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## Pheromone communication and the mushroom body of the ant, *Camponotus obscuripes* (Hymenoptera: Formicidae)

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**Abstract** Communication by means of pheromones plays predominant roles in colony integration by social insects. However, almost nothing is known about pheromone processing in the brains of social insects. In this study, we successfully applied intracellular recording and staining techniques to anatomically and physiologically characterize brain neurons of the ant *Camponotus obscuripes*. We identified 42 protocerebral neurons that responded to undecane and/or formic acid, components of alarm pheromones that evoke attraction or evasive behavior, respectively. Notably, 30 (71%) of these neurons were efferent (output) or feedback neurons of the mushroom body, and many of these exhibited different responses to formic acid and undecane. Eight of the remaining 12 neurons had arborizations in the lateral and/or medial protocerebrum, which receive terminations of efferent neurons of the mushroom body and from which premotor descending neurons originate. The remaining four neurons were bilateral neurons that connect lateral accessory lobes or dorsal protocerebrums of both hemispheres. We suggest that the mushroom body of the ant participates in the processing of alarm pheromones. Seventeen (40%) of 42 neurons exhibited responses to nonpheromonal odors, indicating that the pheromonal and nonpheromonal signals are not fully segregated when they are processed in the

protocerebrum. This may be related to modulatory functions of alarm pheromones, i.e., they change alertness of the ant and change responses to a variety of sensory stimuli.

### Introduction

The impressive diversity and ecological dominance of ant societies are in large part due to their efficient social organization and the underlying communication system (Hölldobler 1995). Most communication systems in social insects are based on pheromones, which play a central role in the organization of insect societies. Hence, the study of neural mechanisms of pheromone processing is an important step toward understanding brain mechanisms for colony integration in social insects.

However, there have been few studies on pheromonal processing in the brains of social insects (Galizia et al. 1999; Müller et al. 2002). In ants, the study of the brain has been largely restricted to histology because of its tiny size (Gronenberg 2001), and electrophysiological studies are restricted to visual neurons in the optic lobe (Labhart 2000). This study is the first to anatomically and physiologically characterize neurons in the protocerebrum (PR) of the ant and is the first to examine central processing pathways for alarm pheromone information.

### Methods

Workers of ants, *Camponotus obscuripes*, were used in this study. Each ant was anesthetized and then fixed on a chamber with wax, and the frontal surface of the brain was exposed. Using a conventional electrophysiological setup, a borosilicate glass electrode was inserted into the PR (Nishino et al. 2003). At the end of recording, the neuron was filled with lucifer yellow by applying a hyperpolarizing current. Brains were fixed in 4% formaldehyde overnight at 4°C and then dissected out, rinsed in saline, dehydrated, and cleared in methyl salicylate. Each stained

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neuron was observed by using a confocal microscope (LSM-510).

An olfactory stimulation system originally developed for the cockroach (Nishino et al. 2003) was used. Stimuli used were: 99% formic acid, 99% *n*-undecane, 10% formic acid (dissolved in water), 10% *n*-undecane (dissolved in liquid paraffin), vanilla, peppermint, hexane, *n*-decyl acetate, and abdominal extract of the same species (dissolved in 50  $\mu$ l hexane for three individuals).

Spontaneous spike frequency was measured as the number of spikes in a 1-s period prior to odor presentation. The response to an odor was referred to as excitatory when the spike frequency during odor presentation was higher than the mean plus standard error (SE) of the spontaneous spike frequency. Relative response strength to an odor was measured as the spike frequency during the 1-s odor presentation minus that during the 1-s period prior to odor presentation. Relative strength of the response to formic acid averaged for all formic-acid-responding neurons ( $n=38$ ) was  $6.4 \pm 1.0$  Hz, and that to *n*-undecane averaged for all *n*-undecane-responding neurons ( $n=28$ ) was  $5.3 \pm 0.9$  Hz. The excitatory response to an odor of a neuron was referred to as strong if the mean minus SE of relative strength of the response to that odor was higher than the mean plus SE of relative response strength averaged for all neurons that responded to that odor. The excitatory response was referred to as weak if the mean plus SE of relative response strength was lower than the mean minus SE of relative response strength averaged for all neurons that responded to that odor. Response latency was defined as the time lag from the onset of odor delivery to an antenna to the onset of spike discharge. For statistical comparison, Wilcoxon test was used.

