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Pheromone Detection by a Pheromone Emitter: a Small, Sex Pheromone-Specific Processing System in the Female American Cockroach

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Abbreviations: AL: antennal lobe; IACT: inner antenno-cerebral tract; MGC: macroglomerular complex; PN: projection neuron.

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Abstract

Many animals depend on pheromone communication for successful mating. Sex pheromone in insects is usually released by females to attract males. In American cockroaches, the largest glomerulus (B-glomerulus) in the male antennal lobe (first-order olfactory centre) processes the major component of sex pheromone. Using intracellular recordings combined with fine neuroanatomical techniques, we provide evidence that the female homolog of the male B-glomerulus also acts as a sex pheromone-specific detector. Whereas ordinary glomeruli that process normal environmental odours are innervated by single PNs, the B-glomerulus in both sexes is innervated by multiple PNs, one of which possesses a thicker axon, termed here B-PN. Both soma size and axon diameter were smaller on B-PNs from females compared to B-PNs from males. The female B-PNs also produce fewer terminal arborisations in the protocerebrum than male B-PNs. Termination fields in the lateral protocerebrum of the female B-PN are mostly segregated from those formed by other uniglomerular PNs innervating ordinary glomeruli. Female B-PN activity was greatest in response to sex pheromone but lower than that in the male B-PN. This specific detection system suggests that sex pheromone affects the behaviour and/or endocrine system of female cockroaches.

Key words: insects; macroglomerular complex; projection neuron; sexual dimorphism; pheromonal communication.

Introduction

In both vertebrates and invertebrates, sex pheromone is used for attracting possible mates. Pheromone communication involves the release of specific chemicals from a pheromone producer (emitter), the environmental transmission of these chemicals to a receiver, and the processing of these chemicals to mediate appropriate behavioural responses in the receiver (Roelofs 1995). For example, the male fruit fly uses the pheromone *cis*-vaccenyl acetate (cVA) to attract females at relatively short distances, while the same chemical causes aggression in other males (e.g. Benton 2007; Wang and Anderson, 2010). Thus, neural systems for processing cVA are developed in both sexes (Benton 2007). In most nocturnal insects such as moths and cockroaches, the female emits sex pheromone with a large range of influence and the male is the recipient (Roelofs 1995; Hildebrand 1996; Mustaparta 1996). The males have elaborate sensory systems for detecting minute quantities of sex pheromone. Long trichoid-type sensilla are specialised for detecting sex pheromone in moths and cockroaches and are a male-only adaptation (Schaller 1978; Steinbrecht 1987).

Axons of pheromone-receptive neurons converge onto a set of enlarged glomeruli called the macroglomerular complex (MGC) in the antennal lobe (the first-order olfactory centre), whereas axons of general odour-receptive neurons project to normal-sized glomeruli (Boeckh et al. 1984; Hillier et al. 2006; Galizia and Rössler 2010). Each glomerulus receives a large number of sensory neurons expressing cognate receptors and these make synaptic connections with a small number of interneurons (Vosshall et al. 2000). The olfactory signals processed in each glomerulus are relayed by one or a few projection neurons (functional homolog to the mitral-tufted cells in the olfactory bulb of vertebrates) to higher-order centres in the protocerebrum (Ernst and

Boeckh 1983). Due to their consistent morphology, the projection neurons from the macroglomerular complex have been used as models to elucidate information processing of specific odours (Christensen and Hildebrand 1987; Mustaparta 1996; Kanzaki et al. 2003; Zhao and Berg 2010).

The question of whether females have specific central neurons for processing their own odours (e.g. pheromones) has been explored in female moths (Ochieng et al. 1995; Rospars and Hildebrand 2000). In the noctuid moth *Spodoptera litoralis* and the tiger moth *Panaxia quadripunctaria*, electroantennogram (EAG) recordings revealed that females can detect their own pheromone component, and this ability was termed “autodetection” (Ljungberg et al. 1995; Schneider et al. 1998). The female glomerulus responsible for processing sex pheromone has a similar location to the male MGC, although it is much smaller than the male MGC (Anton and Hansson 1994; Ochieng et al. 1995). In sphinx moths, the potential female homolog of the “cumulus”, one of the three sex-specific glomeruli, is well-developed and is responsible for processing linalool, a volatile from a host-plant on which they prefer to lay their eggs (King et al. 2000). In the case of the group-living animals, the ability to sense pheromone from emitters of the same sex could be especially important because the existence of nearby rivals must affect mate choice.

A suitable animal model to study is the group-living insect the American cockroach, *Periplaneta americana*. Due to their gregarious habits, different-aged larvae and adults share the same habitat and food resources (Bell et al. 2007). They develop an intricate chemical communication system and use many kinds of pheromones in their nocturnal lives (Gemeno and Schal 2004; Bell et al. 2007). In the cockroach, the MGC consists of two closely located (but separate) A- and B-glomeruli (Nishino et al. 2009) which are specialised for processing the sex pheromone components, periplanone-A and -B, respectively (Burrows et al. 1982; Boeckh et al. 1984). Either periplanone-A or periplanone-B is sufficient to both: 1) attract distant males, and 2) elicit the complete sequence of the male mating display (Seelinger 1985; Okada et al. 1990). The behavioural threshold to periplanone-B is two orders of magnitude lower than to periplanone-A (Okada et al. 1990), suggesting periplanone-B's primary roles as a long-range attractant (Seelinger 1985). Field experiments have shown that periplanone-B attracts males of *P. americana* only, whereas periplanone-A attracts both male *P. americana* and males of the sympatric species, *P. australasiae*, suggesting that periplanone-B is important for reproductive isolation (Waldow and Sass 1984). In fact, the B-glomerulus is the largest glomerulus in the male antennal lobe and nearly three times larger than the A-glomerulus in volume (Nishino et al. 2009).

