A Macroglomerulus in the Antennal Lobe of Leaf-cutting Ant Workers and its Possible Functional Significance

C.J. Kleineidam, M. Obermayer, W. Halbich and W. Rössler

Department of Behavioral Physiology and Sociobiology, Biozentrum, University of Würzburg, Germany

Correspondence to be sent to: Christoph J. Kleineidam, Department of Behavioral Physiology and Sociobiology, Biozentrum, University of Würzburg, Am Hubland, 97074 Würzburg, Germany. E-mail: kleineidam@biozentrum.uni-wuerzburg.de

Abstract

Ants have a well-developed olfactory system, and pheromone communication is essential for regulating social life within their colonies. We compared the organization of primary olfactory centers (antennal lobes, ALs) in the brain of two closely related species of leaf-cutting ants (*Atta vollenweideri*, *Atta sexdens*). Both species express a striking size polymorphism associated with polyethism. We discovered that the ALs of large workers contain a substantially enlarged glomerulus (macroglomerulus, MG) at the entrance of the antennal nerve. This is the first description of an MG in non-sexual individuals of an insect. The location of the MG is laterally reversed in the two species, and workers of different size express a disproportional allometry of glomerular volumes. While ALs of large workers contain an MG, glomeruli in small workers are all similar in size. We further compared electroantennogram (EAG) responses to two common trail pheromone components of leaf-cutting ants: 4-methylpyrrol-2-carboxylate and 2-ethyl-3,6-dimethylpyrazine. At high concentrations the ratio of the EAG signals to 2-ethyl-3,6-dimethylpyrazine versus 4-methylpyrrol-2-carboxylate was significantly smaller in *A. vollenweideri* compared with the ratio of EAG signals to the same two components in *A. sexdens*. The differences in EAG signals and the species specific MG location in large workers provide correlative evidence that the MG may be involved in the detection of the trail pheromone.

Key words: 3-D reconstruction, EAG, olfaction, phenotypic plasticity, trail-pheromone

Introduction

The sense of smell is the most prominent sensory modality for ants (Hölldobler and Wilson, 1990). Chemical communication with pheromones and recognition cues is used to identify colony members, to regulate reproduction in the colony or to alarm each other at danger. Furthermore, trail pheromones enable the colony to exploit new food sources in a very efficient way. Many ant species, including the leaf-cutting ants, recruit colony members to a food source by laying pheromone trails on their way back to the nest. Only minute amounts, in the range of picograms, are necessary to elicit trail following behavior. In leaf-cutting ants, massive recruitment leads to the establishment of impressively long and wide roadways (Moser, 1967; Cross *et al.*, 1979). In the present study we compare the olfactory system of two leaf-cutting ant species in search of specific adaptations to the detection of trail pheromones.

Leaf-cutting ants express an extraordinary size polymorphism associated with polyethism which results in a colony with different worker castes (Wilson, 1980a). Their huge colonies house small individuals (minor workers) which take care of the brood and the fungus, and which are up to 1000 times smaller than soldiers responsible for the nest defense. The symbiotic fungus represents the sole food source for the developing larvae. Foraging workers (major workers) are intermediate in size and responsible for collecting plant material. Trail following, the detection of suitable plants, and cutting and harvesting leaves represent the main tasks for major workers. Thus, only a subgroup of all colony members leaves the nest following pheromone trails, while others perform tasks that are less or not at all dependent on the trail pheromone.

The first identified trail pheromone in ants was methyl-4-methylpyrrole-2-carboxylate (M4MP2C), from the leaf-cutting ant *Atta texana* (Tumlinson *et al.*, 1971, 1972). The source of this major trail pheromone component is the poison gland (Moser and Blum, 1963; Blum and Wilson, 1964). Since then, a number of studies showed the importance of M4MP2C and additional fractions of the venom in other species of the genus *Atta* (Moser and Silverstein, 1967; Riley *et al.*, 1974; Evershed and Morgan, 1983). The major trail pheromone component of *A. vollenweideri* is

not yet known, but behavioral experiments currently done in our laboratory indicate that in this species M4MP2C is the major component too, and only minute amounts are sufficient to elicit trail following behavior (unpublished data). So far, only in *A. sexdens* a different major trail pheromone component (2-ethyl-3,6-dimethylpyrazine, 2E3,6DMP) was found, and in trail following bioassays a mixture of both M4MP2C and 2E3,6DMP proved to be more potent than the single components (Cross *et al.*, 1979; Billen *et al.*, 1992). Leaf-cutting ants do show interspecific trail following behavior, but the ratio of at least these two (main) components makes the trail species-specific (Robinson *et al.*, 1974; Billen *et al.*, 1992).

Ants receive odor information by activation of olfactory receptor neurons (ORNs) located in antennal sensilla. In different castes of *A. texana* this sensory input was measured using electroantennogram (EAG) recordings, suggesting that ORNs in queens, workers and males were sensitive to the major pheromone component (Andryszak *et al.*, 1990).

In insects, axons of ORNs project to the antennal lobe (AL) and terminate in different olfactory glomeruli according to their odor specificity (for reviews, see Homberg *et al.*, 1989; Mustaparta, 1990; Hildebrand and Shepherd, 1997; Hansson and Anton, 2000). Thus, excitation of ORNs results in a spatial representation of different odors by activation of subsets of glomeruli. In insects, this was demonstrated for the first time in the honey bee using functional imaging (Joerges *et al.*, 1997).

