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Responses of protocerebral neurons in *Manduca sexta* to sex-pheromone mixtures

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Abstract

Male *Manduca sexta* moths are attracted to a mixture of two components of the female's sex pheromone at the natural concentration ratio. Deviation from this ratio results in reduced attraction. Projection neurons innervating prominent male-specific glomeruli in the male's antennal lobe produce maximal synchronized spiking activity in response to synthetic mixtures of the two components centering around the natural ratio, suggesting that behaviorally effective mixture ratios are encoded by synchronous neuronal activity. We investigated the physiological activity and morphology of downstream protocerebral neurons that responded to antennal stimulation with single pheromone components and their mixtures at various concentration ratios. Among the tested neurons, only a few gave stronger responses to the mixture at the natural ratio whereas most did not distinguish among the mixtures that were tested. We also found that the population response distinguished among the two pheromone components and their mixtures, prior to the peak population response. This observation is consistent with our previous finding that synchronous firing of antennal-lobe projection neurons reaches its maximum before the firing rate reaches its peak. Moreover, the response patterns of protocerebral neurons are diverse, suggesting that the representation of olfactory stimuli at the level of protocerebrum is complex.

Keywords

moth; pheromone; mixture; ratio; protocerebrum

Introduction

Olfactory cues on which many species of insects depend for location and/or selection of food sources, oviposition sites, and mating partners typically are more-or-less complex mixtures of volatile organic compounds (VOCs), some of which share similar composition. For example, sex pheromones mediating sexual communication among closely related moth species usually are mixtures comprising the same compounds, differing only in their relative proportions in the pheromone blend (Anton et al. 1997; Tillman et al. 1999; Hagström et al. 2012). The insect olfactory system therefore must be capable of discriminating subtle differences among these chemically similar mixtures.

Gas chromatography with coupled-electroantennographic detection (GC-EAD) has shown that olfactory receptor cells (ORCs) in the antennae of various insect species often are sensitive to only some of the VOCs emitted from a source and that the compounds in large quantity may not be the most significant stimuli for behavior (Nojima et al. 2003; Tasin et al. 2006; Piñero and Dorn 2007; Cha et al. 2008; Webster et al. 2008; Riffell et al. 2009a,b).

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Therefore, to extract behaviorally relevant information from natural olfactory stimuli, insects must recognize specific features of these stimuli such as chemical composition, blend proportions, and concentrations of individual components (Bruce et al. 2005; Pickett et al. 2006; Najjar-Rodriguez et al. 2010).

Information about VOC mixtures is encoded combinatorially as well as specifically in the activity of responsive ORCs and transmitted via the axons of those ORCs to the primary olfactory center in the brain, the antennal lobe (AL) (Shields and Hildebrand 2001; Couto et al. 2005; Yao et al. 2005). The ORC response pattern is transformed into a pattern of activity among the glomeruli in the AL. Each glomerulus receives converging input via the axons of ORCs that carry the same olfactory receptor protein (Gao et al. 2000; Vosshall et al. 2000). After synaptic processing, projection neurons (PNs, the output neurons of glomeruli) convey information about the olfactory stimulus to higher-order olfactory centers in the protocerebrum including the mushroom bodies (MBs) and the lateral protocerebrum (LPC) (Kanzaki et al. 1989; Stocker et al. 1990; Anton and Hansson 1995; Seki et al. 2005; Kirschner et al. 2006). Anatomical studies have revealed that considerable convergence and divergence take place at the MB and LPC, multiple PNs converging onto a single postsynaptic neuron and one PN synapsing with multiple postsynaptic neurons, which suggests a reorganization of information at the projection sites of the antennocerebral tracts (Wong et al. 2002; Kanzaki et al. 2003; Jefferis et al. 2007). In moths, the PNs that carry information about sex-pheromone component project to a specific area in the LPC – the inferior lateral protocerebrum (ILPC), clearly different from another LPC area, the lateral horn (LH), where the PNs that convey information about general odors project (Sun et al., 1997; Kanzaki et al., 2003). In addition to the protocerebral neurons that innervate the MB and LPC neurons, some other protocerebral neurons innervating the lateral accessory lobe (LAL), ventral protocerebrum (VP) and superior protocerebrum (SP) are also responsive to sex pheromones (Kanzaki and Shibuya 1992; Lei et al. 2001), indicating an extensive involvement of the protocerebrum in processing this key olfactory signal. In fruit flies, a recent anatomical study revealed previously unknown antennocerebral tracts to the protocerebrum, some of which appear to be inhibitory (Tanaka et al. 2012). In female moths of *Heliothis virescens*, olfactory information reaches the SP neurons directly from the AL and also indirectly via the MB and LPC, providing evidence for parallel processing of olfactory information at this higher level (Løfaldli et al. 2012).

