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# Serotonin-immunoreactive Neurons in the Antennal Sensory System of the Brain in the Carpenter Ant, *Camponotus japonicus*

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Social Hymenoptera such as ants or honeybees are known for their extensive behavioral repertoires and plasticity. Neurons containing biogenic amines appear to play a major role in controlling behavioral plasticity in these insects. Here we describe the morphology of prominent serotonin-immunoreactive neurons of the antennal sensory system in the brain of an ant, *Camponotus japonicus*. Immunoreactive fibers were distributed throughout the brain and the subesophageal ganglion (SOG). The complete profile of a calycal input neuron was identified. The soma and dendritic elements are contralaterally located in the lateral protocerebrum. The neuron supplies varicose axon terminals in the lip regions of the calyces of the mushroom body, axon collaterals in the basal ring but not in the collar region, and other axon terminals ipsilaterally in the lateral protocerebrum. A giant neuron innervating the antennal lobe has varicose axon terminals in most of 300 glomeruli in the ventral region of the antennal lobe (AL) and a thick neurite that spans the entire SOG and continues towards the thoracic ganglia. However, neither a soma nor a dendritic element of this neuron was found in the brain or the SOG. A deutocerebral projection neuron has a soma in the lateral cell-body group of the AL, neuronal branches at most of the 12 glomeruli in the dorso-central region of the ipsilateral AL, and varicose terminal arborizations in both hemispheres of the protocerebrum. Based on the present results, tentative subdivisions in neuropils related to the antennal sensory system of the ant brain are discussed.

**Key words:** 5-HT, social insect, insect brain, mushroom body, antennal lobe, glomeruli

## INTRODUCTION

In social insects, individuals belong to respective castes and comprise a colony. The worker caste needs to perform complex tasks such as nursing, foraging, and defending for the colony and needs to change their behavior readily according to particular circumstances within the colony (Michener, 1974; Hermann, 1979; Engels, 1990; Hölldobler and Wilson, 1990). Such behavioral plasticity and transitions are ascribed to higher brain functions, and hymenopteran social insects such as honeybees are excellent model systems to investigate memory, learning and social behaviors (Menzel, 1999).

The mushroom body (MB) of the insect brain is a prominent neuropil in the protocerebrum and is implicated in integration of sensory signals and in associative memory or learning (Mizunami *et al.*, 1998; Menzel, 1999). The MBs of hymenopteran social insects are particularly large, and the neuronal architectures have been revealed: Kenyon cells,

intrinsic neurons of MBs, extend dendritic arbors connecting with input neurons in the calyces and extend axon terminals connecting with output neurons in the peduncle and lobes (Goll, 1967; Mobbs, 1982). The calyces of social bees, paper wasps and formicine ants appear to be subdivided into concentric subdivisions: lip, collar, and basal ring (Mobbs, 1982; Homberg, 1984; Gronenberg, 1986; Gronenberg, 2001; Strausfeld, 2002). The lip receives input mainly from olfactory afferents, the collar receives input mainly from visual afferents, and the basal ring receives input from afferents of both the olfactory and visual systems (Mobbs, 1982; Gronenberg, 2001; Ehmer and Gronenberg, 2002). Putative gustatory inputs from the subesophageal ganglion innervate the collar just below the lip and the basal ring in the honey-

## ABBREVIATIONS

AL	antennal lobe	LP	lateral protocerebrum
CC	central complex	MB	mushroom body
CIN	calycal input neuron	OL	optic lobe
DC	deutocerebrum	PC	protocerebrum
DL	dorsal lobe	SOG	subesophageal ganglion
DPN	deutocerebral projection neuron		
GAL	giant neuron innervating the AL		

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bee (Schröter and Menzel, 2003). As well as the MB, the lateral horn of the lateral protocerebrum receives axon terminals of the projection neurons from the antennal lobe (AL) and is assigned as a secondary center in the antennal sensory system of the insect brain (Strausfeld, 1976; Boeckh and Tolbert, 1993; Hansson, 1999).

Biogenic amines play a wide-ranging role in insect nervous systems, from peripheral receptor neurons to central brain neurons. (Mercer and Menzel, 1982; Blenau and Erber, 1998; Robinson *et al.*, 1999; Wagener-Hulme *et al.*, 1999; Schulz and Robinson, 2001; Schulz *et al.*, 2003). Serotonin is particularly widespread in the insect brain, and immunocytochemical studies suggest that serotonin-immunoreactive neurons play different roles in functionally discrete neuropils of insect brains (Rehder *et al.*, 1987; Salecker and Distler, 1990; Homberg, 1991; Sun *et al.*, 1993; Nässel, 1988; Iwano and Kanzaki R, 2005; Dacks *et al.*, 2006). Serotonin enhances responsiveness in AL glomeruli and behavioral responses to pheromone in *Bombyx* (Hill *et al.*, 2003; Gatellier *et al.*, 2004). The effects of serotonin on olfactory learning in honeybees have also been reported: direct injection of 5-HT into the brain reduced responsiveness of the honeybee to a conditioned stimulus (Mercer and Menzel, 1982) and reduced the proboscis extension response to a stimulus applied to their antennae (Blenau and Erber, 1998).

Honeybees and ants show respective behavioral specificities (Michener, 1974; Hölldobler and Wilson, 1990). Worker honeybees have excellent visual capability and largely depend on visual cues in their foraging strategies (Srinivasan, 1994; Giurfa and Vorobyev, 1998; Hempel *et al.*, 2001; Fry and Wehner, 2002). On the other hand, worker ants rely heavily on antennal chemoreception (Vander Meer *et al.*, 1989; Lahav *et al.*, 2001; Hara, 2003; Wada *et al.*, 2003; Ozaki *et al.*, 2005). Is this behavioral specificity reflected in neural networks of the brain? Morphological analysis of neuronal elements containing serotonin in social hymenopteran brains could be an important approach for understanding the neural networks underlying memory, learning and social behaviors. In this report, we describe in detail the morphology of serotonin-immunoreactive neurons in discrete neuropils related to the antennal sensory system of the brain of the worker ant, make comparisons to observations in the honeybee brain reported previously, and discuss possible functions of the neurons identified in this study in light of behavioral specificity of the ant.

## MATERIALS AND METHODS

### Animals

Forager ants (*Camponotus japonicus*) were caught around a nest on the campus of Fukuoka University in the autumn and subjected to histological and serotonin-immunohistochemical procedures.

### Standard histology

Ants that had been anaesthetized (by cooling at 4°C) were decapitated, and their brains were dissected out and immersed in cooled normal ant saline (4.8 mM TES, pH 7.4, containing 127 mM NaCl, 6.7 mM KCl, 2 mM CaCl<sub>2</sub> and 3.5 mM sucrose). The brains were fixed with 2% glutaraldehyde and 1% paraformaldehyde, stained with osmium-ethyl gallate, dehydrated in an ethanol series, embedded in Araldite M (Oken, Japan), and serially sectioned at 10

µm (Mizunami *et al.*, 1997). For the reconstructions of brain neuropils and AL glomeruli using tissue autofluorescence, the brains were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0), kept overnight at 4°C, dehydrated in an ethanol series, and cleared in methyl salicylate.

Images of the sections were then taken using a digital camera (Nikon Coolpix) connected to a microscope and saved as TIFF files. The contrast and brightness of the digital images were adjusted using Adobe Photoshop v5.5.

### Serotonin immunohistochemistry

Brains were dissected as described above. The isolated brains were cleaned by removing tracheae and fat tissues, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4), and kept overnight at 4°C. After fixation, the preparations were rinsed several times in PBS containing 0.2% Triton X-100 (PBST) and pre-incubated with 5% normal goat serum in PBST (PBST-NGS) for 3 hr at 4°C under constant agitation. Then the tissue was incubated with rabbit anti-5-HT antibody (Diasorin 20080; diluted 1:1000 in PBST-NGS) at 4°C for 3 days. Following incubation in primary antiserum, the brains were rinsed in PBST and then incubated with Cy3-conjugated secondary anti-rabbit IgG antiserum (Chemicon AP132C; diluted 1:200 in PBST-NGS) at 4°C for 2–3 days. Then the brains were rinsed in PBST, dehydrated in an ethanol series, and cleared in methyl salicylate.

All 5-HT immunoreactivity was lost when the primary antibody was preadsorbed with serotonin-BSA conjugate (ImmunoStar 20081; final concentration of 10 µl/ml) for 20 hr at 4°C prior to application to tissue and when the primary antibody was omitted from the procedure (data not shown).

