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Morphological and developmental analysis of peripheral antennal chemosensory sensilla and central olfactory glomeruli in worker castes of *Camponotus compressus* (Fabricius, 1787)

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ABSTRACT

The antennal lobes of different castes of the ant species *Camponotus compressus* show a marked diversity in the organization of their olfactory glomeruli. Notably, there is a significant difference in the number and size of glomeruli between the reproductives and the workers and among the different worker castes. In this report, we investigate the notion that these caste-specific differences in glomerular number might be accounted for, at least in part, by the differences in numbers of olfactory sensilla that target the antennal lobe. For this, we examine the number of sensilla on the antennal flagella of all the individual castes of *C. compressus*. This analysis reveals a striking correlation between sensillar number and the number of antennal glomeruli in a given caste. As a first step in investigating the causal mechanisms that might give raise to this correlation, we carry out an initial characterization of olfactory system development in the minor workers of *C. compressus*. We analyze the temporal pattern of innervations of the developing antennal lobe by olfactory sensory neuron axons. We document the development of the olfactory glomeruli in the antennal lobe during this process, which occurs during early pupal stages. Our findings provide the basis for future manipulative developmental studies on the role of sensory afferent number in glomerular development of different castes within the same species.

1. Introduction

The olfactory systems of all insects studied to date are characterized by a similar basic organization. Peripheral olfactory sensory neurons (OSNs) are located within sensilla on the surface of the antennal flagella. These OSNs project through the antennal nerve and form a set of identified central antennal tracts to innervate distinct glomeruli in the antennal lobe, where they synapse with local interneurons (LNs) and projection neurons (PNs) (reviewed in Rodrigues and Hummel, 2008; Galizia and Rössler, 2009). The connectivity between the peripheral sensory neurons and their central target neurons in the antennal lobe has comparable structural and functional features in the different insect groups.

Social insects such as ants and bees manifest polymorphisms both in external features and in brain organization which are thought to relate to the specific tasks that they perform within the colony. Some of these aspects have been reported for the mushroom bodies and other neuropils in the ant brain (Gronenberg et al., 1996; Gronenberg and Hölldobler, 1999; Ehmer and Gronenberg, 2004; Brown et al., 2004; Kühn-Bühlmann and Wehner, 2006). Recently, caste-specific differences in organization of the antennal lobe have been reported for the ant species *Camponotus sericeus* and *Camponotus compressus* (Mysore et al., 2009), and these differences may be involved in the variations in the behavioral responses to various stimuli of which olfaction may be a source of major input, that have been reported for genus *Camponotus* (Hölldobler and Wilson, 1990). In *C. sericeus* and *C. compressus*, the number of central olfactory glomeruli was shown to be different for males, queens and workers. Strikingly, there were also significant differences in glomerular number in the three worker castes, the minor workers, medium workers and major workers (Mysore et al., 2009). Among these castes, the largest number of olfactory glomeruli was observed in the minor workers. In *C. sericeus*, minor workers possess 492 ± 6 antennal glomeruli while in *C. compressus*, minor workers have 501 ± 2. In both species, the major workers have the smallest number of glomeruli (344 ± 5 for *C. sericeus* and 408 ± 3 for *C. compressus*) and the medium workers of *C. compressus* possess an

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intermediate number of glomeruli (476 ± 4). Moreover, in minor workers of both the species, it was found that the antennal tracts T4 and T5 innervated a significantly larger number of glomeruli than in the other castes. These marked differences in glomerular number and tract organization in different worker castes are remarkable since all three worker types have a somewhat comparable morphology and importantly share the same genotype. What are the mechanisms that underlie these differences in neuronal architecture of the olfactory system in these worker castes?

In the three cases that have been studied, *Drosophila*, *Apis mellifera* and *Anopheles gambiae*, the overall glomerular number (Laissue et al., 1999; Galizia et al., 1999; Ghaninia et al., 2007) is thought to reflect the number olfactory receptor (OR) genes that are expressed in a given species (Clyne et al., 1999; Hill et al., 2002; Robertson and Wanner, 2006). Work carried out in *Drosophila* indicates that most OSNs express a single olfactory receptor (OR) and connect to a given glomerulus in the antennal lobe; hence the number of glomeruli corresponds roughly to the diversity of OR gene expression (reviewed by Vosshall and Stocker, 2007). If these findings also apply to the organization of olfactory system in *C. compressus*, minor workers might express a larger number of OR genes than medium workers, which in turn might express a larger number of OR genes than major workers. While this is, in principle, possible and would suggest fascinating gene regulation mechanisms, it is also possible that the number of expressed ORs is the same and other mechanisms for glomerular number control occur. These differences could lie in the differences in the number of OSN axons that project from the antenna to the antennal lobe in the three castes.

