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Processing of sting pheromone and its components in the antennal lobe of the worker honeybee

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ABSTRACT

In the honeybee *Apis mellifera*, a sting pheromone produced by sting glands plays an important role in coordinating defensive behavior. This pheromone is a blend constituted by several components. Little is known about the neural substrates underlying sting pheromone processing in the bee brain. Here, we investigated the neural activity elicited by eight components (five acetates and three alcohols) of the sting pheromone, and by real bee stings at the level of the antennal lobe (AL) of worker honeybees. We used *in vivo* calcium imaging to record odor-induced neural activity of 22 identified glomeruli in the AL. We found that acetates mainly activated medial glomeruli while alcohols mainly activated lateral dorsal glomeruli. The sting preparation evoked a glomerular pattern that was clearly distinct from those of individual pheromone components. No particular region of the imaged AL was found to process sting pheromone or any of its components. Further analyses in a putative honeybee olfactory space showed that the neural activity elicited by sting preparation cannot be linearly predicted by those of pheromone components and that such components are not clearly separated from non-sting pheromone odors. We conclude that sting pheromone is processed in the worker honeybee AL following the same principles of general odors so that the chemical structure of odorants is the main determinant of glomerular activation, rather than their pheromonal values. We cannot exclude, however, that the distinctness of sting-pheromone representation with respect to that of its components constitutes a form of specialized neural processing strategy for this kind of substance.

1. Introduction

An important behavioral characteristic of social insects is the defense of the colony upon disturbance by intruders or potential enemies. Alarm pheromone communication among nestmates is critical for effectively organizing such a defense. The defensive behavior has been well studied in the honeybee *Apis mellifera* (Breed et al., 2004; Hunt, 2007). Typically, when guard bees are disturbed near the hive, they exhibit a characteristic behavior in which they raise their abdomen, open the sting chamber and protrude the stinger. Sting pheromone, the most important pheromone in releasing bee defensive behavior, is secreted by the sting sheath glands and the Koschewnikow gland (Cassier et al., 1994; Lensky et al., 1995), and it actually consists of more than 20 different components, primarily acetates and alcohols of low molecular weight (Blum et al., 1978; Pickett et al., 1982; Free, 1987). The first one to be identified was isopentyl acetate (also called isoamyl acetate), a component that is active in recruiting other bees to defensive activities (Boch et al., 1962). Similarly, many of the sting pheromone components are active in inducing alarm responses in bioassays (Collins and Blum, 1982; Free, 1987; Wager and Breed, 2000).

In spite of extensive studies on the behavioral effects of alarm pheromone, we know little about how it is processed in the honeybee brain. Dedicated olfactory structures exist in the brain of some insect species for the processing of certain pheromones, in particular sex pheromones (Masson and Mustaparta, 1990; Hildebrand and Shepherd, 1997; Sandoz, 2006). Some of the sting pheromone components have been tested in optical imaging studies performed at the level of the honeybee antennal lobe (AL), the primary olfactory center in the insect brain. The AL constitutes the invertebrate pendant of the olfactory bulb of vertebrates. Both structures present remarkable anatomical and functional parallels such as the fact that they are both made up from glomeruli, which are the functional units allowing the central coding of odorants. In the honeybee AL, 160 glomeruli can be found and odorants are

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encoded in terms of a specific spatio-temporal pattern of glomerular activation (Joerges et al., 1997; Deisig et al., 2006). Although some sting pheromone components have been characterized in terms of their glomerular activation pattern (Galizia et al., 1999b; Sachse et al., 1999), further research is needed to provide an integral characterization of the AL response to sting pheromone components and to determine whether these substances are processed in a dedicated system (e.g., a common subset of glomeruli) or in an across-fiber pattern, as ordinary odors and other pheromones are. It would also be interesting to know how closely real sting pheromone can be mimicked by individual pheromone components in terms of neural representation in the brain. In other words, whether or not the neural representation of sting pheromone corresponds to the sum of the individual neural representations of the components or has synthetic properties that make it different from the simple sum of its components (the elemental approach vs. the configural approach; see Deisig et al., 2006).

To address these questions, we focused on the worker honeybee AL and used calcium imaging to investigate the neural processing of eight artificial sting pheromone components as well as real bee stings. A honeybee AL atlas is available, which makes it relatively easy to compare neural responses between individuals (Joerges et al., 1997; Galizia et al., 1999a). Neural activity patterns were obtained by recording the responses of 22 glomeruli on the anterior surface of the AL. Neural responses of these glomeruli have proved to be sufficient to account for perceptual and behavioral responses produced by individual honeybees when tested in odor generalization tasks (Guerrieri et al., 2005). The relationships between different pheromone components, and between sting pheromonal odors and non-sting pheromone odors, were further analyzed in a virtual odor space, which was constructed by performing a principal component analysis (PCA) on the physiological data.