### Confocal laser scanning microscopy and reconstruction

Whole-mount preparations of immunostained brains were viewed with a confocal laser scanning microscope system (LSM; Olympus FV-300, Zeiss LSM-510), and all images taken were saved as TIFF files for later analysis. For preparations containing Cy-3-labeled tissue, a filter with an excitation wavelength of 510–560 nm was used. Serial images were taken at intervals of 1–5 µm at a resolution of 1024×1024 pixels. For glomerular or neuropil reconstructions, the preparations were viewed with an LSM using a filter with an excitation wavelength of 488 nm. Serial images were taken at intervals of 2 µm at a resolution of 1024×1024 pixels.

Labeled tracts, neuropils, somata, and neuronal arborizations were reconstructed three-dimensionally using 3-D software (Amira v2.3 and v3.0). Labeled structures were traced manually from subsequent serial images on the monitor screen and then digitized using a digitizing tablet.

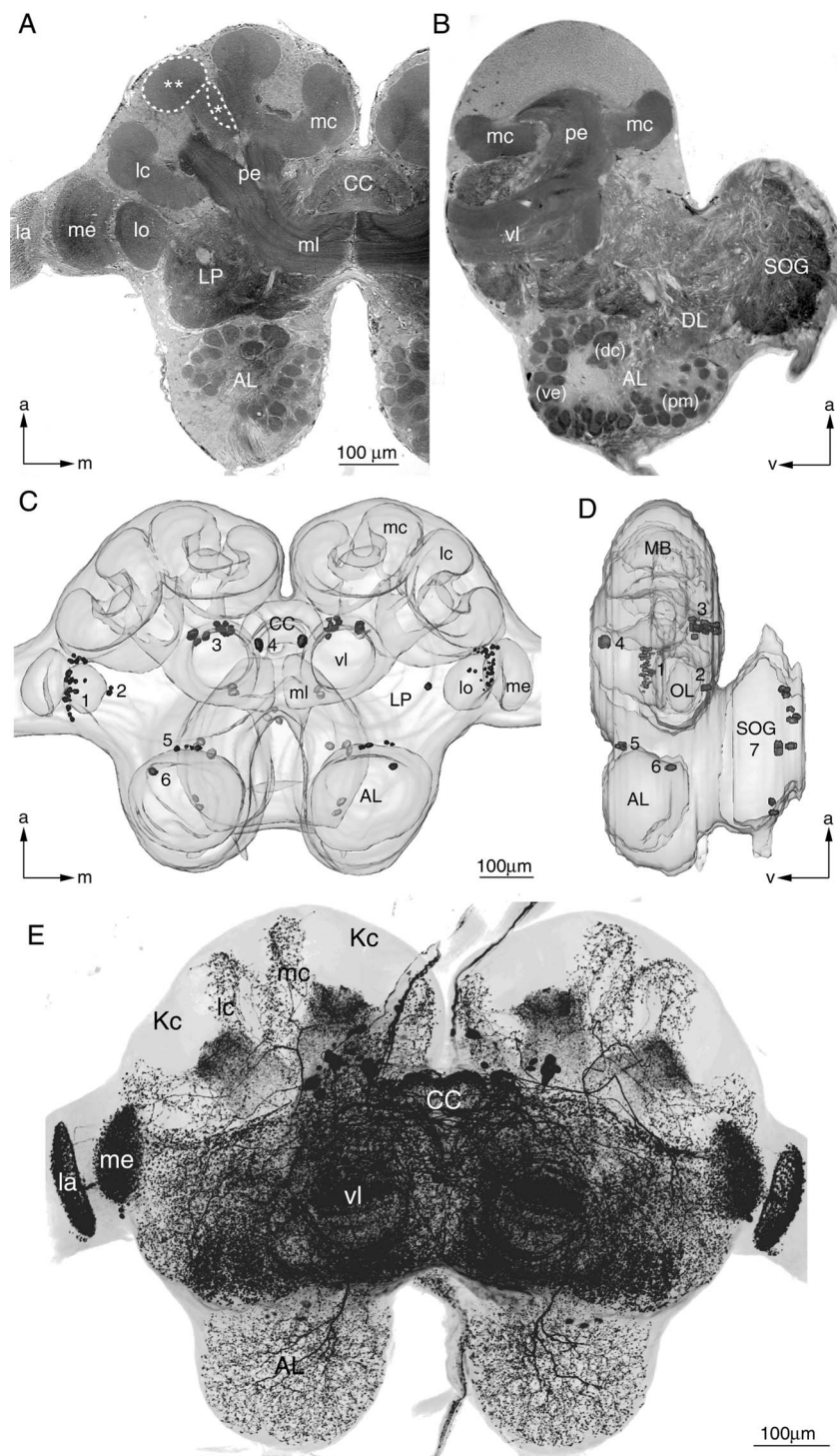
Neuronal arborizations were labeled on subsequent images of the 5-µm sections using photo-imaging software (Adobe Photoshop v5.5) and printed out on a inkjet printer (Epson PM950C). From the printed images, neuronal arborizations were traced manually and then reconstructed for 2-D images.

Unless otherwise stated, orientation of the brain is in the neuraxis and is about 90° tilted against the head-body axis. Abbreviations of orientations in figures are as follows: a, anterior; l, lateral; m, medial; v, ventral.

## RESULTS

### General organization of neuropils in the ant brain

Fig. 1 shows the right hemisphere of the brain viewed ventrally (Fig. 1A) and laterally (Fig. 1B) in osmium-ethyl gallate-stained sections of the brain and subesophageal ganglion (SOG) in the worker ant. The brain is comprised of the protocerebrum (PC) and the deutocerebrum (DC), and the SOG is situated dorsally to the brain (Fig. 1B). The mushroom body (MB) comprises the lateral and medial caly-



**Fig. 1.** The main neuropils of the brain and the subesophageal ganglion (SOG) in ventral (**A**) and lateral side (**B**) views of sections stained with osmium-ethyl gallate. The mushroom body (MB) is the most prominent neuropil in the protocerebrum (PC) and is composed of the lateral calyx (lc), the medial calyx (mc), the peduncle (pe), the vertical lobe (vl), and medial lobe (ml). The calycal neuropils are subdivided into three concentric zones: lip (double asterisks), collar (single asterisk), and basal ring (Gronenberg, 2001). The central complex (CC) is located in the midline of the PC. The optic lobe is the visual neuropil and is composed of the lamina (la), the medulla (me) and the lobula (lo). The deutocerebrum (DC) is composed of the antennal lobe (AL) and the dorsal lobe (DL). (ve), ventral region of the AL; (dc), dorso-central region of the AL; (pm), postero-medial region of the AL. (**C, D**) Distribution of serotonin-immunoreactive somata in the brain and SOG of the worker ant. Ventral (**C**) and lateral side (**D**) views of the brain/SOG complex, three-dimensionally reconstructed. The numbers 1 to 7 refer to the newly defined groups of serotonin-immunoreactive somata. The somata of group 7 in the SOG are indicated by light grey and those of other groups are indicated by dark grey in the ventral view of the brain (**C**). (**E**) Serotonin immunocytochemistry of the brain of the worker ant. Stacked images obtained from optical sections made with a confocal laser scanning microscope (LSM). The image shows a ventral view of a whole-mount preparation of a brain. Anterior is at the top. Immunoreactivity is present in all areas of the brain, PC, and DC but not in Kenyon cells (Kc). The sections of the SOG are excluded from this figure. The arrows indicate the neuronal axis directions (a, anterior; m, medial; v, ventral).

ces (lc, mc; Fig. 1A, B), peduncle (pe; Fig. 1A, B), vertical lobe (vl; Fig. 1B), and medial lobe (ml; Fig. 1A). MBs are composed of Kenyon cells whose cell bodies are situated within and around the calyx cups (Kc; Fig. 1A). The central complex (CC) (Fig. 1A) is a fan-shaped neuropil located anterior to the medial lobe of the MB in the midline of the PC between both hemispheres. The optic lobe (OL) (Fig. 1A) extends laterally from the lateral protocerebrum (LP) and consists of three neuropils: lamina (la; Fig. 1A), medulla (me; Fig. 1A), and lobula (lo; Fig. 1A). The DC consists of a glomerular antennal lobe (AL) (Fig. 1A, B) and the dorsal lobe (DL) (Fig. 1A, B).