In the present study, we investigated the comparative neuroanatomy of the olfactory system of two closely related leaf-cutting ant species (*A. vollenweideri* and *A. sexdens*). In addition to the interspecific comparison, the pronounced size polymorphism within each species allowed an intraspecific comparison of individuals of different sizes to ask the question whether phenotypic plasticity in the central olfactory pathway may underlie caste-specific differences in olfactory guided behavior. To estimate the relative number of receptor neurons sensitive to two species-specific trail-pheromone components, we compared ORN responses in both species to these components by electroantennogram measurements (EAG). We finally correlated the variance in AL morphology with the differences found in the EAG signals. The results are discussed as possible species-specific and intraspecific adaptations of the olfactory pathway for processing of trail pheromone information.

Materials and methods

Animals

Colonies of *A. sexdens rubropilosa* (Forel) and *A. vollenweideri* (Forel) were reared at the Biozentrum, University of Würzburg, in an environmental chamber at 25°C and 50% relative humidity in a 12 h/12 h photoperiod and fed mainly with privet leaves (*Ligustrum vulgaris*) and dog rose (*Rosa canina*). The colony of *A. sexdens rubropilosa* was collected in 1993 in Botucatú, São Paulo, Brazil (by L.C. Forti), and the colony of *A. vollenweideri* was collected in 2002 in El Bagual, Formosa, Argentina (by M. Bolazzi and O. Geissler). At the time of the experiments the fungus garden in both colonies occupied a volume of ~12 l in six plastic boxes (19×19×9 cm) interconnected with plastic tubes.

Neuroanatomical procedures

Workers of different sizes were collected on the foraging trails and from the fungus garden. Animals were anaesthetized with CO_2 and decapitated. In large workers, brains were dissected in ice-cold ant-Ringer solution (127 mM NaCl, 7 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.8 mM TES, 3.2 mM trehalose, pH 7.0). After dissection, brains were immediately transferred in cold 4% formaldehyde in phosphate-buffered saline (PBS, pH 7.2) and stored overnight at 4°C. Brains were rinsed in PBS (4× 10 min), embedded in 5% low-melting-point agarose (agarose type II, no. 210-815; Amreso Inc., Solon, OH), sectioned horizontally at 150 μm with a vibrating Microtome (Leica VT 1000S, Wetzlar, Germany) and rinsed in PBS containing 0.2% Triton X-100 (3 × 10 min). Free-floating agarose sections were pre-incubated in PBS with 0.2% Triton X-100 and 2% normal goat serum (NGS; ICN Biomedicals, no. 191356, Orsay, France) for 1 h at room temperature. To label neuronal filamentous actin (f-actin), which was shown to be aggregated in olfactory glomeruli, sections were incubated in 0.2 units of Alexa Fluor 488 phalloidin (Molecular Probes, A-12379, Leiden, The Netherlands) in PBS for 2 days at 4°C (for further details, see Rössler *et al.*, 2002). To label cell nuclei, sections were incubated for 15 min in 25 μg/ml propidium iodide (Molecular Probes, A-11003) in PBS with 0.2% Triton X-100 at room temperature. Sections were finally rinsed in at least five changes of PBS, transferred into 60% glycerol/PBS for 30 min and mounted on slides in 80% glycerol/PBS. In small workers (<1.4 mm head diameter), the brain was too small for normal dissection and heads were treated as whole mounts. A small window was cut in the head capsule to ease diffusion of different liquids. Brains were fixed, rinsed and preincubated as described above and finally incubated in 0.2 units of Alexa Fluor 488 phalloidin for 3 days. After two rinses in PBS, brains were post-fixed for 1 h in 4% formaldehyde in PBS, rinsed in two changes of PBS, dehydrated in an ascending series of acetone (30, 50, 70, 90, 2 × 100%; 5 min each on ice), and finally transferred and embedded in Spurrs medium. After polymerization overnight at 65°C, preparations were sectioned at 17.5 μm and mounted in Entellan on polylysine-coated slides.

Laser-scanning confocal microscopy and image processing

Preparations were viewed with a laser-scanning confocal microscope (Leica TCS SP) with a 20 × 0.7NA lens (Leica,

imm. HC PL APO). Optical sections were imaged at intervals of 1 5 μm using appropriate pinhole settings. In double-labeled preparations, the two channels were merged with the use of pseudo-colors. Image processing was performed with the following software: Zeiss Image Browser (Carl Zeiss GmbH, Jena, Germany), Corel Photo-Paint and CorelDRAW (Corel Corporation, Ottawa, Ontario, Canada). Three-dimensional reconstructions and measurements were carried out with AMIRA software (Indeed-Visual Concepts GmbH, Berlin, Germany).

