

Neural Pathways for the Processing of Alarm Pheromone in the Ant Brain

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ABSTRACT

Social insects like ants exhibit sophisticated communication by means of pheromones, one example of which is the use of alarm pheromones to alert nestmates for colony defense. In the ant *Camponotus obscuripes*, we have reported that information about formic acid and *n*-undecane, alarm pheromone components, is processed in a set of specific glomeruli in the antennal lobe (primary olfactory center). Alarm pheromone signals are then transmitted, mainly via uniglomerular projection neurons (uni-PNs), to the protocerebrum (PR), where sensory signals are integrated to form motor commands for behavioral responses. In this study, we physiologically and morphologically characterized 63 alarm pheromone-sensitive PR neurons in ants by using intracellular recording and staining techniques. Most of the pheromone-sensitive PR neurons had dendrites in the mushroom body (MB), the lateral horn, or the medial PR. Some neurons with dendrites in these areas responded specifically to formic acid or *n*-undecane and may participate in the control of specific behavioral responses to each pheromone component. Other neurons responded also to non-pheromonal odors, in contrast to uni-PNs, most of which responded specifically to alarm pheromones. Responses to non-pheromonal odors were most prominent in efferent neurons of the MB lobe, suggesting that they may participate in integration of pheromonal and non-pheromonal information. We found a class of PR neurons that receives input in all of these pheromone-processing areas and terminates in a variety of premotor areas. These neurons may participate in the control of pheromone-sensitized aggressive behavior, which is triggered by non-pheromonal sensory stimuli associated with a potential enemy. *J. Comp. Neurol.* 505:424–442, 2007.

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Indexing terms: protocerebrum; mushroom body; lateral horn; aggressive behavior; insect

Pheromones, chemicals that are released by an individual to the outside of its body and received by another individual of the same species to cause specific reactions, play key roles in maintaining social organization. Pheromones are utilized on various occasions, including territory-marking (Hölldobler and Wilson, 1977; Hurst and Beynon, 2004), mating (Karlson and Butenandt, 1959; Kimoto et al., 2005), and parental care (Godfray, 1991; Kölliker et al., 2006). Most sophisticated pheromone communication systems are found in social insects such as ants, termites, and social bees (Vander Meer and Alanso, 1998). Unusual sophistication of pheromone communication in social insects is evidenced by the fact that they possess a diverse array of exocrine glands devoted to social communication, 39 such glands existing in formicine ants and 21 existing in honey bees (Billen, 1994).

Upon facing a potential danger such as a predator, ants secrete alarm pheromones to warn their conspecifics about danger. In the formicine ant *Camponotus obscuripes*,

workers utilize products of the poison gland and Dufour's gland as components of the alarm pheromone, among which formic acid and *n*-undecane play major roles in alarming nestmates (Fujiwara-Tsujii et al., 2006). Behavioral responses of ant workers to alarm pheromones in the context of colony defense are highly complicated and diverse, but, in general, they can be classified into three sequential patterns: 1) initial response to pheromones; 2) alarm behavior; and 3) aggressive behavior against a po-

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tential enemy, which is sensitized by alarm pheromones and is triggered by sensory stimuli associated with the potential enemy, as reviewed by Blum (1985) and Vander Meer and Alonso (1998).

In the initial response to alarm pheromones of *C. obscuripes*, workers that have sensed pheromones reflexively stop their locomotion, swing their antennae, and then come to an alerted posture (N. Yamagata, personal observation), as has been observed in other species of ants (Blum, 1985; Vander Meer and Alonso, 1998). Thereafter, they exhibit alarm behavior of different intensities, which usually includes locomotion of increased speed with alerted posture (Löfqvist, 1976; Blum, 1985; Hölldobler and Wilson, 1990; Vander Meer and Alonso, 1998). Workers of *C. obscuripes* have been shown to exhibit a specific behavioral reaction to each alarm pheromone component, i.e., avoidance of the source of formic acid and attraction toward the source of *n*-undecane (Fujiwara-Tsujii et al., 2006). Therefore, the ratio of alarm pheromone components determines the alarm behavior in this species. Finally, alarm pheromones sensitize aggressive behavior against a potential enemy, which is triggered by olfactory, visual, or tactile stimuli associated with the enemy (Blum, 1969, 1985; Vander Meer and Alonso, 1998). Aggressive behavior is also influenced by many other external and internal factors (Blum, 1985; Vander Meer and Alonso, 1998), including the state of path integrator that tells the ant how far it is away from the nest (Knaden and Wehner, 2004).

Current knowledge of the processing of alarm pheromone information in the brains of social insects is very sparse. In ants, pheromonal and non-pheromonal odors are received by specific clusters of olfactory receptor neurons on the antennae (Dumpert, 1972; Ozaki et al., 2005). The axons of olfactory receptor neurons project to the antennal lobe, the primary olfactory center. Like the vertebrate olfactory bulb, the antennal lobe consists of a number of glomeruli, which are the functional units of the antennal lobe and where axon terminals of each specific class of olfactory receptor neurons make synapses with interneurons, including projection neurons (PNs; Hildebrand and Shepherd, 1997). Notably, ants possess a large number of glomeruli: the number of glomeruli in each antennal lobe is 243 ± 19 in large workers of leaf-cutting ants, *Atta sexdens* (Kleineidam et al., 2005), and about 200 in wood ants, *Formica pratensis* (Goll, 1967), and in carpenter ants, *Camponorus ocreatus* (Gronenberg and Lopez-Riquelme, 2004). The large number of glomeruli in ants may reflect the richness of the kinds of pheromones.

In *C. obscuripes*, we were able to record from eight uniglomerular PNs (uni-PNs) that responded to *n*-undecane and/or formic acid and found that these neurons had dendrites in one of five glomeruli located at the dorsal-most part of the antennal lobe, which we referred to as "AS (alarm pheromone-sensitive)" glomeruli (Yamagata et al., 2006). Moreover, we were also able to record from 15 pheromone-sensitive multiglomerular PNs (multi-PNs) and found that these neurons had dendrites in some of the AS glomeruli, as well as in some other glomeruli (Yamagata et al., 2006). This morphology matched their responses: most of the pheromone-sensitive multi-PNs also responded to non-pheromonal odors, whereas most of the pheromone-sensitive uni-PNs did not respond to the non-pheromonal odors tested. Axons of PNs exit the antennal lobe and project to the protocerebrum

(PR), where sensory signals are integrated to form signals for the control of behavior (Strausfeld, 1976; Mizunami et al., 1998a; Okada et al., 1999). Pheromone-sensitive uni-PNs terminate in the lateral horn and the calyx of the mushroom body (MB), whereas pheromone-sensitive multi-PNs terminate in the lateral horn, ring neuropil around the vertical lobe of the MB, and dorsal PR (Yamagata et al., 2006).

In this study, we examined the morphology and physiology of alarm pheromone-sensitive neurons in the ant PR by using intracellular recording and staining techniques. On the basis of the results, we propose a general scheme of neural pathways for alarm pheromone processing and for the control of pheromone-induced alarm behavior and pheromone-sensitized aggressive behavior. A preliminary part of this study has been reported as a short communication (Yamagata et al., 2005).

MATERIALS AND METHODS

Animals and preparations

Workers of the ant *C. obscuripes* maintained in a laboratory under conditions of a constant light (12-hour) and dark (12-hour) cycle and at a temperature of 25°C were used. Each ant was anesthetized by carbon dioxide for 5–10 minutes and then fixed on a stage with wax, with the frontal surface of the head facing upward. The frontal surface of the brain was exposed by removing a piece of cuticle between the compound eyes. To stabilize the brain, the esophagus and some jaw muscles were removed, and a glass rod was inserted into the esophagus foramen. The brain was immersed in saline formulated for related species, *Camponotus floridanus* (Gronenberg et al., 1996).

Intracellular recording and staining

Borosilicate glass capillaries were pulled on a laser puller (P-2000, Sutter Instrument, Novato, CA) and were filled with 8% Lucifer Yellow (Sigma, St. Louis, MO) dissolved in 1 M LiCl₂ at the tips, with DC resistances of 60–90 MΩ (after Nishino et al., 2003). An electrode was manipulated into various protocerebral areas of the right hemisphere. Electrical signals were amplified with an amplifier (MEZ-8100, Nihon Kohden, Tokyo, Japan) and displayed on an oscilloscope and a digital recorder (Omniace, NEC, Tokyo, Japan). Data were stored on a DAT recorder (RD120-T, TEAC, Tokyo, Japan).

The recorded neuron was filled with Lucifer Yellow by applying a hyperpolarizing current (−0.3 to −2.0 nA for 4–10 minutes). After dye injection, the ant was incubated at 4°C for 3 hours and fixed in 4% formaldehyde in Millonig's buffer overnight at 4°C. Then the brain, together with the subesophageal ganglion, was dissected out, rinsed in saline, dehydrated in ethanol, and cleared in methyl salicylate. Unless otherwise stated, the brain was observed from the ventral (frontal) surface with the use of a confocal laser-scanning microscope (LSM510, Zeiss). The thickness of the optical sections was 4 μm.

Dendrites and axon terminals of neurons could be distinguished as follows. When the microelectrode penetrated a dendrite, responses with membrane fluctuations (synaptic potentials) and superimposed low-amplitude spikes (dendritic spikes) were visible. In contrast, when recordings were made at axons or axon terminals, spikes with prominent after-hyperpolarizations (axonal spikes)

were observed. With high-magnification confocal scanning microscopic observation, dendrites exhibited a number of spines or thorns, whereas axon terminals had numerous varicosities or blebs, as reported previously (Strausfeld and Campos-Ortega, 1977; Okada et al., 2003). The recording site was readily identifiable based on the location of the electrode and dye-spot (showing strong fluorescence) marked on neurites. Orientation of the brain is in the neuraxis (Strausfeld, 2002) and is tilted about 90 degrees against the head-body axis.

Odor stimulation

A continuous airflow system (Nishino et al., 2003) was used to deliver odor-containing air to an antenna of an ant. Air current (5 liters/min) was continuously applied to an antenna. Air that passed through a cartridge containing a filter paper soaked with 40 μ l odorant solution (1 liter/min) could be added to the constant air current without changing the flow rate, by using a solenoid valve. We applied formic acid and *n*-undecane, major alarm pheromone components (Fujiwara-Tsujii et al., 2006), to a filter paper with dilution to 10% by water or liquid paraffin, respectively (designated as 10% formic acid or *n*-undecane), or without dilution (designated as 100% formic acid or *n*-undecane or simply as formic acid or *n*-undecane). Formic acid or *n*-undecane diluted to 1% did not evoke responses in any neurons tested. In an early stage of this study, animals received stimulations of 100% and 10% formic acid, 100% and 10% *n*-undecane, vanilla, peppermint, hexane, extract of the abdomen (three abdomens in 50 μ l hexane), and a 1:1 mixture of formic acid and *n*-undecane (20 μ l each). In a later stage, animals received stimulations of 100% and 10% formic acid, 100% and 10% *n*-undecane, vanilla, peppermint, 1-hexanol, banana, apple, peach, and maple odor.

The sources of the odorants were described previously (Yamagata et al., 2006). The duration of the odor stimulation was 500 msec or 1 second, and each odor was presented two to four times. A sufficient intertrial interval was given (>9 seconds) to avoid sensory adaptation. The residual air was continuously sucked out of the room through a vacuum system. The "latency" of the response in this study included a mechanical delay from activation of the solenoid valve to arrival of the odor-containing air to an antenna of ~250–300 msec.

Light stimulation was performed by turning on and off an illuminating diffuse light. Tactile stimulation to an antenna was performed by gently touching the antenna with a bamboo stick.

Three-dimensional reconstruction

For three-dimensional reconstruction of major neuropil structures in the ant brain, the brain was fixed in 4% formaldehyde solution, dehydrated in an ascending ethanol series, cleared in methyl salicylate, and imaged dorsally by using a confocal microscope. Optical sections were made at ~2.9- μ m intervals throughout the entire depth of each specimen. The brain neuropils (Fig. 1), visualized by using autofluorescence at 488 nm, were outlined and reconstructed three-dimensionally with Amira software (Mercury, San Diego, CA).

Data analysis

Morphological and physiological data were analyzed by Microsoft Excel, its add-in software, Excel statistics, and

Matlab. Optical sections of specimens viewed ventrally showed that all pheromone-sensitive efferent neurons of the vertical lobe have laminar dendritic arborizations in this lobe, which are arranged in parallel among each other (see Fig. 9A). We studied whether the locations of dendritic laminae differed among different types of pheromone-sensitive efferent neurons. First we defined the axis perpendicular to the dendritic laminae as the anteroposterior axis of the vertical lobe, although this axis was slightly tilted against the anteroposterior axis of the brain. Then we measured the length from the posterior edge of the dendritic lamina to the posterior end of the vertical lobe and normalized it to the total length of the vertical lobe in the anteroposterior axis. The location of the anterior edge of the dendritic lamina was plotted against that of the posterior edge for individual neurons (see Fig. 9B), and the possible grouping of the data for different morphological types of efferent neurons was examined by k-means clustering, which seeks to classify the elements into clusters by calculating distances of each element to cluster centroid.