### Serotonin-immunoreactive cell body groups

About 130 ( $131 \pm 4.5$  [mean  $\pm$  S.D.],  $n=4$ ) serotonin-immunoreactive somata were traced in the brain/SOG complex. Their locations within a three-dimensional reconstruction of the brain in ventral and lateral side views are shown in Fig. 1C and D, respectively. We found  $115 \pm 2.2$  ( $n=4$ ) somata in the PC, two somata in the DC, and  $16 \pm 3.7$  ( $n=3$ ) somata in the SOG. The somata were then divided into seven paired groups, named partially according to a previous report on the honeybee brain (Schürmann and Klemm, 1984).

**Group 1.** In each hemisphere, 35 to 40 somata are located at the ventral rim in a cell cluster between the medulla and lobula of the respective OLs. The diameters of these somata are about 5  $\mu$ m. Immunoreactive somata in the anterior and posterior parts of this group send neurites mainly into the lamina and the medulla of the OL, respectively (Fig. 1C, D).

**Group 2.** Two somata are in the dorso-medial region between the lobula and LP in each hemisphere. The somata are about 7  $\mu$ m in diameter. These somata likely send neurites into the lobula (Fig. 1C, D).

**Group 3.** Fourteen somata are located dorso-medial to the peduncle of the MB in the most dorsal area of the PC. The diameters of these somata range from 10 to 15  $\mu$ m. Among the 14 neurons, some thick neurites from the somata bilaterally innervate the LPs or encircle and innervate the ipsilateral vertical lobe. Only one soma of Group 3 in a hemisphere sends a neurite to the calyces of the MB (Fig. 1C, D).

**Group 4.** One large cell is located in the posterior pars intercerebralis, adjacent to the dorsal side of the CC (Fig. 1D) in a hemisphere of the PC. The diameter of this large soma is about 20  $\mu$ m. This soma sends a neurite into the contralateral hemisphere of the PC (Fig. 1C, D).

**Group 5.** There are four cells in the ventro-posterior region of the PC next to the DC in each hemisphere. The somata are about 5  $\mu$ m in diameter (Fig. 1C, D).

**Group 6.** In the DC, only one immunoreactive soma is located in the lateral cell cluster of the AL in each hemisphere. The diameter of the soma is about 15  $\mu$ m (Fig. 1C, D).

**Group 7.** There are about 20 cells in the SOG. Among those, 14 cells lie in the lateral soma rind and about six cells are located in the medial region of the SOG. Some of somata in the SOG send neurites into the DL. The diameters of somata are about 15  $\mu$ m (Fig. 1C, D).

### General aspects of serotonin immunoreactivity in the ant brain

Serotonin immunoreactivity is present in most parts of

the brain (Fig. 1E) and in the SOG (Fig. 2F). It is particularly strong in the PC. Serotonin-immunoreactive fibers are distributed in the MBs, CC, OLs, ALs, DLs, and SOG. However, within each neuropil, the intensity of staining varies over a relatively wide range. No immunoreactivity is present in Kenyon cells, which are the intrinsic neurons of the MBs (Kc; Fig. 1E).

In the MB, the vertical lobes show strong immunoreactivity along several strata in the ventral anterior region (Figs. 1E, 2A). This intensity, however, shows a gradual decrease toward the dorsal posterior region. The medial lobes are almost devoid of immunoreactivity; only in the dorsal region, close to the midline of the PC, is weak staining revealed (Fig. 2B). The anterior part of the peduncles shows relatively strong immunoreactivity, which, however, decreases gradually toward the posterior end (Fig. 1E). Two thin fibers (not shown) from the anterior ends of both peduncles penetrate into the central part of the vertical lobe (vl) and, via the anterior end of vl, extend to the CC of the contralateral PC. There is very subtle immunoreactivity in the region of bifurcation from the peduncle to the two lobes. In the calyces, immunoreactivity is observed in the region of the lip and basal ring, but it is missing in the collar regions (Figs. 1A, 5E). Two immunoreactive elements enter the calyces: one terminates in the lip and the basal ring, and the other terminates in the basal ring only.

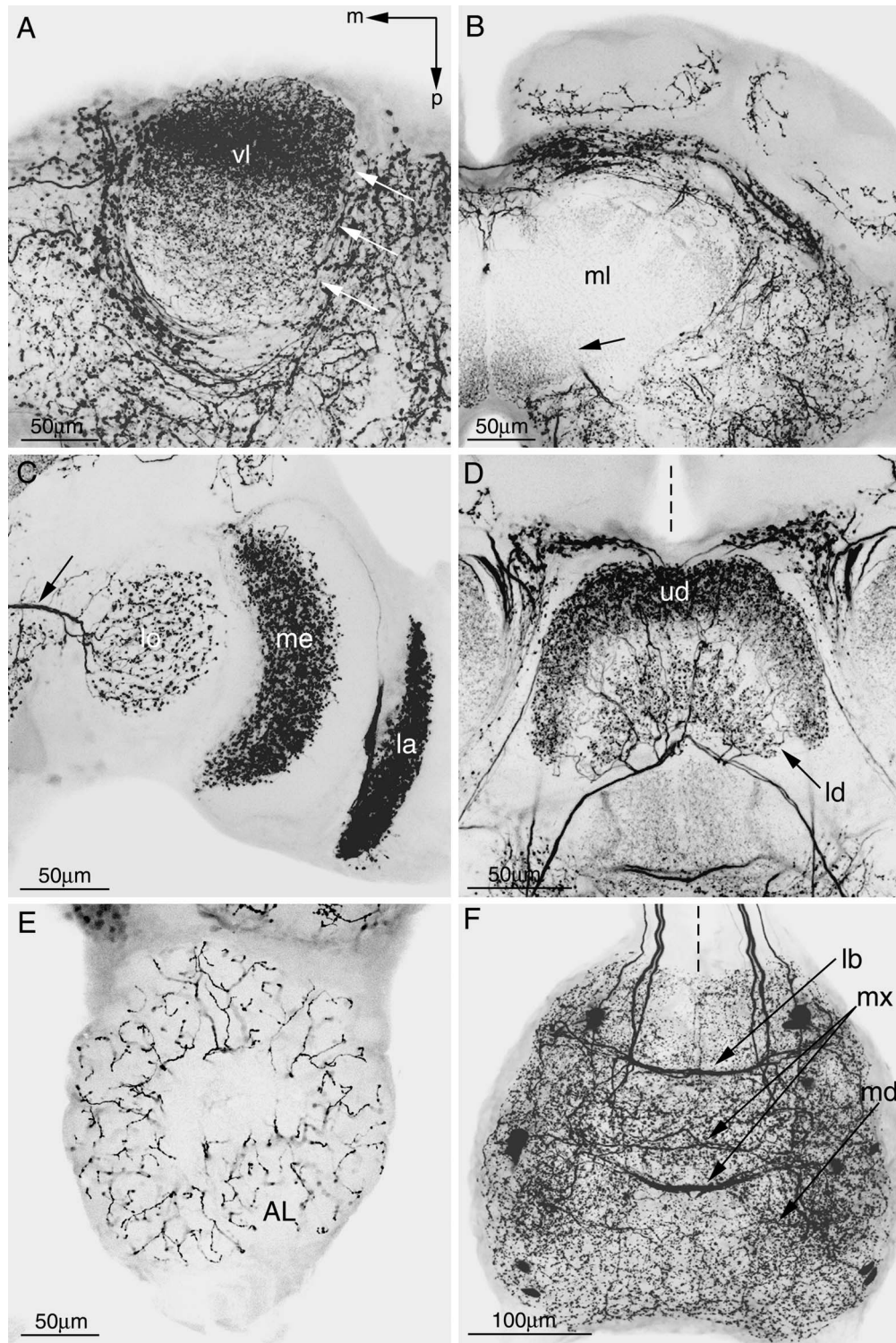
In the OL, immunoreactive neurites from somata in the anterior and posterior parts of group 1 innervate the neuropils of the lamina and the medulla, respectively, and immunoreactive smaller fibers are densely distributed over the two neuropils (Figs. 1C, E, 2C). The lobula appears to be innervated by two different fibers, probably from somata in group 2, which however show a relatively sparse distribution of terminals compared to those in lamina and medulla (Figs. 1C, E, 2C).

According to the staining observed in the CC, several fan-shaped neurons have arborizations in certain layers in the upper and lower divisions of the CC (Figs. 1C, 2D). The upper division shows intense immunoreactivity with dense arborisation, but there is relatively sparse arborization with segmentation in the lower division (Fig. 2D). Immunoreactivity is also observed in the noduli of the CC (not shown).