## Results

As a first step to clarify brain mechanisms of pheromone processing in the ant brain, we anatomically and physiologically characterized neurons in the PR of the brain of the

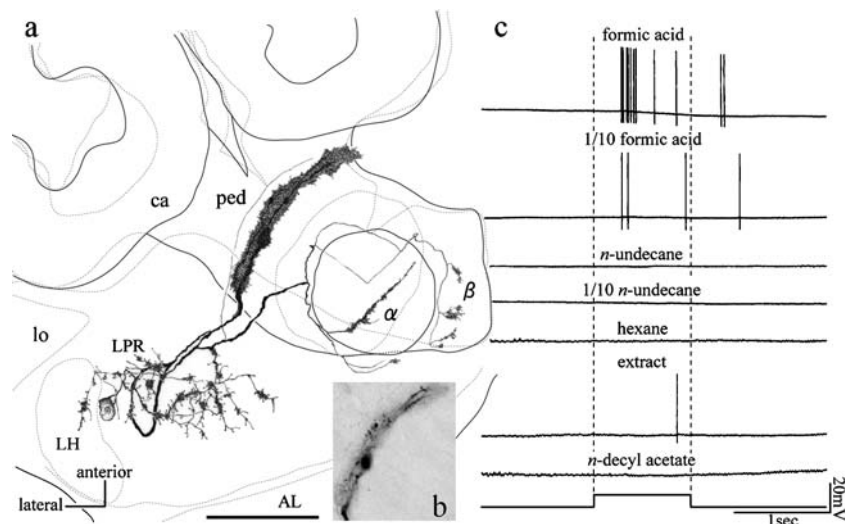
ant *Camponotus obscuripes*. The PR is the area in which sensory signals are integrated to form signals for the control of behavior (Strausfeld 1976). Our study focused on neurons that responded to formic acid and/or *n*-undecane, which are two kinds of alarm pheromones that evoke different behavioral responses, elusion and attraction, respectively (Fujiwara et al. 2004). Intracellular recordings and staining were successfully made for 88 PR neurons. Forty-two of these neurons responded to *n*-undecane and/or formic acid. Notably, 30 (71%) of these 42 neurons were efferent (output) or feedback neurons of the pedunculus or the lobes of the mushroom body (MB), a higher olfactory and associative center (Menzel 1999) (see Figs. 1, 2, 3). Eight of the remaining 12 neurons had arborizations in the lateral and/or medial PR, which receive terminations of efferent neurons of the MB (Li and Strausfeld 1999) and from which premotor descending neurons originate (Okada et al. 2003). The other four neurons were bilateral neurons, which connect the lateral accessory lobes or the dorsal PRs of both hemispheres, where descending premotor neurons give rise to the thoracic ganglion (Okada et al. 2003). Seventeen (40%) of 42 neurons exhibited responses to nonpheromonal odors. Therefore, the pheromonal and nonpheromonal signals are not necessarily fully segregated in the PR. However, the remaining 25 neurons responded specifically to pheromonal odor, at least among the odors we tested.

Here we confine our description to three examples of MB efferent neurons: details of anatomy and physiology of the remaining neurons will be reported elsewhere.