Spontaneous spike frequency of a neuron was measured as spike frequency in a time window of 1 second prior to odor stimulation (two to four trials). Response strength of a neuron was evaluated by subtraction of spontaneous spike frequency from spike frequency during odor stimulation. Responses were referred to as excitatory if the spike frequency during the odor stimulation was higher than the mean plus standard deviation (SD) of spontaneous spike frequency and as inhibitory if the spike frequency during odor stimulation was lower than the mean minus SD of the spontaneous spike frequency.

Some aspects of responses to formic acid and *n*-undecane were compared among uni-PNs, multi-PNs (recorded in a previous study, Yamagata et al., 2006), and different morphological types of PR neurons. To evaluate similarity in the time course of the responses to formic acid and to *n*-undecane, cross-correlation analysis was performed: spike trains for a 2-second period after the stimulus onset were divided into 20 bins (100-msec bin), and the correlation coefficients between formic acid $A = \{a_1, a_2, a_3, \dots, a_{20}\}$ and *n*-undecane $B = \{b_1, b_2, b_3, \dots, b_{20}\}$ were calculated by the formula $C_{AB} = 100 \times A^2/AB$ and averaged for all trial combinations of formic acid and *n*-undecane. Next, the dependence of the response magnitude (spike frequency) on the pheromone concentration was evaluated by measuring the difference in the magnitude of response to 100% formic acid or *n*-undecane and that to 10% formic acid or *n*-undecane. For these analyses, all neurons that responded to at least one of the components (100% or 10% formic acid or *n*-undecane) were included. Finally, to evaluate the specificity of responses to alarm pheromone, the ratio of pheromone-sensitive neurons that also exhibited responses to non-pheromonal odors was compared among different morphological types of neurons.

For statistical evaluation, we used nonparametric tests for all analyses, based on the results of the Kolmogorov-Smirnov test, which evaluates normality of the data. Pheromone information is transmitted to the PR by PNs and is further processed and modified in the PR. Therefore, we performed a priori comparisons between PNs and PR neurons by using the Steel-Dwass test. We did not have any particular hypotheses for the differences among different types of PR neurons and therefore performed

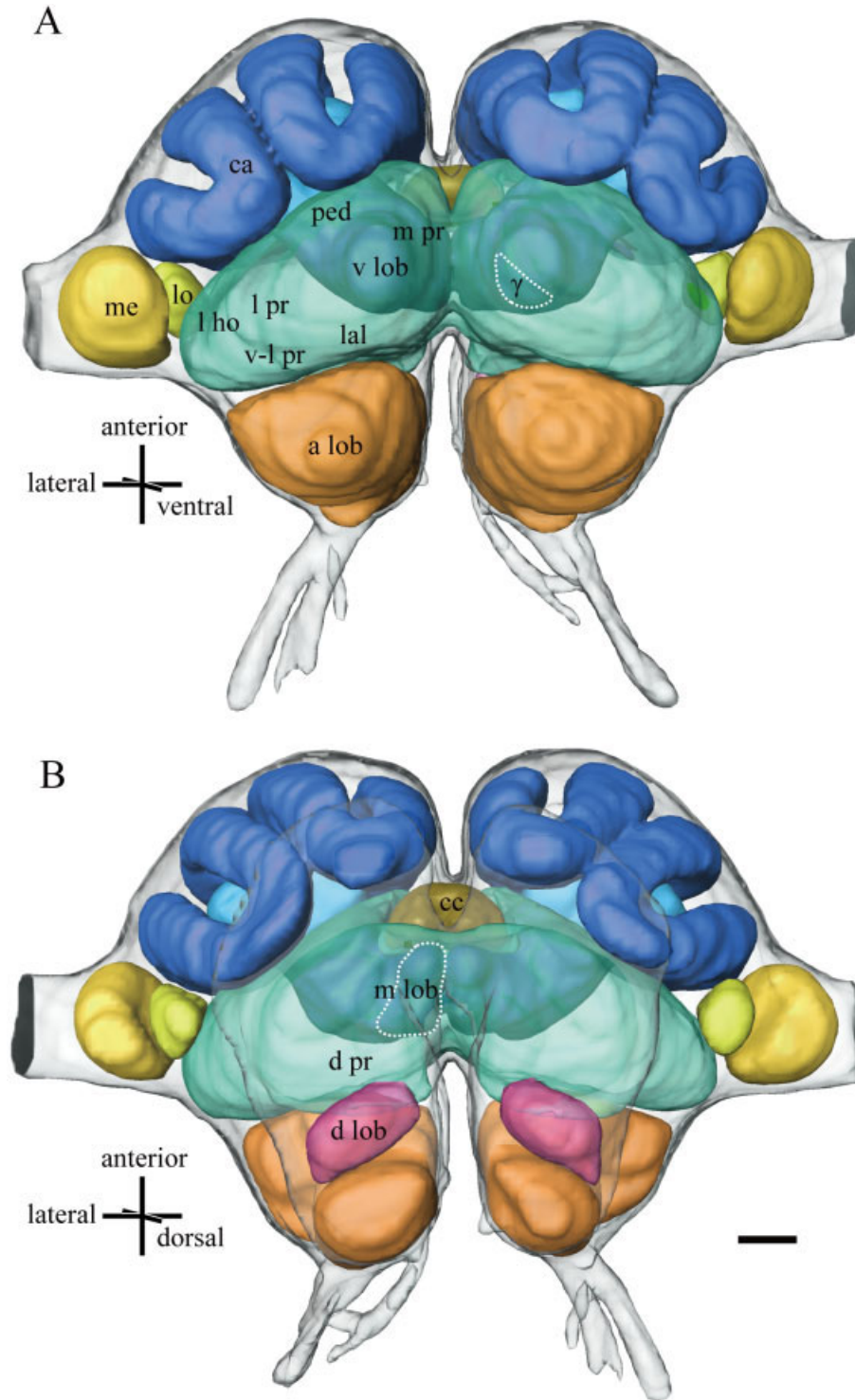


Fig. 1. Three-dimensional reconstructions of major neuropils of the ant brain viewed ventrally (A) and dorsally (B). The protocerebrum (PR: green) consists of the mushroom body (MB), the lateral PR (l pr) that includes the lateral horn (l ho), the ventrolateral PR (v-l pr), the medial and dorsal PR (m pr and d pr), the lateral accessory lobe (lal), and the central complex (cc: khaki). The MB consists of calyces (ca: blue), the pedunculus (ped: light blue), and the ventral and

medial lobes (v lob, m lob: light blue, broken line). The posterior edge of the ventral lobe is called the γ region (broken line). The medulla (med: yellow) and the lobula (lo: light yellow) are the second and third optic neuropils. The deutocerebrum consists of the antennal lobe (a lob: orange) and the dorsal lobe (d lob: magenta). Scale bar = 100 μ m in B (applies to A,B).

TABLE 1. Summary of Responses of Pheromone-Sensitive PNs and PR Neurons in the Ant Brain¹

PNs		Uni-PN ²		Multi-PN ²	
formic acid +		4 (50)		2 (13)	
<i>n</i> -undecane +		1 (13)		1 (7)	
for +/un +		1 (13)		3 (20)	
phe +/non-phe +		2 (25)		9 (60)	
Total		8		15	

PR neurons	Ped efferent	Lobe efferent	MB feedback	m pr efferent	l pr neuron	v-l pr/lal	DN	Total
formic acid +	3 (21)	1 (6)	6 (38)	2 (20)	—	—	—	12 (19)
<i>n</i> -undecane +	—	—	—	1 (10)	—	—	—	1 (2)
for +/un +	6 (43)	1 (6)	5(31)	1 (10)	1 (50)	—	—	14 (22)
phe +/non-phe +	5 (36)	16 (89)	5(31)	6 (60)	1 (50)	1 (100)	2 (100)	36 (57)
Total	14	18	16	10	2	1	2	63

¹Data are numbers, with percents in parentheses. Abbreviations: formic acid + or *n*-undecane +, the neuron(s) responded to formic acid or *n*-undecane, respectively, but not to any of the non-pheromonal odors tested; for +/un +, the neuron(s) responded to formic acid and *n*-undecane, but not to any of non-pheromonal odors tested; phe +/non-phe+, the neuron(s) responded to formic acid and/or *n*-undecane and also to at least one of the non-pheromonal odors tested; v-l pr/lal, local neuron of the ventrolateral PR and lateral accessory lobe; DN, descending neurons; l pr, lateral PR; MB, mushroom body; m pr, medial PR; Ped, pedunculus; PN, projection neuron; PR, protocerebrum.

²Based on Yamagata et al. (2006).

Kruskal-Wallis nonparametric ANOVA among different types of PR neurons; if their variances were nonsignificant, we pooled data from different types of PR neurons and dealt with them as a group for subsequent multiple comparisons with PNs. When we found differences by the Kruskal-Wallis test, we performed post hoc comparisons among different types of PR neurons by the Steel-Dwass test. If we found differences between different types, then we dealt with them separately for subsequent comparisons with PNs.

RESULTS

General organization of the ant brain

To facilitate morphological descriptions of alarm pheromone-sensitive neurons in the brain of the ant *C. obscuripes*, we briefly describe major structures of its brain (Fig. 1). In general, the major structures are similar to those reported for other species of ants (Goll, 1967; Gronenberg and Hölldobler, 1999; Gronenberg, 2001). The brain consists of the PR, deutocerebrum, and tritocerebrum. The deutocerebrum consists of the antennal lobe and the dorsal lobe, the former being the primary olfactory center and the latter being the antennal mechanosensory center.

The PR consists of the MB, central complex (cc), lateral PR (l pr), medial PR (m pr), dorsal PR (d pr), ventrolateral PR (v-l pr), lateral accessory lobe (lal), and several other areas not mentioned here (Fig. 1A,B). The MB, a multi-sensory association center participating in some forms of learning (Erber et al., 1980; Li and Strausfeld, 1997, 1999; Mizunami et al., 1998b; Menzel, 1999; Menzel and Giurfa, 2001; Heisenberg 2003), consists of calyces (ca), a pedunculus (ped), a medial lobe (m lob), and a vertical lobe (v lob). Each calyx consists of lip, collar, and basal ring regions. The pedunculus bifurcates into the vertical lobe and the medial lobe. The vertical lobe of ants protrudes ventrally from the pedunculus, and its posterior portion is called the γ region (Farris et al., 2004), and we refer to the remaining part as the main part of the vertical lobe. Kenyon cells, intrinsic neurons of the MB, extend dendrites in the calyx, and their axons run along the pedunculus and the lobes and make en passant synapses with extrinsic neurons (Goll, 1967).

The medial region of the PR between the two MBs is the medial PR. Within the medial PR, the area around the vertical lobe was specifically referred to as the ring neuropil around the α lobe in honey bees (Abel et al., 2001), and here we refer to it as the ring neuropil around the vertical lobe. The medulla (me) and lobula (lo), the second and third optic neuropils of the optic lobe, delineate the lateral margin of the PR (Fig. 1A,B). The ventrolateral PR is the major termination area of neurons of the optic lobe. The region anterior to the ventrolateral PR and medial to the lobula is the lateral PR. Within the lateral PR, the lateral horn is the termination area of PNs from the antennal lobe (a lob). The dorsal PR (also referred to as the posterior slope) is situated mediodorsally to the lateral PR. The dorsal PR and lateral accessory lobe are major premotor areas from which many descending neurons originate to supply thoracic ganglia (Okada et al., 2003).

Overview of pheromone-sensitive PR neurons

We successfully recorded from and stained 63 neurons of the PR, which responded to formic acid and/or *n*-undecane in the ant *C. obscuripes*. As summarized in Table 1, these neurons are morphologically classified into seven groups: 1) efferent neurons of the pedunculus of the MB (n = 14); 2) efferent neurons of the lobe of the MB (n = 18); 3) feedback neurons from the lobe to the calyx of the MB (n = 16); 4) efferent neurons of the medial PR (n = 10); 5) efferent or local neurons of the lateral PR (n = 2); 6) local neuron of the ventrolateral PR and the lateral accessory lobe (n = 1); and 7) descending neurons from the dorsal PR or the lateral accessory lobe (n = 2). Physiologically, these neurons were divided into three groups based on their responses to formic acid, *n*-undecane, and four to seven kinds of non-pheromonal odors (see Materials and Methods): 1) neurons that responded to one pheromone component (formic acid or *n*-undecane) exclusively (n = 13, 21%); 2) neurons that responded to both pheromone components but not to the non-pheromonal odors tested (n = 14, 22%); and 3) neurons that responded to pheromone components and also to non-pheromonal odors (n = 36, 57%). When only the responses to pheromonal components are considered, these neurons can be classified into 1) neurons that responded specifically to formic acid (n =

