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Glomerular representation of plant volatiles and sex pheromone components in the antennal lobe of the female *Spodoptera littoralis*

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Summary

We studied the projection patterns of antennal lobe (AL) interneurones sensitive to plant volatiles and female-produced sex pheromone components in the female moth, *Spodoptera littoralis*. Ten compounds (eight plant-derived compounds and two sex pheromone components) were singly applied to the antenna and, using intracellular recording and staining techniques, the physiological and morphological characteristics of responding neurones were investigated. In addition, ALs stained with a synapsin antibody were optically sectioned using confocal microscopy, and a three-dimensional map of glomeruli in the anterior aspect of the AL was reconstructed. We used the map as a reference for identification of glomeruli innervated by projection neurones (PNs) that respond to plant volatiles and/or pheromone components. Nineteen PNs, responding to one to seven compounds of the ten tested stimuli, were stained with neurobiotin. These neurones each arborised in a single glomerulus in the frontal side of the AL. PNs responding to the same compound arborised in different glomeruli and PNs arborising in the same glomerulus responded to different compounds. Accordingly, glomeruli harbouring the dendritic arborisations of PNs responding to each of the tested compounds constituted a unique array of glomeruli that were not necessarily adjacent. It was thus clear that, at the output level, a single plant volatile or a sex pheromone component was not represented within a single glomerulus in the AL. We expect complex patterns of glomeruli to be involved in the coding of plant-derived compounds, as well as sex pheromone components, in female *S. littoralis*.

Key words: plant volatile, pheromone component, moth, *Spodoptera littoralis*, antennal lobe interneurone, olfactory processing, glomerular map.

Introduction

The primary neuropil of the olfactory system, both in vertebrate and invertebrate animals, is known for its typical glomerular structure (Strausfeld and Hildebrand, 1999). In insects, glomeruli of the primary olfactory centre, the antennal lobe (AL), house the synaptic contacts between receptor axons and interneurones (Hansson and Anton, 2000 and references therein). Several studies, dealing mainly with receptor neurone input to the AL, have lead to the assumption that morphologically distinct glomeruli represent functionally distinct processing centres for olfactory information. Olfactory receptor neurones (ORNs) tuned to a distinct pheromone component send their axons into a particular compartment of the macroglomerular complex (MGC). This has been elucidated in several Lepidoptera species, e.g. *Agrotis segetum* (Hansson et al., 1992), *Trichoplusia ni* (Todd et al., 1995), *Spodoptera littoralis* (Ochieng' et al., 1995) and *Heliothis virescens* (Hansson et al., 1995; Berg et al., 1998). Molecular studies, revealing specific expression of membrane odour receptors in specific glomeruli in *Drosophila melanogaster*, support the theory of functionally specific input to glomeruli (Gao et al., 2000; Vosshall et al., 2000). For AL output neurones, functionally specific dendritic arborisations have so far been shown only for pheromone-sensitive projection neurones (PNs), arborising in glomeruli in the MGC in *Manduca sexta* and heliothine moths (Hansson et al., 1991; Vickers et al., 1998). However, in the cabbage looper moth *T. ni*, axon terminals of ORNs and the dendritic branches of identified PNs that express similar physiological specificity in response to pheromone components, do not always arborise in the same glomerulus (Anton and Hansson, 1999).

In contrast to the voluminous literature dealing with the central processing of sex pheromone in male moths, the number of corresponding studies on the central processing of plant volatiles is relatively limited. Plant-derived stimuli are, however, no less important for phytophagous insects than is the sex pheromone. Whereas host plant odours induce landing

responses and egg laying by females (Guerin et al., 1983; Barata and Araujo, 2001), odours emitted by naturally resistant plants or by previously infested host plants often deter females as they signal poor nutritional qualities, likely high intraspecific competition and potential risk of predation or parasitism (Langenheim, 1994; Turlings et al., 1995). The noctuid moth, *Spodoptera littoralis* (Boisd.), has been well studied with respect to plant volatile-guided behaviour and detection of compounds involved by ORNs on the antennae. Several plant-derived components have been identified as oviposition deterrents, natural host plant odours or herbivore-induced volatiles, and ORNs responding specifically to these components have been described (Anderson et al., 1993, 1995; Jönsson and Anderson, 1999). Whereas sex pheromone-related information is mostly processed in the MGC in male ALs, plant volatile information is known to be processed in the so-called ordinary glomeruli that are present in both male and female moths (Anton and Hansson, 1994, 1995); but how specifically plant volatile receptors project into the AL and how this information is subsequently integrated is hardly known.

Using Ca^{2+} imaging techniques, studies on *S. littoralis* (Hansson et al., 2000) as well as on other species (Joerges et al., 1997; Galizia et al., 2000; Galizia and Menzel, 2001) suggest that individual plant compounds can be represented in identifiable single glomeruli or specific groups of glomeruli. Anton and Hansson (1994) described arborisation patterns of plant odour-sensitive PNs that vary in their specificity, but accurate identification of the innervated glomeruli demanded an atlas of the AL glomeruli in *S. littoralis*, which was not available at that time.

In addition to presenting a three-dimensional map of the glomeruli within the anterior half of the AL of female *S. littoralis*, the aim of the present work was to investigate whether the dendritic arborisations of plant odour-sensitive PNs convey distinct patterns, representing particular compounds or groups of plant-derived compounds. We also aimed to study the physiological integration of olfactory information in comparison with what is already known from previous studies carried out at the receptor-neurone level in this species.

Materials and methods

Insects

Spodoptera littoralis pupae were obtained from a laboratory culture kept at the Swedish University of Agricultural Sciences, Alnarp, Sweden. Female pupae were kept in a rearing chamber until adult emergence. Moths were prepared for intracellular recordings as described by Anton and Hansson (1995). The moth was restrained in a disposable plastic pipette tip, which was cut to allow passage of the head. The head position was fixed with dental wax (Surgident, Heraeus). The brain was uncovered and the sheath overlaying the AL was carefully removed. During the experiment, the brain was superfused with a saline solution containing 150 mmol l^{-1} NaCl, 3 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} KCl, 10 mmol l^{-1} TES (*N*-Tris-methyl-2-aminoethanesulfonic acid) buffer, and 25 mmol l^{-1} sucrose (pH 6.9) to increase osmolarity and prevent swelling of the brain tissue.

Stimulation

The antenna ipsilateral to the recording site was ventilated by a steady stream of charcoal-filtered and moistened air that passed through a glass tube at a velocity of approximately 0.5 m s^{-1}. Stimuli were presented by inserting a Pasteur pipette, containing a piece of filter paper that carried the stimulus, in the glass tube 20 cm upstream from the end of the tubing where the antenna was placed. A 0.5 s air pulse (4 ml s^{-1}) was then sent through the Pasteur pipette by means of a stimulation device (Syntech). The stimuli were presented randomly at 10 s intervals.

Eight plant volatiles and two sex pheromone components were used as stimuli. The eight behaviourally relevant plant compounds used were: (1) α-humulene (1 ng); (2) β-caryophyllene (10 ng); (3) geraniol (100 ng); (4) eugenol (100 ng); (5) benzaldehyde (1 μg); (6) (±)-linalool (10 ng); (7) (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (1 ng); and (8) α-farnesene (10 ng). The two sex pheromone components were: (9) (*Z*,*E*)-9, 11-tetradecadienyl acetate (*Z*,*E*-9, 11–14:OAc) (10 μg); and (10) (*Z*,*E*)-9, 12-tetradecadienyl acetate (*Z*,*E*-9, 12–14:OAc) (10 μg). Although the behavioural significance of sex pheromone in the life of female *S. littoralis* is not clear, the plant compounds used are known to be emitted naturally from the preferred host plant of the moth, cotton (1–3), oviposition deterrents (4,5) or inducible plant volatiles that signal lower quality and increased resistance of a given plant (6–8) (Anderson et al., 1995; Jönsson and Anderson, 1999). The above doses for sex pheromone components were the same as those used by Anton and Hansson (1994) and are only slightly higher than the threshold in peripheral receptor neurones (Ljungberg et al., 1993). As for α-farnesene, linalool and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, we chose doses that elicit ORN response (Jönsson and Anderson, 1999). For the other five stimuli we determined threshold levels by using three different doses during more than 100 preliminary tests and chose a final amount above threshold for the study. Nine of the compounds were dissolved in hexane and one (α-humulene) was dissolved in paraffin oil. Solvent (10 μl) containing the given amount of chemicals was applied to pieces (5×15 mm) of filter paper in Pasteur pipettes and were renewed every day. A Pasteur pipette containing a clean filter paper or a filter paper with solvent was used as control. Responses to solvent blanks were not different from the mere filter paper blank. Purity of the chemicals was more than 95 % (except α-farnesene, 85 %). Chemicals were purchased from Sigma (St Louis, MO, USA). Not all neurones were exposed to all ten compounds. The first half of the work was carried out using only seven compounds, where α-farnesene and the two sex pheromone components were not included. Whenever a contact could be kept long enough, each stimulus was tested at least twice during the course of an experiment.