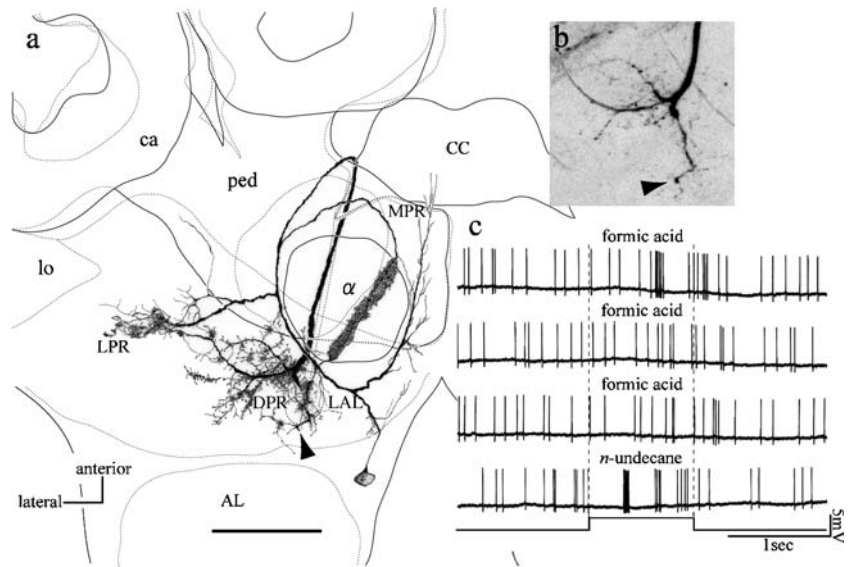
An efferent neuron of the pedunculus that responded to formic acid, but not to *n*-undecane

The soma of the neuron shown in Fig. 1a,b is located in the lateral PR. The neuron extends dense dendritic arborizations in the pedunculus of the MB. High-resolution confocal microscopic observation revealed a number of spines in the arborizations, suggestive of a postsynaptic

**Fig. 1** a–c An efferent neuron of the pedunculus. **a** Drawing from a stack of confocal sections viewed frontally. **b** Confocal sections of dendritic arborizations in the pedunculus. **c** Responses to alarm pheromones. Scale bar=100  $\mu$ m. *ca* calyces, *ped* pedunculus,  $\alpha$   $\alpha$  lobe,  $\beta$   $\beta$  lobe, *LPR* lateral protocerebrum, *LH* lateral horn, *lo* lobula, *AL* antennal lobe



**Fig. 2 a–c** An efferent neuron of the  $\alpha$  lobe. **a** Drawing from a stack of confocal sections viewed frontally. **b** Confocal slice sections of terminal arborizations in the dorsal protocerebrum. *Arrowheads* indicate an axon descending to the subesophageal ganglion (**a, b**). **c** Responses to alarm pheromones. Scale bar=100  $\mu$ m. *MPR* medial protocerebrum, *DPR* dorsal protocerebrum, *LAL* lateral accessory lobe, *CC* central complex



nature. The axon extends laterally and gives off sparse terminal arborizations with varicosities in the lateral PR and the  $\alpha$  lobe (or vertical lobe, Li and Strausfeld 1999) of the MB. Thus, this neuron appears to be a feedback as well as an output neuron of the MB.

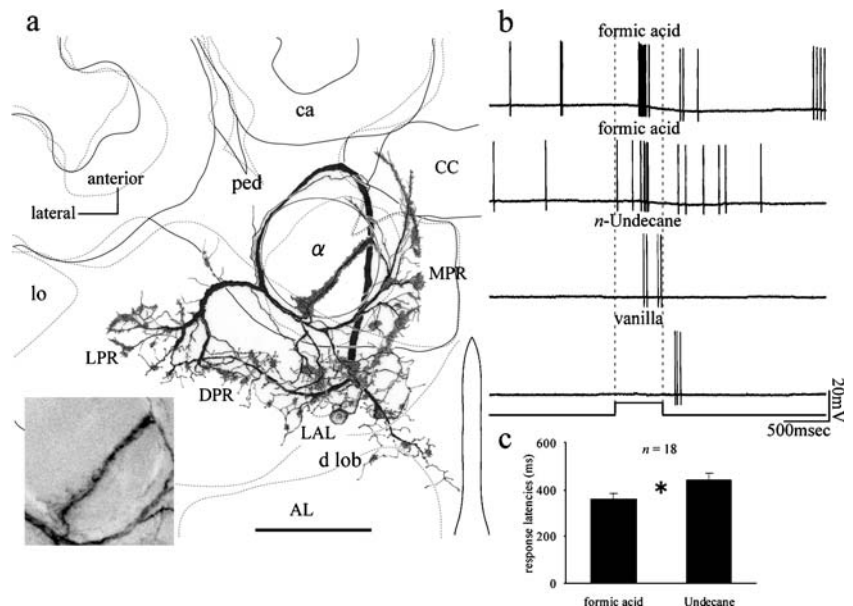
This neuron exhibited no spontaneous spike discharge (Fig. 1c). Formic acid (99%) evoked spike discharges of  $8.3 \pm 1.3$  Hz at  $305 \pm 12$  ms ( $n=3$ ) after the onset of stimulation, and 10% formic acid evoked weak excitatory responses of  $3.0 \pm 0$  Hz, with a similar latency ( $314 \pm 30$  ms,  $n=2$ ). This neuron exhibited no response to 99% *n*-undecane, 10% *n*-undecane, hexane, and *n*-decyl acetate. Abdominal extract evoked one spike in the first trial but not in any of the subsequent six trials. It was unclear whether these spikes represent incidental spontaneous spikes or a rapidly habituating response to formic acid or to other substances contained in abdominal glands.

