Localization of sensory neurons expressing choline acetyltransferase/*lacZ* fusion genes in the leg nervous system of *Drosophila melanogaster*

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Abstract

A summary of the distribution of putative cholinergic sensory neurons in the prothoracic leg of the adult *Drosophila melanogaster* was performed. Putative cholinergic neurons were visualized by X-gal staining of transformed flies carrying a fusion gene consisting of 7.4 kb of 5' flanking DNA from the choline acetyltransferase (ChAT) gene and a *lacZ* reporter gene. The 7.4 kb of ChAT 5' flanking DNA can direct a β-galactosidase expression pattern that is very similar to that of endogenous ChAT protein. The types of sensilla to which the detected neurons belonged were determined by scanning electron microscopy. In the prothoracic legs, the most obvious staining was found in the femoral chordotonal organs. Staining was also detected in the stretch receptors in the tibia. In addition to these internal proprioceptors, the cell clusters corresponding to the sensory neurons innervating the external proprioceptors, including hair plates and campaniform sensilla, were also stained in coxa, trochanter, and tibia segments. In tarsal segments, a number of cell bodies were stained in clusters associated with thin, curved, bractless bristles, the so-called taste bristles. In most cases, four stained cells were recognizable in a cluster. Staining was not detected in the sensory neurons of tactile bristles that function as mechanosensory sensilla. These results strongly suggest that acetylcholine is a neurotransmitter of the sensory neurons of proprioceptors and gustatory sensilla. Key words: cholinergic neurons, ChAT/*lacZ* fusion gene, gustatory sensilla, proprioceptor, *Drosophila*

Introduction

Acetylcholine (ACh) is a dominant neurotransmitter in the nervous system of most animals. In insects, it has long been considered to function as a sensory neurotransmitter. The role of ACh as an excitatory neurotransmitter has been firmly established for synapses between the circal filiform sensory neurons and the giant interneurons in the cockroach[1,2]. Cholinergic monosynaptic connections also have been reported between the proleg tactile sensory hairs and the motoneurons of the tobacco hornworm, *Manduca sexta*[3]. In addition, in

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histochemical studies detecting the activity of acetylcholinesterase (AChE), which is an ACh-degrading enzyme, the anntenal mechanosensory neurons of *M. sexta* were demonstrated to exhibit enzyme activity[4]. This compelling evidence implicates ACh as the primary transmitter in various mechanosensory neurons in insects.

In contrast, much less is known about the transmitters utilized in the insect chemosensory system. It has been proposed that the anntenal olfactory sensory neurons in *M. sexta* are cholinergic based on their AChE activity[5]. Immunocytochemical studies have shown that molluscan cardioexcitatory peptide FMRFamide may serve as a neurotransmitter of non-olfactory chemosensory neurons in the decapode crustaceans[6,7]. Gustatory sensory neurons are also thought to be cholinergic. However, the experimental bases for this proposition are still few. Recently, we have surveyed the overall distribution of putative cholinergic neurons in the whole body of the adult *Drosophila*, and found that not only proprioceptive but also gustatory sensory neurons are probably cholinergic[8,9]. In this study, we attempted further detailed analysis of the distribution of putative cholinergic sensory neurons focusing on the leg sensory system of *Drosophila*.

Cholinergic neurons attain their chemical phenotype by expressing the gene of choline acetyltransferase (ChAT, EC 2.3.1.6) which is responsible for the biosynthesis of ACh. Therefore, ChAT is thought to be the most reliable marker for cholinergic neurons[10]. In the present study, putative cholinergic neurons were visualized by X-gal staining of a P-element transformed *Drosophila* carrying a fusion gene consisting of 7.4 kb of 5' flanking DNA from the *Drosophila* ChAT gene and a *lacZ* reporter gene (7.4 kb-ChAT/*lacZ*). This 7.4 kb of 5' flanking DNA can direct a β-galactosidase expression pattern that is very similar to that of endogenous ChAT protein[11,12]. This fact strongly indicates that the 7.4 kb of 5' flanking DNA of the ChAT gene contains most, if not all, of the information necessary to direct the correct expression of ChAT. Confirming this by utilizing these transformants, we were able to localize the putative cholinergic neurons.

