pattern in response to pheromonal stimulation is observed (Kanzaki 1998). This observation suggests that the head movement itself does not cause the zigzag walking program triggered by the pheromone.

Head movements are mainly controlled by 11 pairs of neck muscles in *B. mori*. Neck muscles and innervation are basically similar to that in the other flying lepidop-

terans such as *Telea polyphemus*, *Manduca sexta*, *Choristoneura fumiferana* and *Danaus plexippus* (Nüesch 1953, 1957; Ehrlich and Davidson 1961; Albert and Seabrook 1970; Eaton 1971, 1974). Based on the anatomy of the neck cuticle and the arrangement of the neck muscles, the head turning is thought to be achieved by the contraction of three pairs of neck muscles (Fig. 2; i.e., v, cv1 and cv2). The right and left cv1 muscles act antagonistically to each other (Fig. 7). Thus, the excitation of the motor neurons innervating these muscles (e.g., cv1 NMN) indicates the direction of head turning, and is correlated to the turning direction of the moth. Therefore, in the present study we used the activity of cv1 NMN which causes head turning as an index for the zigzag behavior.

Functions of the flipflop activity pattern involved in the zigzag behavior

The timing of the alternating activity state in the cv1 NMN was consistently synchronized with that in the flipflop DNs. The cv1 NMN was activated when the ipsilateral 'FF' DNs were in their high firing state. Thus the cv1 NMN was activated unilaterally, and their activation was correlated with that of the 'FF' DNs (Figs. 5, 6). These results demonstrate that flipflop signal of cv1 NMN controls the head turning and that of DNs descending from the brain to the thoracic ganglia may carry neural information for the pheromone-triggered turns.

As shown in Fig. 1, *B. mori* males performed a brief straight-line walk immediately after pheromonal stimulation. This was rarely observed during continuous turning or looping (Kanzaki et al. 1992). During straight forward walking, the head angle was usually fixed at 0 degree (e.g., Fig. 1 open triangle). Such head orientation may be caused when right and left cv1 NMNs are both simultaneously activated just after the pheromonal stimulation (Figs. 7, 8). The state transition of the cv1 NMN from a low-activity state to a high-activity state occurred ca. 0.8–1.4 s after the onset of the pheromonal stimulation (Fig. 8). On the other hand, a longer delay (1.8–2.4 s) was usually observed in a state transition from a high state to a low state (Figs. 7, 8). These temporal differences in state transition could cause a transient equivalency of neural activity in the left and the right cv1 NMNs (Fig. 8). Simultaneous transient excitation in both, the left and right cv1 NMNs, was only observed just after the stimulation (Figs. 7, 8). These results indicate that transient and simultaneous excitation of some DNs in each connective may induces the straight-line walking as well as that of cv1 NMNs cause head movement to 0 degree.

No doubt there are many possible ways to achieve simultaneous activation of the both connectives. We offer two hypothetical explanations based on data from *B. mori* and other species of moth. The first possibility is that the flipflopping DNs can support the straight-line walking as well as the turning. As temporal differences in state transition of cv1 NMN could cause a transient equivalency of neural activity in the left and the right cv1 NMNs, similar temporal differences in state transitions of flipflopping DNs may cause a transient equivalency of the neural activity in both connectives; however, Fig. 8B shows that flipflop DNs alternated state without any overlap of the high-frequency state.

The second possibility is that the brief bout of straight walking are driven by phasic firing DNs (Fig. 9). In the recordings from connective, some DNs elicited phasic excitatory activity in response to every pheromonal stimulation even when the stimulation failed to shift the activity state of 'FF' DNs (Mishima and Kanzaki 1997). Kanzaki et al. (1992) reported that males from which one antenna is completely removed do not show straight-line walking, but rather initially turn in the direction of the stimulated antenna. This observation agrees with the results that when the stimulation is applied to one antenna, only the DNs ipsilateral to the stimulated antenna show the phasic response (Fig. 9) (Mishima and Kanzaki 1997). In the sphingid moth *Manduca sexta*, DNs showing a similar phasic (< 1 s) excitation were recorded when the pheromone blend was applied to the ipsilateral antenna (Kanzaki et al. 1991). These results suggest that the cv1 NMN may receive phasic information as well as flipflop information. Simultaneous phasic firing of left and right cv1 NMNs may cause the head angle fixed to be at 0 degree. Similarly, the phasic DNs descending from the brain to the thoracic ganglion may carry neural information for the straight-line walking observed at the onset of the pheromonal stimulation. Alternatively, the straight-line walking could be generated by the combination of overlapping of flipflopping DNs activity and that of other phasically firing DNs. The neural correlates of pheromone-mediated straight walking are subject of on going studies in our laboratory.

