REVIEW

Representation of Primary Plant Odorants in the Antennal Lobe of the Moth Heliothis virescens Using Calcium Imaging

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Abstract

The primary olfactory centre, the antennal lobe of Heliothis virescens moths, contains 62 glomeruli which process plant odour information and four male-specific glomeruli which form the macroglomerular complex, involved in processing information about pheromone and interspecific signals. Using calcium imaging, we recorded the spatio-temporal activity pattern of the glomeruli in the anterior antennal lobe during stimulation with odorants produced by plants or insects. Each odorant elicited specific excitatory responses in one or a few glomeruli: the major pheromone component did so exclusively in the large glomerulus of the macroglomerular complex and the plant odours exclusively in the ordinary glomeruli. Eight glomeruli, with corresponding plant odour responses and positions, were identified within each sex. Glomeruli responded specifically to linalool, β-ocimene/β-myrcene or germacrene D/α-farnesene. Responses to two essential plant oils covered the response areas of their major constituents, as well as activating additional glomeruli. Stronger activation in the AL due to increased odour concentration was expressed as increased response strength within the odorant-specific glomeruli as well as recruitment of less sensitive glomeruli.

Key words: α-farnesene, germacrene D, glomeruli, linalool, β-ocimene, pheromone

Introduction

It is important for most animals’ survival and reproduction to be able to detect chemical cues in the environment. Receptor neurons (RNs) detect airborne molecules and transduce the information from these chemicals into electrical signals that are brought to the first relay station in the brain, the vertebrate olfactory bulb or the insect antennal lobe (AL). Here the axons of the RNs project to glomeruli, which are thought to be functional units (Hildebrand and Shepherd, 1997; Smith and Shepherd, 1999). As in other lepidopteran species, the macroglomerular complex (MGC) in the AL of Heliothis virescens males receives information about insect-produced signals (pheromones and interspecific signals), while the ordinary glomeruli in both sexes receive information about plant odours, as shown in previous electrophysiological and optical recordings (Berg et al., 1998; Vickers et al., 1998; Galizia et al., 2000; Stranden et al., 2003a,b). Whereas these studies showed a functional organization of the MGC, with each glomerulus processing information about one insect-produced compound, less is known about the functional organization of the ordinary glomeruli. Optical recording (e.g. calcium imaging) is a suitable method for studying odour representation in the AL, as shown in the honeybee Apis mellifera (Galizia and Menzel, 2001). In the female bee, stimulation with plant odours elicits spatial patterns of activity characteristic for each odour, which are conserved within the species and can be mapped to identified glomeruli. Similar calcium imaging studies have also revealed spatial activity patterns for plant odour quality in H. virescens (Galizia et al., 2000) and Spodoptera littoralis (Carlsson et al., 2002).

In recent years knowledge of the specificity of plant odour RNs has increased considerably for H. virescens and other...
species by the use of electrophysiology linked to chemical analyses (Blight et al., 1995; Wibe and Mustaparta, 1996; Røstelien et al., 2000a,b; Stensmyr et al., 2001; Barata et al., 2002; Stranden et al., 2002, 2003a,b; Bichão et al., 2003). These studies generally showed a narrow tuning of the RNs. This is in contrast to results obtained by direct stimulation with selected odorants, which have shown broadly tuned olfactory RNs in vertebrates. Broadly tuned RN types were also found in Drosophila melanogaster, in addition to some specialized RN types (Smith and Shepherd, 1999; De Bruyne et al., 2001). The narrowly tuned RNs obtained by linking gas chromatography to electrophysiology show overlap of molecular receptive ranges only within the same chemical groups, e.g. within monoterpenes or sesquiterpenes, respectively. So far ~20 types of plant odor RNs have been identified in H. virescens (Røstelien et al., 2000a,b; Stranden et al., 2003a,b; Røstelien et al., unpublished data). They are characterized by strong responses to one or two odorants and weak responses to a few others having similar structures. These odorants are defined as primary and secondary odorants, respectively, for each RN type. With one exception for linalool (3,7-dimethyl-1,6-octadien-3-ol), the molecular receptive ranges of these RNs have not shown any overlap. Among RN types for which the primary and secondary components have been identified are neurons responding best to germacrene D [1,6-cyclocdecadiene, 1-methyl-5-methylene-8-(1-methylthyl), E,E], (E,E)-α-farnesene [(3E,6E)-3,7,11-trimethyl-1,3,6,10-dodecatetraene], (E)-β-ocimene [(3E)-3,7-dimethyl-1,3,6-octatriene], geraniol [(2E)-3,7-dimethyl-2,6-octadien-1-ol], or (S)-(+-)linalool, respectively (Røstelien et al., 2000a,b; Stranden et al., 2003a,b; Røstelien et al., unpublished data). The only two odorants that are found to activate two different RN types are (S)-(+-)linalool and (R)-(+-)linalool, both having weak effects on the geraniol RN type, and (R)-(+-)linalool, which has a weak effect on the (S)-(+-)linalool cell (Stranden et al., 2003b; Røstelien et al., unpublished data). Molecular biological studies of olfactory receptor proteins in H. virescens have shown that each receptor subtype appears to be expressed in a distinct population of sensory cells showing no co-expression (Krieger et al., 2002). One would expect that each RN type projects specifically to one or a few glomeruli in the AL in D. melanogaster similar to what is the case for D. melanogaster and in vertebrates (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Gao et al., 2000; Vosshall et al., 2000).

In the present study optical recordings were made for H. virescens males and females, aiming to determine the activity pattern in the antennal lobes during stimulation with different plant odorants. We tested odorants known to be primary or secondary odorants for particular RN types as well as mixtures. The results showed activation of one or a few glomeruli during stimulation with the single odorants. Stimulation with mixtures activated both areas that covered the glomeruli responding to the single constituents and addi-tional glomeruli. Higher odour concentrations usually increased the response strength and sometimes showed a slight activation of additional glomeruli. Eight glomeruli with corresponding positions and plant odour responses were identified within each sex, four of them also corresponded in the two sexes.