We recently found that the female homolog of the male MGC exists in the first larval instar of American cockroaches, and that these glomeruli grow at similar rates in the two sexes until the fifth larval instars (Nishino et al. 2010). From the sixth instar, the growth rate in the next five larval stages is slower in females compared to males, resulting in the female MGC homolog being about 1/30th of the volume of the male MGC in adults (Nishino et al. 2010). Electroantennogram (EAG) recordings from the adult female American cockroach antennae showed that they responded to Periplanone-A and -B, although the magnitude of the response was only a quarter that of adult male antenna (Nishino and Kimura, 1982). In this study, we recorded the activity of single projection neurons with dendrites from the B-glomerulus in both sexes of the adult American cockroach, and characterised their sexual dimorphism with regard to morphology and physiology.

MATERIAL AND METHODS

Animals and gross neuroanatomy

Adult virgin female and male cockroaches, *P. americana* with intact antennae, reared in 12:12h light-dark cycle at 27˚C, were used. Males and females were kept separated from the last instars to prevent mating. The procedures for dissection of animals are identical to those in our previous studies (Nishino et al. 2009). To stain axons from all antennal afferents and uniglomerular PNs differentially, crystals of microemerald (Invitrogen) were inserted manually into the inner antenno-cerebral tract (IACT) after the medial region of the protocerebrum was desheathed (retrograde staining). Then, the antennal sensory afferents were stained by cutting the antennal nerves in the proximal flagellum and placing the proximal cut-end into the broken tip of a tapered glass electrode filled with microruby (anterograde staining; Invitrogen, USA). The dye-injected specimens were incubated in a humid chamber at 4˚C for 12-16 h after which the brain was dissected out and processed for confocal microscopic observations.

Preparation of sex pheromone

As synthetic periplanone-B was not available, we collected natural sex pheromone from virgin females (7 to 15 days after the final moult) using two methods (Nishino and Kimura 1982; Sass 1983). Firstly, naturally released sex pheromone was collected from individual virgin females by placing them on pieces of filter paper in a sealed plastic container (n=10; cylindrical shape, diameter: 10 cm: height: 5 cm) for ten days. The sex pheromone was extracted from the 10 filter papers with hexane. The hexane was filtered, condensed to 5 ml under gentle nitrogen flow, and stored at -20 ˚C. 50 µl aliquots with the hexane evaporated was used for olfactory stimulation. Preliminary behavioural assays were performed. These confirmed that filter paper soaked with this quantity of extract elicited orientation to the odour source in about 90 % of virgin males and 50 % of virgin males also exhibited wing-raising behaviour (Boeckh et al. 1984).

The second method extracted sex pheromone from the putative production site (Abed et al. 1993). The last two abdominal segments of 50 virgin females were dissected out and an acetone extraction was performed. The filtered extract was dried at 50 ˚C. The residue was dissolved in 5 ml hexane and stored at -20 ˚C. Half a female unit (50 µl solvent) was used for odour stimulation tests (Burrows et al. 1982). Preliminary behavioural assays showed that filter paper soaked with this quantity of extract elicited orientation to the odour source in about 60 % of virgin males but did not elicited wing-raising behaviour. Sex pheromone extracted by either method evoked excitatory responses in the male B-PN, substantially similar to those when synthetic periplanone-B was applied (Burrows et al. 1982; Boeckh and Selsam 1984).

Neurophysiology

The method of intracellular recordings and staining with Lucifer Yellow were identical to those previously described (Nishino et al. 2003). The method for olfactory stimulation was adapted to Boeckh and Selsam (1984) and Hösl (1990) to allow comparison with literature data. We used orange, banana (John Wagner and Sons, PA, USA), 1,8-cineole, 1-octanol and 1-hexanol (Wako, Japan) and sex pheromone extract for ordinary odour stimulation. A glass nozzle (tip diameter: 1.5 mm) was placed 10 mm distal to the base of the antenna, at right angles to and 1mm above the proximal flagellum. An air current (1 L. min^{-1}) was passed through a cartridge containing a filter paper (5x40 mm) soaked with either 40 µl odourant solution or 50 µl sex pheromone extract. The residual air in the recording cage was continuously removed using a vacuum system. In preliminary experiments, ordinary uniglomerular PNs tended not to show excitatory responses to direct contact of sex pheromone. Thus, pheromone contact stimuli were used to discriminate between responses of the ordinary uniglomerular PNs versus those of B-PNs when performing intracellular recordings. The pheromone contact stimuli were applied to the proximal antenna by a narrow strip of filter paper (2x40 mm) soaked with 50 µl sex pheromone. The paper was connected to a thin metal pin attached to a strain gauge (TB-612T, Nihon Kohden, Japan) which was used to monitor stimulus duration. A sufficient interval (>1 minute) was set between the same kind of stimuli to avoid sensory adaptation of neurons.

Unless otherwise stated, intracellular recordings were made in the lateral protocerebrum. In three females, recordings from the soma in the antennal lobe were achieved as the axon of the PN with dendrites in the B-glomerulus (B-PN) was extremely thin (see Results). Immediately after the intracellular recordings, anterograde staining of antennal afferents were applied as described above.