Electroantennograms

For EAG experiments, workers were collected only from the feeding site. Therefore, only foragers were investigated. According to their body size, all investigated workers were large workers (head width > 1.4 mm). For EAG recordings an isolated antenna was placed on two differential electrodes. The antenna was cut at the scapus and opened at the last flagellar segment with a small razor blade. Electrical contact to both ends of the antenna was improved using an electrode gel (Spectra 360, Parker, NJ). The EAG recording system consisted of a preamplifier (Universal AC/DC probe 10×) with a data acquisition interface (IDAC-USB) and a data capture software (Autospike 3.0), all from Syntech (Hilversum, The Netherlands). Data were captured at a sampling rate of 1058.2 and band-pass filtered at 0.1 20 Hz. The recordings were slope corrected, aligned, and the signal was quantified using the software Labview 6 (NI, TX). The DC amplitude during the first second after stimulus onset was measured. Only recordings in which the amplitude of the signal to each of both odors was at least 25% larger than to the control stimulus (pure hexane) were used for further analysis. Since signal amplitudes were highly variable between different antennae, we normalized the response by calculating the ratio of the responses to the two pheromone components used. This ratio of response was then pooled for all individual antennae of both species. Statistical analyses were performed with the software Statistica 6.1 (StatSoft, OK).

Stimulus delivery

A continuous air flow of 1 l/min was adjusted by a flow controller (Thomafluid KDM 65, Reichelt, Heidelberg, Germany), humidified in a wash bottle, passed through a solenoid valve (LFAA1201618H, Lee Co., CT) and blown over the preparation through an empty 1 ml syringe. The solenoid, which was controlled by a pulse generator (Master 8, A.M.P.I., Jerusalem, Israel), allowed switching the airflow for 300 ms through a bypass syringe equipped with an odor-loaded filter paper (5 mm × 25 mm). The filter paper was loaded with 10 μl of hexane containing one of two different pheromone components. The following two pheromone components were used: 2-ethyl-3,5-dimethylpyrazine and its isomer 2-ethyl-3,6-dimethylpyrazine as a 1:1 mixture (2E3DMP; in contrast to the single component 2E3,6DMP; Oxford Chemicals, UK) and M4MP2C (Sigma-Aldrich, USA). In a pilot experiment, dose response curves with various dilutions were used to establish the adequate stimulus dosage for a saturated EAG signal (example shown in Figure 2A). Loading 14.45 μg of 2E3DMP and 2.45 μg M4MP2C, respectively, onto the filter paper resulted in saturated EAG signals in both species. For comparison between the two species (*A. vollenweideri* and *A. sexdens*), these two dosages were used in order to excite all specific receptor neurons to a maximal extent. We used this approach to minimize possible species-specific differences in odor sensitivity of the receptor neurons and also because the variance of the EAG signal is lower at high stimulus intensities compared with medium-range stimulus intensities. However, since we compared the ratio of response to both components, stimulus intensity is not of paramount importance as long as the receptor neurons of both species have similar sensitivities to each of the two components. The two investigated species are closely related, and we therefore assume similar sensitivities of the receptor neurons to the two pheromone components.

The pheromone compounds in the two hexane dilutions used for the comparative study were quantified by capillary GC after the experiments. GC analyses were performed on a HP 5890 II gas chromatography (GC; Hewlett-Packard, Avondale, PA) with a flame ionization detector (FID) and a cool on column injection using hydrogen as carrier gas. The GC conditions were as follows: DB-1 column (30 m × 0.32 mm i.d., Agilent J&W Scientific, Palo Alto, CA); temperature program, 40°C for 2 min isotherm, then 3°C/min to 180°C, hold for 2 min isotherm, then 40°C/min to 300°C, hold for 10 min isotherm. As external standards a dilution series with 2E3DMP was used and plotted against the manual integrated peak area of the test dilutions.

Results

Glomerular organization in the antennal lobes

The ALs, the primary olfactory centers in the brain of workers of *Atta vollenweideri* and *Atta sexdens*, occupy a substantial proportion of the brain (Figure 1A,B). This is especially evident in small workers. Here, already visual inspection indicates that the fraction occupied by the ALs is relatively bigger than in large workers (Figure 1A,B and inset). Phalloidin labeling revealed the organization of the AL neuropil in distinct spheroidal glomeruli (Figure 1C,D), indicating a high concentration of f-actin in the synaptic neuropil as was previously found in other species of insects and in vertebrates (Rössler *et al.*, 2002). Individual glomeruli were easy to distinguish in optical sections. In both species the glomeruli were predominantly located in a peripheral layer of two to three glomeruli surrounding a central neuropil which was not labeled with phalloidin. A similar arrangement of glomeruli was also found in the AL of small workers

