Morphology and Physiology of the Serotonin-immunoreactive Putative Antennal Lobe Feedback Neuron in the Male Silkmoth Bombyx mori

Evan S. Hill, Masaaki Iwano, Laureline Gatellier and Ryohei Kanzaki

Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

Correspondence to be sent to: Ryohei Kanzaki, Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan. e-mail: kanzaki@biol.tsukuba.ac.jp

Abstract

In the male silkmoth Bombyx mori, olfactory information is relayed from olfactory receptor neurons in the antennae to the antennal lobe, and then to a variety of protocerebral neuropils. Currently, very little is known about neuromodulators that may affect the dynamics of this olfactory neural network. Immunocytochemical studies have revealed the presence of a serotonin-immunoreactive (SI) neuron that, in several insect species, is thought to provide feedback to the antennal lobe. To date, no studies have revealed details of this neuron’s physiology. Using intracellular recording and staining, the silkmoth SI neuron (in two individuals) was first characterized physiologically and then stained with Lucifer Yellow to reveal morphological details. Immunocytochemical methods were also used to confirm the presence of serotonin. The silkmoth SI neuron branched in many important brain neuropils such as the mushroom body, central body, lateral accessory lobe and antennal lobe. The SI neuron in both individuals fired spontaneous, long duration action potentials, and responded to mechanosensory stimuli to the antennae.

Introduction

The biogenic amine serotonin acts as an important neuromodulator in the nervous systems of many insects (Nässel, 1988). In the hawkmoth Manduca sexta, for example, bath application of serotonin enhances the responses of neurons in the antennal lobe (AL: the first-order olfactory center) to pheromonal and electrical stimuli (Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999). Additionally, serotonin application increases the amplitude and duration of pheromone-evoked local field potentials, as well as the amplitude of potential oscillations in the macroglomerular complex (MGC) of M. sexta (Kloppenburg and Heinbockel, 2000). Similarly, in the silkmoth Bombyx mori, high-speed optical imaging experiments using a voltage-sensitive dye have revealed that both the maximum amplitude and duration of optical responses (to electrical stimulation of the antennal nerve) in the MGC and in the ordinary glomeruli (Gs) are enhanced by bath application of serotonin (Hill et al., 2001). Furthermore, serotonin inhibits two types of K+ currents as well as a voltage-activated Ca2+ current in cultured M. sexta AL neurons (Mercer et al., 1995). It has been proposed that the effects on the K+ currents underlie the serotonin-induced enhancement of AL neurons’ responses to pheromonal stimuli.

In a variety of insect species, immunocytochemical studies have revealed the presence of a pair of serotonin-immunoreactive (SI) neurons with branches throughout one AL as well as in higher order neuropil regions of the brain (Schürman and Klemm, 1984; Kent et al., 1987; Rehder et al., 1987; Homberg and Hildebrand, 1989; Breidbach, 1990; Salecker and Distler, 1990; Sun et al., 1993). Light and electron microscopic studies of these neurons in M. sexta and in the American cockroach Periplaneta americana have revealed that these SI neurons mainly have output synapses in the AL and it has been proposed that the SI neurons may be involved in a feedback system from the protocerebrum (PC) to the AL (Salecker and Distler, 1990; Sun et al., 1993).

At present, nothing is known about the physiology of these SI putative AL feedback neurons. Do they fire spontaneous action potentials? Do they respond to olfactory or mechanosensory stimuli, and if so what is the latency of the response? These are questions that can only be answered by intracellular recording from the SI neuron. We have succeeded twice in recording intracellularly from the SI putative AL feedback neuron in the male silkmoth. Additionally, iontophoretic injections of Lucifer Yellow (LY) were performed to allow us to examine in detail the morphology of this neuron. We found that the silkmoth SI neuron branched in many important brain neuropils including the calyces of the mushroom body, the central body and the lateral accessory lobe (LAL). The SI neuron in both individuals fired spontaneous, long duration action potentials and responded to mechanosensory stimuli.
Materials and methods

Physiology

Adult silkmoth (Bombbyx mori) males were used in 2–4 days of eclosion. The legs were removed, and then the moth was placed in an experimental chamber. The head capsule was opened, and most of the muscles and tracheae were removed. The brain was desheathed using finely sharpened forceps. A glass microelectrode [4% Lucifer Yellow CH (LY, Sigma, St Louis, MO) in the tip; resistance ~150 MΩ] was inserted into the PC in the region of the LAL in order to record from thick branches of the SI neuron. The brain was superfused with physiological saline containing (mM): 140 NaCl, 5 KCl, 7 CaCl2, 1 MgCl2, 4 NaHCO3, 5 trehalose and 5 N-tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) and 100 sucrose (pH 7.3).