An efferent neuron of the  $\alpha$  lobe that responded to *n*-undecane

The soma of the neuron shown in Fig. 2 is located at the boundary between the PR and the deutocerebrum. This neuron extends spiny dendrites with a layered structure in the  $\alpha$  lobe. Its dendrites also cover the medial PR, a part of the lateral PR, and the lateral accessory lobe (Fig. 2a). The axon takes a circuitous route around the pedunculus, leaves the PR, and descends toward the subesophageal ganglion (Fig. 2a,b; arrowheads). It sends off varicose terminal arborizations in the dorsal lobe of the deutocerebrum and the subesophageal ganglion. Perhaps this neuron integrates signals of the MB and lateral and medial PR, and it sends them to posterior parts of the brain and also to the subesophageal ganglion.

This neuron exhibited spontaneous spike activity of  $8.3 \pm 1.2$  Hz ( $n=3$ ). We observed a spike discharge to 99% formic

**Fig. 3 a–c** An efferent neuron of the  $\alpha$  lobe. **a** Drawing from a stack of confocal sections viewed frontally. (**a, inset**) Confocal slice sections of dendritic arborizations in the  $\alpha$  lobe. **b** Responses to alarm pheromones. **c** The average latencies of responses to formic acid and *n*-undecane in 18 MB efferent or feedback neurons that responded to both odors. The average latency for formic acid ( $362 \pm 23$  ms,  $n=18$ ) was shorter than that for *n*-undecane ( $441 \pm 30$  ms,  $n=18$ ). \* $P < 0.05$ , Wilcoxon test. Scale bar=100  $\mu$ m. *d lob*, dorsal lobe



acid in the first trial (12 Hz) but not in the subsequent four trials (Fig. 2c). Whether this spike burst was incidental, spontaneous spikes or rapidly habituating response was unclear. *n*-Undecane (99%) elicited strong excitatory responses of  $15.3 \pm 0.5$  Hz, with a latency of  $340 \pm 14$  ms ( $n=3$ ), and 10% undecane evoked weak responses ( $11.0 \pm 0$  Hz,) with a longer latency ( $765 \pm 15$  ms,  $n=2$ ). No prominent response to 10% formic acid, hexane, abdominal extract or *n*-decyl acetate was observed (not shown).

An efferent neuron of the  $\alpha$  lobe that responded to formic acid and weakly to *n*-undecane

The soma of the neuron shown in Fig. 3 is located at the boundary between the PR and the deutocerebrum. This neuron extends spiny dendrites in the  $\alpha$  lobe with a prominent layered structure (Fig. 3a, inset). It also gives off dendrites in the medial and lateral PR and the lateral accessory lobe (Fig. 3a). The axon extends dorsally and gives off terminal arborizations with varicosities in the lateral and dorsal PR and the dorsal lobe of the deutocerebrum. Perhaps this neuron integrates signals of the MB and lateral and medial PR and sends them to various parts of the brain.