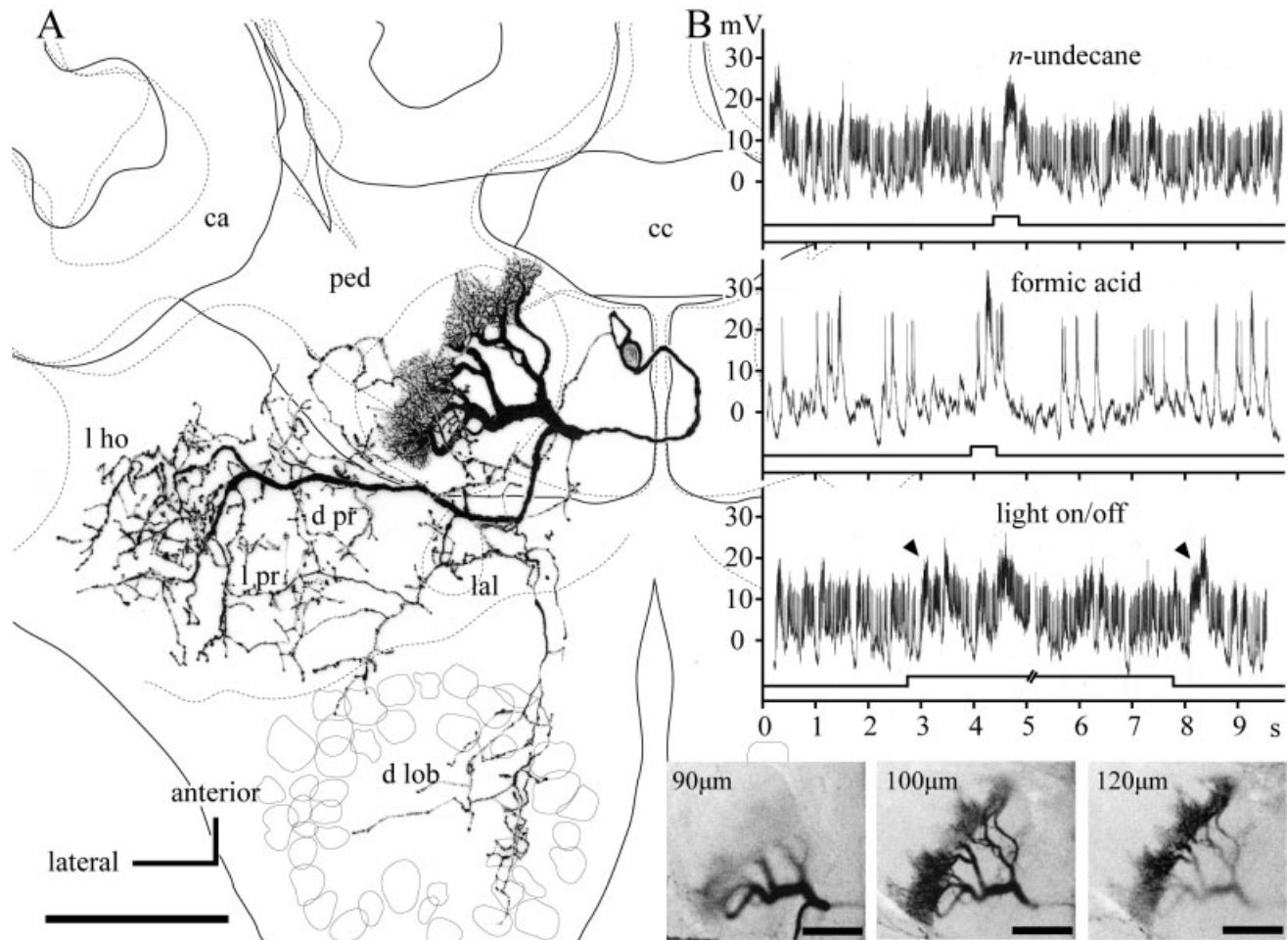


Fig. 2. Pheromone-sensitive efferent neuron of the pedunculus, reminiscent of the Pe1 (Pedunculus-1) neuron of honey bees (Mauelshagen, 1993). **A:** Reconstruction of the Pe1-like neuron from confocal sections viewed ventrally. The soma is located near the midline of the brain. Thick neurites invade the pedunculus-lobe junction and form a laminar dendritic field. The axon traverses the PR and supplies terminal arborizations decorated with many blebs in the lateral PR including the lateral horn, the lateral accessory lobe, the

dorsal PR, and the dorsal lobe. For abbreviations, see legend of Figure 1. Insets (lower right) show confocal images of dendrites in the pedunculus at different depths. **B:** Responses to *n*-undecane, formic acid, and light stimulation. Applications of formic acid and *n*-undecane evoked multiphasic excitatory responses. A hyperpolarizing current of -1.0 nA was applied during recording of the response to formic acid. Turning the light on and off elicited excitatory responses (arrowheads). Scale bar = $100 \mu\text{m}$ in A; $50 \mu\text{m}$ in insets to A.

19, 30%) or *n*-undecane ($n = 4$, 6%); and 2) neurons that responded to both formic acid and *n*-undecane ($n = 40$, 64%). Some of these neurons also responded to visual and/or tactile stimuli. We first describe the morphology and the responses of representative examples of each morphological type of PR neuron and then compare their responses with those of PNs.

Morphologies and responses of pheromone-sensitive PR neurons

Efferent neurons of the MB pedunculus. All pheromone-sensitive efferent neurons of the pedunculus ($n = 14$) possessed laminar dendritic structures in the pedunculus. The majority of these neurons ($n = 9$) had their somata at the dorsolateral edge of the lateral PR, and the somata of other neurons ($n = 5$) were located in various areas of the PR. An example of the major type of neurons, which had terminal arborizations in the lateral

PR, the vertical lobe, and the ring neuropil around it and specifically responded to formic acid, has been reported previously (Yamagata et al., 2005).

Figure 2 shows an impressive efferent neuron with the soma ($\sim 13 \mu\text{m}$ in diameter) situated near the midline of the brain and with dendrites at the junction between the pedunculus and lobes of the MB. The main neurite takes a circular route and forms a very thick dendritic trunk ($\sim 11 \mu\text{m}$ in diameter) at a region anterior to the medial lobe, from which several processes invade the pedunculus-lobe junction and form a band of dense arborizations (insets in Fig. 2), the width being $25 \mu\text{m}$ and the length being $\sim 140 \mu\text{m}$. The dendritic band extends to a depth of $40 \mu\text{m}$. The axon ($\sim 3.5 \mu\text{m}$ in diameter) arising from the dorsal aspect of the trunk proceeds laterally and gives rise to blebbed terminal arborizations in wide areas of the brain, namely, the ring neuropil around the vertical lobe, the lateral accessory lobe, the dorsal PR, the lateral PR in-

cluding the lateral horn, and the dorsal lobe. Many of the morphological features of this type of neuron, like the cell body location and the location and morphology of dendrites and axon terminals, are reminiscent of those of the Pe1 (pedunculus 1) neuron of honey bees (Mauelshagen, 1993; Rybak and Menzel, 1998), and thus we refer to this neuron as Pe1-like neuron. However, there is also a difference: terminal arborizations of the Pe1 neuron of honey bees are confined within the lateral PR and medial PR.

The Pe1-like neuron exhibited vigorous fluctuations of membrane potential on which bursts of spikes were superimposed (Fig. 2B). This obviously reflects postsynaptic potentials, thereby indicating that the microelectrode had penetrated the dendritic region. The frequency of spontaneous spike activities was very high (~ 70 Hz). Reduction of spontaneous spiking activity by application of a hyperpolarizing current (-1 nA; Fig. 2B, second trace) revealed spike doublets and triplets with regular interburst intervals, which are characteristic of activities of Pe1 neuron in honey bees (Rybak and Menzel, 1998). *n*-Undecane induced an increase in spike frequency with a latency of 304 ± 126 msec ($n = 4$), followed by a transient decrease in spike frequency. Application of formic acid during membrane hyperpolarization by a current of -1 nA induced a multiphasic excitatory response with a latency of 289 ± 4 msec ($n = 3$). Other odors were not tested for this neuron. This neuron also exhibited excitatory responses at the onset and the offset of diffuse light stimulation (Fig. 2B, arrowheads).

An example of efferent neurons of the pedunculus with the soma (~ 11 μ m in diameter, arrow) located dorsal to the calyx is depicted in Figure 3. Its dendrites form a layer with a width of ~ 17 – 29 μ m in the pedunculus and a gap at the center (Fig. 3, arrowhead). The dendritic band extends to a depth of 40 μ m. The thick axon (diameter of ~ 3.5 μ m) proceeds medially along the pedunculus and then sharply turns in the opposite direction, proceeding laterally and giving rise to varicose terminal arborizations, which cover a wide area of the lateral PR, including the lateral horn. This neuron exhibited responses to *n*-undecane, formic acid, and 1-hexanol.

Efferent neurons of the MB lobe. Most (16 of 18) of pheromone-sensitive efferent neurons of the lobe also responded to non-pheromonal odor. These neurons were classified into four types from the location of their dendritic and/or terminal arborizations: 1) an efferent neuron of the medial lobe ($n = 1$); 2) an efferent neuron of the γ region of the vertical lobe ($n = 1$); 3) feedback neurons from the MB to the antennal lobe, with dendrites in the main part of the vertical lobe, projecting to the antennal lobe and some premotor PR areas ($n = 4$); and 4) neurons that have dendrites in various PR areas, including the main part of the vertical lobe, ($n = 12$), projecting to various premotor and motor areas. We refer to the last type as wide-field PR neurons rather than efferent neurons of the lobe. Some neurons of the last type had axons descending toward the subesophageal ganglion ($n = 3$). Neurons of types 1 and 2 are described in this section, and those of types 3 and 4 are dealt with in subsequent sections.

The pheromone-sensitive efferent neuron of the medial lobe is shown in Figure 4A. The soma (~ 11 μ m in diameter) is located near the midline of the contralateral hemisphere. The main neurite proceeds medially, crosses the midline, and gives rise to a band of dense dendritic ar-

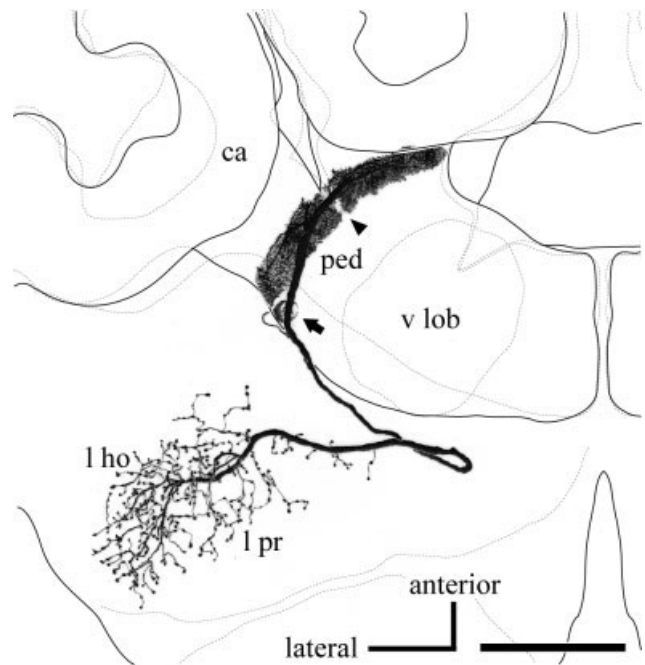


Fig. 3. Pheromone-sensitive efferent neuron of the pedunculus. The soma is located dorsolaterally to the pedunculus (arrow). The neuron extends two sets of laminar dendrites in the pedunculus with a gap at the middle (arrowhead). The axon runs along the pedunculus and gives rise to terminal arborizations in a broad expanse of the lateral PR, including the lateral horn. For abbreviations, see legend of Figure 1. Scale bar = 100 μ m.

borizations of ~ 25 μ m in width in the medial lobe. The main process then bifurcates: one axon proceeds antero-laterally around the vertical lobe and provides varicose terminal arborizations in the anterior (superior) half of the ring neuropil around the vertical lobe, and the other axon proceeds posterolaterally and provides varicose terminal arborizations in the posterior (inferior) half of the ring neuropil. This neuron responded to both 100% and 10% formic acid, exhibiting latencies of 286 ± 20 msec ($n = 2$) and 340 ± 26 msec ($n = 2$), respectively (Fig. 4B). Stimulation with 100% and 10% *n*-undecane elicited responses with latencies of 278 ± 80 msec ($n = 3$) and 420 msec ($n = 1$), respectively. Banana odor evoked strong response with a latency of 336 ± 78 msec ($n = 2$). Tactile stimulation with a bamboo stick to the ipsilateral or contralateral antenna or light stimulation (data not shown) also evoked excitatory responses.

A pheromone-sensitive efferent neuron from the γ region of the vertical lobe (Fig. 5) was recorded only once. This neuron is morphologically similar to efferent neurons of the γ region reported in honey bees (Rybak and Menzel, 1993; Strausfeld, 2002). The cell body (~ 8 μ m in diameter) is located near the midline of the contralateral PR. Its dendrites are densely laminated in the γ region, and its terminal arborizations are distributed in the ring neuropil around the vertical lobe. This neuron responded to formic acid, *n*-undecane, and peppermint odors and to tactile stimulation applied to the ipsilateral antenna and at the onset and extinction of diffuse light stimulation.