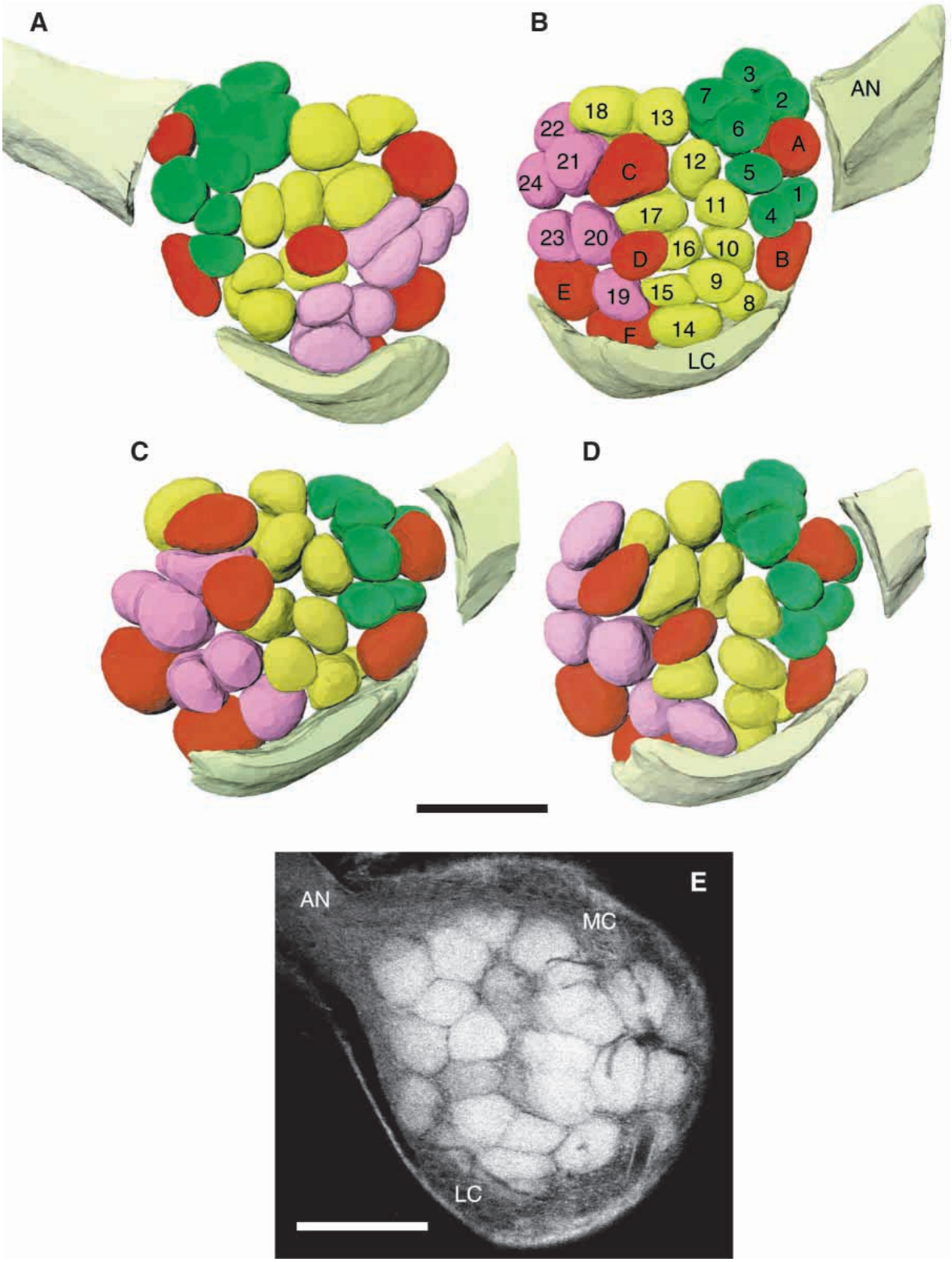

Fig. 1. Reconstructed glomerular structure (A–D) of the anterior aspect of the antennal lobe (AL) of the female *S. littoralis* moth and a typical optical section (E) of the AL. (A) Right and (B) left ALs of the same female, respectively; (C,D) the left ALs of two other different individuals. Landmark glomeruli (red) are denoted by the letters A–F. The other glomeruli are roughly divided into three groups (dark green, yellow and pink) and numbered to facilitate the use of the figure as a reference for the identification of glomeruli targeted by responding neurones (see the text). AN, antennal nerve; LC, lateral cell body cluster; MC, median cell body cluster. Scale bars, 100 μm.

Intracellular recording and staining techniques

Standard intracellular recording and staining techniques were used (Christensen and Hildebrand, 1987; Kanzaki et al., 1989). Using a micromanipulator, a glass recording electrode, with the tip filled with 1 % Neurobiotin in 0.25 mol l^{-1} KCl, was inserted into the AL. The electrode was placed close to the centre of the AL and most successful recordings were obtained with the electrode situated close to the surface. When intracellular contact was established, the ipsilateral antenna was stimulated and the activity of the neurone before, during and after stimulation was observed on a Tektronix digital oscilloscope. The physiological data were stored on video tape for further analysis.

Physiologically characterised neurones were stained with Neurobiotin by passing 0.5–1 nA of constant depolarising current through the recording electrode for 2–10 min. Brains were dissected, fixed in 4 % buffered formaldehyde solution overnight at room temperature, incubated in Alexa NeutrAvidin 488 (Molecular Probes) at 4° C and viewed as whole mounts in a laser scanning confocal microscope (Leica TCS NT). After confocal imaging, selected brains were embedded in Spurr's resin and sectioned at 10 µm. Serial sections were photographed on Fuji Sensia 400 colour slide film, and the neurones were reconstructed from the slides.

Analysis of responses

Action potentials were counted manually from the storage oscilloscope. The number of action potentials counted during a 600 ms period after the stimulus had reached the antenna minus the number of action potentials counted during the preceding 600 ms (representing the spontaneous activity of the neurone) was noted as the net number of action potentials. The net number of action potentials produced in response to the blank stimulus (caused by mechanical stimulation through the applied air pulse) was subtracted from the net number of action potentials produced in response to an odour stimulus, to quantify the response to a specific stimulus in one neurone. A 10–50 % increase in the number of action potentials were considered a weak response (+). An increase of more than 50 % up to 200 % in the action potential number was considered an intermediate response (++). An increase of more than 200 % was considered a strong response (+++).