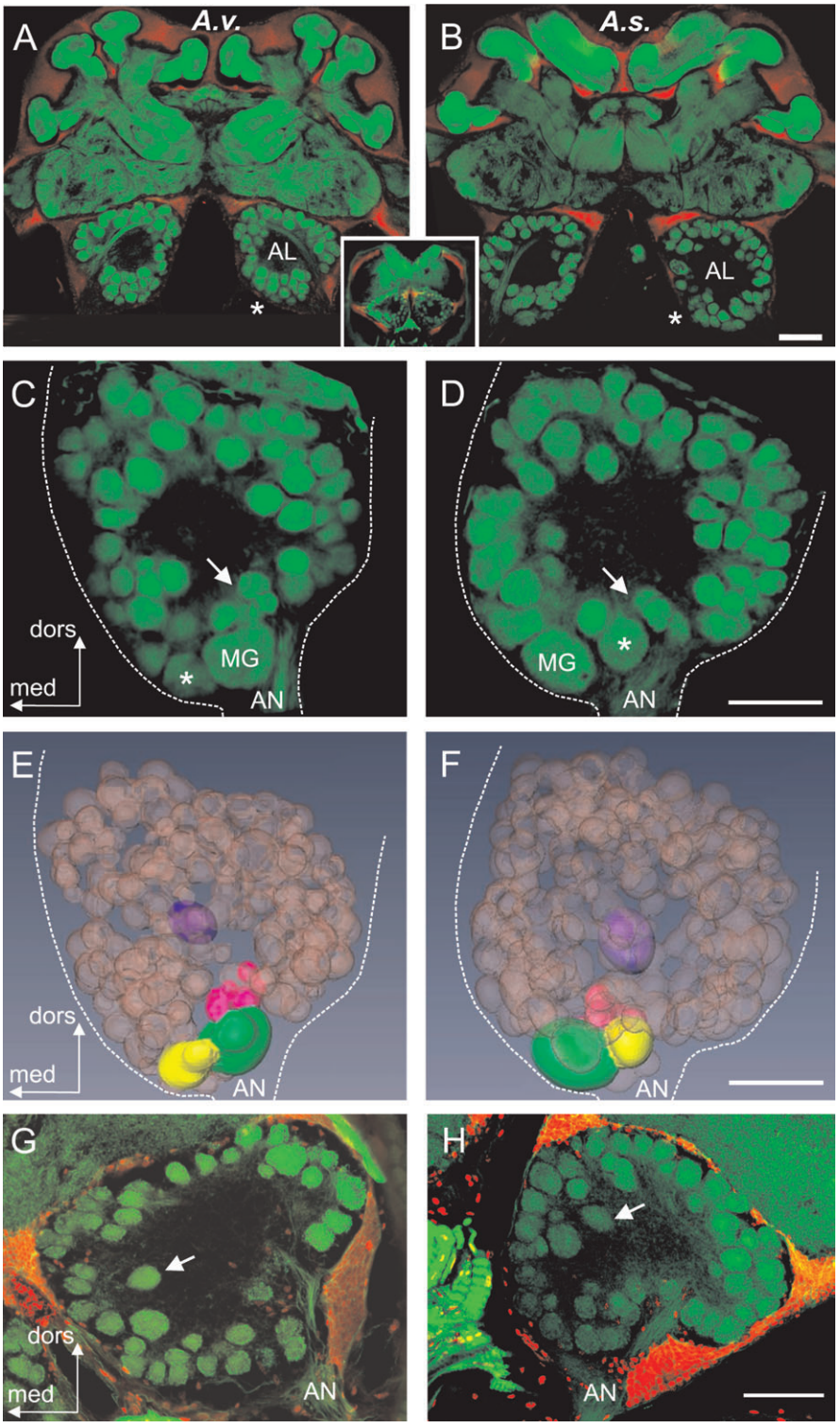

Figure 1 Comparison of the brains and antennal lobes (AL) in large and small workers of *A. vollenweideri* (*A. v.*, left column) and *A. sexdens* (*A. s.*, right column). Laser scanning confocal images of immunofluorescently labeled brains (phalloidin in green and propidium iodide in red). **(A, B)** Overview of the brains of large workers of *A. v.* and *A. s.* and comparison with the brain of a small worker of *A. s.* (inset in the middle). Single optical sections at a central focal plane of the brain in all cases. The asterisks in A and B indicate the left ALs shown at higher magnification in C and D. **(C, D)** High magnification views of the left AL in *A. v.* (C) and *A. s.* (D). Frontal optical sections showing a macroglomerulus (MG) at the entrance of the antennal nerve (AN) and the satellite glomeruli (indicated by asterisks and arrows in C and D). **(E, F)** 3 D reconstructions of the complete sets of glomeruli in the ALs of both species (MG in green, large satellite glomerulus in yellow, small satellite glomeruli in red, and central glomerulus in blue). The general arrangement of glomeruli at the entrance of the AN is similar in both species, but the position of the largest glomerulus (MG, green) is laterally reversed. **(G, H)** Frontal optical sections of the ALs in small workers of *A. v.* (G) and *A. s.* (H). In

(Figure 1G,H). The total number of glomeruli in large workers of *A. vollenweideri* counted in complete confocal image stacks averaged 243 ± 19 (mean ± SD) glomeruli (n = 6 brains), compared with 189 ± 16 glomeruli (n = 6 brains) in ALs of large workers of *A. sexdens*.

Due to difficulties in dissecting the brains of small workers from the extremely small head capsule, brains could not be treated as whole mounts or thick Vibratome sections. Instead, the overall number of glomeruli had to be estimated from complete series of plastic sections. The number of glomeruli (based on counts of one complete series of sections in each case) was found to be in a similar range as in large workers: 223 glomeruli were found in *A. vollenweideri* and 178 glomeruli in *A. sexdens*. The diameter of glomeruli in small workers was clearly smaller compared with large workers. We analyzed the spatial organization and size of glomeruli by visual inspection of a total of five small individuals in *A. vollenweideri* and six small individuals in *A. sexdens* (see below).