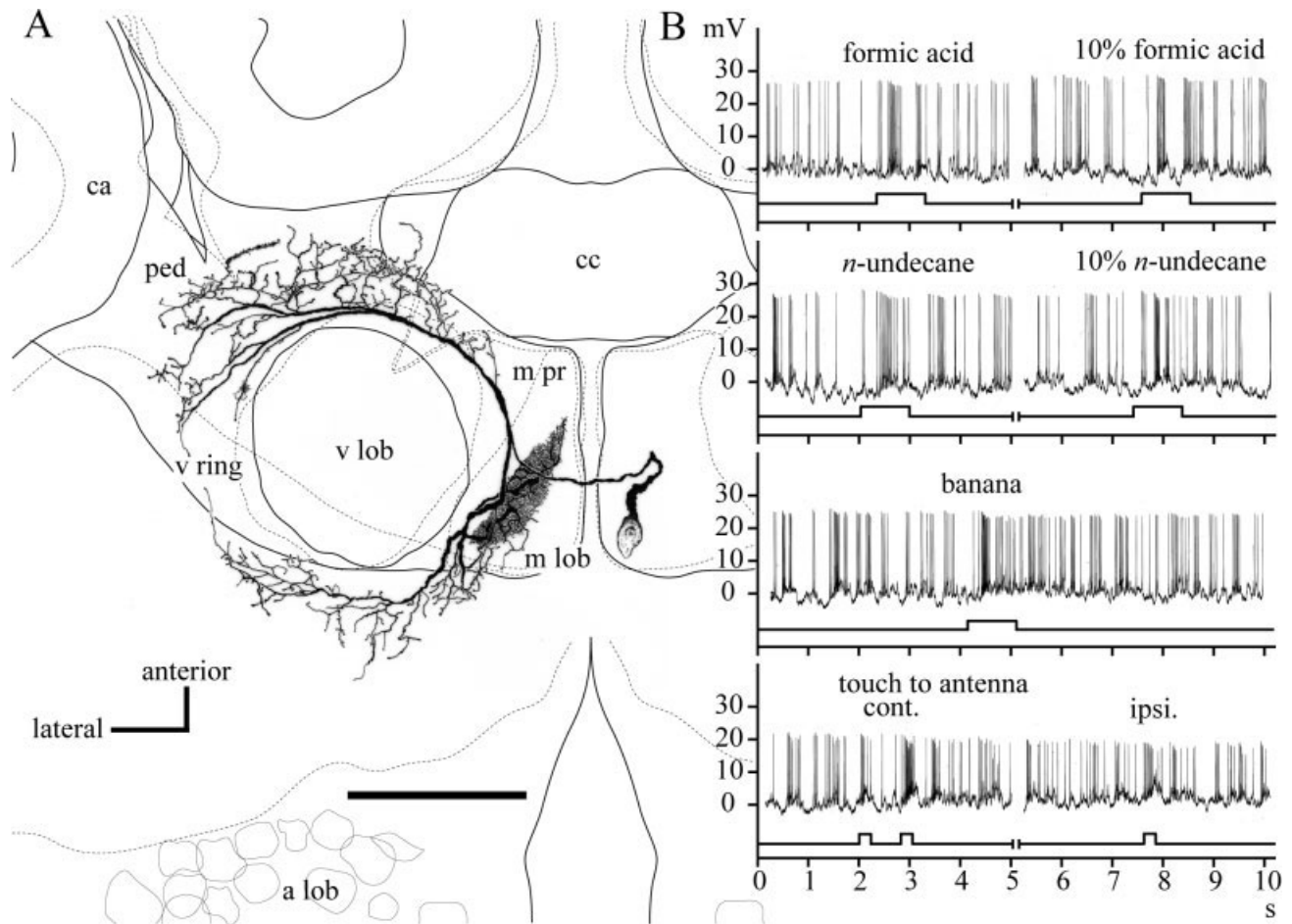


Fig. 4. Pheromone-sensitive efferent neuron of the medial lobe. **A:** Reconstruction of the neuron from confocal sections viewed ventrally. The soma is located in the contralateral hemisphere near the midline. Dendritic arborizations form a layer in the medial lobe. The axon runs along the vertical lobe and provides varicose terminal arborizations in the medial PR, covering the entire region of the ring neuropil

around the vertical lobe (v ring). For abbreviations, see legend of Figure 1. **B:** Responses to 100% or 10% formic acid or *n*-undecane, banana odor or tactile stimulation to an antenna. All of these stimuli evoked transient excitatory responses. A hyperpolarizing current of -0.5 nA was applied during odor and tactile stimulations. Scale bar = 100 μ m in A.

Wide-field PR neurons with dendrites in the vertical lobe. We encountered 12 wide-field PR neurons with dendrites in various PR areas, including the main part of the vertical lobe, and with terminal arborizations in various premotor and motor areas. Notably, most ($n = 11$, 92%) of these neurons responded to non-pheromonal odors, and some also responded to visual and tactile stimuli applied to the antennae. Two examples of these neurons, one responding specifically to *n*-undecane and the other responding to both formic acid and *n*-undecane, have been reported previously (Yamagata et al., 2005). Another notable example is shown in Figure 6A. The cell body (~ 11 μ m in diameter) is located at the boundary between the PR and deutocerebrum. The main process proceeds anterolaterally and forms a thick (~ 6 μ m in diameter) trunk. Collaterals originating from the trunk give rise to spiny dendrites covering wide areas of the brain, namely, the medial PR including the ring neuropil around the vertical lobe, the vertical lobe, the lateral accessory lobe, and a part of the lateral horn. The axon proceeds in a curved route around the pedunculus and gives rise to varicose

terminal arborizations in the lateral and dorsal PR, and it further descends toward the subesophageal ganglion (arrow). This type of neuron has not been reported in any other species of insects. This neuron exhibited excitatory tonic responses to 100% formic acid with a latency of 493 ± 2 msec ($n = 3$; Fig. 6C). It also responded to formic acid at a lower concentration (10%) with a latency of 527 ± 194 msec ($n = 2$). Application of 1-hexanol elicited two clusters of spike burst with a latency of 658 ± 36 msec ($n = 3$). Banana odors elicited excitatory responses with an unusually long latency of 951 ± 135 msec ($n = 3$), which may be off-responses. *n*-Undecane, vanilla, maple, peppermint, apple, and peach odors did not evoke responses.

Feedback neurons from the PR to the antennal lobe. We have encountered four pheromone-sensitive neurons that have complicated systems of dendritic and terminal arborizations, with their dendrites covering the calyces, the pedunculus, the vertical lobe of the MB and medial PR and their axons terminating in the antennal lobe and the dorsal PR, and an example is shown in Figure 7. The cell

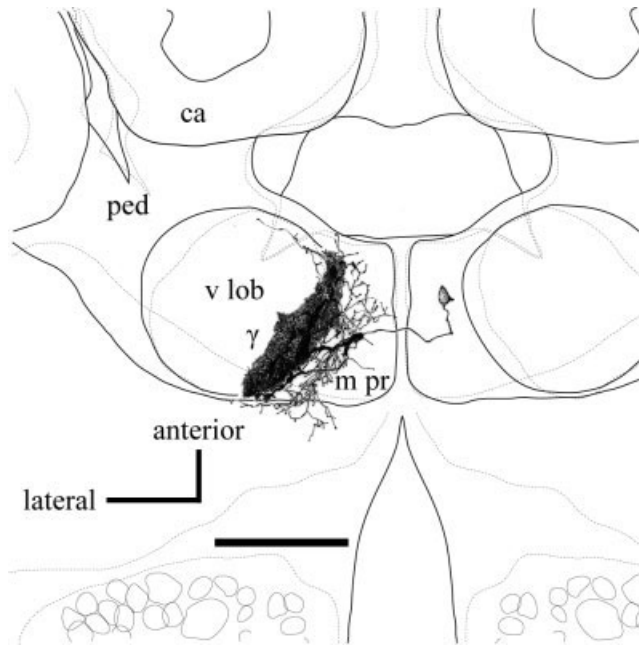


Fig. 5. Pheromone-sensitive efferent neuron with dendrites in the γ region of the vertical lobe. The terminal arborizations are distributed in the medial PR. For abbreviations, see legend of Figure 1. Scale bar = 100 μ m.

body ($\sim 14.5 \mu\text{m}$ in diameter) is located dorsal to the pedunculus (Fig. 7A, arrow). The main process ($\sim 8 \mu\text{m}$ in diameter) gives rise to spiny dendrites in the vertical lobe, a calyx region at the border between the basal ring and the collar, the pedunculus, and the ring neuropil around the vertical lobe. Terminal arborizations are mainly distributed in the antennal lobe, with additional sparse terminal arborizations in the dorsal PR. In the antennal lobe, the arborizations are mainly distributed in the interglomerular region and rarely invade the glomeruli (Fig. 7B). The feedback neuron appears to constitute a feedback (recurrent) loop from the MB to the antennal lobe. Feedback neurons from the MB to the antennal lobe of more or less similar morphology have been reported in honey bees (Rybak and Menzel, 1993; Iwama and Shibuya, 1998). The neuron shown in Figure 7 responded to formic acid and *n*-undecane but not to any of the non-pheromonal odors tested. The other two neurons of the same morphological type, however, responded to both pheromonal and non-pheromonal odors.

Feedback neurons of the MB. Pheromone-sensitive neurons that project from the lobe to the calyx ($n = 16$) have complex systems of dendrites and terminal arborizations. The soma ($\sim 12 \mu\text{m}$ in diameter) of the neuron shown in Figure 8 is located in the lateral margin of the PR. The main process proceeds medially toward the lateral margin of the vertical lobe. There, it gives rise to several processes: some penetrate the vertical and medial lobes and provide numerous spiny collaterals, and the others enter the neck of the pedunculus and provide spiny dendrites (Fig. 8B, arrows). Its laminar dendrites occupy the middle area of the vertical lobe (Fig. 8, inset). The axon runs ventrally toward the calyces via the protocerebral calycal tract. After passing beneath the two calyces, it

bifurcates into two strands, each projecting to the medial or lateral calyx and each giving rise to varicose terminal arborizations in the lip region. This neuron is morphologically similar to feedback neurons from the lobes to the calyx reported in honey bees (Gronenberg, 1987; Grünewald, 1999). This neuron responded to formic acid with a latency of 323 ± 43 msec ($n = 4$; Fig. 8C). It did not respond to *n*-undecane, vanilla, or peppermint odor.

Localization of dendrites of efferent neurons of the vertical lobe. In honey bees, the lip, collar, and basal ring of the calyx receive olfactory inputs, visual inputs, and olfactory and visual inputs, respectively (Gronenberg, 2001), and the axons of the major type (spiny or class I) of Kenyon cells with dendrites in each calycal subdivision occupy separate areas in the vertical lobe, i.e., basal ring area, collar area, and lip area, which are arranged from anterior to posterior, with the posteriormost area (γ region) of the vertical lobe receiving projections of another type (clawed or class II) of Kenyon cells (Farris et al., 2004).

In ants, all pheromone-sensitive uni-PNs innervated the lip region of the MB calyces (Yamagata et al., 2006), and all pheromone-sensitive efferent neurons of the γ region ($n = 1$, Fig. 5) or the main part of the vertical lobe ($n = 25$) exhibited laminar dendritic arborizations. To obtain insights into the internal organization of the ant MB, we studied whether dendrites of different types of efferent neurons with different termination areas are located in different areas of the vertical lobe. Efferent neurons of the main part of the vertical lobe ($n = 25$), which include 10 wide-field PR neurons (Fig. 6), 4 feedback neurons from the MB to the antennal lobe (Fig. 7), and 11 MB feedback neurons (Fig. 8), were used for the study. We measured the lengths from the posterior end of the vertical lobe to the anterior edge (upper edge, Fig. 9A) and posterior edge (lower edge) of the dendritic lamina and normalized the lengths to the total length of the vertical lobe in the anteroposterior axis. Then the location of the upper edge was plotted against that of the lower edge for each neuron (Fig. 9B). All dendritic layers of pheromone-sensitive extrinsic neurons were located in the posterior region of the vertical lobe, i.e., at the coordinate of less than 0.5. This suggests that the posterior region of the vertical lobe receives projections of axons of Kenyon cells with dendrites in the lip region, as reported in the MB of honey bees (Farris et al., 2004). Moreover, *k*-means clustering analysis showed that the locations of dendrites of efferent neurons could be separated into three clusters, which matched separations by morphological types (21 of 24 neurons matched; Fig. 9B). The finding that dendrites of different morphological types of efferent neurons occupy different regions of the vertical lobe suggests, for the first time in insects, that signals from different regions of this lobe are sent to different brain regions.