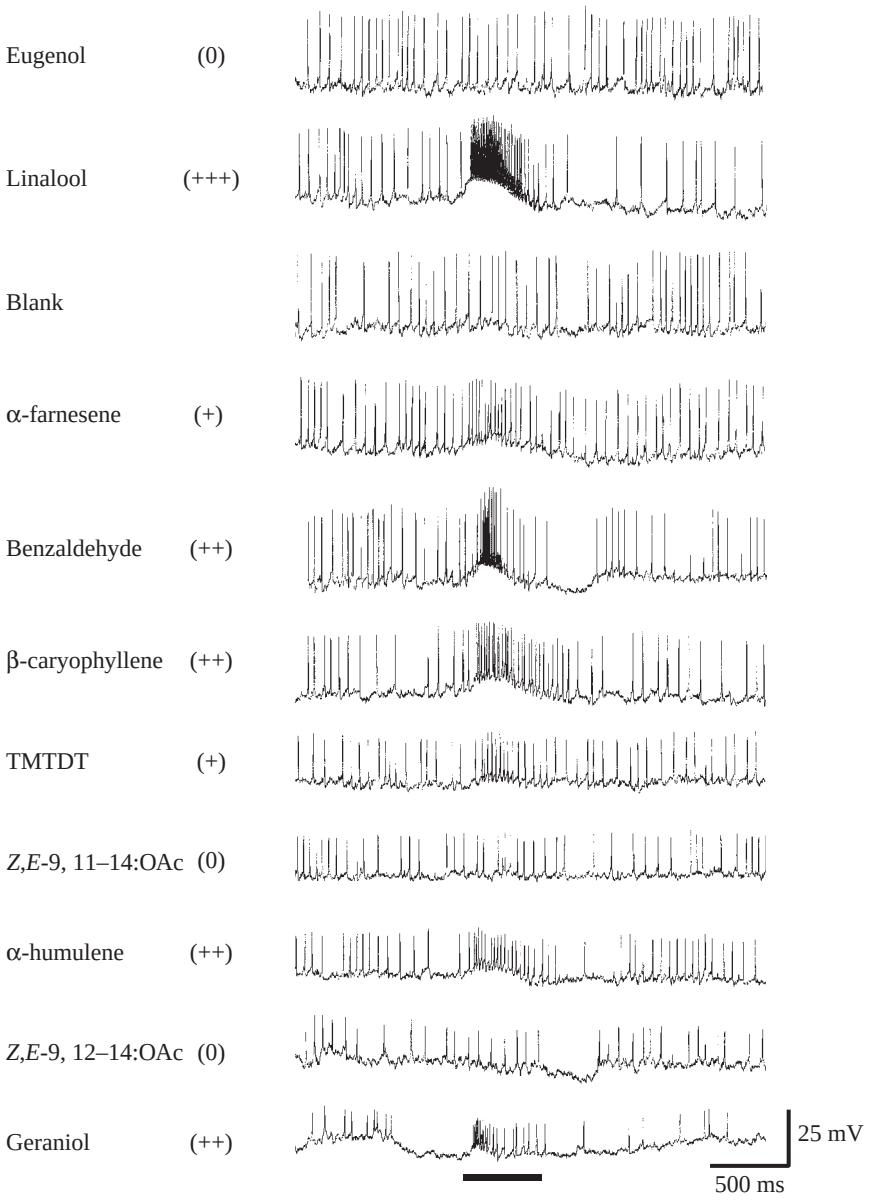

Fig. 2. Responses (action potentials) of a generalist neurone (number 92) to several compounds, indicating degrees of response and the various delays after the application of stimuli. Compounds are given in the order of their application. TMTDT is (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. The thick horizontal bar indicates the time at which the compound was applied. 0, no response; +, a weak response; ++, an intermediate response; +++, a strong response (see Materials and methods for details).

Neuroanatomy

Brains were stained with a synapsin antibody (Klagges et al., 1996) and the ALs were mapped and used as a reference for locating the particular glomeruli innervated by stained neurones in the intracellular experiments. For this purpose, brains of female moths were dissected and fixed in 4 % paraformaldehyde in 0.1 mol l^{-1} phosphate buffer, incubated 5 days in the primary mouse antibody against synapsin (1:50) at room temperature, followed by incubation in the secondary antibody, rabbit- anti-mouse conjugated with FITC (Sigma,

Table 1. *Responses of olfactory interneurones that were specific to one compound and were not stained*

Neurone number	Compound*									
	1	2	3	4	5	6	7	8	9	10
1–6	++	0	0	0	0	0	0	nt	nt	nt
7	++	0	0	0	0	0	0	0	nt	nt
8	++	0	0	0	0	0	0	0	0	0
9, 10	+++	0	0	0	0	0	0	nt	nt	nt
11	0	+	0	0	0	0	0	nt	nt	nt
12, 13	0	+++	0	0	0	0	0	nt	nt	nt
14–16	0	0	+	0	0	0	0	nt	nt	nt
17	0	0	++	0	0	0	0	0	0	0
18	0	0	+++	0	0	0	0	nt	nt	nt
19	0	0	0	+++	0	0	0	nt	nt	nt
20	0	0	0	+++	0	0	0	0	nt	nt
21	0	0	0	0	+	0	0	0	0	0
22	0	0	0	0	++	0	0	0	nt	nt
23	0	0	0	0	0	+	0	0	nt	nt
24	0	0	0	0	0	++	0	nt	nt	nt
25	0	0	0	0	0	++	0	0	nt	nt
26	0	0	0	0	0	0	+	0	0	0
27	0	0	0	0	0	0	++	nt	nt	nt
28	0	0	0	0	0	0	0	++	nt	nt
29	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	+
31	0	0	0	0	0	0	0	0	0	++
32	0	0	0	0	0	0	0	0	0	+++

Responses are classified as weak (+), intermediate (++) or strong (+++). 0, no response; nt, the compound was not tested.

*Compounds in this table, as well as in the subsequent tables, are: (1) α-humulene; (2) β-caryophyllene; (3) geraniol; (4) eugenol; (5) benzaldehyde; (6) (±)-linalool; (7) (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; (8) α-farnesene; (9) *Z*,*E*-9, 11–14:OAc; (10) *Z*,*E*-9, 12–14:OAc.

1:150) for 4 days at 4 °C. Brains were then cleared and mounted in 80 % glycerol and optically sectioned using the confocal microscope.

The AL glomeruli were reconstructed using Imaris 2.7 software (Bitplane), running on a Silicon Graphics computer work station. Right and left ALs from six animals were reconstructed and compared. Glomeruli occupying the anterior aspect of the AL were unmistakably visible in all preparations, whereas posteriorly located ones were less clearly discernible. Unequivocally recognisable glomeruli were reconstructed by manually delineating the borders of each glomerulus in every optical section. A three-dimensional map of the anterior half of the AL was thus created. Of the six maps, the one that had the largest number of clearly delineated glomeruli was chosen as reference.

Every AL with a stained neurone was similarly treated and its three-dimensional map was compared with the reference map to locate the specific glomerulus in which the stained neurone arborised. The orientation of the obtained maps was visually decided depending on the known position of the lateral cluster of cell bodies (Anton and Hansson, 1994), the position of the antennal nerve entrance, the location of landmark glomeruli (see Results) that occupied fixed places in all specimens, and the symmetrical relationship between right and left ALs in each preparation.

Results

Glomerular map of the AL

Brains of six females were analysed. Glomeruli occupying the anterior aspect of the AL on both sides of each brain were reconstructed (see examples in Fig. 1). Glomeruli varied in terms of shape, size and relative position. Most of them were spherical, while some were oval or elongated in shape. Six well-differentiated ‘landmark’ glomeruli were more or less constant in position and shape. They were readily identifiable in all 12 ALs in the six brains examined. We assigned them the labels A–F (Fig. 1). Glomerulus A was spherical and always faced the entrance of the antennal nerve, whereas glomerulus B was oval and located close to the dorsal edge of the lateral cluster of cell bodies. A third glomerulus, D, was distinguishable by its anteriormost location. Glomerulus C was oval and in most cases separated from D by one glomerulus towards the median aspect of the brain. Glomerulus E was remarkably large and more posterior than most of the other identified glomeruli. In a frontal view, its position seemed to align roughly on a straight line with glomeruli D and A, and