In large workers of both species, we found a strikingly enlarged glomerulus close to the entrance of the AL (n = 6 brains in each species; Figure 1C F). Three-dimensional analyses of a complete confocal image stack in each case revealed that the volume of this macroglomerulus (MG) is ~9 10 times the volume of average ordinary glomeruli. In *A. vollenweideri* the MG has a volume of 70.181 μm^3, compared with an average volume of 7577 μm^3 in ordinary glomeruli. The volume of the largest ordinary glomerulus was four times smaller and of the smallest ordinary glomerulus 35 times smaller than that of the MG. In *A. sexdens* the MG had a volume of 73.589 μm^3, compared with an average volume of 7994 μm^3 in ordinary glomeruli. The volume of the largest ordinary glomerulus was four times smaller and of the smallest ordinary glomerulus 36 times smaller than the MG volume. Interestingly, the topographical position of the MG was found to be laterally reversed in the two species (glomeruli indicated with MG in Figure 1C,D; green glomerulus in Figure 1E,F). In *A. vollenweideri* the MG was located near the extension of the midline of the antennal nerve (AN, Figure 1C E). In *A. sexdens* the MG was found in a more medial position compared with its position in *A. vollenweideri*. In both species a characteristic configuration of several satellite glomeruli was found in close vicinity to the MG. In *A. vollenweideri*, a large satellite glomerulus was located medial to the MG (asterisk in Figure 1C; yellow glomerulus in Figure 1E). In *A. sexdens* the position of this glomerulus was laterally reversed to the MG (asterisk in Figure 1D; yellow glomerulus in Figure 1F). Between five and seven small satellite glomeruli were located dorsal to the MG in *A. vollenweideri* (indicated by the arrow in Figure 1C; the red glomeruli in Figure 1E), whereas in *A. sexdens*, a similar arrangement of small satellite glomeruli was found dorsal to the large satellite glomerulus (arrow in Figure 1D; yellow and red glomeruli in Figure 1F). The arrangement of these satellite glomeruli support the idea that the MG (i.e. the largest glomerulus within this group of glomeruli close to the antennal-nerve entrance) is laterally reversed in the two species. Qualitative inspection of the MG and the associated satellite glomeruli in other individuals revealed a similar spatial arrangement (n = 6 inspected brains in each species). Most interestingly, in both species the ALs of small workers completely lacked an MG at the entrance of the AL (Figure 1G,H; observations based on five brains in *A. vollenweideri* and six brains in *A. sexdens*). Analyses of complete series of sections did not reveal any obviously enlarged glomerulus in this and other regions of the AL.

In addition to this characteristic arrangement of glomeruli close to the entrance of the AN into the AL, further anatomical inspection revealed a glomerulus near the center of the AL. In large workers of both species, the volume of this glomerulus was about three times the average volume of ordinary glomeruli, and the spatial position in the AL was similar. In small workers, just as in large workers, we found a glomerulus in the central region of the AL. This glomerulus was, however, not substantially bigger than the other glomeruli, and its position was arranged more medially as compared with the centrally located glomeruli in the ALs of large workers (indicated by the arrows in Figure 1G,H).

Sensory responses to trail-pheromone components

To estimate the dynamic range of the EAG response of antennal ORNs, an odor dose response relationship was measured for large workers of both species. Stimulus intensities ranging over 6 log units showed that even at low odor concentration an EAG signal could be measured in some preparations. This was the case at a loading of the filter paper with 14.45 pg of 2E3DMP and 2.45 pg of M4MP2C, respectively. In a total of 18/19 (*A. vollenweideri*/*A. sexdens*) successful EAG recordings we obtained a clear response to at least one of both components. In 7/10 (*A. v.*/*A. s.*) of the EAG recordings the response to both odors was larger (>25%) than to the control stimulus with pure hexane. Odor responses measured as EAG signals were in the range of 0.5 2 mV. At low odor concentrations EAG amplitude increased with increasing odor concentration, whereas EAG amplitude did not further increase at high odor concentrations. An example of an odor dose response relationship for three different odor concentrations is shown in Figure 2A. Since signal amplitude did not further increase at the highest concentration of the pheromone component, we assume a maximal response of all receptor neurons specific to this

small workers of both species, no MG was found close to the entrance of the AN. All glomeruli in this regions are similarly small in size. The central glomerulus is indicated by arrows in G and H. Axes in C, E and G (also valid for D, F and H): dors, dorsal; med, medial. Scale bars in B, D and F = 100 μm, in H = 50 μm (also valid for the neighboring images of the left column).