Materials and Methods

Animals. The P-element transformed flies were generated by Kitamoto *et al.*[11] and carry a fusion construct consisting of 7.4 kb of 5' flanking DNA from the *Drosophila* ChAT gene driving expression of the *Esherichia coli lacZ* gene. The transformed flies were reared under standard methods at 25°C.

X-gal staining. Prothoracic legs were removed from anesthetized flies, and fixed with 0.5 % glutaraldehyde in phosphate-buffered saline (pH 7.3) containing 0.1 %

Triton X-100 (PBS/T) for 30 min at room temperature followed by washing in PBS/T and 0.1 M Tris-HCl buffer (pH 7.4) for 30 min each at 4°C. The expression of β-galactosidase, derived from the reporter gene, was detected by staining with X-gal as previously described[13]. For whole-mount preparations, the specimens were dehydrated, cleared and mounted with Permount (Fisher Scientific Company).

Scanning Electron Microscopy. Anesthetized flies were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) containing 0.1 % Triton X-100 for 15 hr at 4°C, and post-fixed in 1 % OsO_4 for 1 hr at room temperature. After washing with the same buffer, the specimens were immersed for conductive staining in 2 % tannic acid for 2 hr, washed in the phosphate buffer, then fixed in 1 % OsO_4 for 1 hr. After this treatment, the specimens were processed further by standard methods[14], and observed in a Hitachi S 570 scanning electron microscope.

Results

Sensory organs of the leg

The legs of *Drosophila* bear at least five different types of sense organs: tactile bristles, taste bristles (TBs), hair plates (HPs), campaniform sensilla (CS) and chordotonal organs (COs)[15-19]. Tactile bristles are straight, pointed, bracted hairs innervated by a single neuron and are purely mechanosensory in function. TBs are recurved, open-tipped, bractless bristles. This type of sensillum usually has one mechanosensory and four chemosensory neurons, and functions as a gustatory sensillum[15,18]. The latter three (HPs, CS and COs) have proprioceptive functions[20-22]. Each of these sense organs has a modality-specific structure. HPs are clusters of short mechanosensory hairs that are usually situated near joints, and monitor the position of the limbs. Each hair is innervated by a single sensory neuron. CS are dome-shaped sense organs usually situated near a joint, and they function as proprioceptors by monitoring stress in the cuticle of the leg. COs are located in the femoral segment of the legs and consist of 70 or more scolopes[17]. They code for movements of the limbs, and mediated local leg reflexes. Figure 1 summarizes the proprioceptors on a prothoracic leg identified in this study.

Identification of sensory neurons expressing the ChAT/lacZ reporter gene

X-gal staining visualized β-galactosidase expression directed by the 7.4 kb-ChAT/*lacZ* fusion gene. Staining was restricted to the sensory neurons associated with proprioceptors and TBs, and to leg nerves and thoracico-abdominal ganglion (Fig. 2A).

In the coxa, three clusters of sensory cells were detected close to the joint with the thorax (arrowheads in Fig. 2A). These clusters were associated with three different sets of HPs. The anterior (double-headed arrow in Fig. 2C), middle (arrow in Fig. 2C) and posterior HP (arrow in Fig. 2B) consist of eight, three and four sensory hairs, respectively (Fig. 2A). The number of stained cell bodies equaled the number of observed sensory hairs composing HP (e. g., arrowhead in Fig. 2B).

X-gal staining in the trochanter revealed five cell clusters (Fig. 3A). Three of them belonged to HPs (the clusters labeled as c and d in Fig. 3A), and the remainder to CS (the clusters labeled as a and b in Fig. 3A). Three sets of HPs were found in the proximal region close to the joint with the coxa. Two of them were located on the anterior surface, and had six and seven sensory hairs, respectively (Fig. 3E). The other HP, which was housed on the posterior surface of this articulation, was composed of five sensory hairs (Fig. 3F). In all three HPs, the innermost hairs were in bifurcation (arrows in Fig. 3E and F). The bifurcation of sensory hairs was not observed in the coxal HPs. Two sets of CS were found on the posterior surface of the trochanter (Fig. 3B). One of them, composed of three sensilla, was situated in the vicinity of the joint with the femur (Fig. 3C). The other CS was located in the distal region close to the joint with the coxa. This CS was subdivided into two subsets of CS: an anterior CS containing five sensilla and a posterior one containing eight sensilla (Fig. 3D).