Materials and methods

Animals

Pupae of H. virescens (Heliothinae: Lepidoptera: Noctuo-idae) were kindly provided by Mr Ulrich Ebbinghaus-Kint-scher at Bayer, Germany. Females and males were placed in separate containers and kept in a phase-shifted light–dark photoperiod at room temperature. After emerging, the adult moths were placed in new containers and given honeywater. Insects used in the experiments were 2–5 days old.

Animal preparation and staining of the brain

The insects were prepared for optophysiological recordings as described in a previous work (Galizia et al., 2000). The dye calcium green 2 AM [50 μg dye dissolved in 50 μl Pluronic in DMSO and 950 μl Ringer’s solution (150 mM NaCl, 3 mM CaCl2, 3 mM KCl, 10 mM TES buffer and 25 mM sucrose, pH 6.9); Molecular Probes] was applied in the head capsule of the in vivo preparation for 1–1.5 h. Afterwards, the dye was washed out with Ringer’s solution. The preparation was covered with a plastic cover slip and placed under the microscope (see below). A hole in the plastic cover slip above the brain allowed frequent addition of fresh Ringer’s by a pipette onto the exposed brain during the experiment. The water objective was placed on top of the hole, in contact with the Ringer’s solution.

Odour stimulation

Eight single compounds and two mixtures were tested during the experiments. All plant odorants were tested at five different concentrations (10−1–10−5, where 10−1 corresponded to 10 mg of the component applied to the filter paper for all odorants except for (–)-germacrene D where 10−1 corresponded to 2 mg), whereas the pheromone component was tested with 1 μg of compound on the filter paper. All substances were dissolved in n-hexane (Kebo Lab), evaporated onto filter paper and put into clean glass cartridges. The single odorants tested were (purities tested on gas chromatograph): the major pheromone component Z11–16:Al (Z-11-hexadecenal; provided by Dr J.G. Tumlinson, USDA, Gainesville, FL), the single plant components β-myrcene (7-methyl-3-methylene-1,6-octadiene; Fluka Chemika; purity 90%), β-ocimene (Firmenich; purity 82%), α-farnesene (pro-vided by Dr A.-K. Borg-Karlson, KTH, Stockholm, Sweden; purity 61%), (–)-germacrene D (provided by Dr A.-K. Borg-Karlson; purity 99%) and (R)-(–)-E-β-caryophyllene (bicyclo [7.2.0] undec-4-ene,4,11,11-trimethyl-8-methylene; Fluka Chemica; purity 96%). Four mixtures
were tested, including two enantiomeric mixtures of linalool (one with 47% (R)-(−)-linalool and 48% (S)-(+)linalool, called ‘racemic linalool’ and one containing 96% (R)-(−)-linalool and 2% (S)-(+)linalool, called ‘(R)-(−)-linalool’ (Kock Light Laboratories) and the essential oils of cubebe pepper (Dragoco) and ylang ylang (Firmenich). Cubebe pepper contains mainly sesquiterpenes including germacrene D, but also small amounts of racemic linalool, β-myrcene and (E)-β-ocimene, as well as many other compounds. Ylang ylang contains large amounts of germacrene D and racemic linalool in addition to α-farnesene, (E)-β-ocimene and smaller amounts of other compounds. The animals were placed in a constant clean air stream. A 1 s odour pulse was blown into the constant airflow for stimulation. Stimulus concentration was tested twice on each preparation, the first time focusing on an upper level of the antennal lobe and the second time 100 µm below by shifting the objective with a piezo-control (PiFok; PI-Instruments, Germany). Two cartridges, one containing a filter paper with evaporated hexane and one without hexane were used as controls. The inter-stimulus interval (ISI) was 1 min.

**Calcium measurements**

The prepared moths were imaged under an upright Axioscope Zeiss microscope using a 20× water objective (NA = 0.5; Olympus) or with an upright Olympus BX 50WI microscope using a 20× water objective (NA = 0.95). CCD camera and imaging system was by Till-Photronics. Monochromatic excitation light was 475 nm, dichroic: 500 nm, emission: LP 515. Pixel image size was 2.4 × 2.4 µm, obtained by 2 × 2 binning on chip or 4.8 × 4.8 µm, obtained by 4 × 4 binning on chip. For each stimulus, 100 frames (images) were taken at a frequency of 5 Hz. Exposure time for each frame was between 50 and 100 ms with a binning of 2 and between 10 and 40 ms with a binning of 4. The light was shut off between frames. The odour stimulus was given at frames 25–30, which is 5 s after the first frame, lasting 1 s. During the stimulation we observed patterns of intracellular calcium increase in the antennal lobe of *H. virescens*, followed by a slow, negative phase as found in honeybees (Stetter et al., 2001). Ringer fluctuations outside the AL led in some cases to light intensity fluctuations that appeared as signals in false-colour-coded images (Figure 1). Areas outside the AL were therefore excluded (Figures 2 and 3) or shaded (Figure 4).