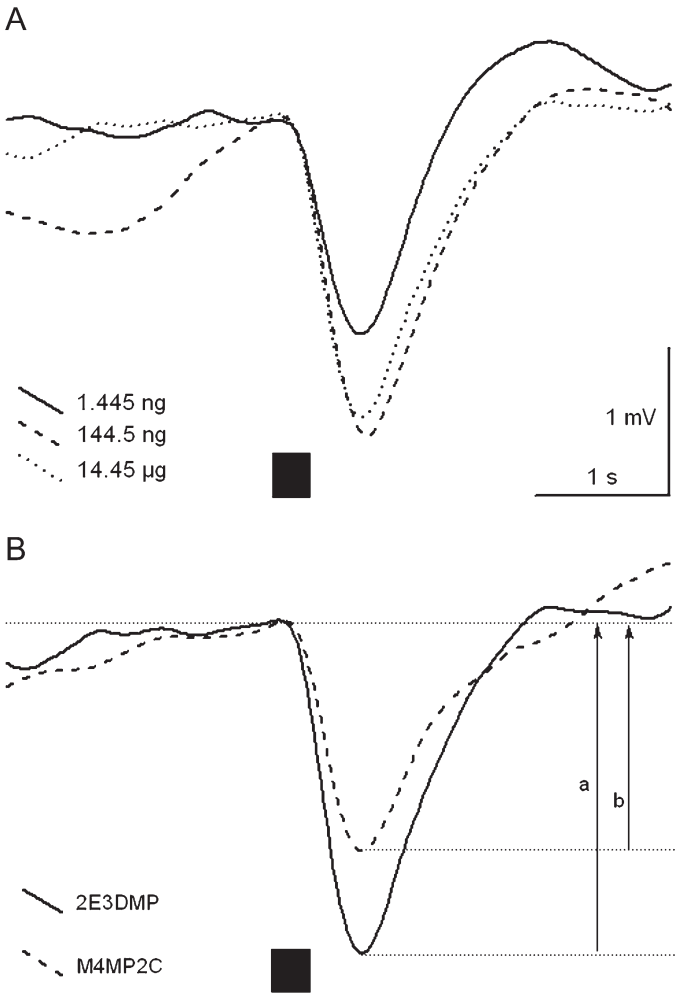

Figure 2 Electroantennogram (EAG) responses of an antenna of *A. sexdens* to the two trail pheromone components tested (curves are least squares estimations). **(A)** With increasing odor concentration (2E3DMP, loading 1.45 144.5 ng) the signal amplitude increases. At high odor concentrations (loading 144.5 ng and 14.45 µg) maximal EAG signal amplitude for this component was reached. **(B)** Comparison of an EAG signal to 2E3DMP and M4MP2C at high odor concentrations (loading 14.45 µg/2.45 µg) show ing a stronger response to 2E3DMP than to M4MP2C. The quotient (*a*/*b*) of the signal amplitude was used in order to normalize the responses to the two components. Black bars denote the stimulation.

pheromone component. For the following comparative study we used only the high odor concentrations, which was a loading of 14.45 µg of 2E3DMP and 2.45 µg of M4MP2C onto the filter paper. An example of the responses of an *A. sexdens* antenna to the two pheromone components is shown in Figure 2B. Since signal amplitude was highly variable between different antennae, we normalized the response by calculating the ratio of the responses to the two pheromone components used. This was achieved by measuring the EAG amplitudes as a response to the two pheromone components and calculating the quotient *a*/*b* (Figure 2B) which allowed us to pool the data from different antennae. The ratios of EAG signals to both pheromone components can be regarded as an estimate of the relative number of receptor neurons activated. Figure 3 shows the median and range of the ratios of the EAG signals to 2E3DMP/M4MP2C. The ratio of the EAG signals did not differ from 1 (similar response to both components tested) in *A. vollenweideri* antennae (*t*-test for single samples, $P > 0.05$). In *A. sexdens* antennae the ratio of the EAG signals was significantly larger than 1 (*t*-test for single samples, $P < 0.01$). Thus, in *A. sexdens* the EAG signal measured as odor response to 2E3DMP was larger than to M4MP2C. The ratio of EAG signals (2E3DMP/M4MP2C) was significantly larger in *A. vollenweideri* antennae compared with the ratio of EAG signals in *A. sexdens* antennae (Mann Whitney *U*-test, exact $P < 0.01$).

Discussion

We found in two phylogenetically closely related species of leaf-cutting ants, *Atta vollenweideri* and *Atta sexdens*, both subgenus Neoatta (Borgmeier, 1959), a prominent, enlarged glomerulus at the entrance of the AL. This glomerulus is 9 10 times larger in volume than average-sized glomeruli, and for this reason we use the term macroglomerulus (MG). Our study shows, for the first time, the existence of an MG in an obligatory non-sexual worker caste. Moreover, our results indicate a phenotypic plasticity of the MG in both species. Only large workers possess an MG, whereas glomeruli in the equivalent region in the AL of small workers are all similarly small in size. We conclude that the first olfactory neuropil of leaf-cutting ants expresses a disproportional allometry of glomerular volume.

The satellite glomeruli that are in close spatial association with the MG could also be functionally related to the MG, like in the macroglomerular complexes (MGCs) found in sex-pheromone-specific glomeruli in the AL of several species of moth. Whether the arrangement of MG and satellite glomeruli represents an MGC needs to be determined in future functional studies. MGs and MGCs are well documented in moths, as well as in the honeybee and the cockroach. In all cases investigated so far the presence of MGs or MGCs were shown to be involved in the detection of sex pheromones (Arnold *et al.*, 1985; Rospars, 1988; Christensen *et al.*, 1995; Cardé, 1997; Anton and Homberg, 1999; Hansson and Anton, 2000).

In the antennal lobe of the female sphinx moth, *Manduca sexta*, two relatively large, sexually dimorphic glomeruli (LFGs) were found, and projection neurons innervating one of these glomeruli were shown to be responsive to linalool, a volatile of host plants of *M. sexta* (Rössler *et al.*, 1998; King *et al.*, 2000; Rospars and Hildebrand, 2000). However, in this case the LFGs are not nearly as prominent as the MGs found in the two *Atta* species of this study. With a diameter of ~100 µm, the LFGs are not substantially bigger than ordinary glomeruli, which range between 45 and 100 µm in diameter (Rospars and Hildebrand, 2000).