In the femur, the most obvious staining was found in CO (double arrowheads in Fig. 4A). In addition, two clusters of sensory cells associated with CS were detected in the dorsal and ventral proximal regions in the vicinity of the joint with the trochanter. The dorsal CS occurred singly (Fig. 4 B and D), and the ventral one contained eleven sensilla (Fig. 4A and C). In the proximal region of the tibia, there was a pair of CS on the anterior surface (Fig. 5C) and three single CS on the posterior surface (Fig. 5D). The cell bodies which belong to these CS were detected by X-gal staining (Fig. 5A and B). In this segment, a stretch receptor-like structure was also strongly stained close to the joint with the tarsus (double arrowheads in Fig. 6A).

The tibia and tarsus bore a number of TBs accompanied by stained cell bodies in cluster (Fig. 6A and C). Figure 6B shows a magnified view of stained cell clusters (arrowheads) and TBs (arrows) found in the tibia. This segment has a depressed shape. This resulted in a lateral view of the stained cell clusters, which made individual cell bodies indistinguishable. In contrast, in a tarsomere, which shows a cylindrical aspect, stained cells were clearly distinguishable in each cluster. In Figure 6D, for example, four cell bodies (arrowheads) make up a cluster in the third tarsomere. In this tarsomere, sexual dimorphism was

observed on a number of TBs : six TBs in the male and four in the female (Fig. 7). In both sexes, each the TB was accompanied by four stained cell bodies. No staining was detected in the sensory cells of the tactile bristles, which serve as mechanosensory sensilla. In addition, single pairs of CS were present on the distal surface of the first, third, and fifth tarsomeres (Fig. 1, CS marked with an asterisk). In this experiment, however, the cell bodies that belong to these CS could not be specified, because a number of stained cell bodies in each tarsomere obscured distinction of their cell body clusters.

Discussion

The 7.4 kb-ChAT/*lacZ* transformants, which were utilized in this study, carry approximately 7.4 kb of 5' flanking DNA from the *Drosophila Cha* gene, and display a β-galactosidase expression pattern that is very similar to that of endogenous ChAT protein in adult flies[8,11]. Putative cholinergic neurons thus can be visualized by staining the 7.4 kb-ChAT/*lacZ* transformants with X-gal. Staining detected in the prothoracic legs was restricted to the sensory neurons associated with TB, and to proprioceptors including HPs, COs and CS. The TB on the legs are multimodal sensory sensilla which usually have one mechanosensory and four chemosensory cells[18]. Four stained cell bodies were recognizable in a cluster associated with TB (Fig. 6D). Buchner *et al.*[23] reported that most sensory bristles in *Drosophila*, including tactile bristles and multimodal TBs, possess only one histamine-like immunoreactive neuron, and that COs or CS are not immunoreactive. Based on these observations, they suggested that the sensory neurons of tactile bristles and the mechanosensory cells of multimodal TB are histaminergic. The results of the present study are consistent with their hypothesis, and imply that the stained cells in TB are chemosensory cells functioning as gustatory neurons. Previous studies using the 7.4 kb-ChAT/*lacZ* transformants have shown that the sensory neurons associated with the olfactory sensilla express the reporter gene in antenna and maxillary palps, in addition to those associated with TB in the labial palps[8,9]. On the basis of the previous and present results, it might be concluded that both of the chemosensory neurons serving olfactory and gustatory functions, and the sensory neurons of proprioceptors are cholinergic. Cholinergic transmissions have been reported between proprioceptive sensory neurons and motoneurons in other insect species[24-26]. An immunocytochemical study using the antiserum against ChAT has also provided evidence that locust femoral chordotonal organs are cholinergic[27]. Concerning the mechanosensory neurons, a cholinergic phenotype has been reported in other insects[1-3], in contrast to the histaminergic phenotype in *Drosophila*[23]. This discrepancy might be attributed to the difference of

species.