Efferent neurons of the medial PR. Pheromone-sensitive neurons with dendrites in the medial PR ($n = 10$) had axon terminals in various premotor areas, including the lateral accessory lobe and dorsal PR. Five of these neurons had axons descending toward the subesophageal ganglion. An example of neurons terminating in the lateral accessory lobe and dorsal PR is shown in Figure 10A. The soma ($\sim 11 \mu\text{m}$ in diameter) is located at the boundary between the PR and the deutocerebrum. The main neurite travels anteriorly close to the midline and provides dendritic arborizations in the medial PR including the ring

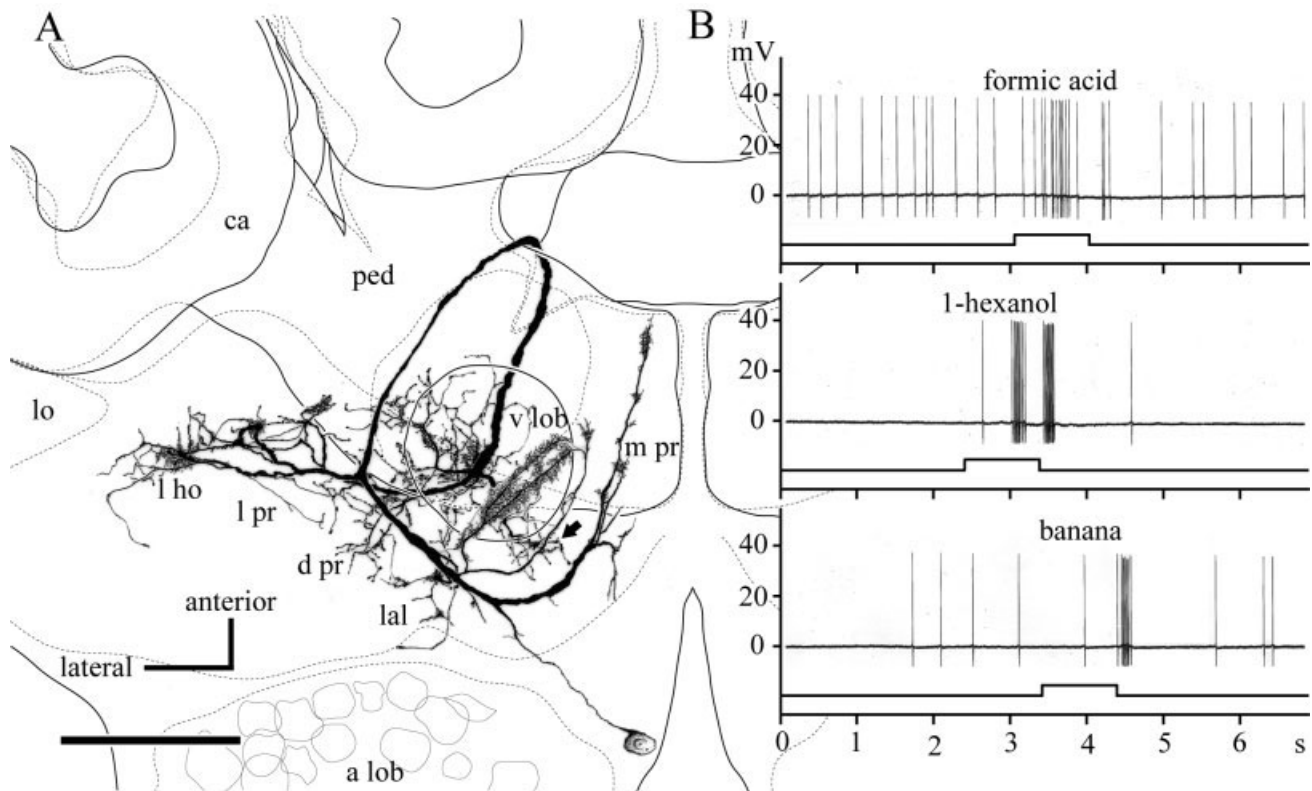


Fig. 6. Pheromone-sensitive wide-field PR neuron with dendrites in the vertical lobe, lateral PR, and medial PR. **A:** Reconstruction of the neuron from confocal sections viewed ventrally. The soma is located at the boundary between the PR and the deutocerebrum. The dendrites cover the vertical lobe, the medial PR, and a part of the lateral horn and the lateral accessory lobe. The axon takes a circular route around the pedunculus and extends terminal arborizations in the

lateral PR and the dorsal PR. One axon descends toward the subesophageal ganglion (arrow). For abbreviations, see legend of Figure 1. **B:** Responses to formic acid, 1-hexanol, and banana odor. Application of formic acid evoked excitatory response. 1-Hexanol evoked biphasic responses, and banana odor elicited a brief spike discharge. Scale bar = 100 μ m in A.

neuropil around the vertical lobe. The axon (~ 3.5 μ m in diameter) proceeds dorsally and sends off terminal arborizations with numerous blebs in the lateral accessory lobe and dorsal PR. The axon reaches the contralateral dorsal PR, where it gives rise to dense terminal arborizations.

This neuron exhibited a brief spike burst in response to 100% formic acid with a latency of 338 ± 48 msec ($n = 3$) and exhibited multiphasic responses to 100% *n*-undecane with latencies of 307 ± 14 msec ($n = 3$; Fig. 10B). Application of 10% formic acid and 10% *n*-undecane also evoked spike discharges with latencies of 655 ± 242 msec ($n = 3$) and 609 ± 25 msec ($n = 2$), respectively. This neuron exhibited responses to several kinds of non-pheromonal odors, i.e., 1-hexanol, banana, apple, peach, and peppermint odors, with similar time courses and latencies (about 600 msec). Tactile stimulation to an antenna and light stimulation also elicited excitatory responses, which often lasted for 1 second or longer.

We noted that dendrites of some types of pheromone-sensitive PR neurons occupy the whole areas of the ring neuropil around the vertical lobe, whereas those of other types of neurons occupy only the medial or lateral half of this neuropil. Dendritic arborizations of efferent neurons of the medial PR and terminal arborizations of Pel-like

neurons and efferent neurons of the vertical or medial lobe cover the whole area of the ring neuropil. In contrast, dendrites of wide-field PR neurons occupy only the medial half. On the other hand, terminal arborizations of multi-PNs and efferent neurons of the pedunculus occupy the lateral half. Therefore, subdivision of this neuropil according to the distribution of dendrites and terminal arborizations of different morphological types of pheromone-sensitive neurons is evident.

We have encountered two pheromone-sensitive neurons that have dendrites in the medial PR and lateral PR with terminal arborizations in premotor areas including the dorsal PR and with axons further descending toward the subesophageal ganglion. These neurons are morphologically similar to the wide-field PR neurons shown in Figure 6, except that these neurons have no dendrites in the lobe of the MB.

Efferent neurons of the lateral PR. Pheromone-sensitive efferent neurons of the lateral PR ($n = 2$) were divided into two types. One type had additional dendrites in the lateral accessory lobe with their axon projecting to the dorsal PR of the contralateral hemisphere, and the other type was a local neuron whose neurites were confined within the lateral PR. The former type is depicted in Figure 11. The cell body (~ 11 μ m in diameter) is located

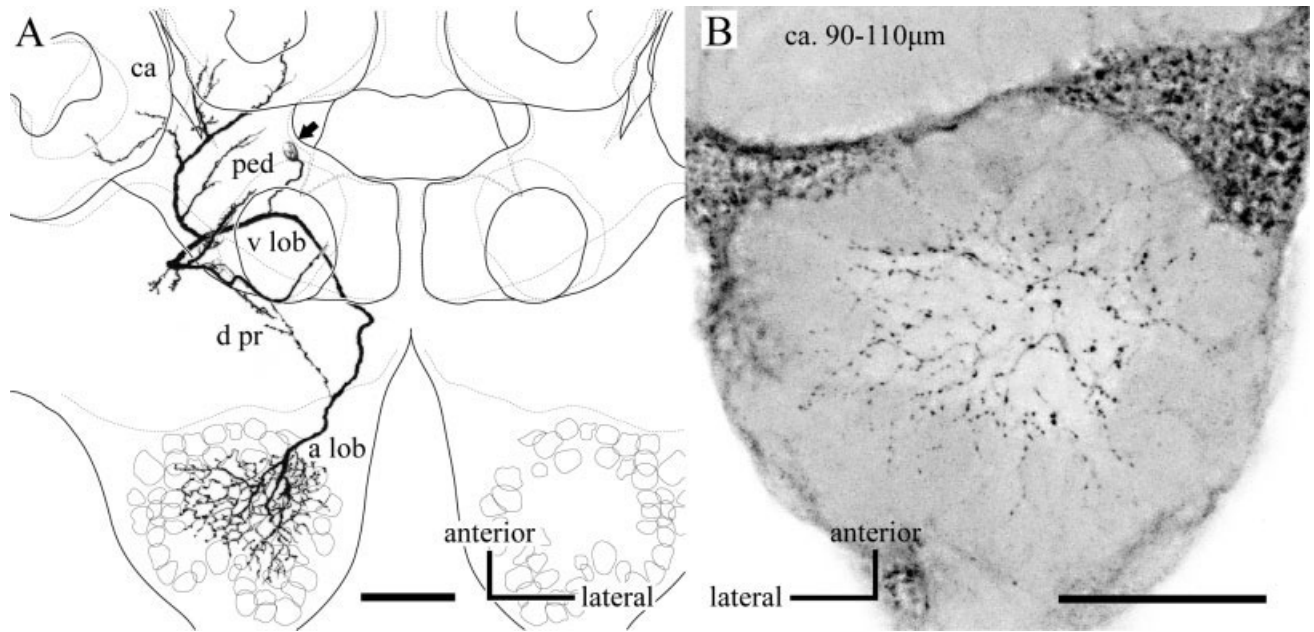


Fig. 7. Phormone-sensitive feedback neuron from the PR to the antennal lobe. **A:** Reconstruction of the neuron from confocal sections viewed ventrally. The soma is located dorsally to the pedunculus (arrow). The dendrites are distributed in the calyces, the pedunculus and the ring neuropil around the vertical lobe. Terminal arborizations are distributed mainly in the antennal lobe, and some terminal ar-

borizations are also seen in the dorsal PR. **B:** A confocal image of terminal arborizations in the antennal lobe. Terminal arborizations with varicosities mainly cover interglomerular regions in the antennal lobe. For abbreviations, see legend of Figure 1. Scale bar = 100 μm in A,B.

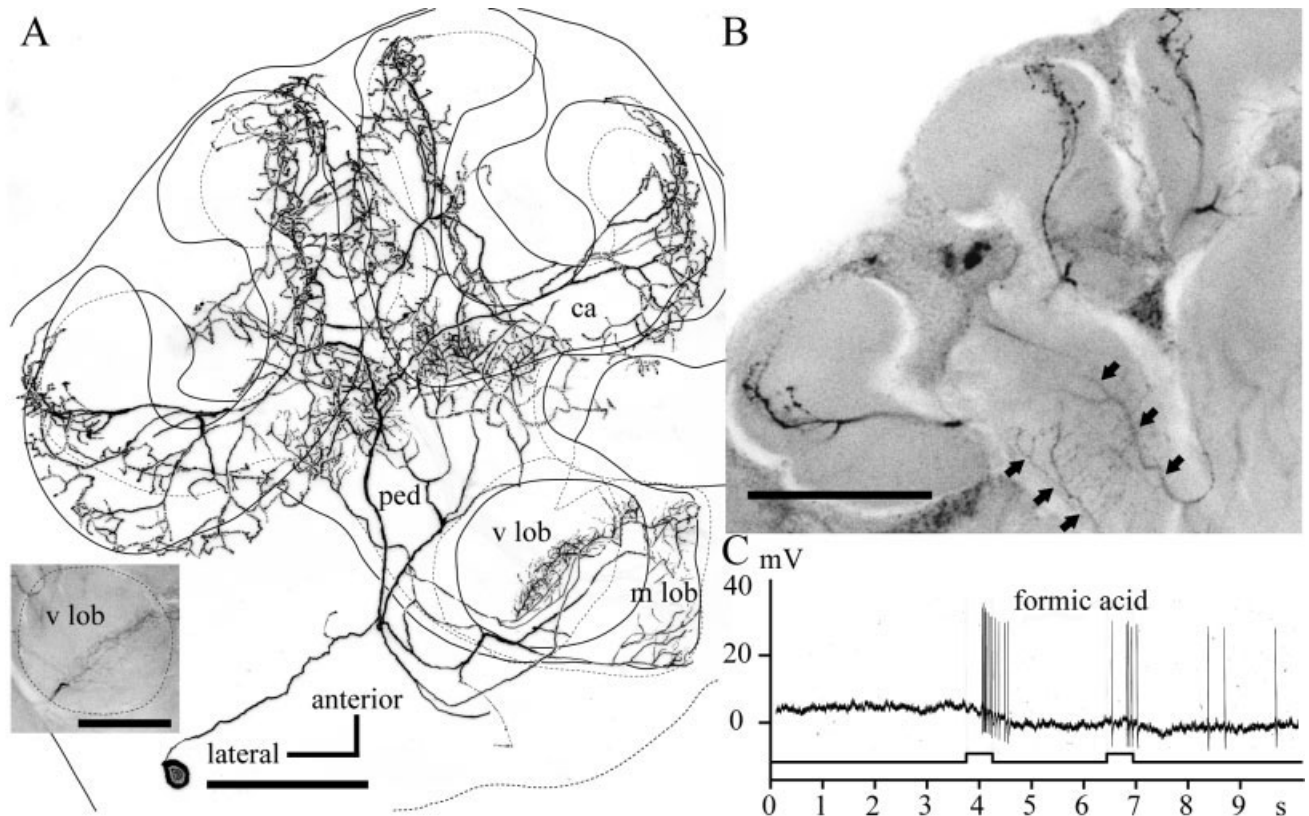


Fig. 8. Phormone-sensitive feedback neuron of the MB. **A:** Reconstruction of the neuron from confocal sections viewed ventrally. The soma is located at the dorsolateral edge of the PR. The neuron extends laminar dendritic arbors in the vertical lobe (inset) and the medial lobe. The axon proceeds through the protocerebral calycal tract and

terminates in the calyces. **B:** On the way to the calyces, some neurites invade the pedunculus and provide dendritic arborizations (arrows, at a depth of ~100 μm). For abbreviations, see legend of Figure 1. **C:** Formic acid evoked a spike burst. Scale bar = 100 μm in A, inset to A, and B.