Confocal microscopy and three-dimensional reconstruction

The brain differentially injected with the two dyes was observed using a confocal scanning microscope (LSM510 Pascal, Zeiss). Projection neurons labelled with Lucifer Yellow or microemerald were visualized using an argon laser with a 505-530 nm band pass filter whereas sensory afferents labelled by microruby were visualized using a helium-neon laser with a longpass filter (>560 nm). Scans were made using three objective lenses: Plan Apochromat 10x0.45NA or 20x0.8NA for low-magnification images and Plan Neofluar 40x1.3NA for high-magnification images. Optical sections made at 1~1.3 µm were reconstructed three-dimensionally with Amira software (Visage Imaging GmbH). The surface rendering function was used for calculating the volume while the volume rendering function was used for creating three-dimensional representations.

Statistical analysis and Terminology

Intracellular recordings of PNs with dendrites throughout the B-glomerulus were obtained from seven animals of each sex. The sample size is shown in Results section. The T-test was used to compare male and female B-PN latencies and spike frequencies in response to cage collected pheromone. The body axis is used as the reference against which position and direction are defined.

RESULTS

Connectivity between glomeruli and projection neurons in antennal lobes of the two sexes

Antennal olfactory receptor neurons send axons via the antennal nerves to about 205 glomeruli in the antennal lobe (AL, Figure 1a,b; Watanabe et al. 2010). Projection neurons with dendrites in each glomerulus (uniglomerular PNs) route their axons to the protocerebrum via the inner antenna-cerebral tract (IACT) (Figure. 1*a*; Malun et al. 1993). Thus, the differential dye injections into the antennal nerves and the IACT (N=7 for each sex) allowed visualisation of the connection patterns between almost all populations of olfactory afferents (magenta) and uniglomerular PNs (green) in both sexes (Figure 1c-i). Immediately after entering the antennal lobe, olfactory afferents from the antennal nerves are bundled into ten thick sensory tracts, each separating gradually into thinner bundles which innervate individual glomeruli (Watanabe et al. 2010). As a general rule, a PN dendritic trunk enters a glomerulus opposite the entry site of the olfactory afferents (Figure 1c-g).

In the male antennal lobe, the A-glomerulus and B-glomerulus are innervated by the proximal branch of sensory tract 4 (T4) (Watanabe et al. 2010; Figure 1d). These glomeruli are conspicuously large and are located antero-laterally to normal-sized, oval-shaped glomeruli termed “ordinary glomeruli” (Figure 1b). The ordinary glomeruli process normal environmental odours (Boeckh et al. 1984; Strausfeld and Li 1999). In all specimens observed, each ordinary glomerulus was innervated by a single uniglomerular PN, which is termed here “ordinary uniglomerular PN” (Figure 1c). Dendrites from each ordinary uniglomerular PN are distributed throughout the entire glomerulus (Figure 1c). In contrast, one thick fibre (white arrow, Figure 1f) and six to seven thin fibre run in parallel on the same focal plane (red arrows, Figure 1f) and enter the B-glomerulus from its medial aspect (Figure 1f). All of these fibres are dendritic trunks leading to somata of different PNs but are not branched dendrites of a single PN. The number of PNs supplying the A-glomerulus was similar to that supplying the B-glomerulus (data not shown). Thus, the total number of PNs innervating the MGC is estimated to be 14-16, which agrees with previous studies (Ernst and Boeckh, 1983; Boeckh et al. 1984). A comparison of arborisation patterns in multiple and single-stained PNs showed that dendrites from thick axonal PNs with large soma (white arrow, Figure 1h) arborised throughout the entire B-glomerulus, whereas those from thin axonal PNs with small somata (red arrows, Figure 1h) innervate specific regions of the glomerulus (Hösl 1990; Malun et al. 1993).

In the female antennal lobe, the homolog of the male MGC is innervated by T4 as in the male MGC (Figure 1e). However, the afferents volume was about 1/30 of the male MGC (Table 1), and similar to that of ordinary glomeruli (Nishino et al. 2010). The female B-glomerulus received innervations from one PN with global arborisations (white arrow, Figure 1g,i) and at least one PN with local arborisations (red arrow, Figure 1g,i). From these observations, we concluded that the PN with dendrites throughout the B-glomerulus is the functional homolog to ordinary uniglomerular PNs, and termed here B-PN.

Sexual dimorphism of the male B-PN and female B-PN

The two-dimensional reconstructions of B-PNs from optical sections showed that the basic morphologies of these neurons are similar in both sexes (Figure 2a,b). Axons of both neurons run in the IACT and supply terminals in similar regions of the mushroom body calyces and the lateral horn (Figure 2a,b). However, there are some differences between the sexes. The soma, dendrites, dendritic trunks, and axon terminals were all much smaller in the female B-PN compared to the male B-PN (Figure 2c-l; Table 1). The afferents versus dendrites volume ratio in the female B-glomerulus was approximately 1:1 whereas that in the male B-glomerulus was approximately 3:1 (Table 1). This implies that the dendrites of the male B-PN distribute more sparsely in the B-glomerulus compared to the female B-PN. The axon terminals in the lateral horn were more concentrated in the antero-medial region in both sexes (Figure 2i-l) but the distribution patterns were different. In the male B-PN, the axon terminals in the antero-medial region were connected to each other by thin filamentous fibres forming triangular-shaped, mesh-like arborisations (Figure 2i). In contrast, in the female B-PN, axon terminals were more diffuse and lacked dorsal arborisations (indicated by white arrow, Figure 2k).

Observations of 21 different ordinary uniglomerular PNs in the female revealed that their termination fields in the lateral horn were almost completely segregated from those of B-PNs. In one typical example (uniglomerular PN with dendrites in the glomerulus I09 in Watanabe et al. 2010), the axon terminals were distributed in the posterior region of the lateral horn and branches were absent from the antero-medial region, where B-PNs give rise to axon terminals (white arrow, Figure 3a). One specimen in which one B-PN and one ordinary uniglomerular PN were simultaneously stained (Figure 3b: anterior view; Figure 3c: dorsal view) shows that axon terminals in the lateral horn were almost completely segregated anterior-posteriorly (Figure 3d,e). Some terminal buttons of female B-PNs and ordinary PNs in the mushroom body calyces were close to each other (Figure 3f), although the termination fields of the B-PN tended to be biased toward the peripheral region of the calyces (Figure 3g).