In *Drosophila*, two genes responsible for specification of the type of sense organ have been identified: *cut* (*ct*) and *pox-neuro* (*poxn*) specify the formation of external sense organs as opposed to chordotonal organs, and the formation of chemosensory organs as opposed to mechanosensory organs, respectively[28-30]. Regarding the transmitter phenotypes of the leg sensory system, histamine is thought to be a transmitter in mechanosensory neurons, but not in proprioceptive neurons[23]. The present study, however provided the evidence that the gustatory and proprioceptive neurons are cholinergic. These facts are interesting in the context of selection of the neurotransmitter phenotype for sensory neurons depending on their function. Therefore, the *Drosophila* leg sensory system offers a good model system for analysis of the molecular mechanism specifying the transmitter phenotype of sensory neurons with relevance to the formation of the specific structure of sense organs depending on their modality.

In the olfactory system of *Drosophila*, we have provided evidence that the primary sensory and the secondary neurons are cholinergic[8,9,31]. The olfactory secondary neurons, called projection neurons (PNs), synapse with intrinsic mushroom body Kenyon cells that are key neurons in olfactory learning and memory in insects[32]. Cholinergic PNs also project into the lateral protocerebrum neuropile, which is a central neural circuit for experience-independent olfactory behavior[33], and terminate as divergent synaptic boutons[34]. In the gustatory system of the fly, by contrast, there is little information on the central neural elements composing this system. The present study has provided evidence that gustatory sensory neurons of TBs are probably cholinergic. X-gal staining of the 7.4 kb-ChAT/*lacZ* transformants also revealed the projection of the gustatory neurons into the central nervous system (data are not shown). Therefore, utilization of these transformants should be useful for analysis of the texture of the neural circuit involved in the chemosensory system in the central nervous system. Future study on the projection of gustatory neurons expressing cholinergic markers may provide clues to understanding the neural mechanism underlying feeding behavior in flies.