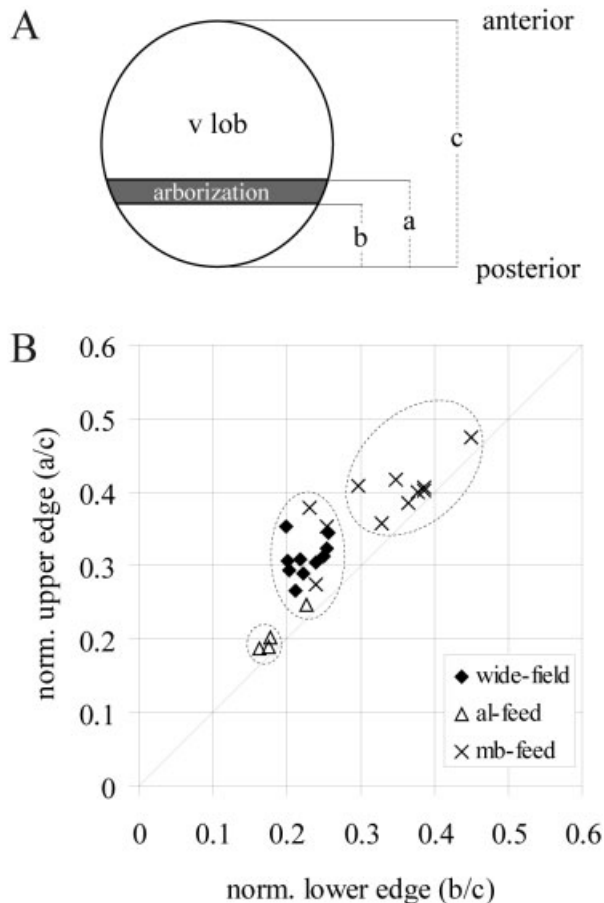


Fig. 9. Analysis of the location of dendrites of efferent neurons of the vertical lobe. **A:** A schema of arborization patterns of dendrites in the vertical lobe and definitions of the upper edge (a) and lower edge (b) of the dendritic lamina. These are defined as the lengths from the posterior end of the vertical lobe and are normalized to the total length of the vertical lobe (c). **B:** The position of the upper edge of dendrites is plotted against that of the lower edge for individual neurons of three types of efferent neurons, with the results of their k-means clustering shown (dotted ellipses). Separation by location of dendrites of efferent neurons generally matched separation by morphological type. al-feed, antennal lobe feedback neuron; mb-feed, mushroom body feedback neuron.

dorsal to the MB (arrow), and the main process gives rise to dendritic arbors in the lateral PR. The axon ($\sim 6.5 \mu\text{m}$ in diameter) proceeds dorsally and gives rise to terminal arborizations in the lateral accessory lobe. The axon further proceeds medially, crosses the midline, and gives rise to terminal arbors in the dorsal PR of the contralateral hemisphere. This neuron responded to formic acid and *n*-undecane but did not respond to any of the non-pheromonal odors tested.

Descending neurons from the lateral accessory lobe or the dorsal PR. We have encountered two pheromone-sensitive neurons with dendrites in the dorsal PR or the lateral accessory lobe and with axons descending toward the subesophageal ganglion. The neuron shown in Figure 12A has the soma ($\sim 12 \mu\text{m}$ in diameter) located dorsally to the antennal lobe, and the main process proceeds anteriorly and gives rise to spiny dendritic arborizations in the

lateral accessory lobe and the medial PR including the ring neuropil around the vertical lobe. The axon ($\sim 5 \mu\text{m}$ in diameter) extends dorsally and gives rise to varicose terminal arborizations in the dorsal PR. Two neurites (arrows) further descend toward the subesophageal ganglion, but they could not be traced further.

This neuron responded to *n*-undecane with long lasting excitation for about 4 seconds with a latency of 343 ± 80 msec ($n = 3$; Fig. 13B). Application of formic acid elicited transient excitation with a latency of 314 ± 8 msec ($n = 3$). *n*-Hexane produced spike bursts for about 1 second with a latency of 639 ± 8 msec ($n = 3$). Application of 10% *n*-undecane evoked a weak and transient excitatory response with a latency of 585 ± 121 msec ($n = 3$).

Comparison of responses of pheromone-sensitive PNs and PR neurons

The majority of pheromone-sensitive PR neurons also responded to non-pheromonal odors. This is in contrast to the fact that most of the uni-PNs, the major type of neurons to transmit alarm pheromone signals to the PR, responded specifically to formic acid and/or *n*-undecane (Table 1; Yamagata et al., 2006). This raises the possibility that PR neurons tend to integrate pheromonal signals with non-pheromonal signals, rather than to further extract specific aspects of pheromonal signals. To evaluate this possibility quantitatively, we compared 1) the cross-correlation (which represents the similarity) between responses to formic acid and those to *n*-undecane; 2) differences in response magnitude (spike frequency) by a 10-fold change in pheromone concentration; and 3) ratio of pheromone-sensitive neurons that also responded to non-pheromonal odors among a) uni-PNs ($n = 8$; Yamagata et al., 2006); b) multi-PNs ($n = 15$; Yamagata et al., 2006); and c) four types of PR neurons ($n = 58$), including efferent neurons of the pedunculus ($n = 14$), efferent neurons of the lobe ($n = 18$), feedback neurons of the MB ($n = 16$), and efferent neurons of the medial PR ($n = 10$). Other types of PR neurons were not used for analysis due to their small sampling numbers. Prior to comparison, we examined data variances by Kruskal-Wallis nonparametric ANOVA among these four types of PR neurons, and we found no significant differences for 1) and 2) and thus dealt with these four types as one "PR neurons" group.

First, the cross-correlation between the response to formic acid and that to *n*-undecane was significantly less in uni-PNs ($n = 5$) compared with that in PR neurons (Steel-Dwass test, $P < 0.05$; $n = 52$). This finding suggests that signals for *n*-undecane and formic acid are more integrated in PR neurons than in uni-PNs (Fig. 13A). No significant difference was found between uni-PNs and multi-PNs ($n = 14$).

Second, the change in response magnitude for a 10-fold change in formic acid and/or *n*-undecane concentration was significantly greater in uni-PNs than that in PR neurons (Steel-Dwass test, $P < 0.05$). No significant difference was found between uni-PNs and multi-PNs. Therefore, PR neurons exhibited more saturated responses to formic acid and/or *n*-undecane than did uni-PNs, possibly because signals from many uni-PNs converge when they are transmitted to PR neurons.

Third, we compared the ratio of pheromone-sensitive neurons that also responded to non-pheromonal signals among different types. In contrast to the prior two analyses, the Kruskal-Wallis test showed significant differences

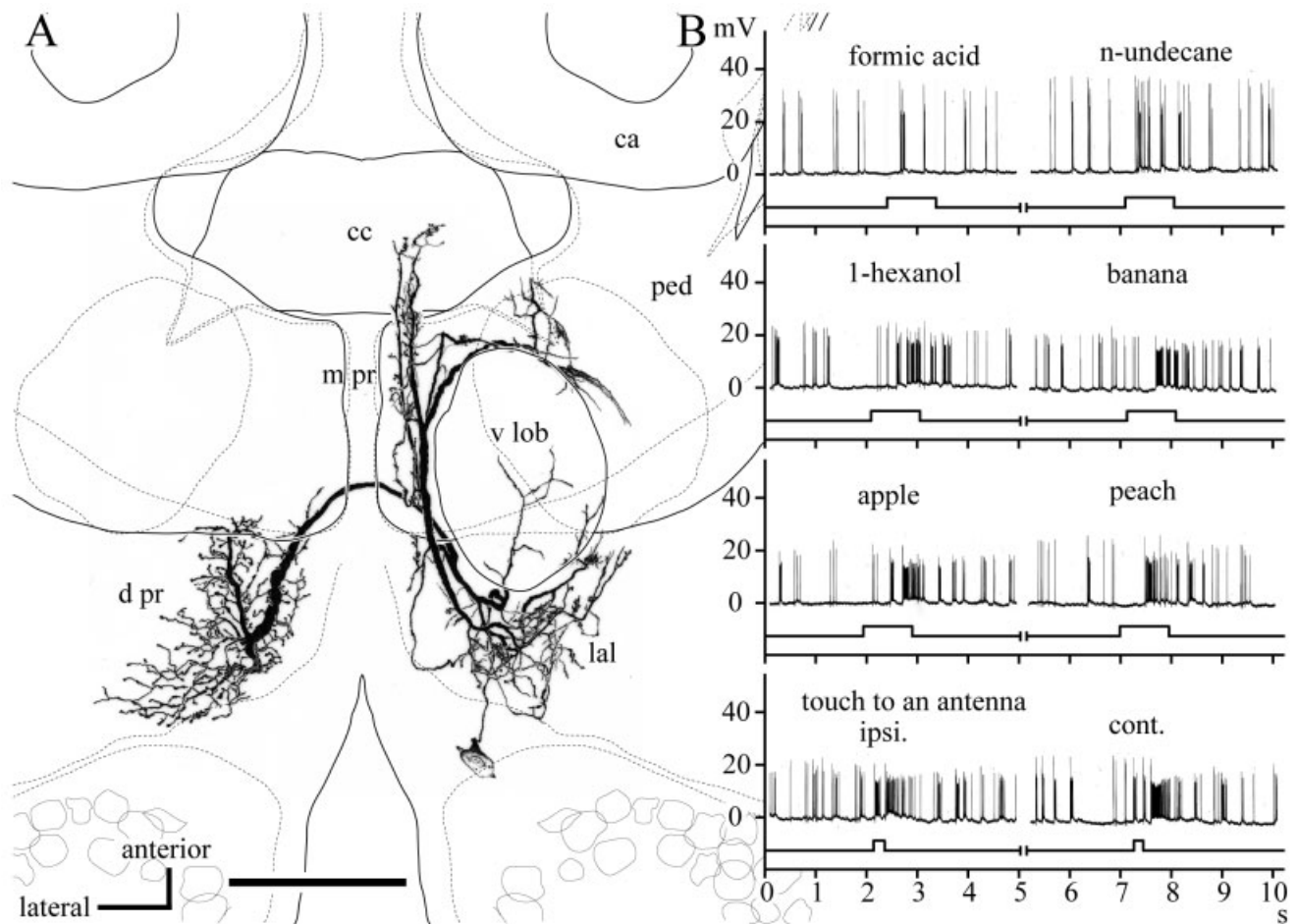


Fig. 10. Pheromone-sensitive efferent neuron of the medial PR. **A:** Reconstruction of the neuron from confocal sections viewed ventrally. The soma is located at the boundary between the PR and the deutocerebrum. Dendritic arbors cover wide areas of the medial PR. A dorsally running axon extends terminal arbors in the lateral accessory lobe and the dorsal PR of the ipsilateral hemisphere and in the contralateral dorsal PR. For abbreviations, see legend of Figure 1.

B: Responses of the neuron to olfactory and tactile stimuli. Application of formic acid and *n*-undecane elicited brief spike bursts, and 1-hexanol, banana, apple, and peach odor, and tactile stimulation to the antenna ipsi- or contralateral to the cell body location evoked multiphasic excitatory responses lasting for about 1 second. Scale bar = 100 μ m in A.

among the four types of PR neurons ($H = 14.0$; $P < 0.005$). A post hoc test (Steel-Dwass test) revealed a significantly higher value for lobe efferent neurons ($n = 18$) than for pedunculus efferent neurons ($P < 0.05$; $n = 14$) and for MB feedback neurons ($P < 0.01$; $n = 16$), and the Kruskal-Wallis test showed no significant differences among pedunculus efferent neurons, MB feedback neurons, and medial PR efferent neurons ($H = 2.23$; $P = 0.33$). Therefore, we examined the ratio of pheromone-sensitive neurons that also responded to non-pheromonal odors among uni-PNs ($n = 8$), multi-PNs ($n = 15$), lobe efferent neurons ($n = 18$), and three other types of PR neurons ($n = 40$). Multiple comparisons with the Steel-Dwass test revealed a significantly higher score in lobe efferent neurons than in uni-PNs ($P < 0.01$) and in the three types of PR neurons other than lobe efferent neurons ($P < 0.01$; Fig. 13C). The results confirm that the lobe efferent neurons tend to integrate pheromonal and non-pheromonal signals.