Physiological properties of the female B-PN in comparison with the male B-PN

The activity of the female B-PN was uniquely characterised by the following physiologic characteristics. Firstly, the rate of background spike discharges was lower in B-PNs compared to ordinary glomerular PNs (Figure 4a,b). The female B-PN discharged single or doublet spikes intermittently (Figure 4a), while ordinary uniglomerular PNs exhibited barrages of 5-10 spikes (Figure 4b). It must be noted that there is great variability between activity profiles of ordinary uniglomerular PNs, and that this comparison is qualitative. Second, increased female B-PN activity occurred almost exclusively in response to sex pheromone. It exhibited strong excitatory responses to sex pheromone collected from the cage (Figure 4c) and somewhat weaker responses to the sex pheromone collected from the abdomen (Figure 4g), and it exhibited no responses or weak inhibitory responses to all other odours tested (Figure 4h-l,n). Third, B-PN activity increased as the sex-pheromone stimulus was applied closer to the antenna. Thus, the direct contact of the filter paper soaked with sex pheromone evoked a strong response in the female B-PN (Figure 4c). The tactile stimulation without pheromone to the antenna did not evoke any detectable responses (not shown). These characteristics were fundamentally the same to those of the male B-PN recorded using the same stimulus conditions (Figure 4d,f).

The physiological distinction between the female B-PN and male B-PNs is due to response latency and intensity differences to sex pheromone stimuli (Figure 4c-f). These are summarized in the peri-stimulus-time histograms with data recorded in response to 500 ms sex pheromone stimulation (18 responses from 6 females and 18 responses from 4 males; Figure 4m). When cage-collected sex pheromone was applied, the response latency of the female B-PN was 312±29 ms (mean±SE), which was significantly longer than that of the male B-PN (169±8 ms, $P<0.05$). The number of spikes during the stimulus was 16±5 in the female B-PN, which was significantly lower than that of the male B-PN (37±4, $P<0.01$).

Discussion

This study shows that the female American cockroach has central neurons specialized for processing sex pheromone. The soma, axons, and terminal buttons of the female B-PN were all smaller than those of male B-PNs (Figure 2; Table 1), and resembled those of ordinary uniglomerular PNs. The termination fields of the female B-PN were largely similar to those of male B-PN, but were also almost completely segregated from those of ordinary uniglomerular PNs (Figure 3). The response identities of the B-PNs of both sexes were substantially similar in that they are narrowly tuned to sex pheromone.

This finding is similar to that observed in the female noctuid moth, and termed “autodetection”. In female moths receptor neurons are tuned to the major female pheromone component and these project to a normal-sized glomerulus at the entrance of the antennal nerve (Ochieng et al. 1995). PNs with dendrites in a glomerulus situated in a similar region exhibit excitatory responses to the female sex pheromone component (Anton and Hansson 1994). Autodetection of female pheromone is an uncommon phenomenon and female antennae of most moth species are apparently anosmic to their own odour (Schneider et al. 1998).

The main differences between male and female American cockroach B-PN responses to equivalent stimuli were that the female responses had a significantly longer latency and fewer spikes. As lower odour concentration results in increased response latency and reduced spike frequency (King et al. 2000), higher concentrations of sex pheromone would be needed for the female B-PN to generate responses equivalent to those in the male. The weak response of the female American cockroach B-PN may be attributable to the physiological properties of the afferents because a considerable number of monosynaptic connections have been found between olfactory afferents and projection neurons (Distler and Boeckh 1997). The EAG response amplitude to periplanone-B in the female antenna was about a quarter that of male antenna (Nishino and Kimura 1982). Larval male antennae possess short type single-walled B sensilla which each contain at least one periplanone-A sensitive and one periplanone-B sensitive neurons. At the imaginal moult, the number of sensilla increases greatly and long type predominate (Schaller 1978). Concurrent with this metamorphic change of the outer cuticular structure, the dendritic branches of the sensory neurons increase in number, and this is thought to underlie the observed increase in sensitivity (Schaller 1978). Electrophysiological recordings from short type sensilla show lower sensitivity to sex pheromone compared to long type (Hartman 1987). As the female antenna has short type single-wall B-sensilla only, we assume that the weak response of the female B-PN reflects the lower number of receptor afferents and their lower sensitivity (Schaller 1978).

Then, what is the functional significance for females to have specific pheromone detection system? No detectable effects of sex-pheromone on female behaviours have been reported in American cockroaches to date. Considering the relatively weak sensitivity of the female B-PN to sex pheromone, the female system appears to detect sex pheromone from nearby females or from herself, in contrast to the male detection system which is adapted to exquisite pheromone detection over long distances. For group-living animals such as cockroaches, the detection of rival females in the pre-mating phase would be important in the competition for males. It may be possible for a female to evaluate their competitors by sensing the amount of sex pheromone released changes depending on age and reproductive status (Sass 1983). In fact, the presence of rival females is known to have a stimulating effect on female sexual activity and the initiation of calling behaviour (i.e. pheromone emission) to male American cockroaches (Abed et al. 1993). When a female detects continually high concentrations of sex pheromone emitted by many females, it may signal a lack of sexually mature males. It is tempting to speculate whether a high concentration of sex pheromone could promote parthenogenesis, which is common in American cockroaches (Gemeno and Schal 2004). As proposed in female moths (Anton and Hansson, 1994; Schneider et al. 1998), the possibility that females need feedback to help them regulate their release of sex pheromone is also plausible.