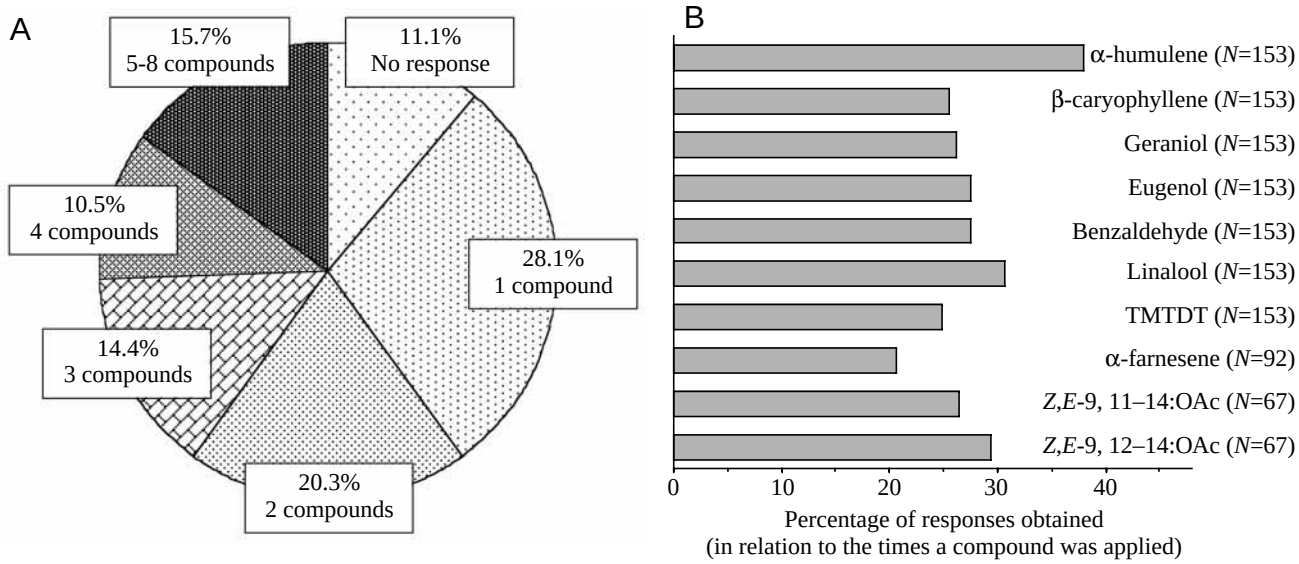

Fig. 3. (A) Degrees of specificity exhibited by the examined interneurones. (B) The percentage of responses obtained for various compounds in relation to the number of times the compound was applied. Recordings were made from 153 neurones.

the axis of the antennal nerve. The largest among the reconstructed glomeruli was the perfectly spherical F, which was distinguished by its most ventral and posterior location.

The remaining reconstructed glomeruli deviated to some extent in size, shape or position between ALs. 23 of these glomeruli were clearly recognised in the specimen chosen as a model and in another AL. In the remaining four ALs, only 22 glomeruli were detected. To facilitate identification, we assigned them to three groups of glomeruli: the first consisted of the two rows adjacent to the entrance of the antennal nerve (dark green, Fig. 1); the second was composed of the next two rows, lying in the middle of AL (yellow, Fig. 1); and the third included the remaining glomeruli in the medioventral part of the AL (pink, Fig. 1). We numbered the glomeruli in the model AL serially, counting every row from the lateral side and moving toward the median aspect of the brain (Fig. 1). The smallest and the largest diameters of the reconstructed glomeruli, including the landmark ones, were 35 and 75 μm, respectively.

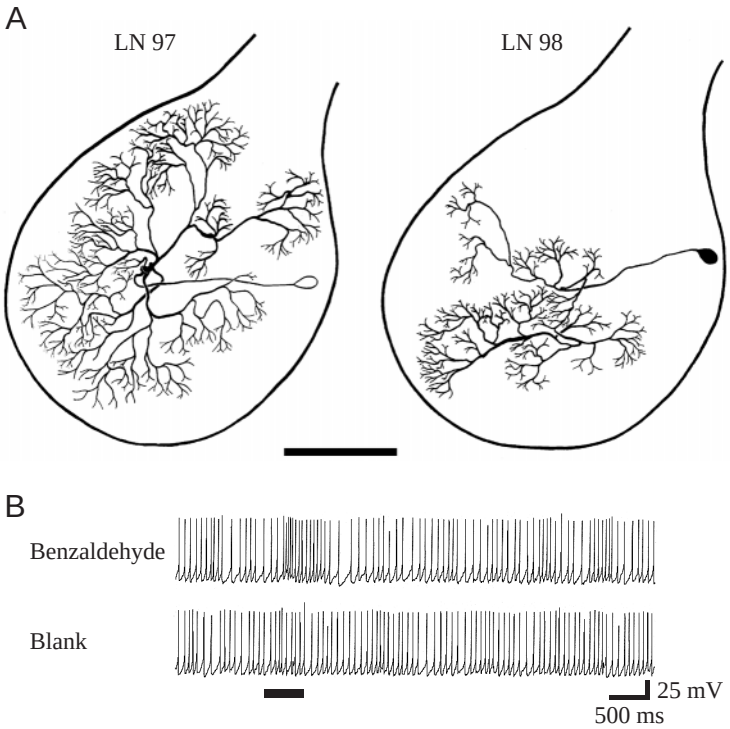

Fig. 4. (A) Morphology of the neurobiotin-filled local interneurones (LNs) reconstructed by projecting optical sections (2.5 μm thick) prepared using a confocal microscope. The two LNs were selected to show homogenous (number 97) and heterogeneous (number 98) branching patterns. Scale bar, 100 μm. (B) Typical response of a local interneurone to benzaldehyde and control (blank). The thick horizontal bar indicates the time at which the compound was applied.

Physiological characteristics of olfactory interneurones

Physiological responses of 153 AL interneurones in 146 *S. littoralis* females were recorded. 17 neurones, in the brains of 12 animals, did not respond to any of the tested odorants. The remaining 136 interneurones (in 134 animals) responded to at least one compound. Responses in PNs and local interneurones (LNs) were characterised by a sudden increase in action potential frequency after the onset of the stimulus with different delays in individual neurones, and sometimes by inhibition after the end of the stimulus, before the level of spontaneous activity was re-established (Fig. 2). Neurone responses never started with initial inhibition and neither were purely inhibitory responses found. Repeated stimulation with the same odour

Table 2. *Response profiles of olfactory interneurones that exhibited various degrees of specificity and were not stained*

Neurone number	Compound*									
	1	2	3	4	5	6	7	8	9	10
Examples of the 31 neurones responding to two compounds										
43	+	0	0	0	0	++	0	nt	nt	nt
44	+++	0	0	0	0	0	+++	0	0	0
45	+++	0	0	0	0	0	0	0	0	+++
46	0	- -	0	- -	0	0	0	nt	nt	nt
47	0	0	+	0	+++	0	0	0	0	0
48	0	0	+	0	0	+	0	nt	nt	nt
Examples of the 22 neurones responding to three compounds										
57	++	0	0	++	0	0	0	0	0	+++
58	++	0	0	0	0	++	0	+++	nt	nt
59	+++	0	+++	+++	0	0	0	nt	nt	nt
60	+++	0	0	0	+	+	0	0	0	0
61	0	+	0	0	+	0	0	0	0	+
Examples of the 16 neurones responding to four compounds										
78	+	++	+	+	0	0	0	nt	nt	nt
79	++	0	+	0	0	0	0	0	++	++
80	++	++	0	0	++	0	0	+++	nt	nt
81	0	+	0	+	0	0	0	++	0	++
82	0	+++	0	++	0	+++	++	0	nt	nt
Examples of the 24 neurones responding to five to eight compounds										
83	+++	0	0	+++	0	+++	++	0	++	0
88	0	0	0	+	0	+	0	+	++	+
89	0	0	++	++	++	++	+++	0	nt	nt
91	+++	+++	+++	+++	0	+++	+++	nt	nt	nt
92	++	++	++	0	++	+++	+	+	0	0
94	++	+	+++	0	++	0	+++	+++	+++	++

Responses are classified as weak (+), intermediate (++) or strong (+++). –, inhibition; 0, no response; nt, the compound was not tested.