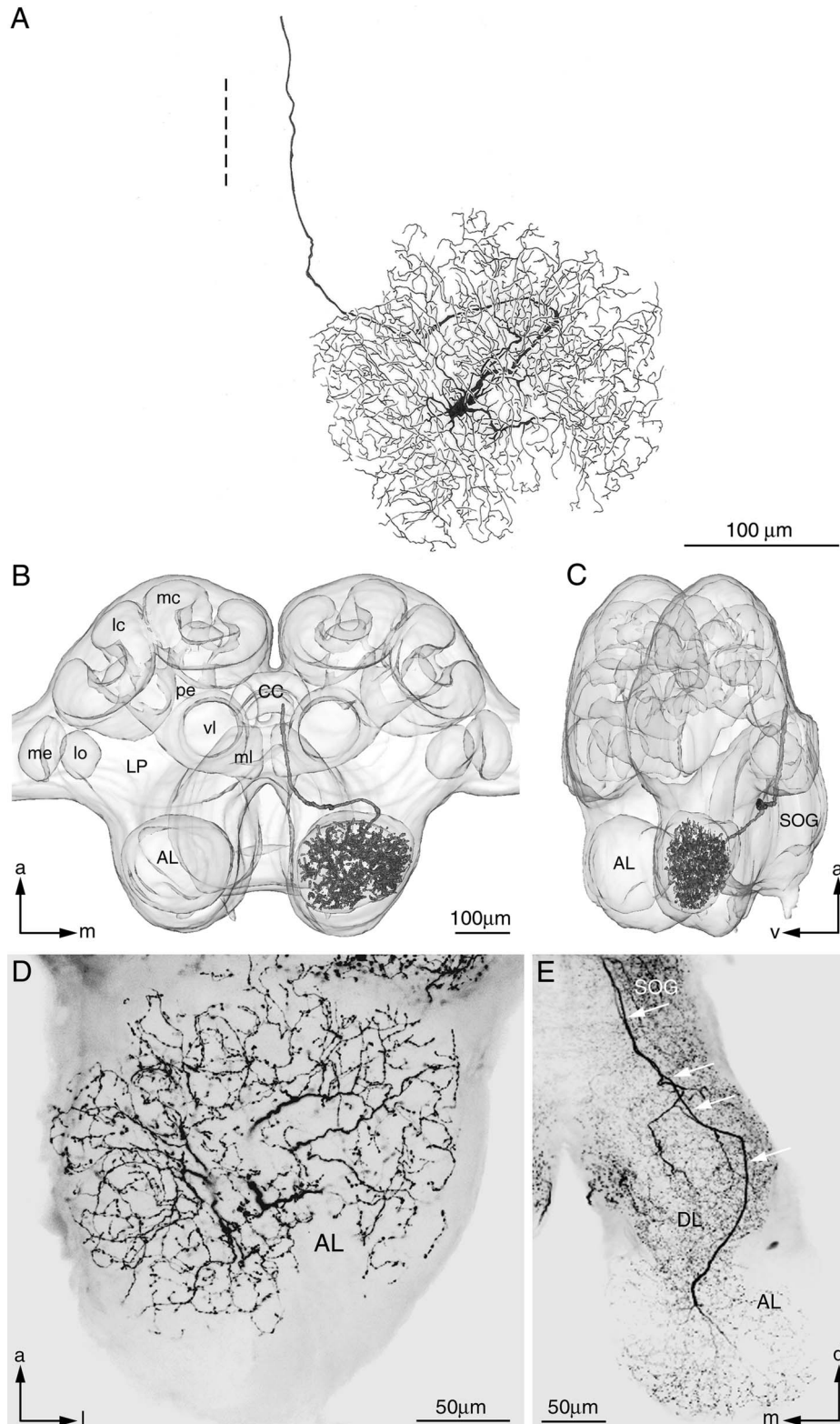
Immunoreactive elements are distributed throughout the AL (Fig. 1E), but the central region of the AL lacks immunoreactivity (Fig. 2E). Immunoreactive terminal branches seem to innervate most of the glomeruli in the ventral region of the AL (Fig. 1B), but each glomerulus has sparse arborization (Figs. 1E, 2E). Few immunoreactive elements are present in glomeruli in the dorso-posterior region of the AL ((dp); Fig. 1B).

In the DL, immunoreactive thin fibers are evenly distributed. Two immunoreactive thick elements from the SOG extend toward the DL. One of them passes through without branching in the DL and terminates in the AL, and the other branches out in the DL and extends into the PC without branching into the AL (Fig. 3E).

Immunoreactivity is distributed in the entire SOG. The SOG contains three commissures, one each for the mandibular, maxillary, and labial neuromeres (Fig. 2F). Several immunoreactive thick elements, the somata of which are located in the respective commissures at the rind of the



**Fig. 2.** Serotonin immunocytochemistry of the main neuropils in the brain of the worker ant. **(A)** Ventral view of the vertical lobe (vl) of the MB in the left hemisphere of the brain. Differing densities of immunoreactivity reveal strata in the vertical lobe (white arrows). The neuronal axis directions (black arrows) are the same in (A–C) and (E) (p, posterior). **(B)** Ventral view of the medial lobe (ml) of the MB in the left hemisphere of the brain. Immunoreactivity in the medial lobe is almost absent, but very weak immunoreactivity is observed in the antero-lateral and postero-medial parts (arrow) of the medial lobe. **(C)** Densely distributed immunoreactive elements are observed in the lamina (la) and medulla (me), but only sparse distribution of immunoreactivity is observed in the lobula (lo). The arrow shows two fibers innervating the lobula probably from somata in group 2. **(D)** The central complex is composed of the upper division (ud) and the lower division (ld). Relatively intense immunoreactivity is found in the upper division, and the lower division reveals a segmental structure. Anterior is at the top. The dotted line indicates the midline of the brain. **(E)** Ventral view of the AL. Immunoreactive varicose terminals are distributed in most glomeruli located in the periphery of the AL. In the central part of the AL, the sensory neurons or deutocerebral interneurons form tracts and show no immunoreactivity. **(F)** Frontal view of the SOG. Anterior is at the top. The dotted line indicates the midline of the brain. The different commissures indicated correspond to the mandibles (md), maxillae (mx), and labium (lb), and their cell bodies are located at the rim of the ganglion.



**Fig. 3.** Serotonin-immunoreactive giant neuron innervating the AL (GAL). **(A)** Ventral view of the neuron traced from 200 consecutive optical sections at intervals of 5  $\mu\text{m}$ . Intense arborizations are observed exclusively in the AL; no soma is located in either the brain or the SOG. The dotted line indicates the midline of the brain. Anterior is at the top. **(B, C)** Ventral **(B)** and lateral **(C)** views of the neuron within the three-dimensionally reconstructed brain/SOG complex. **(D)** Ventral view of the GAL within the AL, reconstructed from stacked LSM images of 14  $\mu\text{m}$  in thickness. The branches terminate with varicosities in most glomeruli in the ventral region of the AL. **(E)** Horizontal view of the neuron, reconstructed from stacked LSM images of 36  $\mu\text{m}$  in thickness. The main process (arrows) runs from the posterior SOG to the DL, sending off processes into the AL. This neuron does not branch in the DL or SOG; staining in these areas originates from other neurons. d, dorsal; l, lateral.



SOG, bilaterally extend across the midline of the SOG (Fig. 2F).

### Profiles of the serotonin-immunoreactive neurons

Three pairs of serotonin-immunoreactive neurons were traced and morphologically identified in the ALs, LPs and MBs. These neurons could be easily traced because of the remarkable thickness of neurites and because they exist as single neurons in a hemisphere.

*Giant neuron innervating the AL (GAL).* Fig. 3 shows a ventral side view of the serotonin-immunoreactive GAL traced on LSM images (Fig. 3A), including its three-dimensionally reconstructed neuronal branches in the brain neuropils (Fig. 3B, C). Since neither the soma nor the dendritic arborizations of this neuron are located in either the brain or the SOG, the neuronal profile of this neuron has not yet been identified in its entirety (Fig. 3). Presumed axon terminals, which are characterized by blebby varicosities (Fig. 3D), are evenly distributed in most glomeruli in the ventral region of the AL ((ve); Fig. 1B). The arborizations within each glomerulus are not closely packed but show sparse branching with varicosities. The GAL is the only neuron showing immunoreactive terminal arborization in most glomeruli in the ventral region of the AL. The central part of this glomerular array is an area consisting mainly of fiber tracts but lacking any glomerular structure, and it shows no immunoreactivity (Fig. 2E). The neuron has a neurite with a thick trunk passing through the DL and the ipsilateral hemisphere of the SOG and has no branches in the DL or SOG (arrows; Fig. 3E). From the SOG, it extends into the thoracic ganglion via the ipsilateral connective.