Olfactory stimuli were delivered using a puff stimulation system (Kanzaki et al., 1989) to both antennae. Two glass stimulant cartridges (Pasteur pipette, 1 mm tip diameter; air flow of 3–5 ml/s) containing a piece of filter paper (1 × 2 cm) bearing either the major pheromone component (sufficient to trigger the complete pheromone searching behavior) bombykol [(E,Z)-10,12-hexadecadienol, 100 ng] or the minor pheromone component bombykal [(E,Z)-10,12-hexadecadienol, 100 ng] were positioned ~2 cm from the antennae. Both bombykol and bombykal were dissolved in n-hexane to allow application of the odorants to the filter paper; after application n-hexane was allowed to dry off before the filter paper was placed in the stimulant cartridge. The blank was n-hexane. The puff duration was 500 ms. Odorants were removed by gentle suction into an exhaust tube positioned behind the preparation. Neurons were also tested for responses to visual stimuli, either light on [from dark (50 lx) to bright (890 lx) or light up gradually (from dark (50 lx) to bright (6100 lx)].

Following the collection of physiological data, LY was injected iontophoretically by 0.2–1.5 nA constant hyperpolarizing current for 1–4 min. After injection of LY, brains were removed surgically from the head capsule and fixed for 1–4 min. After injection of LY, brains were returned to 100% ethanol, and then rehydrated in an ethanol series. Immunocytochemical double-labeled staining on microtome sections.

Confocal microscopy

LY-stained neurons were imaged frontally and dorsally with the brain as a whole mount using a laser scanning confocal microscope. Serial optical sections were acquired at 1–2 µm intervals throughout the entire depth of the neuron. Optical sections were stacked upon each other, giving a three-dimensional reconstruction of the stained neuron. Confocal stacks of images were fitted together and adjusted for contrast and brightness using Adobe Photoshop 5.5.

Following immunocytochemical processing for serotonin, optical sections were imaged with the confocal microscope using appropriate filters to view: first LY and then Cy-3 in the same optical section (excitation: 458 and 543 nm, respectively; emission: longpass 475 and 565 nm, respectively). For scanning of double-labeled sections, optical slices were set at 1.6–1.8 µm. Images were adjusted for contrast and brightness using Adobe Photoshop 5.5.

Results

LY fill

We recorded physiological activity from and stained with LY the silkmoth SI putative AL feedback neuron in two different preparations. Confocal microscopy was used to examine in detail the morphology of these two neurons, and we found that they both resembled morphologically the SI putative AL feedback neurons reported in M. sexta and P. americana (Kent et al., 1987; Salecker and Distler, 1990;
Both neurons had their somata in the posterior portion of the lateral cell cluster of one AL and branched throughout the contralateral AL (both Gs and MGC) (Figure 1). The neurons also had processes in both the ipsi- and contralateral superior PC (Figures 1 and 3C), the ipsilateral LAL (Figures 1 and 4A,B), the calyces of bombus mori

![Figure 1](image-url) Confocal image (frontal view). (A) This neuron has its soma in the posterior portion of the lateral cell cluster of one AL. The primary neurite projects through the ipsilateral AL where it has a few fine branches in the posterior coarse neuropil (see Figure 3D). Branches are present in the ipsilateral LAL and in both ipsi- and contralateral parts of the PC including the calyces (Ca) of both mushroom bodies (in this image branches in the Ca are not visible; see Figure 4C,D), and also in the central body (CB). More extensive branching is seen in the contralateral superior PC. In the contralateral AL, branches are present in every Gs as well as in each compartment of the MGC (this neuron is referred to as SI neuron #1 in the text). Images taken from wholemount preparation. Scale bar = 100 µm. D = dorsal, V = ventral. (B) Schematic diagram of the brain regions innervated by this neuron. AL = antennal lobe, AN = antennal nerve, Ca = calyces of mushroom body, CB = central body, Gs = ordinary glomeruli, LAL = lateral accessory lobe, MGC = macrogglomerular complex, Oe = oesophagus, OL = optic lobe, SOG = suboesophageal ganglion, SPC = superior protocerebrum.
both mushroom bodies (Figure 4C,D), and in the central body (Figures 1 and 4E,F). Examination of individual optical sections revealed that both neurons branched in every ordinary glomerulus, and in each compartment (cumulus, toroid and horseshoe 1 and 2) of the MGC (e.g. Figures 2 and 3A,B). The primary neurite of each neuron projected through the ipsilateral AL where it had a few fine branches in the posterior coarse neuropil region of the AL (Figure 3D).