**Data processing**

Off-line analyses were done with custom programs written in IDL language (Research Systems, CO). Raw data were median-filtered to reduce shot noise (filter size three pixels in one temporal and two spatial dimensions). Responses were calculated as relative changes in fluorescence intensity (∆F/∆F, where F is calculated as the mean of frames 5–24, before stimulus onset at frame 25). We applied a spatial unsharp-mask filter to each frame in the raw data. Specifically, letting A be one raw data image frame and sm(A) the same image treated with a spatial low-pass filter, we then calculated \( A_{\text{sharp}} = (2 \times A) - (\text{sm}(A)) \). This algorithm reduces the effect of scattered light produced by strongly activated glomeruli to neighbouring non- or less-active glomeruli, which would otherwise appear as false-positive glomeruli. This procedure will increase slightly the measured responses for focalized activities, as compared to non-corrected data, since the blurring effect is reduced. For large activity spots no change will occur at the centre of the activity, since \( \text{sm}(A) \) will have the same value as the original image A and the formula will therefore yield the same value as the original measurement A. The time-courses were corrected for bleaching in the following way: for each frame (point in time) the average signal of the entire antennal lobe was calculated. This gives a single function of signal intensity against time. A log-function was fitted to this time-course and the values of the log-function subtracted from each frame. This treatment removes global bleaching and/or photoisomerization effects without changing the spatial components of odour responses. For false-colour display, at each pixel the time-course is calculated as an average of this pixel and its surrounding areas \([13 \times 13 \text{ pixels} = 31.2 \times 31.2 \mu\text{m} (2 \times 2 \text{ binning on chip}) \text{ or } 7 \times 7 \text{ pixels} = 33.6 \times 33.6 \mu\text{m} (4 \times 4 \text{ binning on chip})] \). For calculation of false-colour images the mean of three frames at time frame 22–24 was subtracted from the mean of three frames at time frame 29–31. The false-colour-coded pictures in Figures 2 and 3 were scaled to the upper 50% of their intensity ranges and superimposed on grey-scale images from the measurements (see below).

**Afterstaining**

After the recordings, some of the ALs were treated with a protease (P5459; Sigma) to digest the neurolemma and stained with the membrane-soluble dye RH795 (Molecular Probes) to make the glomerular structures visible in epifluorescent light. The insects were treated with the afterstaining for 2–12 h, sometimes placed on a hot plate for better staining. Morphological images of the ALs were then taken with the CCD camera as z-stacks consisting of 100 images at 1 µm distance. Two overlapping z-stacks were usually chosen, covering the focal depths between 0 and 150 µm in the AL. Exposure time for each image was between 5 and 40 ms. Monochromatic excitation light was 515 nm or 475 nm, emission LP535 or LP515, respectively. Pixel image size was 2.4 × 2.4 µm, obtained by 2 × 2 binning on chip or 1.2 × 1.2 µm, obtained by 1 × 1 binning on chip.

**Reconstruction of the afterstained preparations**

Two-dimensional reconstructions were made for some of the animals based on the morphological images of the different focal depths of the afterstained ALs (usually in a frontal view). The reconstructed glomeruli maps could be posi-
tioned onto the black and white pictures from the calcium imaging experiments, using landmarks such as the AL border, trachea and bright spots. Finally, this black and white picture could be replaced with a false-colour-coded picture showing the responses to an odour so that the response areas could be related to glomerular structures. These 2-D maps were also compared to the 3-D reconstruction of the AL glomeruli (Berg et al., 2002) in order to try to identify the activated glomeruli according to this atlas. Since this was difficult in respect to the ordinary glomeruli, identification of the activated glomeruli across individuals was based on their relative position and the distance (number of glomeruli) between them, as found in the 2-D maps. If the response patterns corresponded in different individuals, the active glomeruli were addressed as identical glomeruli and given the same numbers.

Results

Identification of glomeruli in accordance with the 3-D atlas

The results are based on recordings from antennal lobes of eight males and 16 females that out of 100 tested preparations showed significant signal-to-noise ratios for further analyses. In 16 preparations (three males and 13 females) the glomeruli pattern was visible in the afterstainings, where we used a membrane-permeable dye (see Materials and methods). We made a 2-D reconstruction of the most anterior part by looking at different focal depths (0–150 µm) of the AL (usually in a frontal view). Figure 1 shows the antennal lobe of a male at three focal depths (0, 90 and 160 µm, Figure 1A–C) and the reconstructed glomerular map (Figure 1D). By superimposing the 2-D map of glomeruli on the black and white pictures and the false-colour-coded pictures from the experiments, the activated areas could be ascribed to glomeruli visible in the afterstained preparation (Figure 1E–H, showing the response pattern elicited by racemic linalool). In most of the males the MGC was clearly visible and the cumulus, the dorso-medial glomerulus and one or both of the ventral glomeruli could be identified. It was not possible to identify the ordinary glomeruli according to the glomeruli numbers given in the atlas of the antennal lobe (Berg et al., 2002), except for the two large ventrally located glomeruli that were recognized in a few female antennal lobes. These two glomeruli (data not shown) were identified as glomeruli number 19 (which receives information from the labial pit organ) and 20 in the 3-D atlas. In the present study the activated ordinary glomeruli were labelled with arbitrary numbers, Gm1–Gm8 in males and Gf1–Gf8 in females.

Comparison of the spatial response patterns between individuals

Males

In the males, the MGC was easily identified in most individuals. Since the identification of the ordinary glomeruli according to the 3-D atlas was difficult, the identification of eight activated ordinary glomeruli across individuals was made on the basis of the 2-D maps (Table 1A). This is exemplified in Figure 2, showing the responses to five odours and one control obtained in three male preparations. In these three males the afterstainings were successful and 2-D reconstructions of the glomerular patterns were made. The strong activation of the cumulus (C) by the major pheromone component, Z11–16:AL, which appeared in five males (Table 1A), is shown in the three preparations in Figure 2. Based on the position of the MGC and the 2-D glomeruli map, the relative position of the glomeruli activated by each of the plant odorants was compared among the five males that showed pheromone responses. The positions of eight plant-odour-responding glomeruli were recognized in these males and termed ‘male glomeruli’ Gm1–Gm8 (Figure 2 and Table 1A). Four of them (Gm1–Gm4) were located dorso-medially and three (Gm5–Gm7) ventro-medially of a midline axis from the central cumulus, through the glomerulus situated between Gm2 and Gm5, to the ventro-medial AL. Glomerulus Gm1 was located next to the dorso-medial MGC unit (DM). In preparation 3, Gm1 probably covers the DM glomerulus due to a slightly different orientation of the brain. Glomeruli Gm2 and Gm3 were located next to each other, Gm2 separated from Gm1 and Gm3 from Gm4 by one glomerulus. Next to Gm4 on the midline axis was Gm8.