Table 3. *Responses profiles obtained from brains in which more than one neurone was stained*

Brain number	Compound										Stained neurones*	
	1	2	3	4	5	6	7	8	9	10	PN	LN
122	0	+	0	0	0	0	0	0	nt	nt	2	
123	0	0	0	++	0	0	0	nt	nt	nt	2	
124	0	0	0	+	0	0	0	0	nt	nt	2	1
125	0	0	0	++	0	0	0	0	0	0	1	2
126	++	0	0	0	0	0	0	nt	nt	nt		2
127	+	0	0	0	0	0	0	0	0	0	1	1
128	0	0	0	0	0	0	0	0	+++	0	2	
129	++	0	0	0	++	0	0	0	0	0	1	3
130	0	0	0	0	+	0	+	0	0	0	1	2
131	0	0	0	+++	+++	0	0	0	0	0		2
132	0	0	0	0	0	+	+	0	++	0	2	1
133	0	0	0	0	++	+	0	0	++	+	1	2
134	+++	0	++	0	++	0	0	++	+	0	1	1
135	+	0	++	+	0	0	0	+	0	+		2
136	++	+++	++	0	0	0	0	++	+++	+++	2	

*LN, local interneurone; PN, projection neurone.
Responses are classified as weak (+), intermediate (++) or strong (+++). 0, no response; nt, the compound was not tested.

during the time course of an experiment resulted in similar responses (Fig. 5B). PNs and LNs could not be easily distinguished on purely physiological grounds (see Figs 4, 5). Action potential amplitudes varied between 10 and 45 mV.

Specificity of the responses of AL interneurones to the 10 odorants varied widely. 43 neurones responded to only one compound, whereas 31, 22 and 16 neurones responded to two, three and four compounds, respectively. The remaining 24 neurones responded to several (5–8) compounds (Fig. 3A). None of the examined cells responded to more than eight odorants. Responses to α-humulene were most frequently obtained (38 % of the tested neurones), while responses to most other compounds occurred in approximately 30 % of the tested neurones. The lowest response frequency was observed for α-farnesene. A response was obtained only in 21 % of the neurones when α-farnesene was applied (Fig. 3B).

Profiles of responses to different odorants are shown in Tables 1–4. Neurones responding specifically to each of the ten tested compounds were found. Among the 43 specific neurones, 14 responded to α-humulene (numbers 1–10, 118, 121, 126 and 127) and six neurones responded to geraniol (numbers 14–18 and 120). Only one neurone (number 128) responded specifically to *Z*,*E*-9, 11–14:OAc, while 2–5 specific neurones were found for the remaining compounds. Of the 153 tested neurones, an inhibitory response was observed only in one (number 46). This neurone, inhibited by β-caryophyllene and eugenol, exhibited no response to other tested compounds (Table 2).

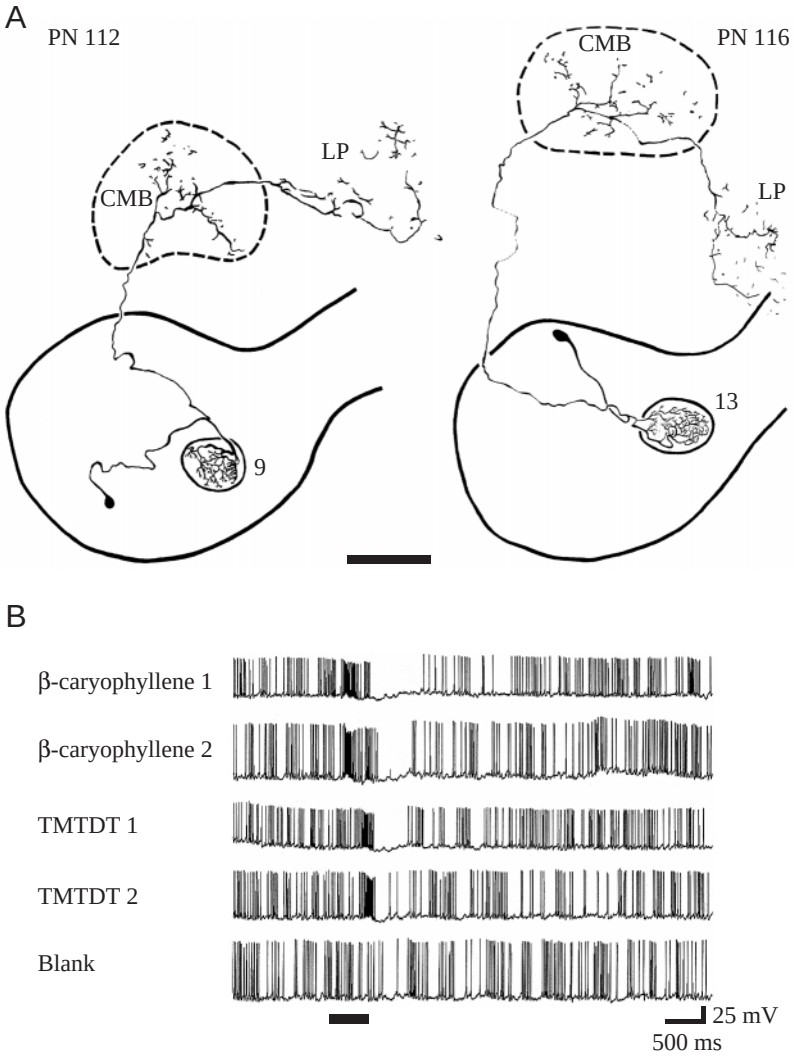

Fig. 5. (A) Morphology of neurobiotin-stained projection neurones (PNs) reconstructed from 10 µm frontal sections. Arborisations within the antennal lobe are uniglomerular. The targeted glomerulus was identified (using the reference in Fig. 1) and indicated by the number alongside. Axons of both neurones leave the antennal lobe through the inner antennocerebral tract. Protocerebral projections arborise within the calyces of the mushroom body (CMB) and then supply the lateral protocerebrum (LP). Broken lines indicate the borders of the CMB. Scale bar, 100 µm. (B) Responses of the generalist neurone (number 112) to repeated stimulation by two compounds. Other stimuli had been tested in between. The thick horizontal bar indicates the time at which the compound was applied.

Morphological characteristics

Of 136 attempted fillings, eight LNs and 19 PNs were successfully stained. In 15 specimens, more than one neurone was accidentally filled (Table 3), and these were not considered in the analysis. Five of the eight stained LNs had their cell bodies in the lateral cell body group (Table 4) and had widespread arborisations with varying densities throughout the AL (Fig. 4), whereas the cell bodies of the remaining three LNs could not be located.

PNs had their cell bodies in the lateral or medial cell body clusters and arborised in one glomerulus each (Table 5). In six preparations, the PN axons were completely stained. According to the nomenclature of Homberg et al. (1988), all of those neurones were of the PIa subtype. Their axons left the AL *via* the inner antennocerebral tract (IACT). Two of those completely stained PNs (numbers 112 and 116) were reconstructed from 10 µm frontal sections. Projecting *via* the IACT, the axons of both PNs branched in the calyces of mushroom bodies, and in the lateral protocerebrum (Fig. 5).

Glomeruli innervated by 14 of the 19 PNs could be individually identified. Among the remaining five PNs, three innervated glomeruli in the central area of the AL (light green, Fig. 1) and two arborised in ventromedial glomeruli (pink, Fig. 1). PNs arborising in the same glomerulus responded to different sets of stimuli, e.g. the three PNs innervating glomerulus C, among others (Fig. 6). These three generalist cells had only the response to linalool in common; otherwise they differed in their response profiles. Conversely, the two neurones (108 and 112) were very similar in their response profiles: each of them responded to the same three out of four compounds. Nevertheless, they arborised in two different and

Table 4. *Response profiles of eight successfully stained local interneurones*

Neurone number	Compound										Cell body position*
	1	2	3	4	5	6	7	8	9	10	
95	0	0	0	+++	0	0	++	nt	nt	nt	?
96	0	0	+++	0	++	0	0	nt	nt	nt	L
97	++	+++	0	0	0	+++	0	0	nt	nt	L
98	0	0	+	0	+++	0	++	0	nt	nt	L
99	++	0	0	0	+++	0	0	0	+	0	?
100	0	+	0	++	++	0	0	0	0	0	?
101	0	0	0	+	++	0	0	++	0	0	L
102	+	0	0	0	+	0	0	+	+	+	L

*L refers to the lateral clusters of cell bodies. ? indicates that the cell body could not be located.
Responses are classified as weak (+), intermediate (++) or strong (+++); 0, no response; nt, the compound was not tested.