*Deutocerebral projection neuron (DPN).* Fig. 4 shows a ventral side view of a complete profile of the serotonin-immunoreactive DPN (Fig. 4A) and the locations of neuronal branches in the brain neuropils reconstructed three-dimensionally (Fig. 4B, C). The soma of this neuron belongs to group 6 (Fig. 1C, D) and is about 15  $\mu\text{m}$  in diameter (Fig. 4D). The primary neurite from the soma runs into the AL (Fig. 4E), and neuronal branches innervate several glomeruli (asterisks; Fig. 4F) that are restricted to the dorso-central region ((dc); Fig. 1B) of the ipsilateral AL close to the DL. From the AL, the axon of the neuron extends towards the ipsilateral PC, continues along the medial side of the PC, and then divides into two branches in the anterior medial region between the medial calyx and the vertical lobe of the MB (Fig. 4B, C). One of the branches extends ipsilaterally into the LP and has terminal arborizations with large dense varicosities in the laterally antero-dorsal region of the LP, which is medially close to the lobula of the OL (Fig. 4G). The other branch extends into the contralateral PC, crossing the midline of the brain in a region antero-dorsal to the CC. This branch has a terminal arborization with large, dense varicosities in the antero-medial region of the PC, which is enclosed by the medial calyx, the vertical lobe of the MB, and the CC (Fig. 4H). The neuron's contralateral branch in the anterior PC terminates in a very restricted ellipsoidal area close to the midline of the brain (Fig. 4H).

*Calycal input neuron (CIN).* Fig. 5 shows a ventral side view of a complete profile of the serotonin-immunoreactive CIN (Fig. 5A) and the locations of neuronal branches in the brain neuropil reconstructed three-dimensionally (Fig. 5B,

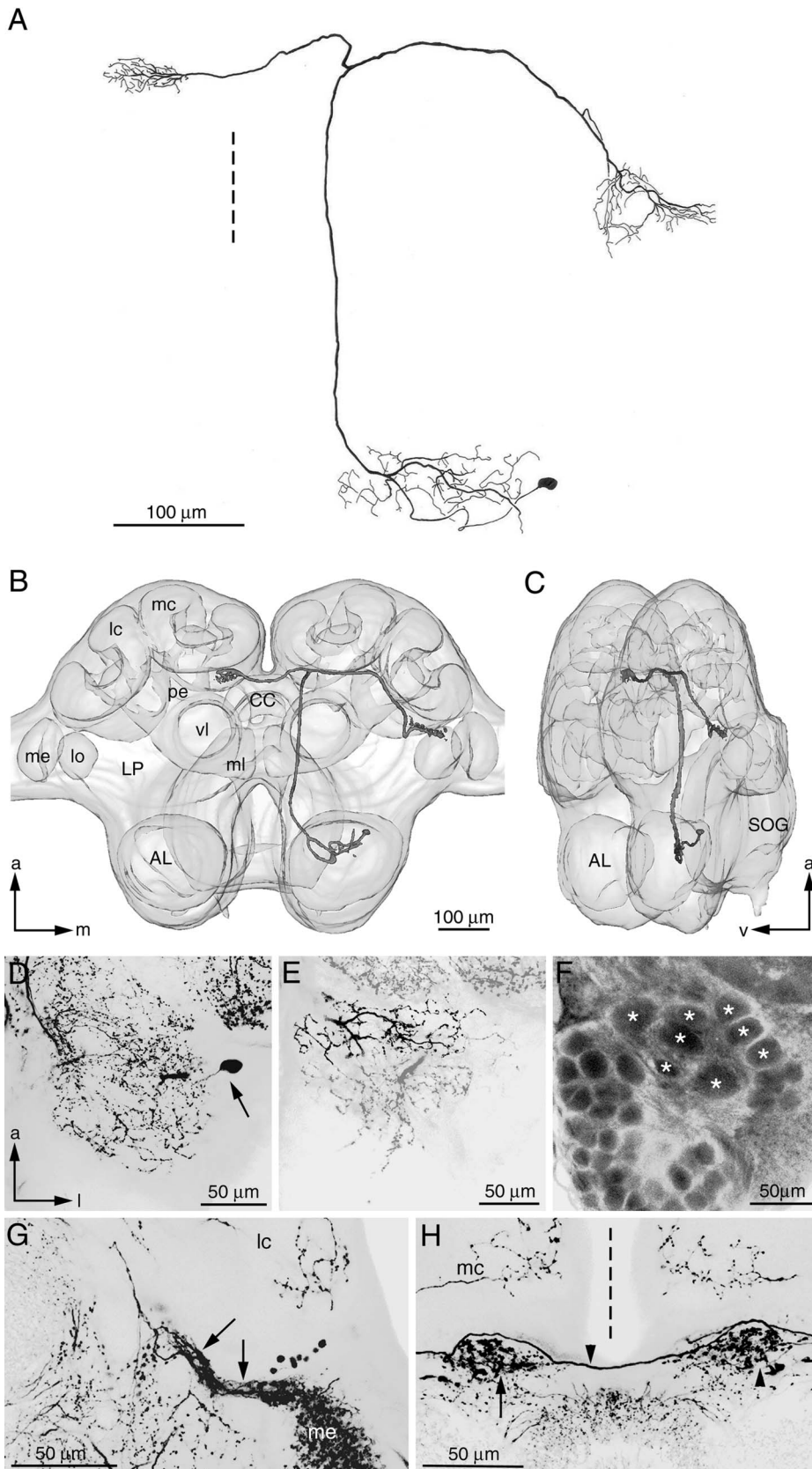
C). The soma of this neuron belongs to group 3 (Fig. 1C, D) and is about 15  $\mu\text{m}$  in diameter (Fig. 5D). This is the only neuron that has terminal branches in the calyces of the MB in a hemisphere (Fig. 5E). The primary neurite of the neuron runs in a posterior direction and then divides into two branches in the dorsal region of the ipsilateral MB peduncle (Fig. 5). These two branches extend bilaterally and terminate in the LPs of both hemispheres. One branch has sparse arborizations and terminates in the antero-ventral region of the LP in the ipsilateral hemisphere (Fig. 5A–C). The other branch passes through the dorso-medial region of the CC, extending to the contralateral PC, and there divides into two further branches dorsal to the peduncle of the MB (Fig. 5). One branch terminates with sparse arborizations in the postero-ventral region of the LP in the contralateral PC (Fig. 5A–C), and the other branch extends from the dorsal side of the MB peduncle to the calyces and branches into the medial and the lateral calyces (Fig. 5A–C). These branches penetrate into the basal rings of both calyces with small co-laterals (arrowheads; Fig. 5E) and terminate with varicose arborizations in the outer region of the lip (arrows; Fig. 5E) of both calyces, but no branch invades into the collar regions (single asterisk; Fig. 5E). The ipsilateral terminal region is antero-medially located in the LP, and the contralateral terminal region is postero-laterally located in the LP; however, both terminal regions are at almost the same level on the ventral-dorsal axis (Fig. 5A–C).