2. Materials and methods

2.1. Honeybee preparation and staining with calcium dye

Guard or young foraging worker honeybees (*A. mellifera* L.) were captured at the entrance of outdoor hives. They were individually anesthetized on ice, and then secured by beeswax in a holding tube. A rectangular window was cut in the cuticle between the two compound eyes, and between the antennae and ocelli. The small piece of cuticle was removed, along with any gland, membrane and trachea covering the brain, to expose the ALs. The exposed brains were kept moist with bee saline (130 NaCl, 6 KCl, 4 $MgCl_2$, 5 $CaCl_2$, 160 sucrose, 25 glucose, 10 HEPES, in mM; pH 6.7, 500 m Osmol; all chemicals were from Sigma-Aldrich Pty. Ltd., Australia). For *in vivo* calcium imaging, we introduced in saline solution the calcium indicator Calcium-Green 2 (Molecular Probes, Eugene, OR, USA) in the form of cell-permeable acetoxymethyl (AM) esters. Calcium-Green 2 AM (50 μg) was firstly dissolved in 30 μL DMSO (Sigma-Aldrich) containing non-ionic detergent Pluronic F-127 (20% (w/v); Molecular Probes) which assisted the dispersion of the AM esters in aqueous medium; the stock solution was then diluted in saline solution to a final concentration of 130 μM. About 15 μL of the dye solution was introduced into the head capsule to stain the brain; the incubation normally lasted for 40–50 min at room temperature. After staining, the brains were washed in indicator-free saline solution to remove any dye that was non-specifically associated with the tissue surface, and then incubated for a further 20 min to allow complete de-esterification of intracellular AM esters.

2.2. Calcium image recording and odor delivery

Fluorescence images were recorded from the left AL. Before starting the recording, the abdomen was removed and the mouthparts secured with insect pins to avoid interferences from movements. The bees were then positioned under a 20 × (NA 0.5, WD 2.0 mm) water immersion lens of an epi-fluorescence microscope (Eclipse E600FN, Nikon Corp., Tokyo, Japan) equipped with the filter set Chroma Yellow GFP (Excitation 500 ± 10, Dichroic 515, Emission 535 ± 15, in nm; Chroma Tech. Corp., Brattleboro, VT, USA). Sequential fluorescence images were captured at five frames per second (each frame was exposed for 200 ms) by a 12-bit, cooled CCD camera (Photometrics HQ, Tucson, AZ, USA). Customised programming with image software V++ (Digital Optics Ltd., Auckland, New Zealand) was used for capturing the calcium images and analyzing the image data. Each frame in the captured image sequence was composed of 118 × 162 pixels after on-chip binning and trimming the edges, and each pixel corresponded to a 2.5 μm square area of the bee brain. During optical recording, the exposed brain was constantly perfused with oxygen-saturated saline to maintain vitality.

Eight artificial compounds present in bee sting pheromone and preparation of real bee stings were used as stimuli in image recording. Five compounds were acetates: benzyl acetate (BZA), butyl acetate (BTA), hexyl acetate (HXA), isopentyl acetate (IPA), and octyl acetate (OTA). Three compounds were alcohols: 1-butanol (1BL), 1-octanol (1OL), and 2-nonanol (2NL). Four non-sting pheromone odors were also tested in order to compare the relationship between the sting pheromonal odors and other odors, not involved in alarm behavior. The four odors are: citral (CIT), clove oil (CLV, >85% is Eugenol), geraniol (GER), and limonene (LIM), all of them are primarily found in plants. In addition, CIT and GER have also been identified as major components of the Nasonov pheromone, which acts as an attractant in a variety of behavioral contexts. All substances were purchased from Sigma-Aldrich in liquid form. They were diluted to 1:4 in mineral oil to obtain a concentration of 0.25 (v/v). In a preliminary experiment, 1OL and 2NL were not tested, and all other pheromone components were used at a concentration of 0.05 (v/v). Cartridges for delivering the odors were made from a 1 mL syringe containing a small piece of filter paper soaked with 3 μL of each substance. When the sting preparation was used as stimuli, the sting apparatuses of five guard or young forager bees were excised. Care was taken to remove all other tissues and glands, leaving only the stingers and their attached glands. These were placed on a piece of filter paper and slightly squeezed to allow the venom absorbed by the filter paper, and then immediately inserted into a syringe. As a control for the odor stimuli, pure mineral oil was delivered in the same way through a control cartridge. Throughout the recording, a constant stream of air (0.4 L/min), driven by pressure, was passed through a cartridge, and delivered to the antennae via a plastic nozzle (3.5 mm in diameter) which was placed at a distance of 1 cm from the antenna. The antenna was oriented toward the side and secured by beeswax and pins; thus allowing the air stream to blow onto the distal end of the left antenna, and not onto the saline solution between the head opening and the lens. A computer-controlled valve directed the air stream through either the odorant cartridge or the control cartridge, as desired. During the experiment, air was always blown through the control cartridge, except during the 1 s odor stimulation period when it was diverted to the odorant cartridge by the valve. All odors were sequentially tested in random order, with 4-min intervals in between. Constant air flow was maintained during the intervals to avoid any carryover effects between stimulations. After testing all odorants once, another random sequence containing all odors was tested again. Hence, for