Table 5. *Response profiles and morphological characteristics of 19 stained projection neurones*

Neurone number	Compound										Glom	CB	Tract
	1	2	3	4	5	6	7	8	9	10			
103	+++	0	+++	+++	0	0	+++	0	0	0	A	M	IACT
104	++	++	0	0	+++	+++	0	nt	nt	nt	C	M	ns
105	+++	0	0	+++	0	+++	++	0	+++	0	C	M	ns
106	0	++	++	0	+++	+	+++	0	++	0	C	M	ns
107	+	0	0	0	0	++	0	+	+	++	D	L	ns
108	0	+++	0	++	++	0	+++	0	0	0	E	L	ns
109	++	0	0	++	0	+++	0	0	nt	nt	2	M	ns
110	0	0	0	0	0	+++	0	0	0	++	3 ?	M	IACT
111	0	0	0	0	+	+	0	0	0	0	6 ?	M	ns
112	0	+	0	0	++	++	++	0	0	0	9	M	IACT
113	++	0	0	0	0	0	0	+	nt	nt	10	L	IACT
114	0	0	++	0	0	++	0	0	nt	nt	11	L	ns
115	+	+++	++	+++	0	+++	0	0	++	+++	11	M	IACT
116	0	0	0	0	0	++	0	0	nt	nt	13	L	IACT
117	0	0	0	0	0	0	0	++	0	0	Y	L	ns
118	+	0	0	0	0	0	0	nt	nt	nt	Y	M	ns
119	++	0	0	0	0	0	++	nt	nt	nt	Y	M	ns
120	0	0	+	0	0	0	0	nt	nt	nt	P	L	ns
121	+++	0	0	0	0	0	0	nt	nt	nt	P	L	ns

Numbers followed by question marks indicate that identification of the glomerulus could be incorrect; if so, the correct glomerulus must be adjacent to the identified one. Y and P indicate that the targeted glomerulus is one of the yellow or pink coloured groups of glomeruli (see the model in Fig. 1).

CB, cell body position; Glom, input glomerulus; M, median and L, lateral clusters of cell bodies; IACT, inner antennocerebral tract; ns, the axon was not stained.

Responses are classified as weak (+), intermediate (++) or strong (+++). 0, no response; nt, the compound was not tested.

distantly located glomeruli (Fig. 7). Two neurones specific for α-humulene (118 and 121) innervated two different glomeruli; one of them had its arborisation in a glomerulus in the middle part of the mapped region, whereas the other innervated a different glomerulus residing in the medioventral area of the AL (Table 5). Neurones with completely different response profiles, such as 113 and 114, arborised in two adjacent glomeruli (Fig. 8). Projection patterns for neurones responding to each of the 10 compounds are compiled in Fig. 9: glomerulus C and glomerulus 11 were more often innervated by PNs responding to the tested compounds than other glomeruli. Each of them harboured the arborisations of neurones responding to seven different stimuli. Glomerulus 13, which lies adjacent to glomerulus C, was innervated by only one neurone specific to linalool.

Discussion

Identification of innervated glomeruli

In the present study, we found that in female *S. littoralis*, PNs responding to the same plant volatile may arborise in different glomeruli, and a single glomerulus seems to

PN 104	
Linalool	(+++)
α-humulene	(++)
β-caryophyllene	(++)
Benzaldehyde	(+++)
TMTDT	(0)
Z,*E*-9, 11–14:OAc	(0)
Eugenol	(0)
Geraniol	(0)

PN 105	
Linalool	(+++)
α-humulene	(+++)
β-caryophyllene	(0)
Benzaldehyde	(0)
TMTDT	(++)
Z,*E*-9, 11–14:OAc	(+++)
Eugenol	(+++)
Geraniol	(0)

PN 106	
Linalool	(+)
α-humulene	(0)
β-caryophyllene	(++)
Benzaldehyde	(+++)
TMTDT	(+++)
Z,*E*-9, 11–14:OAc	(++)
Eugenol	(0)
Geraniol	(++)

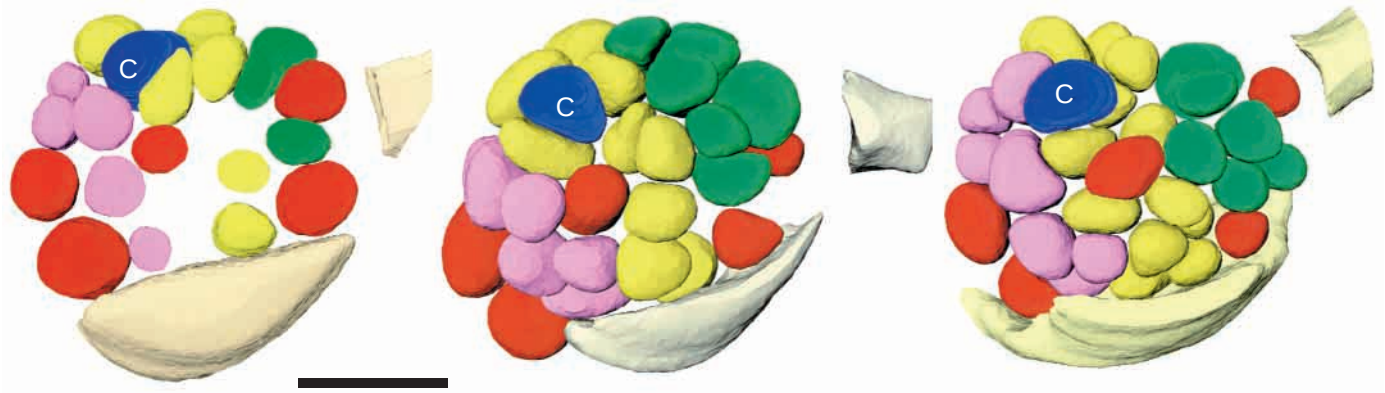

Fig. 6. Reconstructed antennal lobes indicating the glomerulus targeted by a specific projection neurone (PN). The three neurones arborised in the same glomerulus, C (blue). Although some glomeruli were not reconstructed in number 104, the targeted glomerulus was easily identifiable because it is one of the landmark glomeruli. For reference see Fig. 1. +++, strong response; ++, moderate response; +, weak response; 0, no response. Scale bar, 100 µm.

participate in the processing of several odour compounds. For the identification of the innervated glomeruli, we created a map of the anterior aspect of the AL of the moth. The 30 glomeruli we reconstructed in our model represent almost half of the total number of glomeruli, as estimated earlier by Anton and Hansson (1994). The glomeruli included in the map were clearly distinguishable, while the borderlines of the remaining ones were not clear. One of the landmark glomeruli, labelled F, had the shape, relative size and position to indicate that it is most likely to be homologous to the glomerulus called 'the labial pit organ glomerulus', which receives input from receptor neurones of the labial pit organ in a number of moth species (Kent et al., 1986, 1999; Rospars and Hildebrand, 1992, 2000). In addition to the 30 reconstructed glomeruli, a few more were visible from the front but they were not mapped because their borders could not be unequivocally defined.