Figure 1  Frontal view of a male AL, showing the most anterior glomeruli. (A–C) Pictures of the AL at three focal depths (0, 90 and 160 µm), afterstained with a membrane-soluble dye. (D) A two-dimensional (2-D) reconstruction of the visualized glomeruli. (E) Black and white picture of the same AL taken during the experiments. (F) The 2-D map of glomeruli superimposed on the black and white picture. (G) A false-colour coded picture of the AL during stimulation with racemic linalool. (H) The 2-D map of glomeruli superimposed on the false-colour coded picture. The directions medial (M), lateral (L), dorsal (D) and ventral (V) are indicated. C, cumulus; DM, dorso-medial MGC glomerulus; VL, ventro-lateral MGC glomerulus; VM, ventro-medial MGC glomerulus.

Figure 2  Spatial representation of five odours and one control in three male preparations shown as overthresholded false-colour-coded response patterns superimposed on a morphological view of the AL. For preparation 3, the time-traces of the responses in six glomeruli are shown. The response to the major pheromone component, Z11–16:AL, was exclusive in the large MGC glomerulus (C). The plant odorants mainly showed responses in 1–3 ordinary glomeruli, β-ocimene in Gm2, Gm3 and Gm4 and racemic linalool in Gm1, Gm2 and Gm5 in preparation 3. Responses in these and additional glomeruli (Gm6–Gm8) are shown for preparations 1 and 2. The concentration of each odorant is indicated in the pictures. Except for three odorants tested in preparation 1 (β-ocimene, β-myrcene and racemic linalool) all responses are shown for the focus level of 100 µm depth. C, cumulus; DM, dorso-medial MGC unit; glomeruli number 1–8, Gm1–Gm8. The ΔF/F maximum values are 2.0% for Z11–16:AL and cubeb pepper in preparation 1 and 1.3% for the others. The directions in relation to the midline axis are indicated by dm = dorso-medial and vl = ventro-lateral.
which was only activated in preparation 2. The three glomeruli Gm5, Gm6 and Gm7, ventro-lateral to the midline axis, were located next to each other; Gm5 separated both from Gm2 and from the MGC by one glomerulus. For the remaining three males lacking 2-D maps and pheromone response, some of the active foci were identified as glomeruli Gm1–Gm8 based on their location in the AL and specific responses to each plant odorant.

Stimulation with single odorants activated one or a few glomeruli (Table 1A). Only minimal overlap was found for the response patterns elicited by different odorants. β-Ocimene strongly and specifically activated Gm3 (two individuals), Gm4 (four individuals) and Gm8 (one individual). Gm3 was in one case weakly activated by β-myrcene [a secondary odorant of the (E)-β-ocimene RN type]. Linalool elicited strong and specific responses in Gm5 (six individuals) and Gm6 (two individuals). Somewhat weaker responses to linalool and cubebe pepper (containing small amounts of β-myrcene in many males, as shown in preparations 1 and 2, Figure 2), suggesting that this glomerulus was specified for other constituents of cubebe pepper than the single odorants tested in these experiments. All together, the results showed many similarities in response patterns across individuals. Some differences appeared, e.g. the lack of response to β-ocimene in glomerulus Gm3 in preparation 1 and the additional response to β-ocimene in one ventral glomerulus, Gm8, in preparation 2.

Table 1 Percentage of individuals showing responses in specific glomeruli to the different test odours

<table>
<thead>
<tr>
<th>Location</th>
<th>Glomerulus number</th>
<th>Odour</th>
<th>Z11–16:AL</th>
<th>(–)-Germa-crene D</th>
<th>α-Farnesene</th>
<th>β-Ocimene weak responses</th>
<th>Linalool</th>
<th>Caryophyllene</th>
<th>Cubebe pepper</th>
<th>Ylang</th>
<th>Hexane minor responses</th>
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<tr>
<td>(A) Males</td>
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<tr>
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<td>0 (0/8)</td>
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<tr>
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<td>75 (6/8)</td>
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<td>44 (16/16)</td>
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<td>7 (1/15)</td>
<td>6 (1/16)</td>
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<td>38 (6/16)</td>
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When >=40% of the individuals show odour responses in a glomerulus, the box is shaded in grey. The numbers of responding individuals out of those tested are indicated in parentheses.
**Females**

In 13 female preparations, the afterstainings were successful and 2-D reconstructions of the glomerular patterns were carried out. Based on the response patterns elicited by the different odorants and the 2-D map of glomeruli in each animal a comparison of active female glomeruli, G1–G8 (Table 1B), could be made across individuals in the same way as for males. This was based on the position of a dorso-medially located glomerulus, G1, which in nine preparations specifically responded to germacrene D, α-farnesene and/or to the two mixtures cubebe pepper and ylang ylang, which both contain these odorants (Figure 3 and Table 1B). Responses to (–)-germacrene D and α-farnesene were obtained in six of the seven preparations tested for the two single odorants and a response to α-farnesene was obtained in an additional individual not tested for germacrene D. In the remaining males, not tested for the two single odorants, two showed responses in G1 to the mixtures. Figure 3 shows the spatial and temporal response patterns elicited in the AL by the different odorants and two controls in one of the females. Two glomeruli located medially to G1 responded to β-ocimene, β-myrcene and linalool in most preparations and were named G2 and G3 (Table 1B). Stimulation with the two controls (filter paper with or without evaporated hexane) showed only slightly increased calcium levels in the AL of most of the females, as shown in Figure 3. A few females had a somewhat higher control (hexane) response in glomeruli G2 and G3, suggesting that the activation of these glomeruli was caused partly by either hexane or the mechanical component of the stimulus. In one preparation, G2 and G3 had more specific odour responses (Figure 3). β-Occimene and β-myrcene activated only G1, while stimulation with linalool, both the (R)-(–)-linalool and the racemic linalool, activated G3 (strongest) and G2. Looking more carefully at the responses and the location of the two glomeruli presented in Figure 3, G3 seemed to be the major glomerulus responding to linalool. Being located somewhat deeper and partly under G2, the activation of G3 might have given the impression of responses in both glomeruli. Another glomerulus, G4, located ventro-laterally, next to G2 and G3 (Figure 4), was activated in five preparations only by (–)-germacrene D and the mixtures cubebe pepper and ylang ylang (Table 1B). Lateral to G4 was glomerulus G5 that responded only to linalool and the mixtures.