Odor-oriented flight behavior has been investigated in several moth species (Baker and Kuenen 1982; Kennedy 1983; Willis and Arbas 1991, 1995, 1996; Arbas et al. 1993; Mafra-Neto and Cardé 1994). The most widely accepted explanation of upwind flight to a pheromone source involves the integration of two main

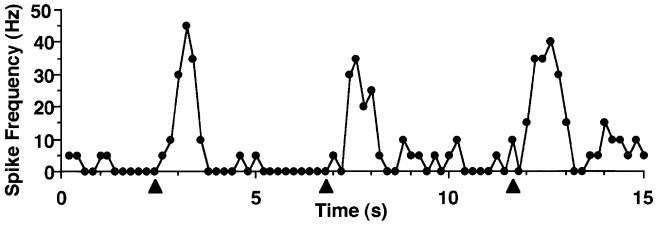

Fig. 9 Spike-frequency histogram (0.2-s bins) of phasic-activated DNs (modified from Mishima and Kanzaki 1997). The DNs showed phasic excitatory activity in response to every pheromonal stimulation (200 ms)

mechanisms, optomotor anemotaxis and self-steered counter-turning, both triggered by contact with the pheromone plume (Kennedy 1983; Willis and Arbas 1991, 1995, 1996). This model views zigzagging upwind flight and crosswind casting as a behavioral continuum. Upon contact with a pheromone plume in flight, an optomotor anemotaxis which causes the male to keep the angle of track leg between turn with some 'preferred' values using visual feedback, and internal program of counterturn which causes to turn back and forth across the wind, are activated. A combination of both counterturn and upwind anemotaxis caused by contact with pheromone should result in zigzagging upwind flight, and counterturning and a more crosswind anemotaxis caused by loss of pheromone resulting in crosswind casting. Even when the encounter rate of pheromone increases, there is little or no effect on the flight track (Willis and Arbas 1991). In contrast, Baker (1990) has proposed a model of a two-part control system underlying odor-oriented flight. In this system, upon contact with odor two separate but interacting control systems are activated. One is a tonically driven system which drives an internal program of counterturns, the other is phasically driven and is transiently activated upon each encounter with a filament of pheromone. In two flying moth species, pheromone-modulated flight activated by a single-pulsed pheromonal stimulation was examined and represented as a template (Mafra-Neto and Cardé 1994, 1996; Vickers and Baker 1992, 1994, 1996). These results show that when the male moth intercepts a pheromone pulse during casting flight, after a delay of 0.2–0.3 s, a straighter upwind surge (0.6–0.7 s in *Cadra cautella*, 0.4 s in *Heliothis virescens*) is activated, followed by a return to the casting behavior. Thus, the male moths fly straighter upwind to fast-pulsed plumes (4.7 Hz) than slow-pulsed plumes (0.6 Hz) due to reiterative switching from casting to upwind flight (Mafra-Neto and Cardé 1994). In the latter view, the male moths have abilities to react to each pheromonal stimulus and alter the flight pattern between casting and upwind flight. In *B. mori* males, a single pulsed pheromonal stimulation to both antennae elicited 0.2–0.5 s of straight-line walking followed by series of continuous left and right turning (Fig. 1). Moreover, we have demonstrated a strong possibility that the pheromone-triggered zigzag turns are induced by flipflopping DNs and proposed that the straight-line walking may be induced by another descending system (phasic system). In *M. sexta*, Kanzaki et al. (1991) reported the two types of DNs; one is a state-dependent activity for which the response characteristics are similar to those of the flip-flop activity, the other is a phasic activity.

The results of the present study may support Baker's phasic-tonic model. Although *B. mori* males cannot fly, judging from the arrangement and number of flight muscles, and the wing motion pattern during the zigzag behavior, the flight motor system seems similar to those of flying moth species (Ariyoshi and Kanzaki 1996; Kanzaki 1998). During turns, the wing on the inside of the turn is consistently retracted to greater extent than the wing on the outside of the turn in *Bombyx* (Kanzaki 1998). The timing of the wing retraction pattern seem to be similar to that of *M. sexta* (Kammer 1968, 1971; Kammer and Nachtigall 1973; Rheuben and Kammer 1987; Wendler et al. 1993; Kanzaki 1998). The wing on the inside on the turn is retracted at the top of the stroke, and the wing on the outside of the turn is retracted after the downstroke at the bottom of the stroke. This suggests that the odor-oriented zigzagging behavior of a walking *B. mori* corresponds to a flying moth species and is induced by similar neural mechanisms to those of flying moth species.

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