PN 108	
β-caryophyllene	(+++)
Benzaldehyde	(++)
TMTDT	(+++)
Eugenol	(++)
Linalool	(0)

PN 112	
β-caryophyllene	(+)
Benzaldehyde	(++)
TMTDT	(++)
Eugenol	(0)
Linalool	(++)

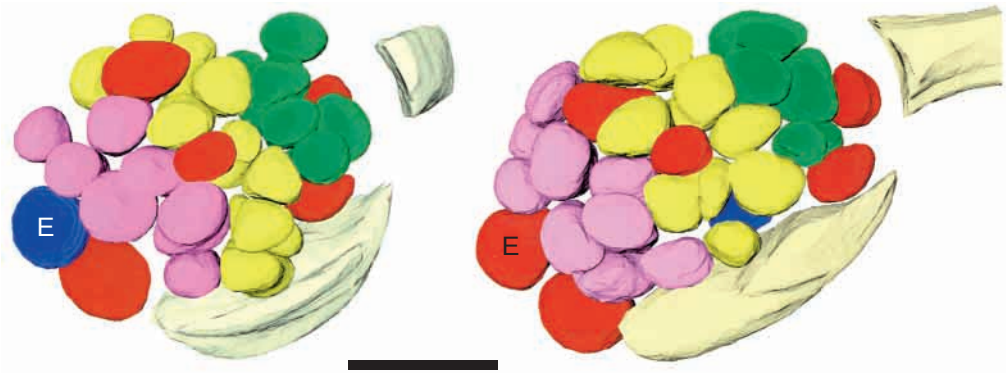

Fig. 7. Reconstructed antennal lobes with two physiologically similar projection neurones (PNs) arborising in different, and distantly located, glomeruli (blue). The glomerulus innervated by neurone 108 is the landmark glomerulus E. Response profiles are given as in Fig. 6. Scale bar, 100 µm.

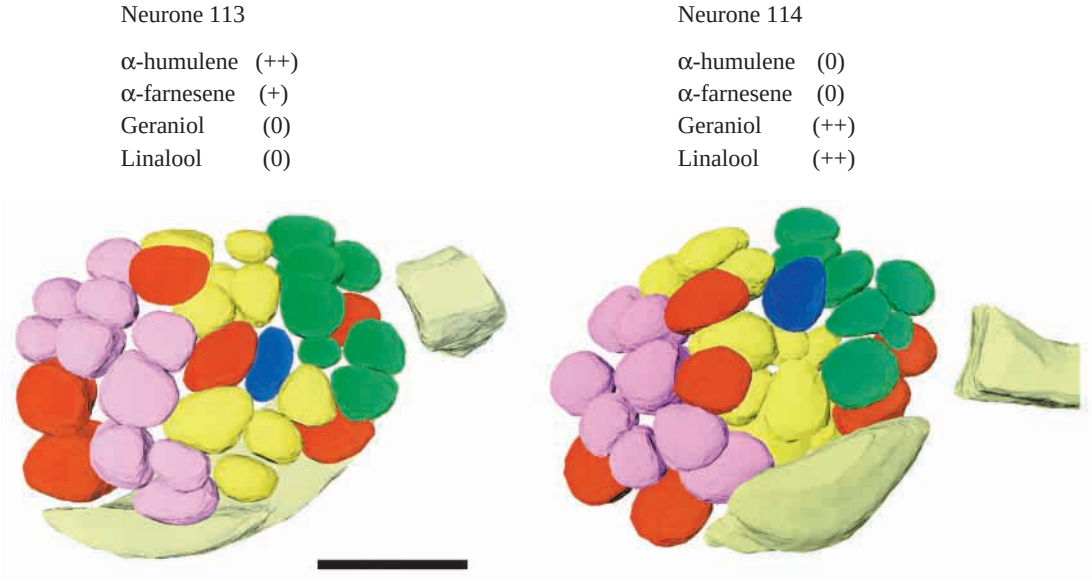

Fig. 8. Reconstructed antennal lobes with two physiologically non-overlapping types of neurones arborising in two different but adjacent glomeruli (blue). Response profiles are given as in Fig. 6. Scale bar, 100 μm.

Apart from what we considered to be 'landmark glomeruli', individual small differences were observed in the mapped part of ALs in the size, shape and location of identified glomeruli. They result from the normal biological variability (Rospars, 1988; Rospars and Chambille, 1989; Todd and Baker, 1996). However, anomalous variations, e.g. the occasional absence of some glomeruli, were also found. They are more difficult to interpret because, besides true biological differences, artefacts may occur. Methodological factors that may contribute to a certain ambiguity in the identification of non-landmark glomeruli include delicate differences in the strength of antibody binding or difficulties in discerning glomerular borders when their surfaces coincide with the plane of section. Such irregular variations have been reported in several insect species (e.g. Arnold et al., 1985; Chambille and Rospars, 1985; Flanagan and Mercer, 1989; Stocker et al., 1990; Galizia et al.,

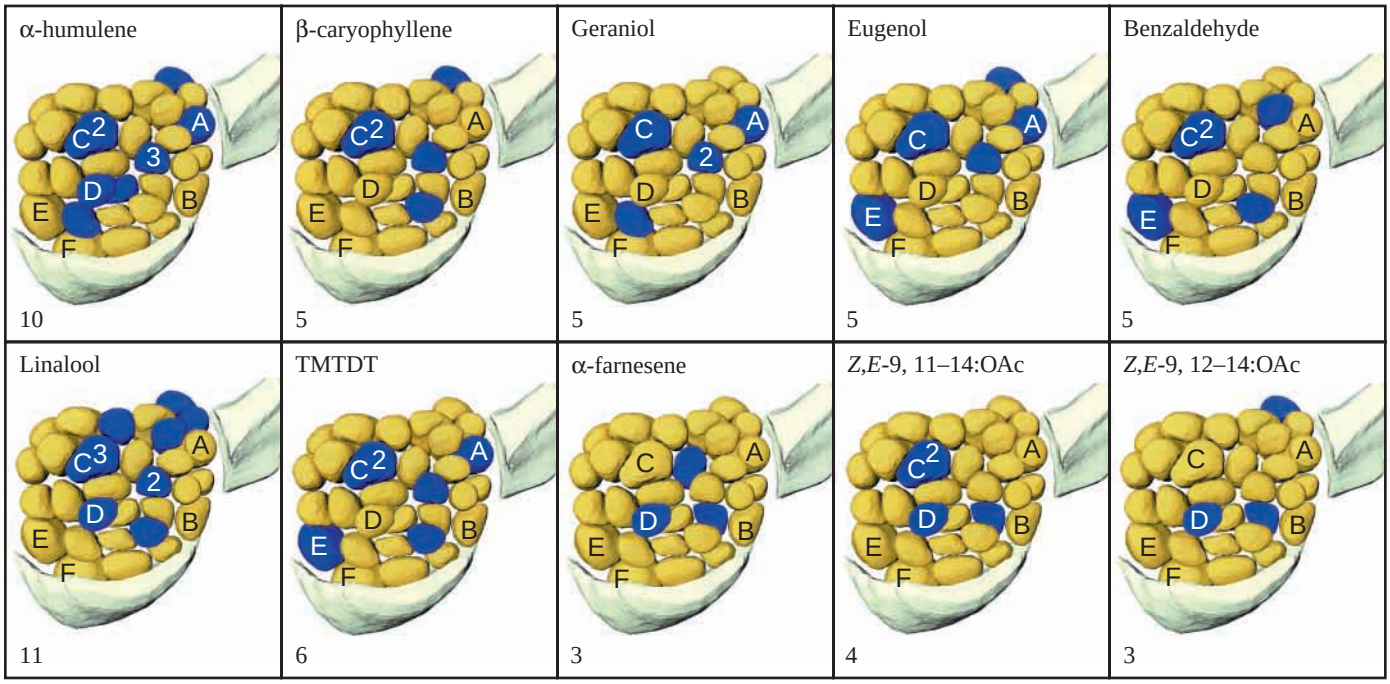

Fig. 9. Representation of the ten tested odours in the AL glomeruli. Patterns are drawn based on the morphology of 19 stained projection neurones, where glomeruli targeted by responding neurones are blue. For glomeruli harbouring the dendritic arborisations of more than one neurone, the number of innervating neurones is given on the glomerulus. Numbers in the lower left-hand corners are the numbers of stained projection neurones for a given compound.