Typically, the response areas of the two essential oils covered the response areas elicited by their major constituents. In accordance with the responses to the single odorants, ylang-ylang, containing a large amount of germacrene D, α-farnesene and linalool, strongly activated G1 and G3 in the individual shown in Figure 3. Only weak responses to ylang-ylang were elicited in G2, in accordance with the small amount of (E)-β-ocimene and β-myrcene in this mixture. The much stronger activation in G1 than in G2 and G3 elicited by cubebe pepper in Figure 3 might be due to the high germacrene D content and only small traces of linalool, (E)-β-ocimene and β-myrcene. The two mixtures also elicited responses in additional glomeruli, indicating that other constituents of the mixtures, not tested in these experiments, caused the activation. One ventral glomerulus, G6, separated from G3 by two glomeruli was strongly activated in eight preparations only by cubebe pepper (Figure 3). Two neighbouring glomeruli (G7 and G8), located ventro-medially of G6, separated by one glomerulus, also showed response only to cubebe pepper (four preparations) and ylang ylang (six preparations), respectively (Table 1B).

Taken together, the results from the female moths showed strong activation by the single odorants in glomeruli located nearby. Two glomeruli, G1 and G4, were ascribed to germacrene D and α-farnesene, and germacrene D alone, respectively. Another two neighbouring glomeruli (G2 and G3) were activated by β-ocimene, β-myrcene and the two linalool stimulants, and were in one preparation separated into one linalool and one β-ocimene/β-myrcene glomerulus. One lateral glomerulus (G5) was, in a few individuals, activated only by linalool and the essential oils. Three more ventral glomeruli (G6–G8) were ascribed to unknown constituents of the two mixtures ylang ylang and cubebe pepper.

**Comparison of spatial response pattern in males and females**

Some similarities were found in males and females when spatial response patterns were compared. Activation by β-ocimene, β-myrcene and two linalool stimulants was found in two neighbouring glomeruli, located dorso-medially in the AL of both sexes (Gm2/Gm3 and G2/G3). In addition, one glomerulus responding to linalool was found lateral in the AL (Gm5 and G5). More ventrally located glomeruli, which specifically responded to cubebe pepper and not to the single components tested, were also found in both sexes (Gm7, G6 and G7). Differences between the sexes were also found, e.g. by the two dorso-medially located glomeruli, Gm1 responding exclusively to linalool and Gm4 to β-ocimene, which both were only found in males (Figure 2). (–)-Germacrene D and α-farnesene were tested only in the females.

**Responses to the racemic linalool and the enantiomer (R)-(–)-linalool**

Comparison of responses to racemic linalool and the 96% pure (R)-(–)-linalool (containing 2% (S)-(++)-linalool) could be made in 15 females and eight males tested for the same concentrations. In general, no difference was found between the responses to these two linalool stimulants (Figure 4). Only in two females and one male was a slightly stronger response obtained for the racemic mixture than for the single enantiomer. In the female shown in Figure 3 a somewhat stronger response was found to the sample (R)-(–)-linalool than to the racemic mixture in the glomeruli G2 and G3. In one female and two males the racemic mixture elicited an
Figure 3  Spatial representation of eight odorants and two controls in one female AL (frontal view). False-colour-coded overthreshold response patterns are superimposed onto the morphological views of the ALs and time-traces of the responses in four glomeruli (Gf1–Gf3 and Gf6) are shown for each odour stimulus. Glomerulus Gf1 was activated by (−)-germacrene D, α-farnesene and the ylang ylang and cubebe pepper mixtures, Gf2 by β-ocimene and β-myrcene, Gf3 by racemic linalool, (R)-(−)-linalool and the ylang ylang mixture and Gf6 by cubebe pepper. The activation by (R)-(−)-linalool in Gf2 was probably due to the fact that Gf3 was located partly under Gf2. All responses are from the 100 µm focus level. Except for cubebe pepper (concentration 10⁻³), the concentration was 10⁻⁵ for all odours. Glomeruli number 1–8, glomeruli Gf1–Gf8. The ΔF/F maximum values are 3.0% for (R)-(−)-linalool and cubebe pepper and 2.3% for the others. The directions in relation to the midline axis are indicated by dm = dorso-medial and vl = ventro-lateral.

Figure 4  Comparison of responses to racemic linalool and the 96% pure (R)-(−)-linalool sample [containing 2% (S)-(+)linalool]. Responses in a female AL to the racemic mixture of linalool (A) and the (R)-(−)-linalool sample (B). (A, B) show the difference between the two responses obtained by subtraction. Only negligible differences were found. The concentration of the two odorants was 10⁻⁵ and focal level 100 µm. The 2-D map of the AL of this female is shown on the left, with five of the glomeruli (Gf2–Gf5 and Gf8) that could be identified across individuals.
additional small response in one area not activated by the 
(R)-(−)-linalool sample alone.