This neuron exhibited irregular spontaneous spike activity of  $0.3 \pm 0.4$  Hz ( $n=8$ ) (Fig. 3b). Formic acid (99%) evoked excitatory responses of  $6.5 \pm 3.0$  Hz, with a latency of  $288 \pm 30$  ms ( $n=4$ ), and 99% *n*-undecane evoked weak excitatory responses ( $2.5 \pm 1.1$  Hz), with a latency of  $375 \pm 90$  ms ( $n=4$ ). Vanilla odor evoked weak responses ( $2.3 \pm 0.8$  Hz), with a latency of  $601 \pm 30$  ms ( $n=4$ ). It was not determined whether the response was an excitatory response with a long latency or a postinhibitory rebound response associated with the offset of the stimulus. Peppermint odor could not provoke any responses (not shown).

Latencies of responses to formic acid and *n*-undecane in MB efferent or feedback neurons

Eighteen MB efferent or feedback neurons exhibited responses both to formic acid and *n*-undecane. The average latency of responses of these neurons to formic acid ( $n=18$ ) was found to be significantly shorter than that to *n*-undecane ( $n=18$ ) ( $U=99$ ;  $P<0.05$ ) (Fig. 3c).

## Discussion

This study is the first to anatomically and physiologically characterize neurons in the PR of the ant brain. Although the processing of sex pheromones in the brains of nonsocial insects has been studied in considerable detail (Kanzaki et al. 2003), there have been few studies on the processing of nonsexual pheromones in the brains of social insects (Galizia et al. 1999; Müller et al. 2002). The only study in

which the activity of brain neurons in ants was examined is an optical recording study of calcium responses to alarm pheromones and nonpheromonal odors from the antennal lobe (primary olfactory center) of the deutocerebrum by Galizia et al. (1999).

Among 42 PR neurons that responded to formic acid and/or *n*-undecane in the ant brain, 30 (71%) were efferent (output) or feedback neurons of the MB. Many of these neurons exhibited different responses to formic acid and *n*-undecane: some neurons responded only to one of these substances (see Figs. 1 and 2), and other neurons responded differentially to formic acid and *n*-undecane with respect to spike frequency, response latency, or temporal patterns of spike activities (see Fig. 3). Evaluation of the average latencies of responses of 18 MB efferent or feedback neurons that responded both to formic acid and *n*-undecane showed that the latency of response to formic acid was significantly shorter than that to *n*-undecane (Fig. 3c). One of the roles of formic acid and *n*-undecane is to evoke each specific behavioral response, i.e., elusion and attraction (Fujiwara et al. 2004). Thus, we suggest that the MB participates in the processing of alarm pheromones, possibly leading to appropriate behavioral responses. The shorter response latency to formic acid may allow for rapid elusion.

We found that many (40%) of the pheromone-sensitive neurons of the PR, including efferent or feedback neurons of the MB, responded also to nonpheromonal odors. Galizia et al. (1999) observed that pheromonal and nonpheromonal odors evoked similar spatio-temporal patterns of calcium response in the antennal lobe, the primary olfactory center, of the ant *Camponotus rufipes*, and they argued that processing at a more central level is necessary to segregate pheromonal and nonpheromonal signals. Similar observations were made in honeybees at the level of projection neurons from the antennal lobe to the lateral horn and the MB of the PR. Müller et al. (2002) observed that projection neurons that responded to alarm pheromones also responded to a variety of nonpheromonal odors in honey bees. We showed that pheromonal and nonpheromonal signals in ants are not fully separated into different neurons even at the level of the PR, including the MB. This is not surprising considering the second behavioral role of alarm pheromones, namely, to change the alertness of ants and to change responses to a variety of sensory stimuli evoked by potential enemy. Li and Strausfeld (1999) observed that efferent neurons of the MB of the cockroach respond to multisensory stimuli and argued that MBs may play roles in the evaluation of general olfactory signals in multisensory context. In agreement with this suggestion, we observed that some pheromone-sensitive efferent neurons of the ant MB responded not only to nonpheromonal odors but also to visual and mechanosensory stimuli (data not shown). Therefore, PR neurons that respond both to pheromonal and nonpheromonal odors, especially those in the MB, may be related to modulatory functions of alarm pheromones.

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