### Tentative subdivisions of the AL and LP

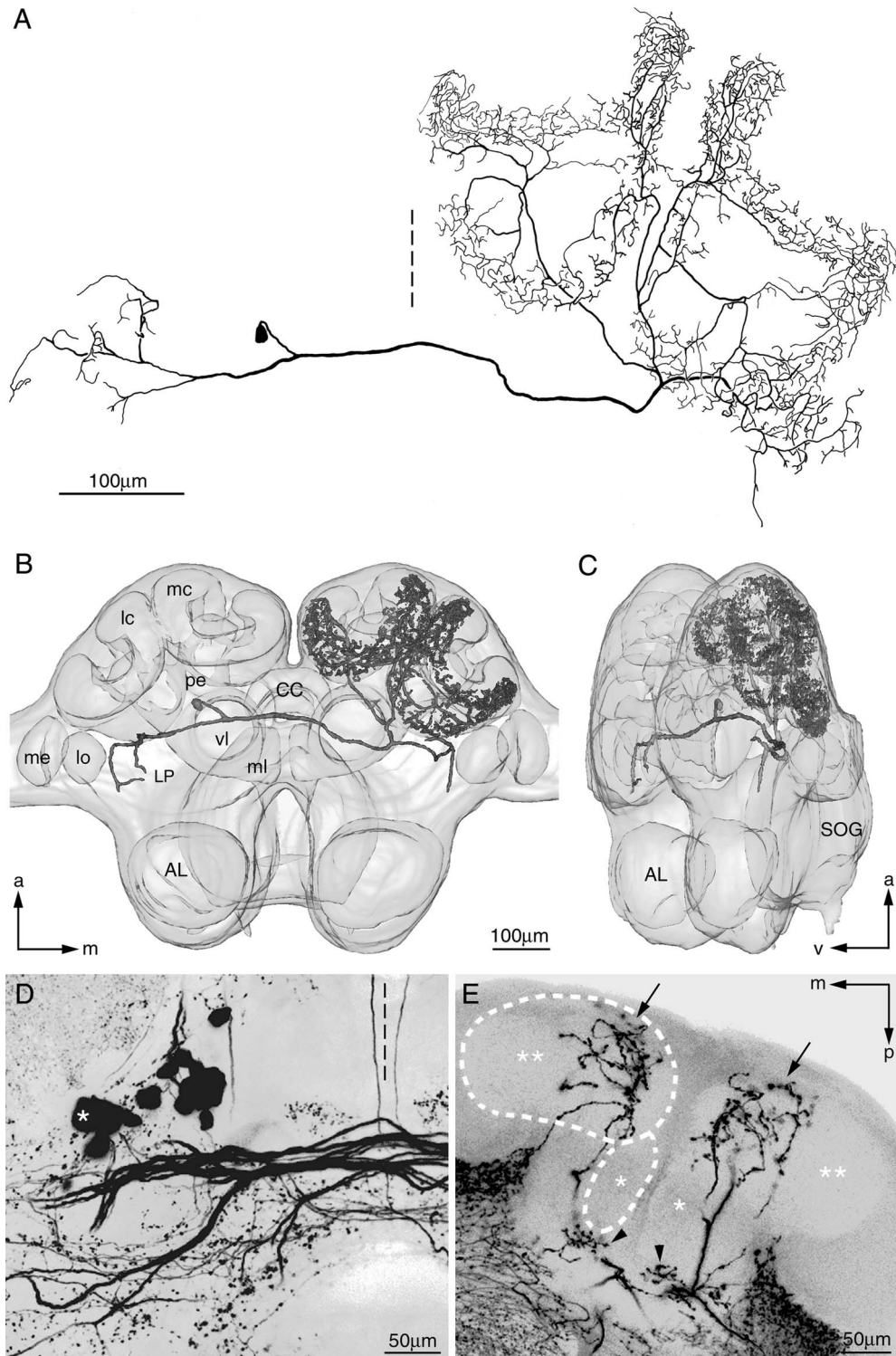
There are over 400 glomeruli in the AL of the worker ant (Misaka *et al.*, 2006). Most of the 300 glomeruli in the ventral region ((ve) in Fig. 1B) of the AL had varicose terminals of the GAL and were colored blue in three-dimensionally reconstructed AL glomeruli (Fig. 6A, B). At most, 12 glomeruli in the dorso-central region ((dc) in Fig. 1B) of the AL next to the DL had sparse branches of the DPN and were colored red in three-dimensionally reconstructed AL glomeruli (Fig. 6B). The glomeruli in these two clusters seemed to be different in shape and size. About 300 glomeruli of the ventral cluster were relatively small and spherical-shaped, and they occupied the major part of the AL. At most, 12 glomeruli of the dorso-central cluster were relatively large and ellipsoidal or deformed, and they occupied a small part of the AL next to the DL. Over 100 glomeruli of the postero-medial region ((pm) in Figs. 1B) in the AL were almost free from serotonin immunoreactivity (Fig. 4E) and were colored yellow in three-dimensionally reconstructed AL glomeruli (Fig. 6A, B). Most of the glomeruli in the AL were tentatively divided into these three clusters according to whether they had serotonin-immunoreactive neuronal elements of the GAL or DPN, or no serotonin-immunoreactive neuronal elements.

Dendritic and axonal arborizations of the DPN and CIN were mapped together in the LP of left hemisphere reconstructed three-dimensionally (Fig. 6C). The terminal branches of DPN are indicated by red in the laterally antero-dorsal region of the LP, the spiny branches of CIN are indicated by green in the medially antero-ventral region, and the blebby terminals of CIN are indicated by blue in the laterally postero-ventral region. No overlapping area was found among these arborizations in the LP (Fig. 6C).

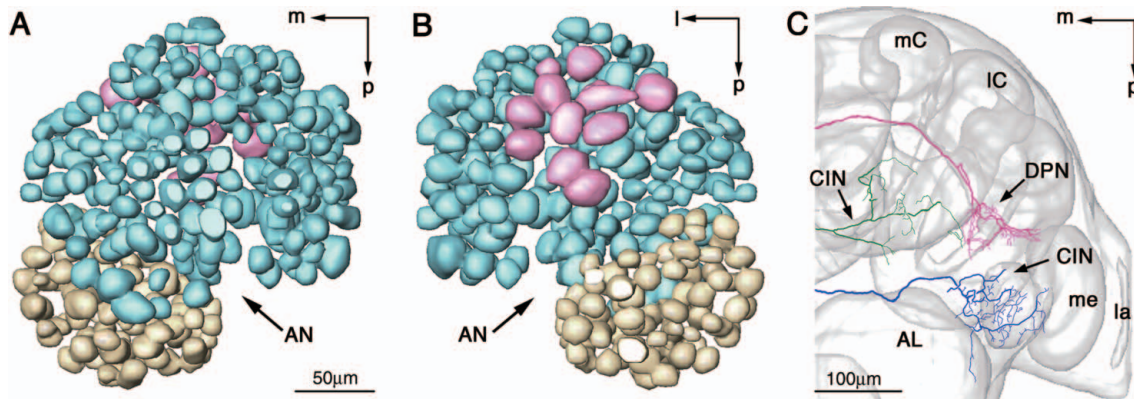




**Fig. 4.** Serotonin-immunoreactive deutocerebral projection neuron (DPN). **(A)** Ventral view of the neuron traced from 200 consecutive optical sections at intervals of 5  $\mu\text{m}$ . The dotted line indicates the midline of the brain. Anterior is at the top. **(B, C)** Ventral **(B)** and lateral **(C)** views of the neuron within the three-dimensionally reconstructed brain/SOG complex. **(D)** Ventral view of the DPN in the AL, reconstructed from stacked LSM images of about 10  $\mu\text{m}$  in thickness. The soma of the DPN (arrow) is the only 5-HT-immunoreactive one in the lateral cell body group of the AL, and it extends a neurite into the AL. Terminal branches of the GAL are also seen in the AL of this figure. **(E)** Ventral view of the neuron, reconstructed from stacked LSM images of 10  $\mu\text{m}$  in thickness. The branches of the DPN in several glomeruli in the dorso-central region of the AL close to the DL are indicated by black. The branches of the other neurons are indicated by grey. **(F)** LSM image of AL glomeruli in almost the same area as that in **(E)**. The asterisks indicate the corresponding glomeruli having branches of the DPN in the dorso-central region of the AL. **(G)** Ventral view of the neuron, reconstructed from stacked LSM images of 8  $\mu\text{m}$  in thickness. The neuron bifurcates at the dorso-medial region of the ipsilateral PC; one branch terminates at the antero-dorsal region in the LP of the ipsilateral PC. Many large varicosities are observed at the terminals (arrows). **(H)** Ventral view of the neuron, reconstructed from stacked LSM images of 8  $\mu\text{m}$  in thickness, show the second branch terminating at the dorso-medial region of the contralateral PC. The terminal arborization has many large varicosities (arrow). Arrowheads indicate the axon and terminal arborizations of the other DPN in the contralateral hemisphere. Anterior is at the top. The dotted line indicates the midline of the brain.