**General responsiveness and temporal response patterns in males and females**

In males the maximum response within each individual was in the range of 1.0–2.4% (mean = 1.64%, SD = 0.48), while the hexane control had a maximum response in the range of 0.5–1.0% (mean = 0.79%, SD = 0.19). In females the values were 1.2–4.2% (mean = 2.33%, SD = 0.78) and 0.6–2.1% (mean = 1.14%, SD = 0.40), respectively. The preparations with the highest maximum response also showed the strongest responses to the hexane control, which could be ascribed to a small activation by hexane or to the mechanical component of the stimulus. In general, linalool [either the racemic linalool or the (E)-(−)-linalool] elicited a strong response in all individuals, being the strongest stimulant among the single odorants in 10 of the 16 females and in six of eight males studied. The cubebe pepper and ylang ylang mixtures, both containing linalool, slightly exceeded the linalool response in most of the 10 females, whereas in males the cubebe pepper response exceeded the linalool response only in one of the six individuals. The typical response latencies were short (up to 100 ms) in both sexes. The maximum responses were reached 1–2.0 s after stimulus onset in females and after 1–2.5 s in males when including all individuals, whereas within each individual the variation was minimal. Only a few responses in some of the individuals showed deviation of the upstroke pattern, such as for the β-ocimene response in Figure 3, showing a steeper response curve in glomerulus G2. In both sexes the variation in temporal response patterns was independent of odour quality and glomerular position. Typically the responses in females (14 out of 16) and males (six out of eight) had a relatively short duration and were often followed by a drop below baseline that started 2–5 s after stimulus onset in the two sexes, respectively (Figure 5). Usually the response peaks were reached slightly later for the strongest responses than for the weaker ones, i.e. a variation in the range of 0–0.5 s. Less variation was seen among the males than in the females. Increased response duration was often seen along with increasing response strength. In the remaining two males and females both brief and long-lasting responses appeared which were not followed by a drop below baseline. The long-lasting responses were most evident in the two males, which also showed peaks appearing later and slower response decay with increasing response strength (Figure 5C).

The responsiveness of females and males was compared using the mean values of the maximum odour responses and the mean values of the maximum responses to cubebe pepper (concentration $10^{-3}$) and control (hexane) obtained in each preparation. In females the mean value was 2.33% for the max odour responses, 1.65% for the maximum cubebe pepper responses and 1.14% for the maximum control responses (SEM = 0.195, 0.198 and 0.100, respectively). In comparison, the values in males were 1.64% (SEM = 0.170), 1.16% (SEM = 0.169) and 0.79% (SEM = 0.066), respectively. These significantly different values of odour and control responses [independent-samples t-test (equal variances not assumed), $P = 0.015$ and 0.008 respectively] and the trend toward significantly difference for cubebe pepper ($P = 0.075$), showed that, in general, the females had a higher responsiveness than the males. Since this difference was not quite so evident for cubebe pepper, it implies that the response to this odour was in fact relatively higher in the males than in the females.

**The effects of increased odour concentrations**

In the glomeruli with the strongest responses to the tested odorants, increased response strength was mostly found with increased concentration. This increase was in a few cases followed by saturation or a small decrease of response strength at the highest concentration. A decrease of responses with increased concentrations, or concentration-independent responses appeared occasionally. At higher concentrations more glomeruli became active and similar variations of dose–response relationships were also
observed for these glomeruli. Figure 6 shows the time-courses of responses to increased odor concentrations in four glomeruli of one female: two strongly activated glomeruli (Gf2 and Gf3) and two weakly-activated glomeruli (Gf4 and Gf8). Responses to four or five concentrations of each odorant [β-ocimene, β-myrcene and (R)-(−)-linalool] are shown. For the two strongly activated glomeruli, an increased response with increased concentration appeared for β-ocimene. For the stronger responses to (R)-(−)-linalool, a slight response increase was followed by saturation in Gf3, while saturation was already reached at the lowest concentrations in Gf2. The weaker responses to β-myrcene showed a slight increase in Gf3 and concentration independence in Gf2. The different dose–response relationships were also observed in the weakly activated glomeruli Gf4 and Gf8. Increased response strength with increased β-ocimene concentration was clearly found in Gf8. The other dose–response relationships showed either a slight increase followed by saturation or a decreased response, dose–response independence or response only at the highest concentration. Thus each glomerulus exhibited different response characteristics for different odours and each odour elicited a different dose–response relationship in each of the four glomeruli. This is apparent for glomeruli both with high and low responsiveness to these odorants. The results indicate that the dose–response characteristic is not a property of the glomerulus, but a property of a given odour in a given glomerulus and the relative pattern across glomeruli differs with increased concentration of one odorant.

**Discussion**

The major intention in the present study was to investigate how the quality of identified odorants mediated by specific RNs is represented in the AL of heliothine moths (Mustaparta, 2002) and knowledge of the principle of convergence in the AL of each RN type (Vosshall et al., 2000; Gao et al., 2000), one would expect to find a specific activation of one or a few glomeruli when stimulating with single odorants. In accordance with this, the present calcium imaging study of the antennal lobe in *H. virescens* did show that the primary odorants activate one or a few glomeruli, some representing specific odorants. For instance, specific linalool responses were in both sexes found in one ventro-lateral glomerulus (Gm5/Gf5) and in two other glomeruli in males, Gm6 (next by) and Gm1 (dorso-medially), in addition to specific β-ocimene responses in the two dorso-medial male glomeruli Gm3 and Gm4. Ventrilaterally located linalool and dorso-medially located ocimene responses were also recorded in a previous calcium imaging study of this moth species (Galizia et al., 2000). Some overlaps of the spatial response patterns of different odorants were found in the present study, which might not be expected since the different RN types have shown no overlap for the tested odorants. According to this, the only expected overlap was for β-ocimene and β-myrcene, both of which activate the (E)-β-ocimene RN type (Røstelien et al., 2000b; Strand et al., 2003b). In the present study the two compounds elicited responses in the same glomeruli, β-ocimene somewhat stronger than β-myrcene, in accordance with the receptor neuron sensitivity. Unexpected overlap of activation was found in one or two dorso-medial glomeruli in both sexes by β-ocimene/β-myrcene and linalool (Gm2, Gf2 and Gf3) as well as by (−)-germacrene D and α-farnesene in one glomerulus (Gf1) in females. Only in one female preparation could Gf2 and Gf3 be functionally separated in one β-ocimene/β-myrcene glomerulus and one linalool glomerulus, respectively (Figure 3). In the males the glomerulus showing overlapping responses (Gm2) was located next to one of the β-ocimene/β-myrcene-specific glomeruli (Gm3; Figure 2, preparation 3).