A recent study on *Camponotus japonicus*, examined the number of sensory neurons housed in different types of sensilla; *s. basiconica* are innervated by in > 130 OSNs, the *s. trichodea* have only 8–9, while the *s. trichodea curvata* have 50–60 sensory neurons (Nakanishi et al., 2009). We determined the number of chemosensory sensilla on the antennal flagella of the three worker castes using scanning electron microscopy. This revealed a significant correlation between the total number of sensilla and the total number of antennal lobe glomeruli. This correlation was also found for the three different types of sensilla analyzed in the present study, *s. basiconica*, *s. trichodea* and *s. trichodea curvata*, as well as for the number of sensilla on each of the 12 antennal segments in all three worker types. Different number of chemosensory sensilla on the antennal flagella implies different number of total sensory neurons and partially might influence the organization of the antennal lobe in ants.

To investigate the processes that give rise to this correlation between antennal sensillar number and antennal lobe glomeruli number, a study of the development of the olfactory system in the three worker castes is required. As a first step towards this end, we traced the projections of the OSNs from the antennal flagella to the antennal lobe during pupal metamorphosis in minor workers of *C. compressus*. Our findings document the formation of the projections from the OSNs into the antennal lobe and show that the axonal terminals of the OSNs prefigure the formation of the protoglomeruli. Moreover, they reveal a striking increase in the size of the antennal lobe that occurs once the OSN axons invade it. These findings support the notion that major morphological features of the antennal lobe are dependent on the innervating OSN axons and provide the basis for further investigation of mechanisms that determine glomerular numbers in these different worker castes.

2. Materials and methods

2.1. Insects

Mated queens of Camponotus compressus were collected from the fields in the vicinity of National Centre for Biological Sciences (TIFR), Bangalore, India [13°, 43′ N, 77°, 35′ E] during the month of March. They were kept in plastic containers and constantly maintained at 12/12 h photoperiod at 25 °C. They were fed with 20% sucrose solution and a diet prescribed by Bhatkar and Whitcomb (1970); an additional supplement of 10% honey water improved their survival and egg-laying. These colonies produced adults which were >99% (observation from 10 colonies for a year) minor workers.

When sensillar numbers were to be estimated, the worker castes were collected from the field between April and May, while the reproductives were collected outside the colony during March; but the ages of individuals are unknown.

2.2. Scanning electron microscopy

Individuals were classified into castes as described by Mysore et al. (2009). The size and structure of the antennal flagella was analyzed by scanning electron microscopy (SEM). Antennae ($n = 10$ for workers; $n = 6$ for males; $n = 6$ for queens) were ablated and fixed overnight at 4 °C in 4% paraformaldehyde (PFA – Electron Microscopy Sciences, Hatfield, PA.) in phosphate buffered saline (PBS). Samples were taken through an ascending series and stored in 100% methanol: they were air dried, mounted vertically using superglue and coated with a thin layer of gold on all sides for SEM (Leica 440i, Leica Microsystems, Germany). The samples were placed in the chamber, tilted 90° and were imaged across diameter. Each segment was subjected to 4 horizontal scans at an interval of 90°.

The scans were overlapped using clear landmarks to minimize errors in counting of the sensilla. Only *sensilla basiconica*, *sensilla trichodea* and *sensilla trichodea curvata* were analyzed from the worker antenna. These sensilla were described as chemosensory earlier by Fresneau (1979), Hashimoto (1990) and Renthal et al., (2003).

Differences between two groups of samples were computed using Student's unpaired *t*-test with 0.001 as the significance level. Data comprising more than two groups were compared by one-way ANOVA and Scheffe's post-hoc tests using Origin 6.0 software. At least 10 samples were used in each case.

2.3. Analyses of the developing olfactory sense organs

Pupal stages were determined as described by Ishii et al. (2005; Table S1). Briefly, cocoon- spinning larvae (L4s), pre-pupae and pupae were removed from the nest and incubated individually at 25 °C and 60% relative humidity. Pupae were aged to 40 h (~7% of pupal life), 75 h (~12% of pupal life) and 125 h (20% of pupal life) and removed from the pupal case. An incision was made along the axis of the antenna using a fine microsurgical knife and a few crystals of micro-ruby (Cat. No. D7162, Molecular Probes, Invitrogen Corporation, CA) were loaded on a fine capillary and applied on the incisions. Preparations were left in a dark and moist chamber for 8 h at room temperature. The antennae were fixed in 4% paraformaldehdye (PFA) overnight, washed extensively in PBS and mounted in 70% glycerol. Imaging of micro-ruby stained profiles was carried out using an Olympus FV 1000 confocal microscope (Olympus Corporation, Japan).

2.4. Developmental analysis of antennal lobes by anterograde tracing and immunohistochemistry

Timed pupae were removed from their case, the antenna ablated and a few crystals of micro-ruby loaded on a fine capillary applied on the cut end of the antenna; preparations were left in a humid