DISCUSSION

We characterized the morphology and physiology of 63 PR neurons that responded to formic acid and/or *n*-undecane in the ant brain, as a first step to elucidate brain mechanisms underlying pheromone communication by social insects. Many of these were efferent or recurrent neurons of the MB, and some responded to other modalities of stimuli as well. Behavioral responses of ant workers to alarm pheromone in the context of colony defense are extremely diverse but can be categorized into 1) termination of ongoing behavior and preparation for alarm behavior; 2) induction of alarm behavior, including orientation toward or away from the pheromone source; and 3) sensitization of aggressive behavior toward a potential enemy (Blum, 1995; Vander Meer and Alonso, 1998). The critical question to be addressed is how each of the recorded neurons participates in each aspect of the ant's behavior, but discussion on this point is very difficult because 1) we

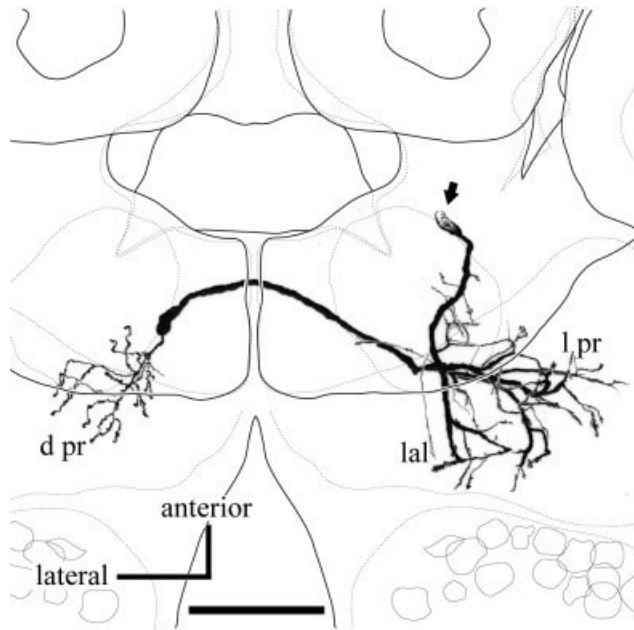


Fig. 11. Pheromone-sensitive efferent neuron of the lateral PR. The soma is located dorsal to the pedunculus (arrow). The dendritic arborizations cover the lateral PR. The axon runs dorsally, gives rise to terminal arborizations in the lateral accessory lobe, and further proceeds into the contralateral hemisphere and gives rise to terminal arborizations in the dorsal PR. For abbreviations, see legend of Figure 1. Scale bar = 100 μm .

have sampled only a small portion of alarm-pheromone sensitive PR neurons, due mainly to the difficulties in attaining intracellular recordings from brain neurons; 2) in-depth analysis of sensory responses of pheromone-sensitive PR neurons has not been achieved, due mainly to limited recording time: insect PR neurons often exhibit complicated response properties, probably processing stimulus context, coincidence, and history (Li and Strausfeld, 1999) and therefore sophisticated analyses are needed to characterize them fully; and 3) external (sensory) and internal factors controlling pheromone-induced alarm behavior and pheromone-sensitized aggressive behavior are not yet fully understood in *C. obscuripes*.

Considering these limitations, we confine our discussion to 1) a comparison of the anatomy and physiology of pheromone-sensitive PR neurons with those of uni-PNs and multi-PNs and with those of PR neurons reported in other species of insects; and 2) the proposal of major neural pathways in the ant PR for alarm pheromone processing and for the control of each aspect of the ant's behavior in the context of colony defense.

We have reported that alarm pheromone signals are transmitted from the antennal lobe (primary olfactory center) to the PR by two sets of morphologically and physiologically different neurons, uni-PNs and multi-PNs (Yamagata et al., 2006). Alarm pheromone-sensitive uni-PNs had dendrites in one of five glomeruli, which are referred to as "AS (alarm pheromone-sensitive)" glomeruli, and most of these neurons exhibited no responses to the non-pheromonal odors tested (Table 1). In contrast, many of the pheromone-sensitive multi-PNs also responded to non-pheromonal odors (Table 1), in accordance

with their dendritic morphology: they had dendrites in AS glomeruli as well as in other glomeruli. Notably, termination areas of pheromone-sensitive uni-PNs differed in large part from those of multi-PNs: uni-PNs terminate in the calyx of the MB, known as the multisensory center participating in learning and memory (Menzel and Giurfa, 2001; Heisenberg, 2003), and the lateral horn, whereas multi-PNs terminate in the lateral horn, the ring neuropil around the vertical lobe, and the dorsal PR. It is likely that pheromone-sensitive multi-PNs provide a rapid sensorimotor pathway for termination of ongoing behavior and preparation of behavioral reactions to alarm pheromone, whereas uni-PNs comprise a major pathway to convey detailed signals for alarm pheromones to the PR for the control of pheromone-induced alarm behavior and pheromone-sensitized aggressive behavior.

Comparison of responses of PNs and MB neurons

We noted differences in the responses of pheromone-sensitive uni-PNs and PR neurons. First, the ratio of neurons that responded to both formic acid and *n*-undecane was higher in PR neurons (63.5%) than in uni-PNs (37.5%). Second, although the majority (75%; Table 1) of pheromone-sensitive uni-PNs did not respond to non-pheromonal odors, the majority of pheromone-sensitive PR neurons also responded to non-pheromonal odors (57%). These results suggest that signals for formic acid and *n*-undecane and those for pheromonal and non-pheromonal odors are integrated in the PR. Indeed, quantitative comparison of the responses showed that four major types of pheromone-sensitive PR neurons (i.e., efferent or feedback neurons of the MB and medial PR neurons) exhibited responses that less well discriminate 1) formic acid and *n*-undecane (Fig. 13A); and 2) different concentrations of pheromone components (Fig. 13B), compared with uni-PNs. We also noted that responses to non-pheromonal odors are more prominent in efferent neurons of the MB lobe, compared with other types of PR neurons and with uni-PNs (Fig. 13C), and thus lobe efferent neurons may play roles in the integration of pheromonal signals with non-pheromonal signals. Moreover, we often observed responses to visual and/or tactile stimuli in pheromone-sensitive MB efferent neurons, although we did not systematically test such non-olfactory sensory stimuli in this study. These observations are in agreement with results of previous studies in cockroaches showing that many of efferent neurons of the lobes of the MB responded to multimodal sensory signals (Mizunami et al., 1998a; Okada et al., 1999; Li and Strausfeld, 1997, 1999). In ants, such multisensory signals may be sent to downstream premotor PR areas to set the context for aggressive behaviors, as is discussed below.

It should also be noted that more than 20% of pheromone-sensitive PR neurons specifically responded to *n*-undecane or formic acid (Table 1), thereby suggesting that signals for each pheromone component are retained in some PR neurons. Such segregated signals may be used for the control of each specific behavioral response to *n*-undecane and formic acid, i.e., attraction toward the odor source in the case of the former and avoidance of it in the case of the latter (Fujiwara-Tsujii et al., 2005).

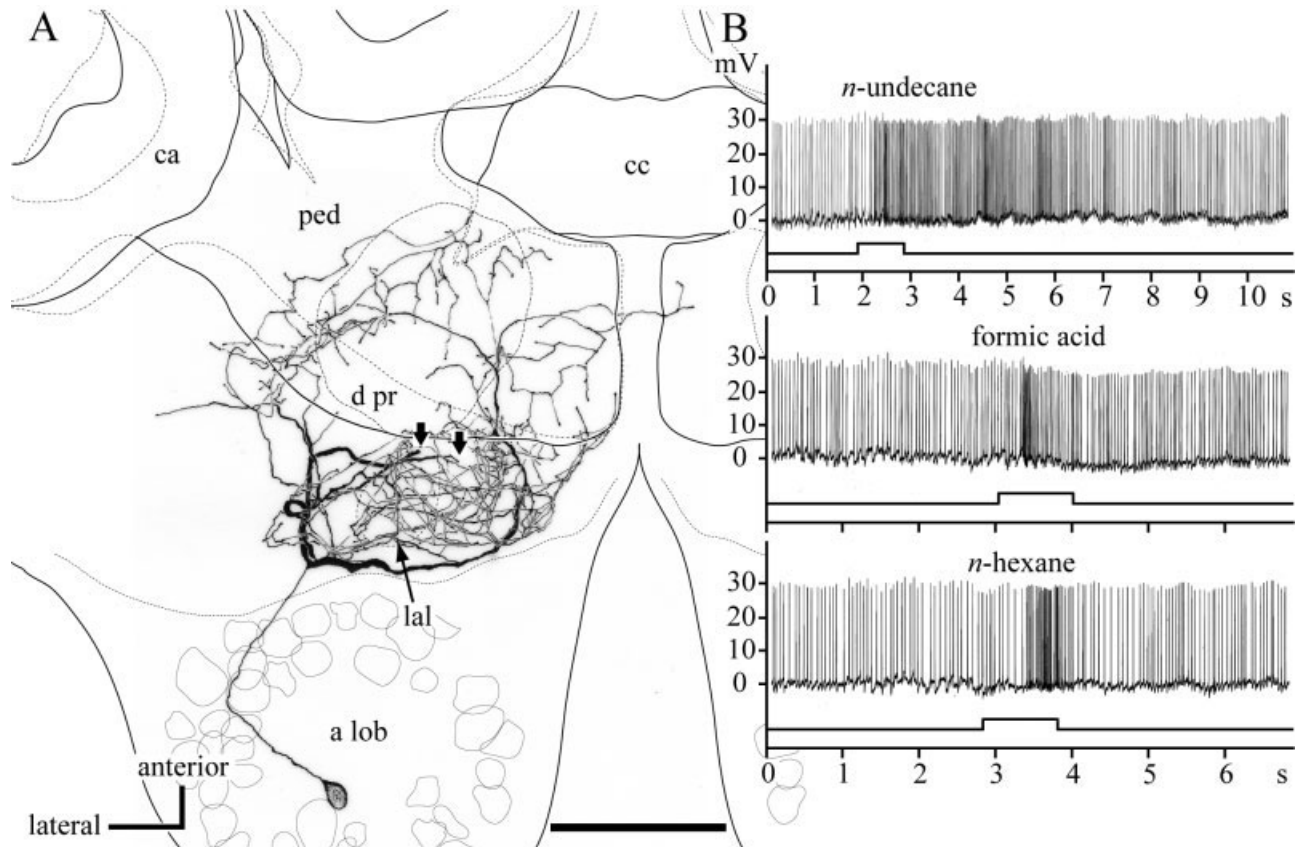


Fig. 12. Pheromone-sensitive neuron of the dorsal PR with a descending axon. **A:** Reconstruction of the neuron from confocal sections viewed ventrally. The soma is located near the dorsal edge of the deutocerebrum. The dendrites cover the medial PR, the lateral accessory lobe, and a part of the dorsal PR. Several processes invade the dorsal PR, where they provide varicose terminal arborizations. Two of the branches further descend toward the subesophageal ganglion (ar-

rows). For abbreviations, see legend of Figure 1. **B:** Responses to *n*-undecane, formic acid, and *n*-hexane. Application of *n*-undecane evoked a long-lasting excitatory response lasting for over 4 seconds. *n*-Hexane evoked a tonic excitatory response lasting for about 1 second. Formic acid evoked transient excitatory responses. A hyperpolarizing current of -0.7 nA was applied during recordings. Scale bar = 100 μ m in A.

Comparison to PR neurons of other species of insects

The Pe1-like neuron depicted in Figure 2 is morphologically similar to the Pe1 neuron of honey bees, except that there is a slight difference in the distribution of terminal arborizations (see Results). Pe1-like neuron in ants exhibited a high frequency of spontaneous spike discharge with spike doublets and triplets as observed for the Pe1 neuron in honey bees (Rybak and Menzel, 1998). Notably, the Pe1 neuron in honey bees is implicated in the processing of short-term olfactory memory, because it exhibited a change in response to an odor for a few minutes after the odor was paired with sucrose reward (Mauelshagen, 1993). Because sensitization, a form of short-term memory, of alarm pheromone signals is critical for the control of aggregation behavior toward a potential enemy (Blum, 1969), it is tempting to speculate that Pe1-like neurons participate in the processing of short-term retention of alarm pheromone signals for the control of aggressive behavior. It is most likely that Pe1-like neurons also play roles in the control of behaviors other than aggression, because Pe1 neurons of honey bees responded to any kinds of sensory stimuli tested (Mauelshagen, 1993) and this

property is suited to serve as a part of general memory system rather than to control a particular behavior.

Pheromone-sensitive feedback neurons from the PR to the antennal lobe (Fig. 7) are morphologically similar to feedback neurons to the antennal lobe reported in honey bees (Iwama and Shibuya, 1998; Kirschner et al., 2006), in that both neurons have dendrites in the vertical lobe and in the ring neuropil around the vertical lobe. Moreover, the location of the dendrites in the vertical lobe (Fig. 9) appears to match that of feedback neurons in honey bees (Kirschner et al., 2006). However, feedback neurons to the antennal lobe in honey bees do not possess dendrites in the calyx, and their terminal arborizations invade most of the glomeruli in the antennal lobe, features that are different from those of feedback neurons to the antennal lobe in ants.

Pheromone-sensitive feedback neurons from the lobe to the calyx (Fig. 8) are morphologically similar to feedback neurons of the MB reported in honey bees (Gronenberg, 1987; Grunewald, 1999). The only difference is that the majority of these neurons possess dendrites in the ring neuropil around the vertical lobe in honey bees (Gronenberg, 1987), but such dendrites are absent in MB feedback

neurons in ants. These neurons have been shown to be immunoreactive to γ -aminobutyric acid (GABA) in honey bees. In ants, the majority of MB feedback neurons (11 of 16) responded specifically to alarm pheromone compo-

nents, whereas the majority of efferent neurons of the MB lobe (16 of 18) also responded to non-pheromonal odors. It should be determined whether this physiological difference is related to the difference in the location of their dendrites in the vertical lobe (Fig. 9).

We noted that all dendrites of pheromone-sensitive neurons of the MB vertical lobe were confined to its posterior region (Fig. 9). In honey bees, the posterior region of the vertical lobe is composed of axons of Kenyon cells, which have dendrites in the lip region of the calyx and receive olfactory signals (Farris et al., 2004). This similarity may indicate that the basic pattern of projection of Kenyon cells from the calyx to the vertical lobe is conserved between honey bees and ants.