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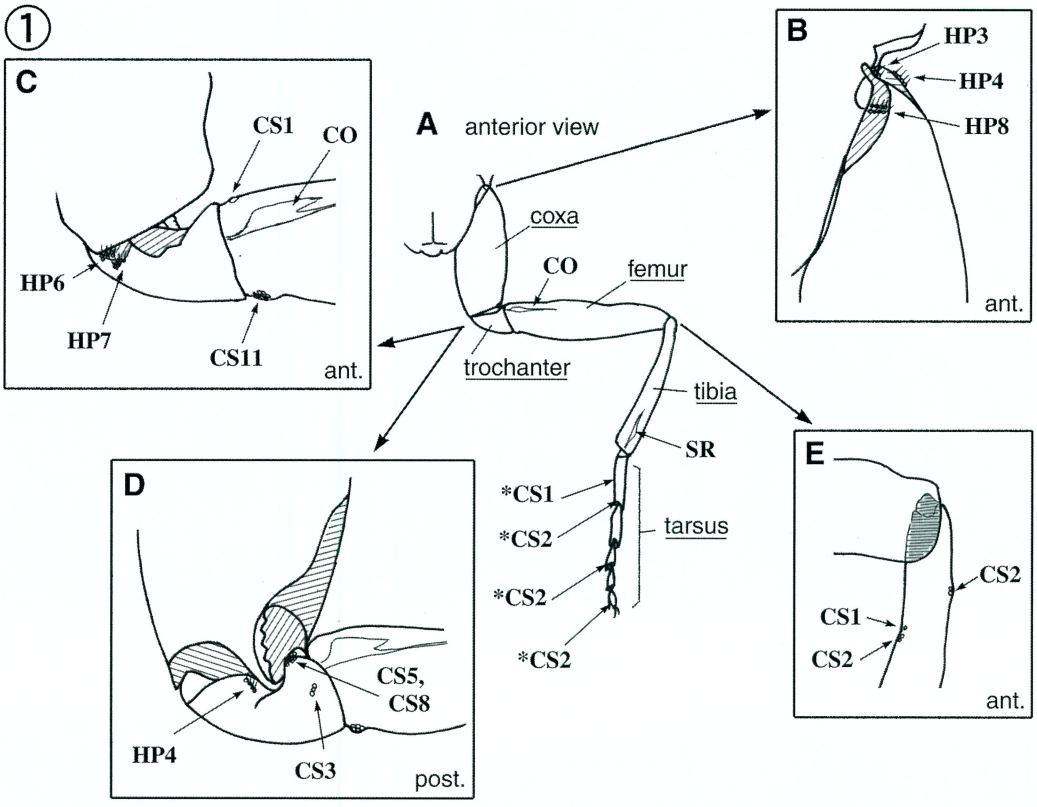