**Fig. 5.** Serotonin-immunoreactive calycal input neuron (CIN). **(A)** Ventral view of the neuron traced from 200 consecutive optical sections at intervals of 5  $\mu\text{m}$ . The neuron sends branches into the ventral region of the LP in both hemispheres of the PC. The dotted line indicates the midline of the brain. Anterior is at the top. **(B, C)** Ventral **(B)** and lateral **(C)** views of the neuron within the three-dimensionally reconstructed brain/SOG complex. **(D)** Ventral view of the CIN, reconstructed from stacked LSM images of about 50  $\mu\text{m}$  in thickness. The soma (asterisk) is located in immunoreactive soma group 3 (Fig. 1C, D) at the dorso-medial region of the PC. Among group 3 somata in a hemisphere, this is the only soma with terminals in the calyces of the MB. Anterior is at the top. The dotted line indicates the midline of the brain. **(E)** Ventral view of the neuron, reconstructed from stacked LSM images of 20  $\mu\text{m}$  in thickness. Arrows show the terminal branches with varicosities restricted to the outer layer of the lips (double asterisks), and arrowheads show the axon collaterals in the basal rings in the medial and lateral calyces of the MB in the contralateral PC. There is no immunoreactive element in the inner layers of the lips and collar (single asterisk) in the medial and lateral calyces.



**Fig. 6.** Ventral (A) and dorsal side (B) views of the left AL glomeruli reconstructed three-dimensionally. Most of the glomeruli in the ventral region (blue-colored) had branches of the GAL, and several glomeruli in the dorso-central region (red-colored) had branches of the DPN. Most of the glomeruli in the postero-medial region (yellow-colored) had little serotonin immunoreactivity. The arrow indicates the direction of antennal nerve input. (C) Ventral side view of the left LP reconstructed three-dimensionally. The branches of the DPN and CIN are tentatively mapped together in the LP. The probable axonal arborization of the DPN (red) located in the laterally antero-dorsal region, the probable axonal arborization of CIN (blue) located in the laterally postero-ventral region and the probable dendritic arborization of CIN (green) located in the medially antero-ventral region of the LP are shown.

## DISCUSSION

### Serotonin-immunoreactive cell body groups

About 130 serotonin-immunoreactive somata were divided into six paired groups in the brain and one group in the SOG of the ant (Fig. 1C, D). About 75 or more serotonin-immunoreactive somata have been reported in the honeybee brain (Schürmann and Klemm, 1984; Rehder *et al.*, 1987; Bicker, 1999). The distribution patterns of serotonin-immunoreactive somata in the ant seem to be similar to those in the honeybee. However, the somata of Group 5 and six somata in the medial region of the SOG observed in the ant have not been reported in the honeybee (Schürmann and Klemm, 1984; Rehder *et al.*, 1987; Bicker, 1999).

### Serotonin-immunoreactive neurons in the mushroom body

General aspects of serotonin immunoreactivity in the brain of forager ants seem to be similar to those already described for the honeybee (Schürmann and Klemm, 1984; Rehder *et al.*, 1987; Bicker, 1999). However, prominent differences between the ant and the honeybee can be seen in the profiles of arborization in the calyces of the MBs (Figs. 1E, 5A, E). In the honeybee brain, the calyces are devoid of serotonin immunoreactivity (Schürmann and Klemm, 1984; Rehder *et al.*, 1987; Bicker, 1999). In the ant brain, there are immunoreactive branches with varicosities at the lip and the basal ring in the lateral and medial calyces (Fig. 5E). We have also examined serotonin immunoreactivity in the brains of honeybee workers using the same technique as that used in the ant brain: no immunoreactivity was detected in the calyces of the MB in the honeybee (unpublished data).

Is the fact, then, that the elements are present only in the lip and the basal ring of the ant significant? Nestmate recognition is of crucial importance for social insects (Vander Meer *et al.*, 1998; Hara, 2003). Particularly in ants, the nestmate cuticular-hydrocarbon (CHC) profile changes with time (Vander Meer *et al.*, 1989; Lahav *et al.*, 2001). Old nestmate CHC profiles induce aggressive behavior in 50%

of nestmates in *C. japonicus* (Wada *et al.*, 2003). The antennal sesillum responding to CHC has been identified and revealed to contain about 200 sensory cells (Ozaki *et al.*, 2005). Ants seem to use a highly complicated processing system for chemical communications, in which MBs including CIN elements might be involved.

In the MB calyces, axon terminals of the CIN are restricted to the outer half of the lip region (Fig. 5E). Some types of Kenyon cells have dendrites in this restricted lip region of the ant MB (Gronenberg, 2001). In *Drosophila* and *Bombyx*, projection neurons from specific AL glomeruli supply distinct zones in both the lateral horn and MB calyces; hence, the two secondary olfactory centers confer comparable odor maps (Tanaka *et al.*, 2004; Seki *et al.*, 2005). In ants, axons from alarm pheromone-sensitive projection neurons terminate in the lip regions of the MB and the lateral horn (Yamagata *et al.*, 2006). Although comprehensive odor maps in the MB calyces have not been revealed in ants, the outer half of the lip region may have connections to particular glomerular subsets.

Calyxal neuropils in insect MBs consist of intrinsic and extrinsic neurons that connect to each other and make small glomerular structures called microglomeruli (Mobbs, 1982). In calyxal glomeruli of *Drosophila*, synaptic connections were revealed for three major neuronal elements: (i) local intrinsic neurons, Kenyon cells, (ii) extrinsic presumed cholinergic neurons, and (iii) presumed GABAergic extrinsic neurons (Yasuyama *et al.*, 2002). However, no serotonin-immunoreactive neuronal elements have been identified in the calyxal glomeruli of *Drosophila*. In calyxal neuropils of *Periplaneta*, serotonin-immunoreactive neuronal elements were observed (Salecker and Distler, 1990), and also GABA-like immunoreactive calyxal giant neurons are thought to have an inhibitory role in microglomeruli of MB calyces (Schürmann, 1973; Nishikawa *et al.*, 1998; Nishino and Mizunami, 1998; Yamazaki *et al.*, 1998; Strausfeld and Li, 1999). Small glomerular structures are also seen in calyxal neuropils of ant MBs (Goll, 1967), but it is not yet confirmed whether the calyxal glomeruli in ants contain

GABA-like or serotonin-immunoreactive neuronal elements. Considering the terminal profile of the CIN in the calyces, serotonin as a neuromodulator might affect the calycal glomeruli.

Probable dendritic elements lacking varicosities are located in the medially antero-ventral region of the LP in the ipsilateral hemisphere, and probable axon co-laterals with varicose arborizations are located in the laterally postero-ventral region of the LP in the contralateral hemisphere (Fig. 5A–C). These branching patterns in the LPs in the two hemispheres are different, but both arborization areas are located in a relatively ventral region of the LP. Alarm pheromone-sensitive PNs with axon terminals in the lateral horn, which is the terminal area of olfactory PNs in the LP, have been reported in the ant *Camponotus obscuripes* (Yamagata *et al.*, 2006). The axon-terminal area of the CIN in the LP is very similar to the lateral horn of *C. obscuripes*. In *Drosophila*, the major targets of the olfactory or mechanosensory projection neurons are different in both the LP and the MB, and also axon terminals of projection neurons from specific AL glomeruli supply distinct zones in both the LP and the MB (Tanaka *et al.*, 2004). Based on the results obtained in *Drosophila* (Tanaka *et al.*, 2004) or *C. obscuripes* (Yamagata *et al.*, 2006), bilateral arborization areas of the CIN in the LPs may have connections to different kinds of chemosensory information from specific AL glomeruli rather than antennal mechanosensory information.