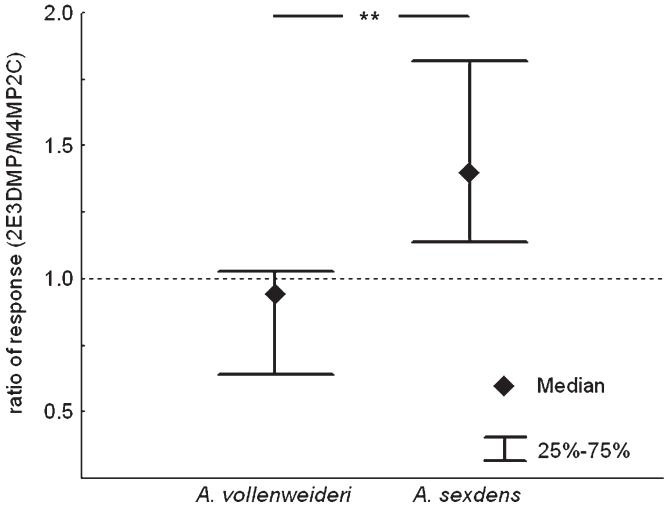

Figure 3 Median ratio of EAG signals in response to 2E3DMP/M4MP2C from antennae in *A. vollenweideri* and *A. sexdens*. At a ratio of 1.0 the EAG signals to both components have the same amplitude. In *A. vollenweideri* ($n = 7$) the EAG signal to 2E3DMP is smaller than to M4MP2C, whereas in *A. sexdens* ($n = 10$) the EAG signal to 2E3DMP is larger than to M4MP2C. The ratio of EAG signal to 2E3DMP/M4MP2C is significantly larger than 1 in A. sexdens (*t* test for single samples, $P < 0.01$). The difference in the ratio of EAG signals between *A. vollenweideri* and *A. sexdens* antennae is signif icant (Mann Whitney *U* test, exact $P < 0.01$).

In other ant species (e.g. *Camponotus vagus*, *Formica pratensis* and *Mesoponera caffraria*) the AL architecture allows the classification of clusters or subsets of glomeruli, but no MG was found (Goll, 1967; Masson, 1972). Similarly, subsets of glomeruli were defined in the honeybee based upon innervation by different antennal nerve tracts (Mobbs, 1982; Flanagan and Mercer, 1989), but MGs were found only in the AL of drones and are assumed to process queen pheromones (Brockmann and Brückner, 2001). Glomeruli are located in a peripheral layer of the AL, which in both *Atta* species is arranged in up to three piled rows of glomeruli. Such an arrangement of glomeruli was also found in other ant species (see above) and in wasps (Masson and Strambi, 1977; Smid *et al.*, 2003). However, it differs from the peripheral layer of glomeruli in the AL of the honeybee, which comprises only a single row of glomeruli (Galizia *et al.*, 1999).

In both species, strong labeling of glomeruli with fluorophore-conjugated phalloidin indicates that also glomeruli in the AL of leaf-cutting ants are highly enriched with neuronal f-actin (Rössler *et al.*, 2002). Rössler *et al.* (2002) showed that in insects f-actin is present in axons of ORNs terminating within the glomeruli of the AL. This suggests that ORN axons may be capable of undergoing structural changes and may be involved in structural plasticity of olfactory glomeruli in insects. Interestingly, in vertebrate glomeruli f-actin was shown to be localized exclusively in postsynaptic neuronal elements, indicating that the presence of high concentrations of f-actin in ORN axons may be a special feature of insect olfactory glomeruli.

Despite a difference in the total number of olfactory glomeruli between the investigated species, our results show a very characteristic arrangement of the MG and associated satellite glomeruli (terms after Hansson, 1997) at the entrance of the antennal nerve. The results further show that the position of the MG (i.e. the largest glomerulus within this arrangement) is laterally reversed in the two species. At this point we cannot state whether the disproportional allometry of the MG in large workers represents a monophasic or, for example, a diphasic polymorphism, since we did not systematically map the body size and volumes of a large enough number of individuals. It is very likely that the difference in the presence of an MG is the result of developmental differences between the two castes rather than an age or activity dependent plasticity. In the honeybee it was shown that glomeruli may undergo age and activity related changes in volume (Winnington *et al.*, 1996; Sigg *et al.*, 1997), but these changes were in the range of ~30%. The difference in size between the MG and other glomeruli described in this study is much larger, and we found the MG in all large workers we investigated. However, the examined individuals were not age marked and, according to their coloration, they were at least 1 week old. Therefore, we cannot exclude that additional age- or experience-related differences in glomerular volume may be present in workers.

We assume that in ants ORN types segregate into different glomeruli, as has been shown for *Drosophila*, the honeybee and several species of moths with pheromone-sensitive ORNs (Brockmann and Brückner, 1995; Hansson, 1997; Vosshall *et al.*, 2000). In moths, e.g. *Heliothis virescens*, ORNs tuned to the major sex pheromone component (which account for the largest proportion of receptor neurons on a male antenna) terminate in the cumulus, the largest glomerulus within the AL (Hansson *et al.*, 1995; Berg *et al.*, 1998). This indicates a correlation between glomerulus size and the number of ORNs terminating in it. In the AL, ORNs converge onto relatively few output neurons, the projection neurons (PNs), and the neuronal network of the AL shapes the assembly code carried to higher centers by PNs (Sachse and Galizia, 2003).