Double labeling demonstrates that these neurons are SI

Immunocytochemical processing for serotonin revealed that one of the LY-stained neurons (SI neuron #1) was in fact SI. Although we did not perform immunocytochemical experiments on the second neuron stained with LY, its morphology was identical to SI neuron #1, therefore we will refer to the second LY stained neuron as SI neuron #2. Most parts of SI neuron #1 showed serotonin immunoreactivity. Figure 2 shows double-labeling in the contralateral MGC (Figure

![Double labeling in the contra- and ipsilateral AL. LY staining shown in the left, serotonin immunostaining in the middle, and both superimposed in the right column. (A–C) Every process that was stained with LY in the contralateral MGC also showed serotonin immunoreactivity. (D–F) In the contralateralGs, every LY-stained process showed serotonin immunoreactivity. A SI process that was not stained with LY (arrows in E and F) presumably belongs to the counterpart of the SI neuron or an unknown SI neuron. (G–I) The LY-stained soma adjacent and lateral to the ipsilateral AL showed serotonin immunoreactivity. The primary neurite, however, was only very weakly SI. SI processes observed in the ipsilateral AL (arrows in H, I) presumably belong to the counterpart SI neuron. Images shown are stacks of 11 individual optical sections; images taken from microtome sections. Scale bar = 100 µm. D = dorsal, L = lateral, M = medial, V = ventral.](image-url)
2A–C), the contralateral Gs (Figure 2D–F) and in the ipsilateral AL (Figure 2G–I) of SI neuron #1. LY images are shown in the left-hand column, serotonin immunostaining in the middle column and double-labeling is shown in the right-hand column. Virtually every LY stained process in the contralateral MGC also showed serotonin immunoreactivity (Figure 2A–C). In the contralateral Gs, again, virtually every LY stained process also showed serotonin immunoreactivity (Figure 2D–F). In the contralateral Gs, a process that was not stained with LY also showed serotonin immunoreactivity (arrows in Figure 2E,F). In the ipsilateral AL, double-labeling of the LY-stained soma can be observed (Figure 2G–I). The primary neurite was only very weakly serotonin immunoreactive (Figure 2H). In the ipsilateral AL, many SI processes that were not stained with LY can be observed (arrows in Figure 2H,I). These processes can be attributed to the counterpart of the LY-stained SI neuron.

In other brain regions, double-labeling was also observed, but in some cases only parts of processes showed serotonin immunoreactivity and some cases, fine LY-stained processes did not show serotonin immunoreactivity (data not shown).

**Detailed morphology of the SI neuron revealed by intracellular staining**

Examination of the LY-stained SI neuron in two individuals with a confocal microscope revealed that in both cases the SI neuron had extensive branchings in important neuropils including the calyces of both mushroom bodies, the ipsilateral LAL, the central body, the contralateral AL, and both the ipsi- and contralateral superior PC.

The SI neuron’s processes in both the contralateral Gs and MGC (Figure 3A,B), and in the ipsilateral LAL (Figure 4A,B) were thicker and more varicose than those seen in other brain regions (Figures 3C,D and 4C–F). Figure 3C shows fine processes in the ipsilateral superior PC (anterior to the calyx of the mushroom body). The SI neuron also had fine processes in the posterior coarse neuropil region of the ipsilateral AL (Figure 3D). In the contralateral AL the SI neuron's branches were restricted to the inner portions of Gs and MGC compartments (Figure 2C,F).

The SI neuron had extensive processes in the ipsilateral LAL. These processes were located in the dorsal region of the LAL (Figure 4A). In Figure 4B, varicose processes can be seen in a dorsal view of the LAL. Both SI neurons had many fine processes throughout the calyces of both mushroom bodies. Numerous fine processes can be seen in both the ipsilateral (Figure 4C) and contralateral (Figure 4D) calyces. Interestingly, the fine processes were not stained by serotonin immunocytochemistry (data not shown). Therefore, the presence of fine processes throughout the calyces of both mushroom bodies could only have been detected by intracellular staining of the SI neuron. The SI neuron had fine processes throughout the central body. In Figure 4E,F, two confocal stacks throughout the central body are shown.