### Serotonin-immunoreactive neurons in the antennal lobe

The AL of the worker ant contains only one serotonin-immunoreactive soma in the lateral cell body group (Group 6; Fig. 1C, D) and two types of immunoreactive neuronal branches that belong to the GAL and DPN. Since no soma of the GAL was detected in either the brain or in the SOG, the complete neuronal profile of this neuron has not yet been identified. A serotonin-immunoreactive neuron was identified in the AL of the honeybee brain: a soma located in the lateral cell body group of the AL sends blebbed fibers into the glomeruli of the AL, interconnects the AL and the DL with the SOG, and from there descends into the ventral nerve cord (Rehder *et al.*, 1987). In the ant brain, the arborizations with varicosities detected in most of the 300 glomeruli in the ventral region of the AL are very likely to be axon terminals of the GAL (Fig. 3D). The neurite with branches in the AL extends into the thoracic ganglion, thereby passing through the SOG via the connective. Its soma and dendritic elements are presumably located in the thoracic or abdominal ganglion. Although the neurons of the two insects have different soma locations, serotonin is probably released from blebbed axon terminals in AL glomeruli of both insects. In the ant, the glomeruli in the postero-medial region of the AL are almost free from immunoreactive elements, whereas such glomeruli have not been reported in the AL of the honeybee. It was reported for *Manduca sexta* that serotonin-immunoreactive neurons in the AL, which have varicosities in all glomeruli, might not receive direct input from sensory neurons and might modulate the activity of neurons in the AL rather than the activity of sensory neurons (Sun *et al.*, 1993). Since the axon terminals of the GAL are sparsely distributed in each glomerulus and have relatively large varicosities, this neuron might not

have direct connections with the enormous number of antennal sensory neurons in each glomerulus. We speculate that serotonin released by the GAL might act as a neuromodulator diffusing throughout an entire glomerulus but not act simultaneously over relatively long diffusion distances. Axonal elements of the GAL terminate in the major part of glomeruli in the ventral region, while few immunoreactive elements are present in the postero-medial region of the AL (Figs. 1B, 6). The serotonin released by the GAL may have an effect simultaneously on each glomerulus in the ventral region of the AL but not on the glomeruli in the postero-medial region. Most of the glomeruli with GAL elements, which are located in the ventral region of the AL, are likely to be ordinary glomeruli that receive antennal olfactory signals in other insect species (Boeckh and Tolbert, 1993; Hansson, 1999). Although we cannot be certain about the afferent input to the GAL, this neuron might regulate the responsiveness of some neuronal elements in the glomeruli and/or the activity level in the processing of antennal olfactory signals through input from posterior ganglia.

The DPN connects the AL with the PC bilaterally and has sparse branches innervating at most 12 glomeruli in the dorso-central region of the AL close to the DL (Fig. 4E). Most of the AL glomeruli in insects receive antennal olfactory afferents (Boeckh and Tolbert, 1993; Hansson, 1999). In some insects, antennal sex-pheromone-sensitive neurons project into male-specific glomeruli, which are enlarged and called the macroglomerular complex or macroglomerulus (Boeckh and Tolbert, 1993; Hansson, 1999). In *Periplaneta*, antennal hygro- and thermosensory axons also project into several glomeruli, which are relatively large, ellipsoidal or crescent shaped, and restricted to the dorso-central region of the AL close to the DL (Nishikawa *et al.*, 1995; Nishino *et al.*, 2003). Five alarm pheromone-sensitive glomeruli have been identified in the dorsalmost part of the ventral cluster of the AL in the ant, *C. obscuripes* (Yamagata *et al.*, 2006). Those glomeruli seem to be located very close to the glomeruli of the DPN. Most of the ordinary glomeruli in the ant AL probably receive antennal olfactory inputs. Only one serotonin-immunoreactive DPN in the AL innervates a small number of particular glomeruli, which are ellipsoidal or crescent shaped and larger than ordinary spherical glomeruli: the diameter of the former is about 30  $\mu\text{m}$  and that of the latter about 15  $\mu\text{m}$  (Figs. 4F, 6A, B). The ipsilateral terminal area of the DPN was the laterally antero-dorsal region in the LP (Fig. 4G). These morphological properties of the DPN, the location of the AL glomeruli and axon terminals in the LP, suggest a connection with the processing network for peculiar modalities rather than ordinary olfactory signals in the ant brain. The other branches of the DPN in the contralateral PC terminate in a restricted, small region, which has not yet been functionally identified in any insect brain (Fig. 4H).

### Tentative subdivisions of the AL and LP

The AL glomeruli and LP neuropils were tentatively subdivided in this study based on the branching area of the serotonin-immunoreactive neurons (Fig. 6).

The GAL had probable axon terminals in most glomeruli of the ventral region of the AL (Figs. 1B, 3). These glomeruli occupy the major part of the AL (Figs. 1B, 6A) and probably

receive axon terminals of antennal olfactory neurons, like ordinary glomeruli in other insect species (Boeckh and Tolbert, 1993; Hansson, 1999). The DPN, on the other hand, extended sparse branches in a small number of glomeruli of the dorso-central region of the AL next to the DL and projected into very restricted areas of LPs (Figs. 1B, 4). Based on the loci, shape and size of these glomeruli, some of them might receive peculiar modalities of antennal sensory signals other than ordinary olfactory signals. Most of the glomeruli in the postero-medial region of the AL in the ant were almost free from immunoreactive elements and seemed to be located outside the arrays of other glomerular regions (Figs. 1B, 6A, B). Although the sensory modality has not yet been identified, these glomeruli might have a special function different from those of glomeruli of the ventral or dorso-central regions. Thus, the AL glomeruli of the ant might be subdivided into three functionally different regions.

The DPN had a probable axonal arborization in the laterally antero-dorsal region of the LP (Fig. 4). The probable dendritic and axonal arborizations of the CIN were located medially in the antero-ventral region and laterally in the postero-ventral region of the LP, respectively (Fig. 5). These arborizations were tentatively mapped together on the three-dimensional brain hemisphere and showed no overlapping area in the LP (Fig. 6C). Subdivisions of the lateral horn or LP were revealed by intensive analysis based on sensory modalities of projection neurons connecting between subsets of glomeruli in the AL and corresponding terminal regions of the lateral horn or LP in *Drosophila* and *Bombyx* (Tanaka *et al.*, 2004; Seki *et al.*, 2005). Alarm pheromone-sensitive uniglomerular projection neurons have been identified in the ant, the dendritic arbors of which were observed in several glomeruli in the dorsalmost part of the ventral cluster of the AL and the axon terminals of which were observed in the lip regions of calyces of the MB and the lateral horn of the LP (Yamagata *et al.*, 2006). The probable axonal arbors of the CIN are likely located in the lateral horn of the LP. In the cockroach, axon terminals of hygro- or thermosensitive PNs are located in restricted regions of the lateral horn and are different from those of ordinary uniglomerular projection neurons (Nishino *et al.*, 2003). Terminal areas of the DPN and CIN in the LP suggest functionally different subdivisions connecting to different glomerular subsets for antennal olfactory or other sensory modalities.

### Presumptive roles of serotonin-immunoreactive neurons in the ant brain

Biogenic amines play important roles as neurotransmitters, neurohormones, and neuromodulators in insect brains. Particularly in social insects, workers have to perform complex tasks and adapt their behavior according to the conditions of their colony. The role of several biogenic amines in the modulation of behavior has already been investigated in a wide range of species, including social insects. In the honeybee, direct injection of 5-HT into the brain reduced responsiveness to a conditioned stimulus (Mercer and Menzel, 1982) and significantly reduced the proboscis extension response to a stimulus applied to the antennae (Blenau and Erber, 1998). Mapping of serotonin immunoreactivities in the AL and SOG of the honeybee suggested that a serotonergic pathway between these two neuropils might influence the

response properties of their neurons (Rehder *et al.*, 1987). However, 5-HT injection had no effect on the initiation and maintenance of the bees' foraging behavior (Schulz *et al.*, 2003). Based on these results, it is speculated that 5-HT has the function of regulating activity in sensory-processing systems rather than changing behaviors in social insects.

Electrophysiological analyses of the effects of biogenic amines on peripheral and/or central brain neurons have been investigated (Kloppenburg and Hildebrand, 1995; Kloppenburg *et al.*, 1999; Grosmaître *et al.*, 2001; Pophof, 2002; Molaei and Lange, 2003). In adult *Manduca sexta*, 5-HT modulates the responses of local interneurons and projection neurons: it not only reduces excitatory responses at low concentrations but also enhances the responses at high concentrations by increasing the cell input resistance and thereby leading to a broadening of action potentials and increased cell excitability in many AL neurons (Kloppenburg and Hildebrand, 1995). Concentration-dependent effects of the amine were also reported in optical responses in AL glomeruli and in behavioral responses to a pheromone in *Bombyx* (Hill *et al.*, 2003; Gatellier *et al.*, 2004). Each neuronal profile identified in this study suggests the function of regulating activity in processing for respective antennal sensory signals connecting to a particular area of the AL, MB and LP.

The distribution of serotonin immunoreactivity in insect brains is quite broad; however, individual neurons still show very specific features within the prominent neuropils. Some neuropils of the insect brain, such as the OL (Nässel, 1988), CC (Homberg, 1991), AL (Rehder *et al.*, 1987) and MBs (Homberg *et al.*, 1989), show dense immunoreactivity, indicating an important role of 5-HT in these neuropils. The functional properties of biogenic amines in insect brains still need to be investigated further: individual neurons containing particular biogenic amines should be examined in detail physiologically and morphologically, and their target neurons should be identified.

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