Cockroaches are representatives of primitive neopteran insects and it is apparent that there is evolutionary conservation in the nervous system. The antero-medial region of the lateral horn is targeted by the sex pheromone-receptive PNs in the cockroaches and the evolutionally more modern insects like moths and flies (Figure 2; Kanzaki et al. 2003; Benton 2007; Datta et al. 2008). Additionally, the typical triangular-shape of the sex pheromone-processing region is common to the male cockroach and the male silkmoth (Seki et al. 2005). It has been suggested that the lateral horn mediates innate behaviours in *Drosophila* (Heimbeck et al. 2001). If this is also the case in cockroaches, and given that there is a clear separation of pheromone-processing and normal environmental odour-processing regions in the lateral horn of cockroaches (Figure 3; Nishino et al., 2003; 2010), this suggests that the antero-medial region of the lateral horn may be important for mediating pheromonal orientation and courtship behaviour in males. Sexual dimorphism in the branching pattern of B-PNs in the lateral horn may relate to the mediation of different motor responses to sex pheromone.

Our previous study suggested that axonal arborisations in the antero-ventral region of the lateral horn emerge during the eighth larval instars in males (Nishino et al. 2010). The location and size of the adult female B-glomerulus were equivalent to those of the male MGC precursor at mid-larval stages (Nishino et al. 2010), and the projection patterns of PNs in the lateral horn were similar in adult females and mid-larval males (Nishino et al. 2010). Thus, one might speculate that the female B-PN is homologous to the state of the male B-PN at mid-larval stages. Maturation of the male B-PN may be achieved by extension of additional branches to the antero-dorsal region of the lateral horn. It has been shown that formation of male-specific, long sw-B sensilla is promoted by suppression of juvenile hormone released from *corpola allata* (Schafer and Sanchez 1976). The effect of juvenile hormone stimulation on development of the male B-PN needs investigating because this would answer how the neural circuits that control sex-specific behaviours are remodelled during postembryonic development in this primitive, slowly-developing insect.

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Figure Legends

Figure 1. The olfactory afferents (magenta) and uniglomerular projection neurons (green) in the right antennal lobes of both sexes, viewed anteriorly. **(a)** Diagram illustrating the antennal nerves and the antennal lobe (AL) of the adult cockroach brain. **(b)** A three-dimensional reconstruction of glomeruli in the male antennal lobe, viewed frontally (modified from figure 1b in Nishino et al. 2009). A- and B-glomeruli are highlightend with colors. **(c)** The antero-dorsal region of the antennal lobe showing projection neurons (PNs) innervating single glomeruli. **(d, e)** A-glomerulus (A-glom) and B-glomerulus (B-glom), innervated by sensory tract 4 (T4), are about 30 times larger in males (d) than in females (e). **(f, g)** High-resolution confocal images using an oil-immersion objective. B-glomeruli receive multiple innervations from one thick axonal PN (white arrow) and one or more thin axonal PNs (red arrow) depending on sex (6-7 in the males (f) and one in females (g)). **(h,i)** Intracellular dye injections in the lateral protocerebrum reveal that one thick axonal PN (white arrow) and one or more thin axonal PNs (red arrows) innervate the B-glomerulus. Scale bars: (a) 1 mm; (b,d,e) 100 µm; (c,f-i) 50 µm.

Figure 2. Sexual dimorphism of B-PNs. **(a, b)** Diagrams showing all B-PNs reconstructed from optical sections. The axon projects to the calyces of the mushroom body and lateral horn via the inner-antennocerebral tract. **(c, e)** Soma and dendrites of B-PNs (green) and sensory afferents (magenta). **(d, f)** Dendritic arborisations reconstructed three-dimensionally from optical sections viewed anteriorly. **(g, h)** Axon terminals in the antero-medial calyx of the mushroom body. White broken lines indicate

the outline of the calyx. **(i-l)** Axon terminals in the lateral horn viewed anteriorly (i,k) and laterally (j,k). Scale bars: (a,b,c,e) 100 µm; (d,f,g-l) 50 µm.

Figure 3. Different termination fields of axons of female B-PNs and ordinary uniglomerular projection neurons. **(a)** A uniglomerular projection neuron with dendrites in a posterior glomerulus (termed I09 in Watanabe et al. 2010) in the female. **(b, c)** A B-PN and one uniglomerular PN with dendrites in a medio-central glomerulus (termed F16 in Watanabe et al. 2010), double stained, viewed anteriorly (*b*) and dorsally (*c*). **(d, e)** A three-dimensional reconstruction of axon terminals of a B-PN (magenta) and a uniglomerular PN (cyan) in the lateral horn (see "b" for the reconstructed region), viewed anteriorly (d) and laterally (e). **(f, g)** A three-dimensional reconstruction of axon terminals of a B-PN (magenta) and a uniglomerular PN (cyan) in the medial calyx (see "b" for the reconstructed region), viewed anteriorly (d) and dorsally (e). Scale bars: (a-c) 100 µm; (d-g) 50 µm.