Thus, in both sexes there were two neighbouring dorso-medially located glomeruli that received information about the three odorants. Whether there is a functional separation of them in all individuals, as indicated in the single female preparation, remains to be shown. The overlapping activity as found in this study of both sexes rather gives the impression that one glomerulus in males and two glomeruli in females receive input from both the (E)-β-ocimene and the (S)-(+) linalool RN types. As expected, no overlapping spatial response patterns were found between the monoterprenes (linalool, β-ocimene and β-myrcene) and the sesquiterpenes [(−)-germacrene D and α-farnesene]. However, overlapping responses to (−)-germacrene D and α-farnesene were found in Gf1 (but not Gf4) in most of the female preparations tested for the two odorants, (−)-germacrene D eliciting a slightly stronger response. This is surprising, since the germacrene D and the α-farnesene RN types appear in separate sensilla and have shown no overlap of their molecular receptive ranges. Thus, the results suggest that this glomerulus (Gf1) receives input from both RN types, while Gf4 receives input only from germacrene D RNs. From the large number of germacrene D receptor neurons identified in the electrophysiological recordings one might have expected activation of one large glomerulus. Instead, activation of one or two normal-sized neighbouring glomeruli was found in this study. These glomeruli were located relatively close to the entrance of the AL nerve. Interestingly, the existence of the same germacrene D RN type has been shown in an electrophysiological study of a related species, *Helicoverpa assulta*. Moreover, the projection of the germacrene D RN has been indicated in a corresponding position (Strand et al., 2003a). All together the spatial response patterns have shown glomeruli with specificity to single primary odorants as well as glomeruli that seem to be activated by two primary odorants.

Another interesting aspect is how odour mixtures are represented as compared to the single odorants in the AL. In this study the comparison was made between the complex
Figure 6 The response ($\Delta F/F$) changes with increased concentrations of the three odorants, (R)-(−)-linalool, β-ocimene and β-myrcene. Increases or saturation of the responses with increased concentration are shown for two medially located glomeruli (Gf2 and Gf3) which are highly sensitive to these odorants. Recruitment of two less sensitive glomeruli (Gf4 and Gf8) is also shown. The positions of the four glomeruli are shown in the 2-D map below. 2–8, Gf2–Gf8.
plant essential oils (ylang ylang and cubebe pepper) and some of their major constituents, which were tested individually. In addition the racemic mixture of linalool was compared with the mixture containing 96% (R)-(–)-linalool and 2% (S)-(+) linalool [called (R)-(–)-linalool]. In all the cases the mixtures covered the response areas of the major constituents tested. Comparison of the response strengths was in general difficult since the concentrations tested as single components were higher than in the mixtures. Interestingly, ylang ylang elicited a higher response than (–)-germacrene D in the germacrene D/α-farnesene glomerulus (G1,1) in some preparations (Figure 3). This may indicate a mixture effect with a non-additive increase caused by the two components germacrene D and α-farnesene, that are both present in large amounts in the ylang ylang essential oil. Also the racemic linalool showed the same spatial response pattern as the mixture containing 96% (R)-(–)-linalool. This might be expected since (R)-(–)-linalool constitutes ~50% of the racemic test sample. The question here is whether (S)-(+) linalool and (R)-(–)-linalool activate the same or different RN types. Indeed, it is shown that the two enantiomers have different effects on two types of RNs. (S)-(+) linalool is the primary odorant for one neuron type which responds much more weakly to (R)-(–)-linalool (Røstelien et al., unpublished data). In addition, both enantiomers induce weak responses in another RN type specified for geraniol, (R)-(+)-linalool somewhat stronger than the other enantiomer (Stranden et al., 2003b). This means that the responses to linalool obtained in the present study might have been caused by activation of two different RN types. However, it seems likely that the responses recorded originated from neurons tuned to (S)-(+) linalool. This may be due to the fact that the linalool responses in most cases had reached saturation for all concentrations tested, and the (R)-(–)-linalool test tubes contained ~2% (S)-(+) linalool. The low sensitivity to (R)-(–)-linalool by the geraniol RN probably caused only a minimal response. This explains the similar response patterns obtained for the linalool samples with different ratios of the enantiomers.

In general, this method allows measuring changes in the calcium level of neurites only in the upper parts of the AL. The possibility still exists that those glomeruli located deeper respond to the tested odorants, e.g. to germacrene D, which have been found to activate ~50% of the recorded plant-odor-sensitive RNs in heliothine species (Stranden et al., 2003a). Furthermore, only a limited number of RN types have been classified according to relevant odorants. Thus, we do not know whether the tested odorants might have secondary effects on other RN types, although this has not yet been shown. Comparison with results from intracellular recordings is interesting and can help resolving the glomerular response mapping. An interesting example is the lateral linalool glomerulus identified in this study and in a parallel study using intracellular recordings. A projection neuron excited by linalool in males had a dendritic arborization in one lateral glomerulus, in a corresponding position to the linalool response in the present study (Rø, unpublished data). A differently positioned linalool glomerulus is found in the sphinx moth Manduca sexta. Here the ‘lateral large female glomerulus’ (latLFG) located at the antennal nerve entrance was activated by linalool (King et al., 2000). Female heliothine moths also have two large glomeruli at the antennal nerve entrance, ‘the central large female glomerulus’ (cLFG) corresponding to the latLFG in M. sexta (Berg et al., 2002). Since the cLFG was not in focus in this study, we do not know the specificity of this glomerulus in H. virescens. In another moth, S. littoralis, a glomerulus responding to linalool is found in a more central (frontal) location (Carlsson et al., 2002). Thus, linalool is represented in variously located ordinary glomeruli of the ALs in the three moth species.