**Physiology**

In both individuals, bombykol and blank stimuli elicited very similar responses from the SI neuron in terms of spike frequency and number of spikes fired. A mechanosensory response is a response to the air puff associated with an odor and not to the odor itself. Therefore we believe that the responses of the SI neuron in both individuals are actually mechanosensory in nature. SI neuron #1 responded to bombykol and blank stimulation (Figure 5A) with a burst of 6–10 action potentials with a peak frequency between 35 and 55 Hz (n = 6), which was higher than the background frequency level of ~4 Hz. SI neuron #2 responded to bombykol, bombykal and blank stimulation (Figure 5B) with a brief increase in spike frequency. The responses had a peak spike frequency of ~10 Hz, which was higher than the background frequency of ~2.5 Hz. Neither SI
neuron responded to visual stimuli (data not shown). The average latency of the responses of SI neuron #1 was 157.1 ± 12.5 ms (n = 6), and the average latency of the responses of SI neuron #2 was 204.3 ± 7.0 ms (n = 6). In both cases, there were no significant differences in the latencies of the responses to bombykol, bombykal or the blank.

The action potentials recorded in the SI neuron in both individuals had considerably longer durations than those of typical silkmoth PC neurons (PCNs). In Figure 6A, action potentials recorded from the SI neuron are shown. The action potentials in SI neuron #1 had an average duration of 9.3 ± 0.5 ms (n = 25) and those in SI neuron #2 had an average duration of 4.5 ± 0.3 ms (n = 25), whereas the action potentials recorded from three other typical silkmoth PCNs had much shorter durations (PCN #1: 1.6 ± 0.1 ms (n = 25), PCN #2: 1.4 ± 0.08 ms (n = 25), PCN #3: 2.6 ± 0.2 ms (n = 25) (Figure 6B). PCNs were identified as such by confocal examination of LY fills.

Discussion

The silkmoth neuron from which we, in two individuals, recorded physiological activity and stained with LY resembles a SI putative AL feedback neuron that has been reported in several insect species using immunocytochemical methods (Schürman and Klemm, 1984; Kent et al., 1987; Rehder et al., 1987; Homberg and Hildebrand, 1989; Breidbach, 1990; Salecker and Distler, 1990; Sun et al., 1993). Furthermore, double-labeling with serotonin immunocytochemistry confirmed the presence of serotonin in one of these neurons (SI neuron #1). In both individuals, the SI neuron responded to mechanosensory stimuli and not to visual stimuli. Examination of the LY stains of the SI neuron with a confocal microscope revealed extensive
Figure 6. The SI neuron in both individuals fired spontaneous, long action potentials. (A) Action potentials recorded from the SI neuron. Scale bars = 15, 10 mV, 10 ms. (B) The SI neuron in both cases fired action potentials with longer durations than those recorded in typical B. mori PCNs. See text for details.

branches in areas of the brain that had not been reported in immunocytochemical studies of similar SI neurons in other insects (Kent et al., 1987; Salecker and Distler, 1990; Sun et al., 1993).

Where does the SI neuron receive synaptic input and where does it output? A definitive answer to this question can only be obtained using electron microscopy and is beyond the scope of this study. However, based on the appearance of LY-stained processes we can make inferences as to potential pre- and postsynaptic regions similar to those made in previously published studies (Kondoh and Hisada, 1986; Mishima and Kanzaki, 1999; Lei et al., 2001). In these studies the authors proposed that thick or blebbby processes could represent presynaptic processes and that thin or smooth processes could be postsynaptic processes. Furthermore, similar inferences have been drawn in determining the possible synaptic polarity of crayfish neurons (Kondoh and Hisada, 1986). Branches in the contralateral AL and in the LAL of the SI neuron in both individuals appear thicker and more varicose than processes in all other regions of the brain. Previous electron microscopic studies (Salecker and Distler, 1990; Sun et al., 1993) of similar SI neurons in P. americana and M. sexta reported that these neurons make mostly output synapses in the contralateral AL. Therefore, the thick, varicose appearance of processes in the contralateral AL is consistent with this hypothesis and suggest that the SI neuron in B. mori may also have mainly presynaptic processes in the contralateral AL. The varicose appearance of the processes in the LAL leads us to speculate that these may also represent output synapses.

Some local interneurons (LNs) in B. mori have branchings in the AMMC (antenno-mechanosensory motor center), the GSs, and in the posterior coarse neuropil region of the AL (Y. Seki, personal communication). These LNs may provide a link between the AMMC and the AL, causing AL neurons to respond to mechanosensory as well as olfactory stimuli. It is interesting to note that the fine processes of the SI neuron are also located in the posterior coarse neuropil region of the ipsilateral AL. The fine appearance of the SI neuron’s processes in this region suggests that these may be postsynaptic in nature.