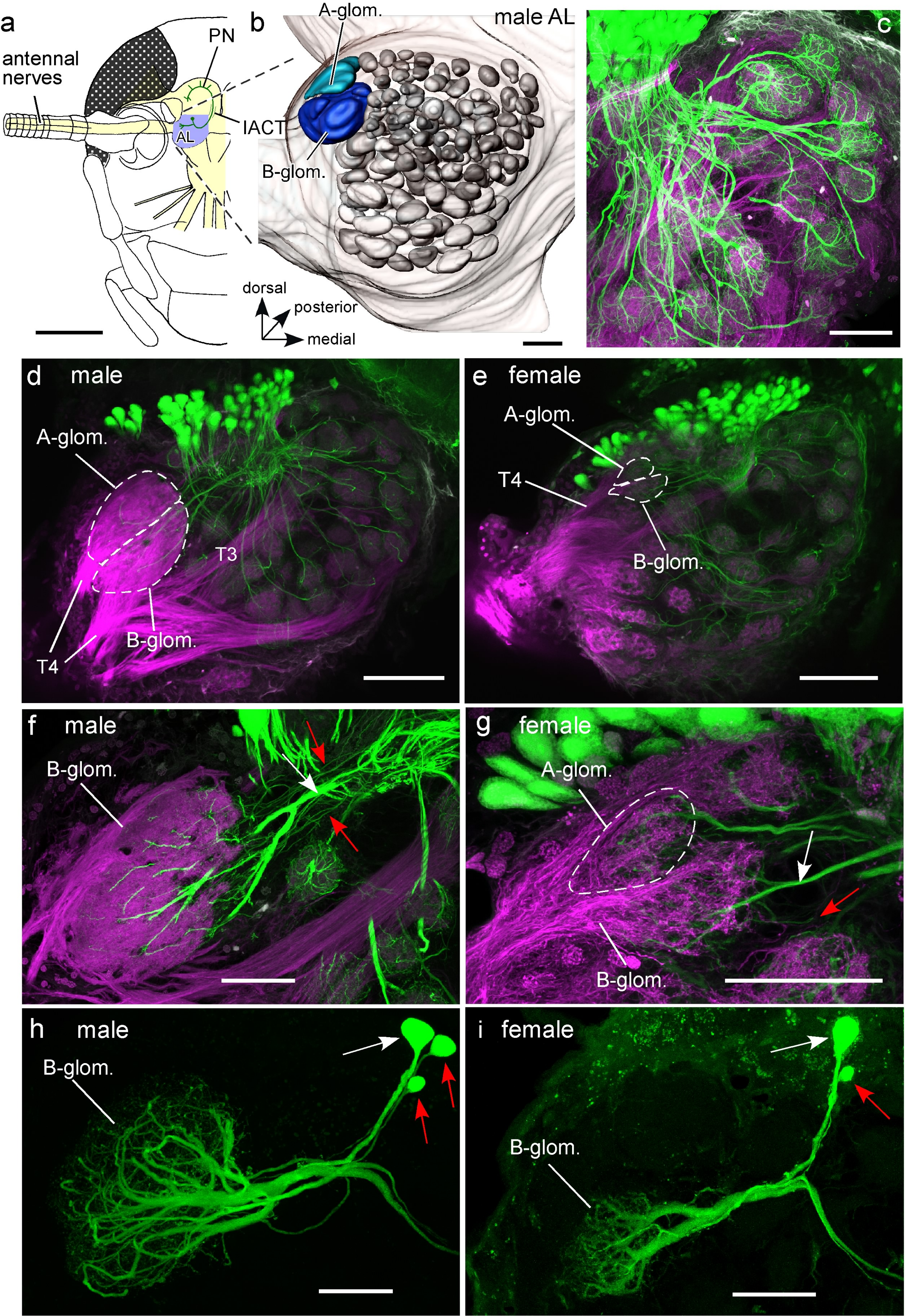
Figure 4. Spontaneous firing activity and firing activity in response to stimuli in female and male B-PNs. (**a**) Spontaneous activity of the female B-PN. (**b**) Spontaneous activity of an ordinary uniglomerular PN with dendrites in a lateral glomerulus (termed E09 in Watanabe et al. 2010) in the female. (**c-l**) Responses to various odour stimuli. c,e and g-l are from females (f), and d, and f are from males (m). **(m)** Peri-stimulus-time-histogram (PSTH) showing spike responses to sex pheromone stimuli with 500 msec duration (shaded area) in the male (thin solid line) and the female (thick solid line). 18 stimuli derived from 6 females and 18 stimuli derived from 5 males are averaged. The vertical bar shows standard error. **(n)** PSTH showing the spike responses to banana, orange,