One intention of this study was to identify the glomeruli across individuals on the basis of the 3-D atlas (Berg et al., 2002). Because of the spherical form of the antennal lobes of heliothine moths only few glomeruli were in focus at each level, in contrast to the larger and differently oriented honeybee AL. This made the identification of glomeruli more difficult in the moth. Small variations of the antennal lobe orientations in different preparations contributed to the difficulties in identifying the glomeruli. Another complicating factor is the small individual differences in glomerular shapes/sizes, positions and/or numbers which is seen in heliothine moths and other insect species (Flanagan and Mercer, 1989; Rospars and Hildebrand, 1992; Galizia et al., 1999; Laisisse et al., 1999; Berg et al., 2002). Only a few exceptionally large glomeruli could be identified, like the MGC glomeruli in the males and the labial pit organ glomerulus and a neighbouring glomerulus in both sexes. However, in spite of the difficulties in identifying the glomeruli according to the 3-D atlas, eight glomeruli with corresponding positions and responses were identified across individuals within each sex by the use of the 2-D map, four of them in similar positions in both sexes. This suggests that a conservation of odour responses in the AL also exists in heliothine moths.

The temporal response patterns observed in the present study had the same properties as the responses previously recorded by optical imaging in this and other insect species (Galizia et al., 2000; Carlsson et al., 2002; Fiala et al., 2002). No inhibition was ever found in this study as a response to the test odorants, but following many of the excitatory responses we generally observed a signal drop below baseline, as also seen in honeybees (Stetter et al., 2001). These response characteristics match those found in bees when using a bath application of the calcium-sensitive dye, where the responses are thought mainly to represent the activity of the RNs (Sachse and Galizia, 2002). The results obtained in the present study are in accordance with the electrophysiological recordings from RNs in heliothine moths, showing only excitatory responses to odorants with a phasic–tonic
response pattern often showing a firing stop occurring a short time (100–200 ms) after stimulation ends. The concentration-dependent latencies of the RNs are in the range of 10–300 ms and the peak response frequencies are reached after 30–150 ms. Considering the summation in a glomerulus of activity in the large number of RNs located at different distances from the AL, the temporal response pattern of the recorded calcium responses in this study seems to be in accordance with the RN responses (Almaas and Mustaparta, 1990; Skiri, 1999; Stranden et al., 2003b). Differences between the sexes appeared mainly as a generally lower responsiveness to plant odorants in males than in females, which can be explained by a considerably higher number of plant odor-sensitive sensilla on the female antennae (Almaas and Mustaparta, 1990).

Response changes with increased concentrations appeared both as increased response strength of the already-activated glomeruli and as recruitment of additional glomeruli. Similar dose–response relationships have been shown in other calcium imaging studies of H. virescens (Galizia et al., 2000), the moth S. littoralis (Carlsson and Hansson, 2003), the bee A. mellifera (Sachse and Galizia, 2003), as well as the mouse (Fried et al., 2002). Increased response strength in the glomeruli was expected, since all activated olfactory receptor neurons of this and other species respond to increased concentrations with increased firing rates. In some preparations a decrease was also found in the responses to the highest concentration of a few odorants. This might be due to lateral inhibition within the AL, or to over-saturation of the RNs. The saturation over several decade-steps of concentrations shown for linalool indicated that H. virescens is highly sensitive to this odorant, also indicated by electro-antennogram recordings (unpublished). Interestingly, appetitive learning experiments have shown that this moth performs equally well over the same concentration range of linalool (Skiri et al., unpublished data). These results suggest that a larger number of linalool receptor neurons are present on the antennae of H. virescens than indicated so far in electrophysiological recordings of single RNs.

The activation of an increased number of glomeruli with increased odour concentrations has been well documented in a recent honeybee study (Sachse and Galizia, 2003). It is suggested that for each odorant a few strongly-activated glomeruli are responsible for the coding of odour quality, whereas intensity is coded by additional activation of less-sensitive glomeruli. The recruitment of additional glomeruli in the present study of H. virescens can be explained in two possible ways. The common explanation is that increased odour concentration secondarily activates additional types of RNs. This is, for instance, shown for insect-produced components in H. virescens, where overlapping molecular receptive ranges of two RN types correlated with the activation at high concentrations of two MGC glomeruli (Almaas and Mustaparta, 1990; Berg et al., 1998; Galizia et al., 2000). Since the molecular receptive ranges of the identified plant odour RN types have shown no overlap, except for linalool (Stranden et al., 2003b; Røstelien et al., unpublished data), this interpretation does not seem obvious for all the odorants tested in this study. An alternative explanation is that more glomeruli receive input from the same type of receptor neurons, perhaps some targeted by highly sensitive and other less sensitive RNs. Different sensitivity of RNs specified for the single odorants has been shown for pheromone as well as for plant odour components, in addition to different sensitivity of antennal lobe neurons responding to pheromones in H. virescens. This suggests a recruitment of less sensitive RNs and projection neurons in the detection of higher odour concentrations (Almaas and Mustaparta, 1991; Christensen et al., 1995; Stranden et al., 2003a). Whether the plant odour RNs with different sensitivities project in different glomeruli remains to be shown. Obviously, the specificity and axon projections of more receptor neuron types must be characterized to answer fully the questions about coding of odour quality and odour concentration in H. virescens. In future experiments, better techniques will also have to be developed that allow unambiguous identification of glomeruli in physiological measurements according to the available 3-D atlas.

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References


blends are represented in distinct combinations of uniquely identifiable glomeruli. J. Comp. Neurol., 400, 35–56.


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