A similar SI neuron in P. americana was also reported to have branches in the calyx of the mushroom body (Salecker and Distler, 1990), however no such branches were reported in the M. sexta SI neuron (Kent et al., 1987). The fine appearance of the branches of the B. mori SI neuron in the calyces suggests that they may serve a postsynaptic function. We found that these fine processes were either only weakly serotonin immunopositive or did not show serotonin immunoreactivity at all. Coupled with the fact that brilliant serotonin immunoreactivity was observed throughout the calyces of both mushroom bodies (data not shown), it would have been impossible to confirm the presence of the SI neuron’s branches in the calyces of the mushroom body relying solely upon immunocytochemical methods. This may explain why no report of processes in the calyces of the mushroom body was made in M. sexta in studies relying solely, or mainly, upon immunocytochemical methods to characterize the SI neuron (Kent et al., 1987; Sun et al., 1993). Additionally, this may also explain why previous studies of a similar SI neuron in M. sexta reported no branches in the coarse neuropil region of the ipsilateral AL or in the ipsilateral LAL. Alternatively, species-specific differences could also account for the differences in the branching areas between B. mori and M. sexta.

The present data are the first report of the SI neuron having branchings in the LAL, thought to be a convergence center for multi-modal neural processing in which many descending interneurons, which link the brain to the thoracic motor center, have branches (Kanzaki et al., 1991a,b; Kanzaki and Shibuya, 1992; Mishima and Kanzaki, 1999; Lei et al., 2001). The varicose appearance of LY-stained processes in the LAL leads us to speculate that these may be presynaptic in nature. Consequently, the possibility that the SI neuron may output in both the contralateral AL and in the ipsilateral LAL arises. Intracellular recording studies have demonstrated that serotonin enhances the olfactory responses of both AL neurons and PC neurons with branches in the LAL (Kloppenburg et al., 1999; Hill and Kanzaki, 2000). The enhancement of olfactory responses of interneurons in these two important neuropils would have a multiplicative effect, and the sensitivity of the moth to
olfactory stimuli would be increased much more than if interneurons in only one (i.e. the AL) of the two neuropils were affected. On the other hand, until an electron microscopic study of the processes in the LAL is performed, the possibility that the processes are postsynaptic in nature remains. In such a scenario, neural information would be relayed from the last olfactory processing neuropil in the insect brain (the LAL) to the first (the AL).

Electron microscopic studies of similar SI neurons in *P. americana* and *M. sexta* have demonstrated the presence of both input and output synapses in the AL (Salecker and Distler, 1990; Sun *et al*., 1993), suggesting that the SI neuron may participate in local processing in the AL, in addition to centrifugal processing. Until electron microscopic analyses of the protocerebral branches of the SI neuron are performed, the possibility that the SI neuron also participates in local processing in various protocerebral neuropils must be considered. For instance, it is conceivable that rather than acting as a centrifugal neuron, the SI neuron could be involved in local processing (input–output) in each of the neuropils in which it branches.

The SI neuron in both individuals exhibited low frequency (~2.5–4 Hz) spontaneous firing of long duration action potentials and responded to mechanosensory stimuli. The long duration of the action potentials recorded in the SI neuron is similar to those observed in *B. mori* neurosecretory neurons (Ichikawa, 2001). While the SI neuron in both individuals responded to mechanosensory stimuli with increases in spike frequency, the responses of SI neuron #1 were much greater in terms of peak spike frequency and number of spikes in the burst. These discrepancies could be due to differences in the air-flux of the stimuli, or it is possible that there may be some individual differences in the responses of the SI neuron. Since the SI neuron in both cases responded to mechanosensory stimuli, we speculate that the SI neuron may mediate an increase in serotonin levels in neuropils in which it outputs in response to mechanosensory stimuli. Such increases in serotonin levels would potentially lead to an increased sensitivity to subsequent exposure to pheromonal or general odor stimuli. Mechanosensory stimuli are abundant during the course of the male silkmoth's pheromone searching behavior. Due to the fact that odors are carried to the moth's antennae by wind, there is the 'passive' mechanosensory stimulus of the air movements. Next, the wing fluttering associated with *B. mori* pheromone-triggered upwind walking is an example of an 'active' mechanosensory stimulus. Both of these stimuli could, in theory, cause the SI neuron to increase its firing rate briefly, thus increasing transiently the levels of serotonin in certain neuropils. Such increases in serotonin levels would potentially increase the moth's sensitivity to the pheromone source it is tracking.

The presence of a similar SI neuron in a variety of insects suggests that serotonergic modulation, and the flexibility it confers upon neural processing, is of great importance for insect olfaction.

**Acknowledgements**

This work was supported by the Program for the Promotion of Basic Research Activities for Innovative Biosciences and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**References**


20 March 2002