Earlier studies examined the olfactory basis of the behavioral effectiveness of specific VOC mixtures. For example, single-sensillum recording from olfactory sensilla on the antennae of the moth *H. virescens* showed that mixing cognate and non-cognate sexpheromone components suppressed the responses of ORCs to individual components (Hillier and Vickers 2011). On the contrary, Ochieng et al. (2002) found that spiking activity of *Helicoverpa zea* ORCs evoked by Z11-hexadecenal (Z11-16:Ald), a primary pheromone component, was increased when that component was mixed with linalool, a common plant VOC. Furthermore, single-sensillum recordings from the Japanese beetle, *Popillia japonica* revealed that on one type of sensilla, a mixture of a pheromone component and a behavioral antagonist compound resulted in enhanced response; but the same mixture produced suppressed response on a different type of sensilla (Nikonov and Leal 2002). In male *H. virescens*, the suppressive mixture interactions between sex-pheromone components and plant VOCs are believed to result from an inhibition of calcium signal at the ORC level (Pregitzer et al. 2012).

In our investigations of olfactory information processing in the hawk moth *Manduca sexta*, intracellular and extracellular recordings from the ALs demonstrated synchronous spiking of PNs when antennae were stimulated with behaviorally effective VOC mixtures (Lei et al.

2002; Lei et al. 2004; Riffell et al. 2009a,b). Most recently, using an extracellular recording method, Martin et al. (2013, this issue) found that AL PNs associated with the male-specific macroglomerular complex in *M. sexta* show maximally synchronized spiking upon antennal stimulation with a mixture of the two key pheromone components, *E*10-*Z*12-hexadecadienal (bombykal or BAL, the major pheromone component of this species) and *E*10-*E*12-*Z*14-hexadecatrienal (or EEZ, the second major component) at the natural 2:1 blend ratio. That synchronous activity decreases when the mixture ratio deviates from the natural 2:1. It is unclear, however, whether such selectivity for the natural mixture ratio is maintained at the level of downstream centers in the protocerebrum.

In this study, we used intracellular recording and staining methods to survey physiological and morphological characteristics of protocerebral neurons that responded to antennal stimulation with pheromone components and mixtures. The neurons in this limited sample innervated various regions of the protocerebrum, including the superior, lateral, and ventral protocerebrum, lateral accessory lobe, and optic lobe. While many of them showed no difference in responses to single pheromone components and mixtures, some responded differently to the natural (2:1) blend ratio than to other ratios or to single pheromone components.

Materials and Methods

Preparation

Male *M. sexta* (Lepidoptera: Sphingidae) moths were raised in the laboratory on artificial diet (modified from that of Bell and Joachim 1976) under a long-day photoperiod (LD 17:7), as previously described (Sanes and Hildebrand 1976; Tolbert et al. 1983). For physiological experiments, animals were prepared for experiments as described by Christensen and Hildebrand (1987). In brief, moths were restrained with wax in a closely-fitting plastic tube. The labial palps and proboscis were removed, and a window was cut in the dorsal head capsule. The cibarial pump and other muscles were excised to allow access to the brain. To eliminate movement, the head was isolated and pinned to an algar-coated petri dish, with the ALs oriented anteriorly. The dorsal region of protocerebrum behind an AL was carefully desheathed with a pair of fine forceps. The preparation was superfused with a saline solution (150 mM NaCl, 3 mM CaCl₂, 3 mM KCl, 10 mM TES buffer, and 25 mM sucrose, pH 6.9; Christensen and Hildebrand 1987).