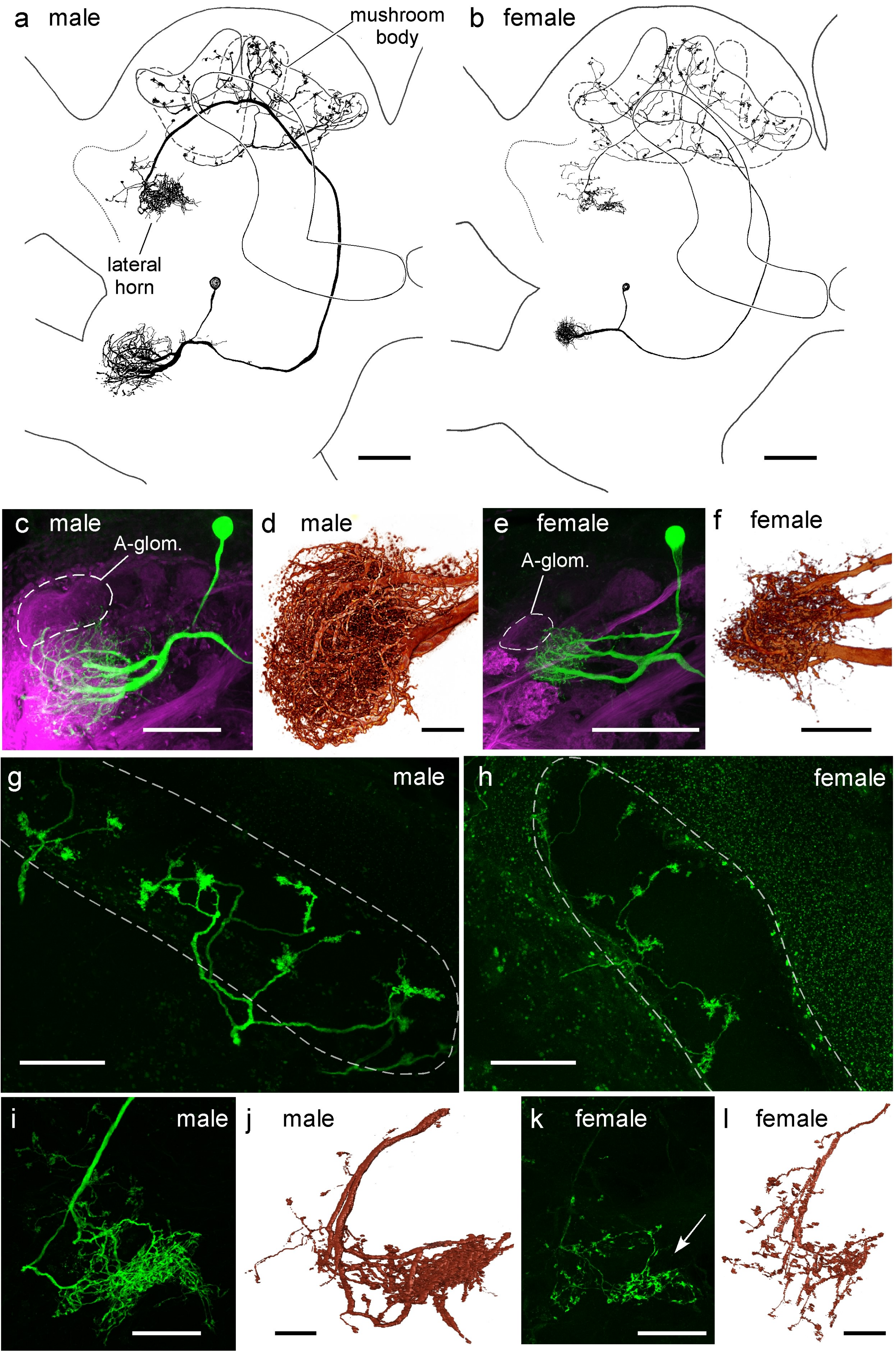
1-hexanol, 1-octanol, and 1,8-cineole stimuli with 500 msec duration (shade area) in the female.

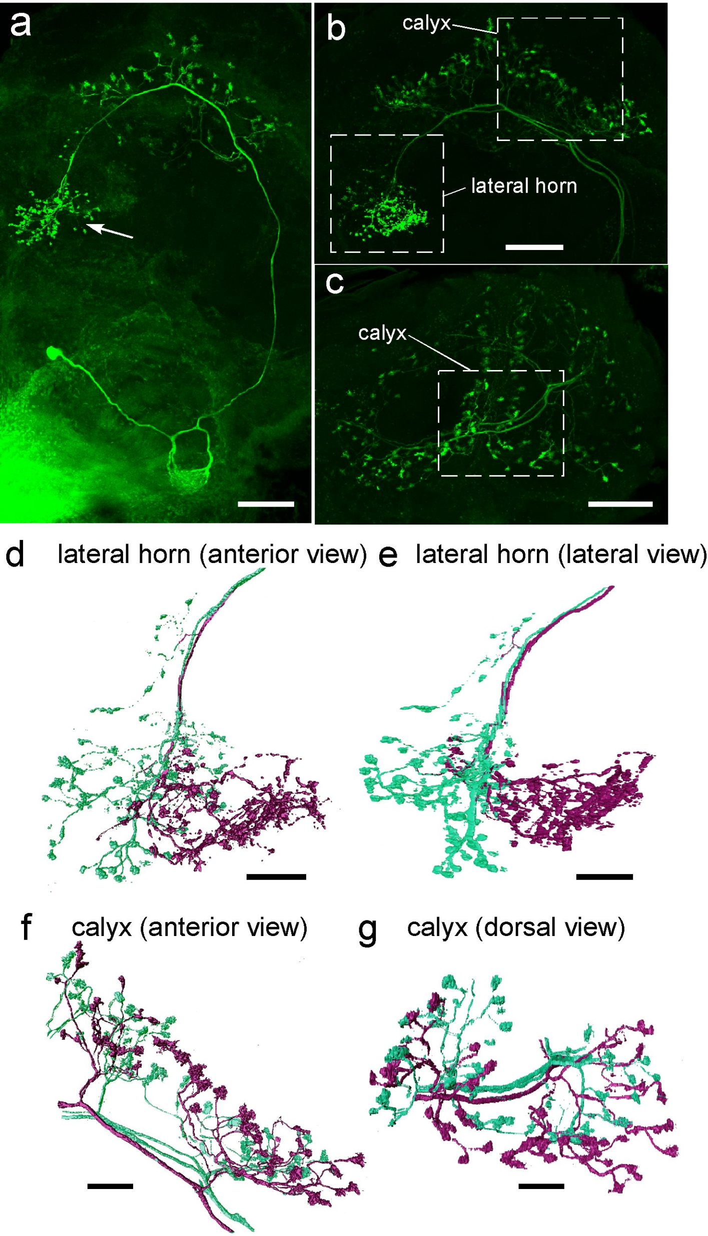
Table 1 Morphometric measurements of afferents and parts of B-PN.