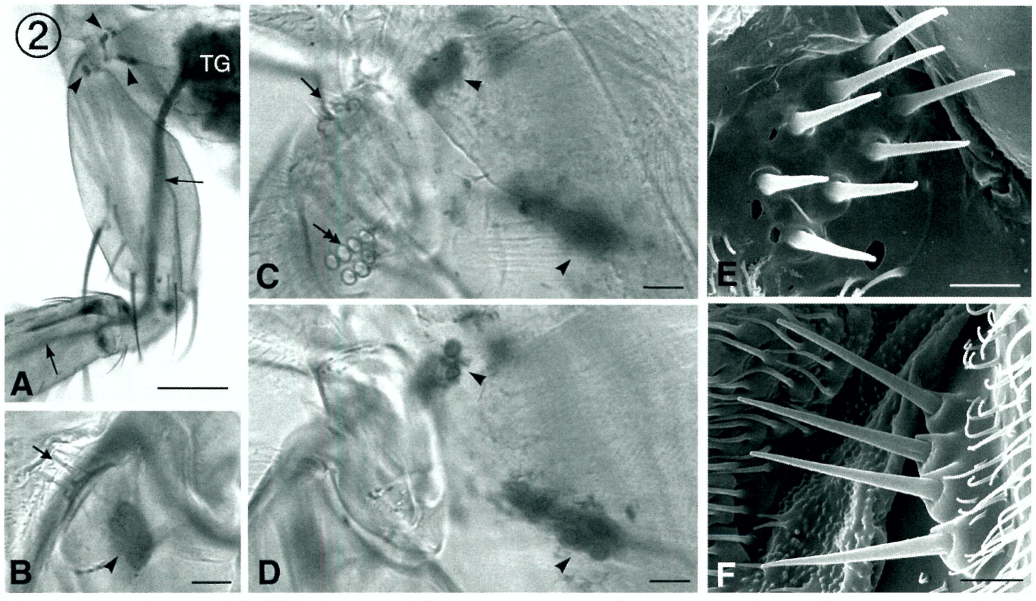
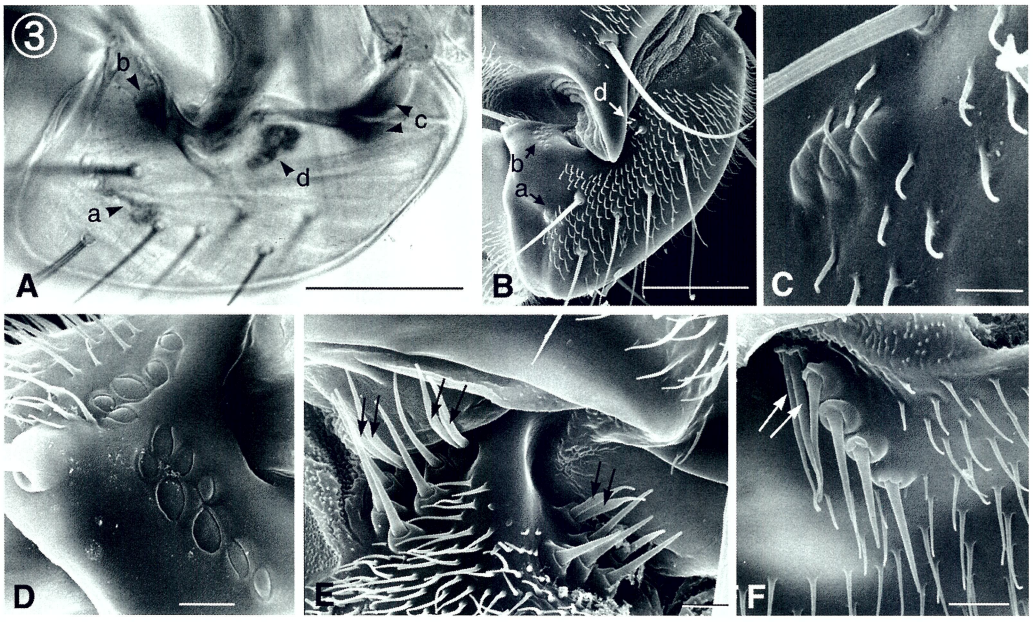
Figure Plates