Intracellular recording and staining

Sharp microelectrodes were made from borosilicate glass capillaries with filament (1 mm outer diameter, 0.58 mm internal diameter, Sutter Instruments Co., Novato, CA) with a laser puller (P-2000, Sutter Instruments Co., Novato, CA). The tip of the micropipette was filled with a solution of Lucifer Yellow CH (65 mM, Sigma-Aldrich, St. Louis, MO) in 200 mM LiCl, or with a solution of Alexa Fluor 568 hydrazide (10 mM in 200 mM KCl, Molecular Probes, Eugene, OR), and the shaft with 2 M LiCl; microelectrodes had resistances in the range 100–350 M Ω . The electrophysiological activity of an impaled neuron to stimulation of the ipsilateral antenna was amplified 10- to 50-fold with an Axoclamp-2A amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA) coupled to a DC amplifier (LPF 202A, Warner Instruments, Hamden, CT), and digitized at 20 kHz (via Datapack, Run Technologies, Mission Viejo, CA).

After physiological characterization, neurons were injected with either Lucifer Yellow or Alexa 568 by passing hyperpolarizing current (0.2–1 nA) for 5–15 min. Upon completion of an experiment, the brain was excised and immersed in 2.5% formaldehyde fixative solution

(pH 7.2) overnight at 4° C, dehydrated through a graded series of aqueous ethanol solutions (from 50 to 100%), and cleared with methyl salicylate (Sigma-Aldrich, St. Louis, MO). Cleared brains were imaged as whole mounts with a laser-scanning confocal microscope using a 20X objective lens (Carl Zeiss 510 Meta equipped with a 457-nm Argon laser and a 543-nm Green HeNe laser).

Mass staining

Microruby (Invitrogen©, Life Technologies, Grand Island, NY) fluorescent dye (4% in distilled water) was introduced into the MGC using an electroporation method as described in (Fujiwara et al. 2009) but with some modifications. In short, a stimulus isolator (World Precision Instruments, Sarasota, Florida, USA) was used to generate about -80 Volts DC potential, which was applied to patch-type electrodes with 5–20 MΩ resistance. The electrode, back-filled with microruby dye, was carefully inserted into the MGC area of a dissected moth brain which was perfused with physiological saline solution as in intracellular recording experiments. Twenty-five electric pulses with 1 sec pulse duration and 2 sec inter-pulse interval were delivered to the electrode. The moth brain was then given three hours in room temperature immersed in physiological saline solution for the dye to diffuse. Then the brain was fixed, dehydrated, cleared and imaged following the protocol used for intracellular recording.

Stimulus protocol

Pheromone components, *E10-E12-Z14*-hexadecatrienal (EEZ) and *E10-Z12* hexadecadienal (Bombykal or BAL), were diluted in cyclohexane to a concentration series ranging from 0.1–1000 ng/μl and 0.2–2000 ng/μl, respectively. From these stocks, binary blends were made, centering around the natural 2:1 ratio and altering the concentration of one component (BAL:EEZ = 20:0.1, 20:1, 20:10, 20:100 and 20:1000; or BAL:EEZ = 0.2:10, 2:10, 20:10, 200:10 and 2000:10). One μl of each solution was deposited on a piece of filter paper that was contained in a 0.5 ml glass syringe. Each syringe was used as a stimulus cartridge to deliver stimuli via a solenoid-controlled air stream. The complete set of stimuli included: solvent control, EEZ (10ng), EEZ (10ng) + BAL (0.2ng), EEZ (10ng) + BAL (20ng), EEZ (10ng) + BAL (200ng), EEZ (10ng) + BAL (2000ng), BAL (20ng), BAL (20ng) + EEZ (0.1ng), BAL (20ng) + EEZ (100ng), BAL (20ng) + EEZ (1000ng), and undiluted hibiscus oil (Select Oil, Litchfield, CT). The highest concentrations of pheromone components and hibiscus oil were not tested on every neuron.

Stimuli were delivered in train of 5 or 10 pulses with duration of 100 ms and interpulse interval of 2 sec. At least 30 seconds of line-flushing time were given before switching to a different stimulus. The air speed in the flushing line was about 1 liter per minute and the air pressure imposed on the solenoid was about 20 psi. In this system it takes about 150 ms for stimulus molecules to reach the antenna.