1999; Laissue et al., 1999) and in at least one case the presence of an anomalous supernumerary glomerulus in one individual has been documented (Rospars and Hildebrand, 2000). In the present study, the posterior glomeruli were less clearly visible than anteriorly situated ones. Glomeruli in the AL of *S. littoralis*, as well as in other Lepidoptera species, are known to be arranged around a non-glomerular central fibre core (Anton and Hansson, 1994). Penetration of the tissue was, however, not the cause of the low visibility of the posteriorly situated glomeruli, as brains cut in half showed the same results. Mapping the anterior half of the AL was sufficient for the purpose of our study, as all of the stained PNs innervated glomeruli that were situated within the mapped region. This is probably due to electrode placement, although no responses to the tested stimuli were obtained when the electrode was inserted deeper within the AL. A complete map of the AL of *S. littoralis* is, however, indispensable for a better understanding of odour coding in the insect olfactory system. Work towards this goal is under way.

Physiological response of interneurones

Both PNs and LNs examined in the present study varied in their specificity, from specific neurones that respond to single compounds, to less specific ones that respond to two or three compounds, to generalists that responded to a variety of up to eight compounds. Plant odour-sensitive ORNs in insects have originally been considered as generalists, responding to a broad spectrum of odours (Visser, 1986). However, highly specific ORNs for single plant odours have been described in several species, including *S. littoralis* (Dickens, 1990; Todd and Baker, 1993; Anderson et al., 1995; Hansson et al., 1999). In our study, more than a quarter of the investigated interneurones were highly specific and about 20% responded to only two compounds. Considerable numbers of interneurones that respond specifically to plant volatiles and sex pheromone components have been demonstrated earlier in the female, as well as in the male *S. littoralis* (Anton and Hansson, 1994, 1995). The specificity of receptor neurones observed in both sexes of the insect (Ljungberg et al., 1993; Anderson et al., 1995) seems, therefore, to be remarkably preserved at the AL interneuronal level. However, information stemming from different specific receptor neurones definitely converge on more integrative interneurones. Neither generalist nor specific receptor neurones in *S. littoralis* that respond to plant-derived compounds respond to any of the sex pheromone components (Anderson et al., 1995; Jönsson and Anderson, 1999). Many of the generalist interneurones described in the present study did, however, respond to plant volatiles and pheromone components. These neurones must, therefore, receive input from both types of receptor neurones. It is worth noting that the variation in specificity among the investigated interneurones was observed for both PNs and LNs. The discrimination between LNs and PNs was based on the morphological features of the successfully stained neurones. However, for the unstained neurones, we were unable to discriminate between LNs and PNs, as their physiological characteristics did not differ clearly.

The highly specific interneurones found in this study indicate that not all specific ORN types might yet have been studied on the antenna. ORNs responding to α-farnesene, together with other compounds, have been described, but the presence of ORNs tuned to either α-farnesene only or benzaldehyde only has not been reported in studies in the periphery (Anderson et al., 1995; Jönsson and Anderson, 1999). However, two specific interneurones that respond only to benzaldehyde, and two others that respond only to α-farnesene were found. Receptor neurones specific for α-farnesene and for benzaldehyde are, therefore, very likely to exist on the antennae of female *S. littoralis*, unless specificity of central neurones is obtained through properties of the AL circuitry.

Characteristics of local interneurones and pheromone sensitive projection neurones

The entirely stained LNs exhibited different degrees of complexity. Some cells innervated more glomeruli than others, but in all cases large numbers of glomeruli were innervated by one LN. In an earlier study, Anton and Hansson (1994) described multiglomerular LNs with homogenous arborisations throughout the AL and oligoglomerular arborisations whose branches invade relatively few glomeruli. In addition to these two types, we found also multiglomerular LNs with heterogeneous arborisations that were asymmetrically distributed within the glomeruli of the AL.

PNs responding to the major sex pheromone component arborised in three different anteromedially located glomeruli. Interestingly, these three glomeruli did not include any of the glomeruli that were shown to receive inputs from receptor neurones tuned to the same pheromone compound in female *S. littoralis* (Ochieng' et al., 1995). The relationship between axonal arborisations of receptor neurones and the dendritic branches of PNs that display identical response characteristics to female pheromones is particularly well studied in male moths. Receptor neurones on the male antennae often project specifically to different MGC compartments in the AL (Hansson et al., 1992; Ochieng' et al., 1995; Todd et al., 1995; Berg et al., 1998). In some moths, e.g. *Heliothis virescens*, PNs seem to innervate precisely the same glomerulus or subunit of the MGC to which physiologically identical ORNs send their axonal branches (Vickers et al., 1998). However, in several other noctuid species, the correspondence between the PN dendrites and the axonal terminals of corresponding ORNs is limited (Hansson et al., 1994; Anton and Hansson, 1995; Wu et al., 1996; Anton and Hansson, 1999).

Characteristics of projection neurones sensitive to plant odours

The axonal arborisation patterns of ORNs specific to plant-derived compounds in female *S. littoralis* are unknown. However, the projection patterns of plant odour-sensitive PNs investigated in the present study were not restricted to a particular glomerulus or group of glomeruli in a restricted area of the AL that depended on the response spectrum. Each of the

19 stained PNs responded to one or more of the plant-derived compounds and they innervated in all at least 13 different glomeruli scattered throughout the whole anterior aspect of the AL. This finding can be interpreted in the framework of the hypothesis of 'across-glomeruli pattern' (Rospars, 1983; Rospars and Fort, 1994): according to this hypothesis, a large number of odours can be discriminated by a few glomeruli because a small set of interconnected, anatomically identified and functionally unspecific glomeruli can give rise to a large number of different patterns of activity, each pattern characterising a specific odour (e.g. n such glomeruli in two states can give rise to 2^n patterns). This is also consistent with what has been recently revealed in *S. littoralis* using optical imaging techniques (Hansson et al., 2000; Carlsson et al., 2001; Meijerink et al., 2001). Similar results have also been reported in a number of other insect and vertebrate species, where an odour molecule elicits activity in several glomeruli and each glomerulus participates in the evoked pattern of several odours (for a review, see Galizia and Menzel, 2001). Molecular studies on *Drosophila* have shown that the number of expressed odour receptor genes (Clyne et al., 1999; Vosshall et al., 1999, 2000; Gao et al., 2000) is comparable with the number of glomeruli in the AL of the fly (Laissue et al., 1999). Each receptor neurone is believed to express a single receptor gene and the axons of ORNs expressing the same gene all converge onto one or two glomeruli in the AL. However, what is clear from our observations, is that not only one or two, but several glomeruli are innervated by PNs responding to a single compound, emphasising that one compound might bind to several odour receptor types and that LNs might play an important role as mediators between ORNs and PNs in the AL.

It has recently been shown that specific glomeruli in the female moth, *Manduca sexta* have characteristic limited molecular receptive ranges, harbouring the dendritic arborisations of output neurones that respond only to one compound or chemically related compounds (King et al., 2000). In the present study, successfully stained PNs that arborised in the same glomerulus had wider response spectra. However, their response profiles suggest that a single glomerulus in the AL of *S. littoralis* could be innervated by output neurones responding to compounds that are neither behaviourally nor chemically related. PNs responding to compounds of different behavioural roles, e.g. oviposition deterrents, host plant odours and sex pheromones, or compounds with different functional groups, e.g. alcohols, aldehydes and sesquiterpenes, arborised in the same glomerulus. This is consistent with what has been found in the honeybee, where glomeruli are preferentially activated by one functional group over another, but can also be activated by other functional groups, even though the level of activation is weaker (Sachse et al., 1999; Galizia and Menzel, 2001).

In conclusion, our work shows that a single plant volatile compound is not represented within a single glomerulus in the AL of the female *S. littoralis*. Similarly, neither compounds with the same functional group nor those that play the same behavioural role are represented in one glomerulus or one group of adjacent glomeruli. We, thus, expect complex patterns of glomeruli to be involved in the processing of olfactory information concerning both plant-derived compounds and pheromones in this species.

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