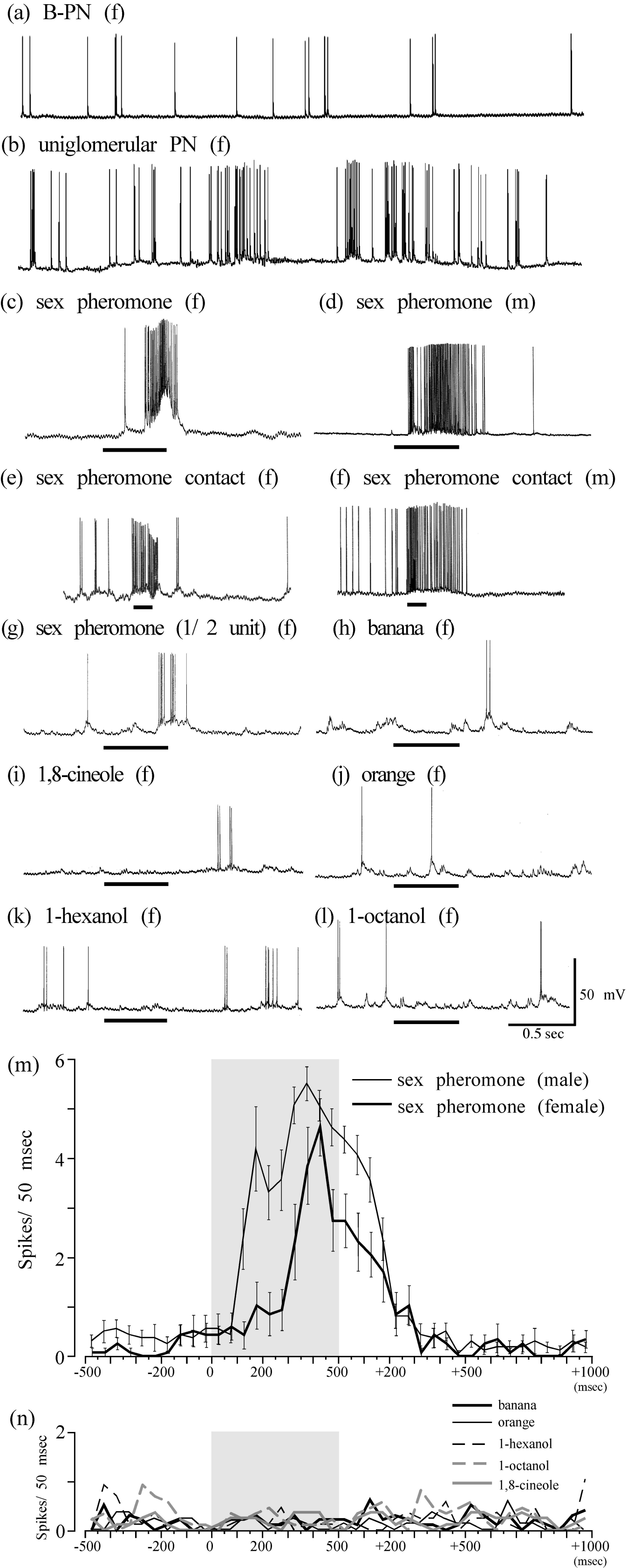
	Afferents Volume (μm^3)	Soma diameter (µm)	Dendritic volume (μm^3)	Thickness of dendritic trunk (µm)	Axonal diameter (µm)	Axon terminals volume in the lateral horn (μm^3)
Male 1	331615	29	111329	18	8	40968
Male 2	390481	28	130031	15	9	42567
Male 3	348002	24	102348	15	8	39864
Mean	**356699**	**27**	**114569**	**16**	**8.5**	**41133**
Female 1	11831	19	11847	10	4	14001
Female 2	8959	18	8408	7	3	9022
Female 3	11200	18	11943	8	3	13107
Mean	**10663**	**18.3**	**10732**	**7-9**	**3.3**	**12043**

Mean values are derived from three PNs intracellularly stained for each sex. The thickness of dendritic trunk was measured at its thickest region. Axonal diameter of B-PN was measured at the exit point from the antennal lobe.


a
antennal nerves
PN
lACT
AL
b
A-glom.
male AL
B-glom.
dorsal
posterior
medial
c
d
male
A-glom.
T3
T4
B-glom.
T4
e
female
A-glom.
T4
B-glom.
f
male
B-glom.
g
female
A-glom.
B-glom.
h
male
B-glom.
i
female
B-glom.


a
male
mushroom body
lateral horn
b
female
c
male
A-glom.
d
male
e
female
A-glom.
f
female
g
male
h
female
i
male
j
male
k
female
l
female


a
b
calyx
lateral horn
c
calyx
d
lateral horn (anterior view)
e
lateral horn (lateral view)
f
calyx (anterior view)
g
calyx (dorsal view)


(a) B-PN (f)
(b) uniglomerular PN (f)
(c) sex pheromone (f)
(d) sex pheromone (m)
(e) sex pheromone contact (f)
(f) sex pheromone contact (m)
(g) sex pheromone (1/ 2 unit) (f)
(h) banana (f)
(i) 1,8-cineole (f)
(j) orange (f)
(k) 1-hexanol (f)
(l) 1-octanol (f)
50 mV
0.5 sec
(m)
sex pheromone (male)
sex pheromone (female)
Spikes/ 50 msec
6
4
2
0
-500
-200
0
200
500
+200
+500
+1000
(msec)
(n)
banana
orange
1-hexanol
1-octanol
1,8-cineole
Spikes/ 50 msec
2
1
0
-500
-200
0
200
500
+200
+500
+1000
(msec)