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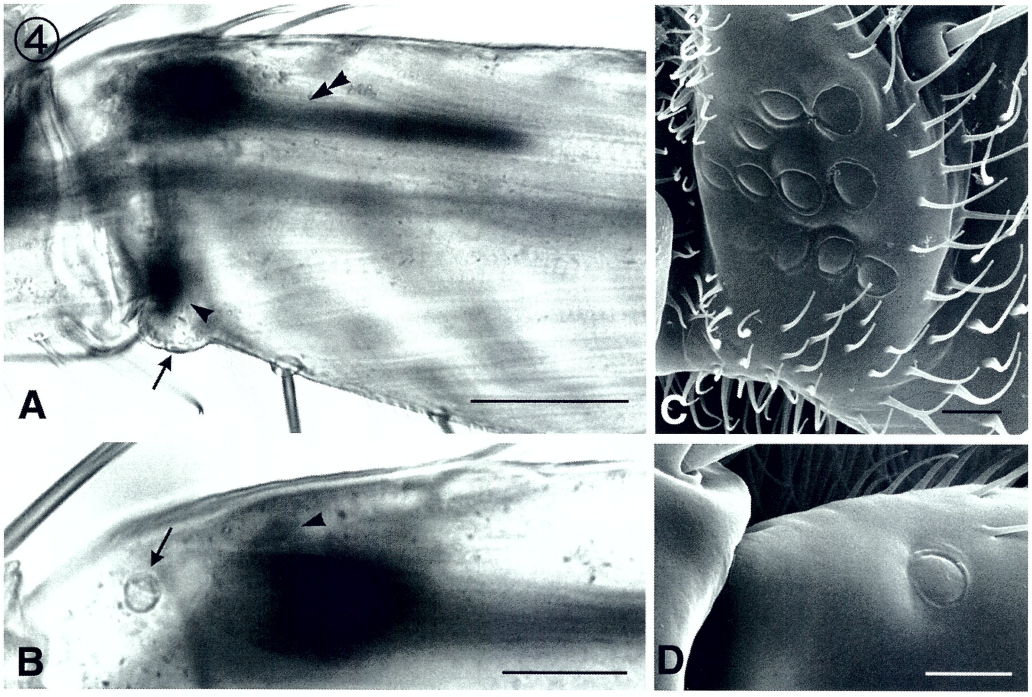
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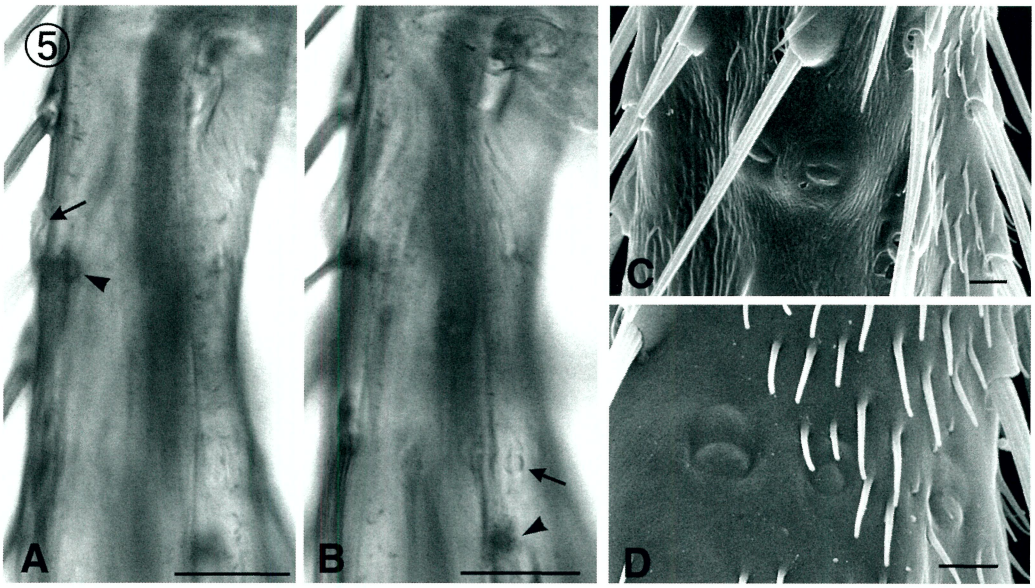
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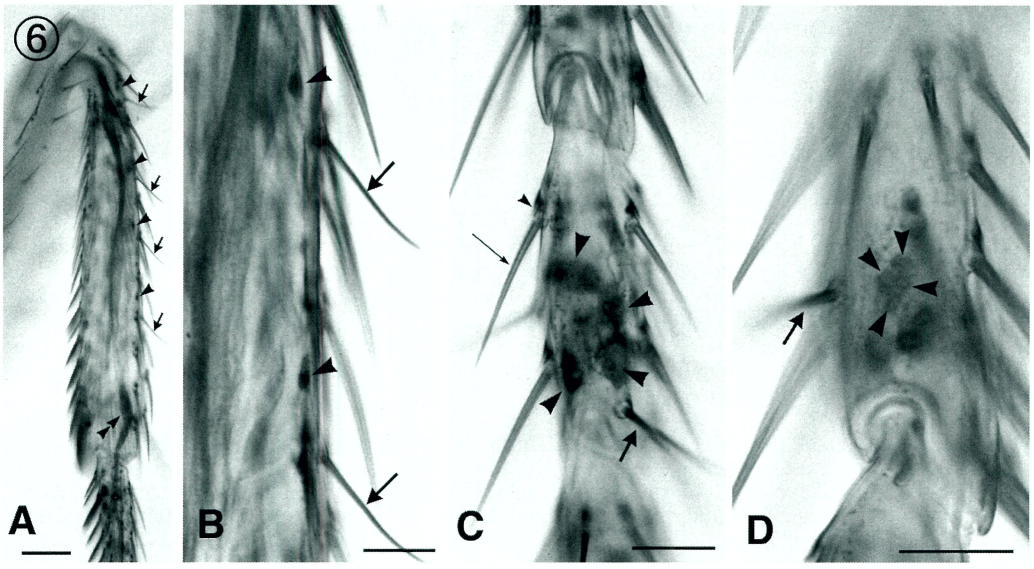
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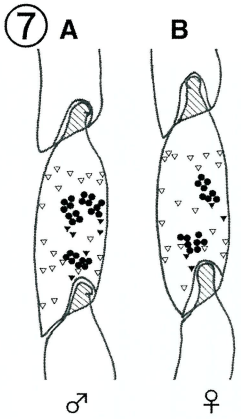