Processing pathways for alarm pheromone signals in the ant brain

We propose a general scheme for processing pathways for alarm pheromone signals in the brain (Fig. 14), on the basis of observations of 63 alarm pheromone-sensitive PR neurons. In this scheme, we have incorporated our proposal on the basic organization of the cockroach brain, to facilitate functional interpretation of the pheromone-processing pathways. In cockroaches, we have proposed that brain areas can be classified into primary sensory areas, association areas, and premotor areas: sensory areas are defined as areas that receive sensory afferents, premotor areas are areas from which descending neurons originate to supply thoracic ganglia, and association areas, which include the MB and the lateral horn, are areas that intervene between sensory and premotor areas (Okada et al., 2003).

In this scheme, we suggest two parallel sensorimotor pathways for pheromone processing. In the first pathway, the lateral horn intervenes between the sensory area and premotor/motor areas; in the second pathway, the MB intervenes between them. The first pathway in which the lateral horn intervenes is implicated in some stereotyped behaviors in fruit flies (Heimbeck et al., 2001; Suh et al.,

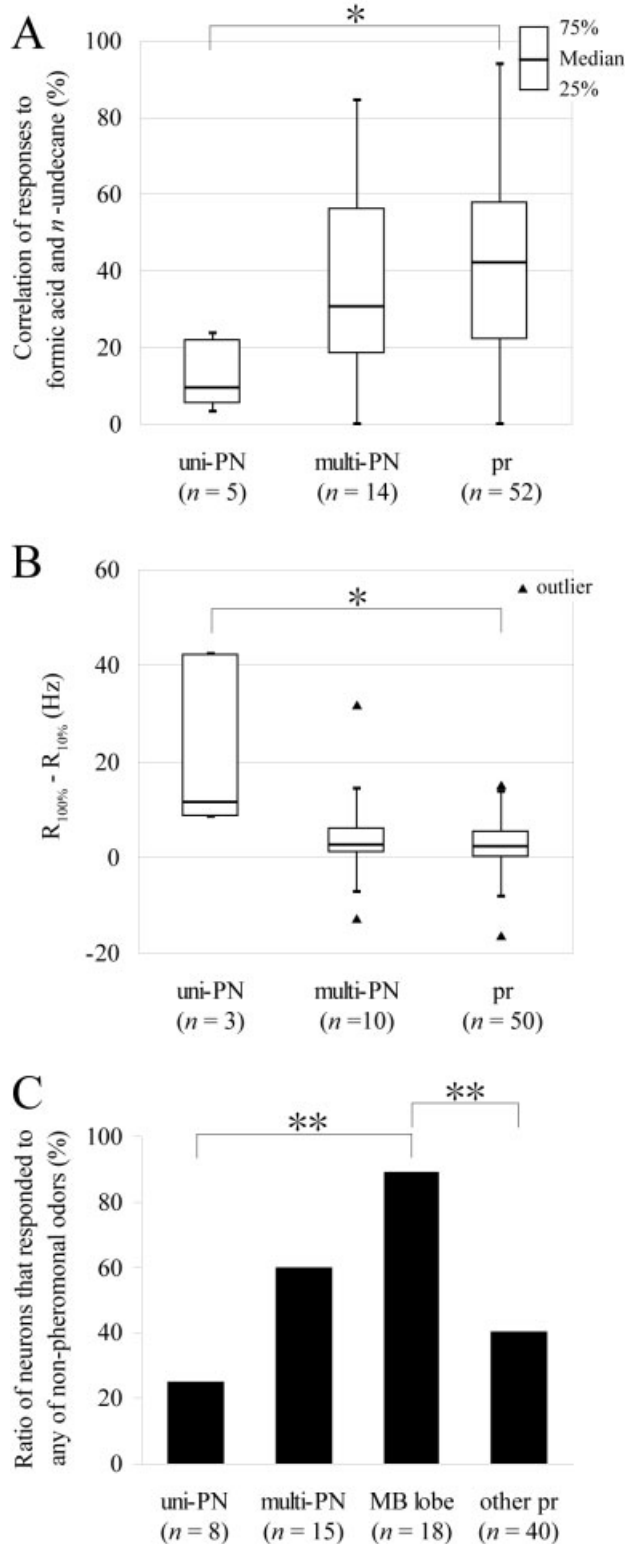


Fig. 13. Comparison of responses of pheromone-sensitive projections neurons (PNs) and protocerebrum (PR, pr) neurons. **A**: Correlation between the response to formic acid and that to *n*-undecane of uni-PNs, multi-PNs, and four types of PR neurons (pedunculus efferent neurons, lobe efferent neurons, MB feedback neurons, and medial PR efferent neurons). Correlation coefficients of spike train data for a 2-second period after formic acid stimulation and that after *n*-undecane stimulation are shown as box-plots with the median and interquartiles, as a measure of similarity in the responses. Whiskers are 1.5 times of the box lengths, or simply maximum or minimum of variances. Correlation between the response to formic acid and that to *n*-undecane in uni-PNs ($13 \pm 9\%$: mean \pm SD) was significantly lower than that in the four types of PR neurons ($42 \pm 24\%$) but did not significantly differ from that in multi-PNs ($36 \pm 25\%$). **B**: Effects of 10-fold change in concentration of formic acid or *n*-undecane on magnitude of the response. The increase in response magnitude for a 10-fold increase in concentration in uni-PNs (20.8 ± 18.8 Hz: mean \pm SD) was significantly greater than that in the four types of PR neurons (3.0 ± 5.4 Hz) but did not significantly differ from that in multi-PNs (4.9 ± 10.2 Hz). **C**: Ratio (%) of pheromone-sensitive neurons that also responded to non-pheromonal odors for uni-PNs, multi-PNs, efferent neurons of the lobe, and three other types of PR neurons. The ratio in efferent neurons of the mushroom body (MB) lobes (89%) was significantly greater than in uni-PNs (25%) or the other three types of PR neurons (40%). *, $P < 0.05$; **, $P < 0.01$, Steel-Dwass test.

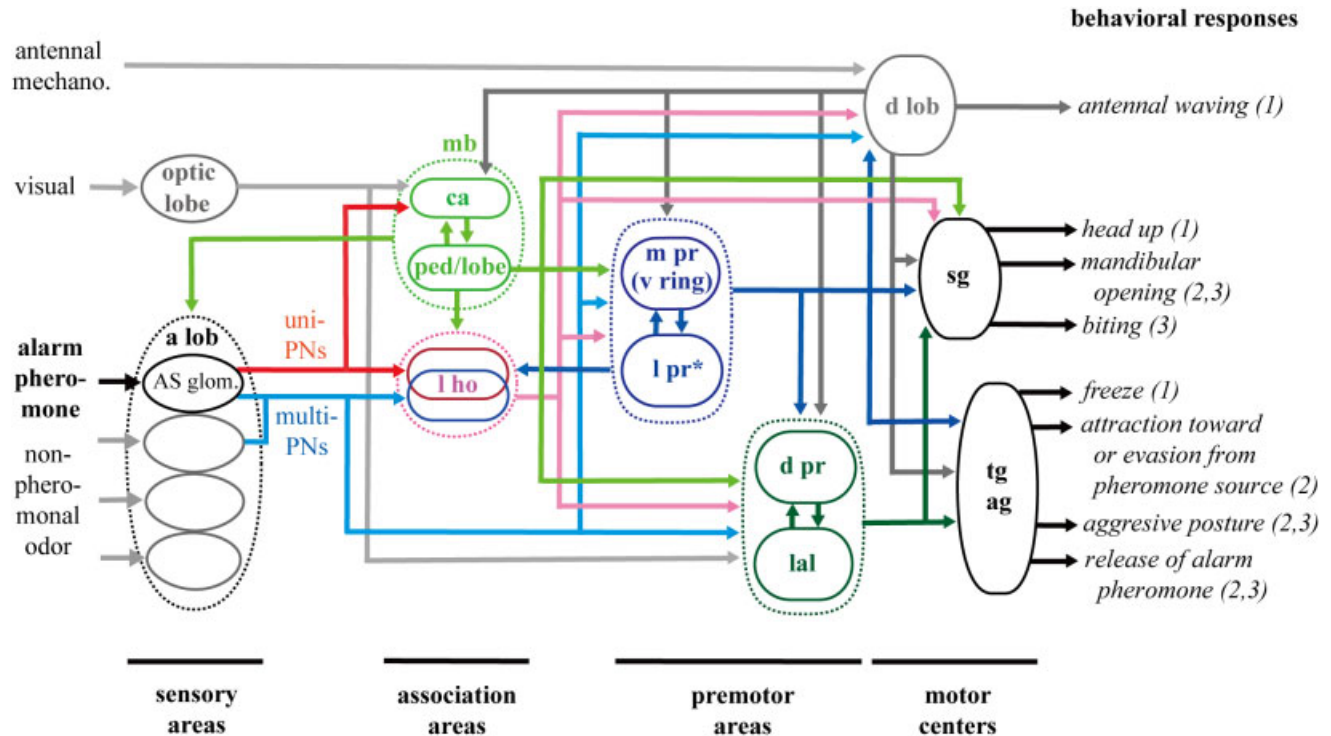


Fig. 14. Schematic model of processing pathways for alarm pheromone signals in the ant brain. The pathways are based on observations of 63 pheromone-sensitive neurons. Visual pathways to the dorsal protocerebrum (PR, pr) and to the lateral accessory lobe and mechanosensory pathway to the dorsal lobe are based on our unpublished observations. ag, abdominal ganglia; AS glom, alarm pheromone-sensitive glomeruli; PN, projection neuron; sg, subesophageal ganglion; tg, thoracic ganglia; v ring, ring neuropil around the

vertical lobe. For other abbreviations, see legend of Figure 1. *l pr in this figure indicates the lateral PR excluding the lateral horn. The distinction of sensory, association, premotor, areas is based on studies of basic organizations of the cockroach brain (Okada et al., 2003). To facilitate interpretation of data, we classified behavioral responses into 1) initial preparatory phase of alarm behavior; 2) alarm behavior; and 3) aggression against a potential enemy, as reviewed by Blum (1985) and Vander Meer and Alonso (1998).

2004). In contrast, the MB and its downstream PR areas are supposed to underlie behavioral plasticity, learning and memory, and the processing of contextual signals (Li and Strausfeld, 1999; Heisenberg, 1998, 2003). We propose that the alarm behavior, which is more or less stereotyped, is mediated via the first pathway, whereas aggressive behavior, which is controlled by contextual information like the presence of alarm pheromones (Blum, 1985) and in which learning and memory play important roles (Yurkovic et al., 2006; in fruit flies), is controlled by the second pathway involving the MB. To elucidate the validity of our proposal, further sophisticated analyses of responses of PR neurons are necessary.

Another interesting question is whether alarm pheromone-sensitive neurons occupy specific areas within these neuropils to form "alarm pheromone foci." In cockroaches (Boeckh and Ernst, 1987; Nishino et al., 2003) and moths (Kanzaki et al., 2003), two types of PNs, namely, macroglomerular PNs, which originate from the macroglomerulus and convey signals for female sex-pheromone, and uni-PNs, which originate from ordinary glomeruli and convey signals for general environmental odors, occupy specific areas in the lateral horn. Comparison of termination areas of alarm pheromone-sensitive and -insensitive PNs in the lateral horn, and also in the calyx of the MB and the medial PR, is needed to address this issue.

The ring neuropil around the vertical lobe is subdivided into medial and lateral halves according to the distribu-

tion of dendrites and terminal arborizations of pheromone-sensitive PR neurons. Terminal arborizations of multi-PNs and efferent neurons of the MB pedunculus innervated only the lateral half, and dendrites of wide-field PR neurons with the vertical lobe innervated only the medial half. On the other hand, terminal arborizations of the Pe1-like neuron, efferent neurons of the MB lobe, and dendrites of medial PR efferent neurons occupied both halves. Because this neuropil is a major downstream target of efferent neurons of the MB, it is tempting to speculate that different aspects of signals processed in the MB are sent to different subdivisions of this neuropil and are further transmitted to downstream premotor areas.

We suggest that wide-field PR neurons with dendrites in the vertical lobe, lateral PR, and medial PR are the best candidates for control of aggression behavior against a potential enemy. This is because 1) multisensory integration, which is essential for the control of aggressive behavior, is prominent in efferent neurons of the vertical lobe, including this type of neuron; 2) this type of neuron also responds to non-pheromonal odors; and 3) this type of neuron possesses dendrites in all three major pheromone-processing areas in the PR, sends axons to various premotor and motor areas, and thus may be suited for making a decision about initiation and maintenance of particular behavior.

One type of pheromone-sensitive descending PR neuron exhibited long-lasting activity to alarm pheromone stim-

ulation, as reported for sex-pheromone-sensitive descending PR neurons of the silkworm (Kanzaki et al., 1994). In the silkworm, descending neurons from the lateral accessory lobe exhibited long-lasting activity after sex pheromone stimulus, and it has been suggested that these neurons participate in the control of orientation behavior toward the source of sex pheromone (Kanzaki et al., 1994). Therefore, alarm pheromone-sensitive descending neurons may convey signals for the control of orientation behavior toward or away from the sources of pheromone.

Future perspective

Communication by means of pheromones is highly sophisticated in social insects, and the present study provides a starting point toward elucidating brain mechanisms underlying pheromone communication in ants. Future studies should include analysis of multisensory integration by wide-field PR neurons, which may play essential roles in the control of aggression behavior. Pheromone communication is widespread across animal phyla, and studies of neural mechanisms underlying pheromonal communication by ants will provide hints for understanding brain mechanisms involved in the control of social behavior of other groups of animals, including humans.

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