The major pheromone component in *Atta texana* and *A. cephalotes*, as well as in the smaller leaf-cutting ants *Acromyrmex octospinosus* and *A. subterraneus*, is M4MP2C (Tumlinson *et al.*, 1971; Riley *et al.*, 1974; Cross *et al.*, 1982; do Nascimento *et al.*, 1994). A different major pheromone component was found only in the leaf-cutting ant *Atta sexdens*. In this species, 2E3,6DMP was identified as major component and is present in smaller amounts (secondary main component) in the venom of the other species of the genus *Atta* as well (Cross *et al.*, 1979; Evershed and Morgan, 1983). The ratio of 2E3,6DMP to M4MP2C in *A. sexdens* is 14:1, and this ratio is more potent than single components in behavioral assays (Billen *et al.*, 1992). The major trail pheromone component of *A. vollenweideri* is not yet known, but from the findings in the other leaf-cutting

ant species we expect that both major components (2E3,6DMP and M4MP2C) are present in the venom. Behavioral assays indicate that M4MP2C is likely to be the major component of the trail pheromone of *A. vollenweideri* since workers do follow an artificially laid trail with M4MP2C but, in contrast to *A. sexdens* workers, do not follow an artificially laid trail with 2E3DMP alone (Cross *et al.*, 1979; unpublished data).

With the EAG measurements of sensory responses of the antennae in both species we gained correlative evidence that the MGs may be involved in detection of the trail pheromone, and that the major component of the trail pheromone may be represented in the MG. An EAG reflects the response of a large number of receptor neurons, thus, integrates the sensory input of many functional units (sensilla) on the antenna (Schneider, 1957). Since we used high odor concentrations for stimulation, all receptor neurons tuned to the tested component should be excited. The resulting saturation in the EAG signals to both components support this assumption. The ratio of the EAG signals to both components is a measure of the relative number of receptor neurons responding to these odors, if the specificities of the receptor neurons do not differ between the two species. We compared closely related species, and therefore we assume that the sensitivity of the ORNs tuned to the two pheromone components is similar.

At high odor concentrations the EAG signals of *A. sexdens* antennae were larger to 2E3DMP than to M4MP2C. This supports the idea that there are more ORNs tuned to 2E3,6DMP than tuned to M4MP2C on the antennae of *A. sexdens*. This, in turn, would suggest that the glomerulus in which ORNs tuned to 2E3,6DMP terminate is larger than the glomerulus in which ORNs tuned to M4MP2C terminate. The pronounced size difference found between the MG and all other glomeruli is in line with the idea that the major component 2E3,6DMP is represented in the MG of *A. sexdens*. The EAG signals measured in *A. vollenweideri* antennae alone did not support the idea that the putative major trail pheromone component (M4MP2C) is represented in the MG. However, this approach is more sensitive to variation in odor concentration than the following comparative approach between the two species. If the used odor concentration of, for example, M4MP2C did not elicit a maximal response of all receptor neurons tuned to this component in all experiments, an overestimation of the ratio of EAG signals in both species might occur. In addition, possible responses of unspecific ORNs might reduce the difference in EAG signal as response to the two tested trail pheromone components.

The comparison between species showed that the ratio of EAG signals to 2E3DMP/M4MP2C was lower in *A. vollenweideri* antennae than in *A. sexdens* antennae. This indicates that the relative numbers of ORNs tuned to the two pheromone components differ between *A. vollenweideri* and *A. sexdens*. We cannot separate the following two possibilities with the comparative approach: The number of ORNs on the antennae of *A. vollenweideri* tuned to 2E3,6DMP is lower than on the antennae of *A. sexdens*, and/or the number of ORNs on the antennae of *A. vollenweideri* tuned to M4MP2C is larger than on the antennae of *A. sexdens*.

The laterally reversed positions of the MG and its spatial arrangement in the AL of *A. vollenweideri* and *A. sexdens* further supports the idea that different odors are represented in the MG of both species. The representation of odors could be spatially conserved in the two closely related *Atta* species, as was shown for the MGC of closely related Heliothine moths (Vickers *et al.*, 1998; Vickers and Christensen, 2003).

The colony structure in leaf-cutting ants with its pronounced polymorphism and polyethism is among the most complex colony structures found in all ants (Wilson, 1980a,b; Hölldobler and Wilson, 1990). AN MG in large workers but not in small workers further adds to this complexity at the level of the central nervous system. In general, size polymorphism drives division of labor and improves the efficiency of foraging (Wilson, 1980b; Fowler, 1983). The different AL architecture of small and large leaf-cutting ant workers may further promote division of labor by modulating the behavioral response to olfactory signals. However, so far there are no data available which show that small individuals are limited in any way compared with large individuals when challenged with odor tasks. In contrast to large workers, the smallest workers never leave the nest and therefore may not be specialized for trail detection (Wilson, 1980a). Indeed, behavioral experiments currently done in our laboratory show that small and large workers differ in trail following behavior and discrimination performance to different trails (unpublished data). Future combined neuroanatomical and neurophysiological studies will focus on the representation of trail pheromone components in the AL in order to elucidate the possible role of the MG in neuronal processing of pheromone components.

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