Figure legends

Fig. 1 Diagrams of the position of proprioceptors on an adult prothoracic leg. A-C, E: Anterior view. D: Posterior view. CO, chordotonal organ; CS, campaniform sensillum; HP, hair plate; SR, stretch receptor. Each number appended to CS or HP represents the number of sensilla composing each cluster of campaniform sensilla or hair plates.

Fig. 2 Expression of the 7.4 kb-ChAT/*lacZ* fusion gene in cell bodies associated with HPs on a coxa revealed by X-gal staining (A-D), and scanning electron microphotographs of the corresponding HPs related to the stained cells (E, F). A: Three clusters of stained cell bodies (arrowheads) in the joint with the thorax. Thoracico-abdominal ganglion (TG) and leg nerve (arrows) are also stained. B-D: High magnification views of the cell clusters (arrowheads) and HPs (arrows). The posterior (arrow in B), middle (arrow in C) and anterior HP (double-headed arrow in C) are composed of four, three and eight sensory hairs, respectively. The number of stained cell bodies equals the number of the hairs. In B, four cell bodies equal to the number of hairs are visible. In D, some cell bodies are out of focus. E: Anterior HP. F: Posterior HP. Scale bars = 100 μm in A; 10 μm in B-D; 5 μm in E, F.

Fig. 3 Expression of the 7.4 kb-ChAT/*lacZ* fusion gene in cell bodies associated with sensilla on a trochanter revealed by X-gal staining (A), and scanning electron microphotographs of the corresponding sensilla related to the stained cells (B-F). A: Five cell clusters (arrowheads labeled as a-d) detected by X-gal staining. B: Posterior view of a trochanter. Sensilla corresponding to the stained cell clusters in A are indicated by arrows labeled a, b and d. Sensilla corresponding to the cell cluster c in A are located on the anterior surface and not seen. C: CS composed of three sensilla labeled as a in B. D: Two sets of CS, labeled as b in B, composed of five and eight sensilla, respectively. E: Two sets of HPs corresponding to the cell cluster c in A. One of them consists of six sensory hairs, and the other seven. Arrows indicate the bifurcated hairs. F: HP, marked as d in B, was composed of five sensilla. Scale bars = 50 μm in A, B; 5 μm in C-F.

Fig. 4 Expression of the 7.4 kb-ChAT/*lacZ* fusion gene in cell bodies associated with sensilla on a femur revealed by X-gal staining (A, B), and scanning electron microphotographs of the corresponding sensilla related to the stained cells (C, D). A: The stained cell cluster (double-arrowheads) composing CO, and the stained cell bodies (arrowhead) associated with CS (arrow) in the ventro-proximal region. B: A single CS (arrow) in the dorso-proximal region, and a single cell body associated with it (arrowhead). C: Ventro-proximal CS composed of eleven sensilla. D: Dorso-ventral CS. Scale bars = 50 μm in A; 20 μm in B; 5 μm in C, D.

Fig. 5 Expression of the 7.4 kb-ChAT/*lacZ* fusion gene in cell bodies associated with CS on a tibia revealed by X-gal staining (A, B), and scanning electron microphotographs of the corresponding CS related to the stained cells (C, D). A: The stained cell bodies (arrowhead) associated with CS (arrow) on the anterior surface of the proximal region. B: The stained cell body (arrowhead) associated with CS (arrow) on the postero-proximal surface. C: A pair of CS in the antero-proximal region. D: Three single CS in the postero-proximal region. Scale bars = 20 μm in A, B; 5 μm in C, D.

Fig. 6 Expression of the 7.4 kb-ChAT/*lacZ* fusion gene in cell bodies associated with taste bristles (TB) on a tibia (A, B) and on a tarsus (C, D) revealed by X-gal staining A : Distribution of the stained cell bodies (arrowheads) associated with TB (arrows). Double-arrowheads indicate a stained stretch receptor. B : High magnification view of A. Each the stained, spindle-shaped cell cluster (arrowheads) is located proximal to the corresponding TB (arrows). C : Distribution of stained cell clusters (arrowheads) in the third tarsomere. The thick arrow indicates TB. Tactile bristle (thin arrow) is accompanied by a bract (small arrowhead) D : High magnification of a cell cluster in the third tarsomere. Four stained cell bodies (arrowheads) are recognizable in the cluster. TB to which the stained cluster belongs is shown by the arrow. Scale bars = 50 μm ; 20 μm in B-D.

Fig. 7 Distribution of TB and cell body clusters revealed by X-gal staining on the third tarsomeres of 7.4 kb-ChAT/*lacZ* transformants. A : The third tarsomere of a male fly. B : The tarsomere of a female fly. The number of TB (solid triangles) is six in the male and four in the female. The number of the stained cell clusters is equal to the number of TB. In both sexes, each TB is accompanied by four stained cell bodies (solid circles). The number of tactile bristles (open triangles) is twenty in both sexes, and the sensory cells of this type of bristles are not stained.

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