Data analysis

Time stamps of recorded spikes were extracted within Datapack (Run Technologies, Mission Viejo, CA) and subsequently exported to Neuroexplorer (Nex Technologies, Littleton, MA) to generate peristimulus time histograms (PSTHs) and raster plots. The width of the response window for each neuron was determined in the following way. First, we generated PSTHs of responses to all stimuli in a single neuron with a bin size of 5 ms and a starting point of 200 ms before the onset of stimulation and 1500 ms after it; second, we obtained an averaged PSTH for this neuron and smoothed the histogram with a Gaussian method; third, we determined the response window of this neuron after visually inspecting the grand average of the PSTHs of this neuron. The after-inhibition period was included in

the response window but might not be accurate for all responses. However, this inaccuracy had little effect on the chosen parameters of responses (see below). Across the entire dataset, the response windows varied from 0.3 to 0.8 sec. The same width, however, was used for one neuron across all responses. Excitatory responses were determined if the peak value of PSTHs was greater or equal to the standard deviation of the spontaneous activity by a factor of two. Because inhibitory responses with measurable hyperpolarizations were rare in our data set, no particular parameters were designed to quantify this type of response.

We initially selected 3 parameters to quantify the neural responses: (1) firing rate, i.e. the mean instantaneous firing rate within a response window, calculated by averaging the inverse of inter-spike intervals (ISI) for all the spikes within the response window; the averaging process was stopped when an ISI greater than 5 times of preceding ISI was encountered; (2) response delay, i.e. the time between the onset of stimulus and the onset of the response, which was defined as the time point before the peak PSTH where the maximum of second derivative was found; this process was aided with visual inspection; (3) number of spikes within response window. To assess the consistency of these parameters, we calculated the coefficient of variation (CV), i.e. standard deviation divided by mean, for all 34 neurons that responded to the pheromone mixture with the natural ratio, using all three parameters. A lower CV value indicated more consistent responses. The CV values derived from all neurons were then arranged in a symmetric matrix. The main diagonal of the matrix comprised CV values generated in each neuron, whereas all other parallel diagonals were based on pairwise comparisons of the neurons (supplemental figure 1). Placed at the top right corner of the matrices were bar graphs that displayed the averaged CV values of each diagonal of the matrix. When using the firing rate within response window, response delay and number of spikes within response window (panels a–c of supplemental figure 1) to quantify the responses, the within-neuron variation was markedly less than the between-neuron variation (arrows pointing to the middle section of the bar graphs). Because the number-of-spikes parameter (panel c) produced a matrix similar to that based on the firing-rate parameter (panel a) did, the firing rate and response delay were finally chosen for all subsequent analysis.

The Kruskal-Wallis nonparametric test was used to assess the statistical significance of these two measurements among stimuli, followed by Tukey-Kramer multicomparison procedure. Based on the PSTHs, principal-component analysis was performed to examine the population response. Additionally, Euclidean distances were calculated to compare the differences embedded in the population response between pairs of stimuli. All of these multivariate analyses were conducted using Matlab (The Mathworks, Natick, MA). Because of the small number of neurons that were partially or completely stained intracellularly, and the fact that each labeled neuron had unique morphological features, we made no attempt to categorize these neurons. Instead, we focused on one functional perspective – whether or not the neurons could distinguish among pheromone mixtures of various ratios. One advantage of using the PSTH-based multivariate analyses was that the subtle differences embedded in the response profile were taken into account by the algorithms on a bin-by-bin basis. These differences are usually difficult to capture in terms of parameters. Although the chosen multivariate analyses do not clarify the mechanisms by which different mixtures are discriminated, they do show if at all the protocerebral neurons could collectively distinguish these stimuli, despite that they may have vastly different response patterns.

Results

Thirty-four protocerebral neurons were examined physiologically, of which 11 were partially or completely stained with fluorescent dye (Supplemental Table 1). None of the 11

protocerebral neurons innervated the mushroom bodies and the central body complex. Twenty-six neurons differed statistically when comparing their responses, measured either as the firing rate during response window or as response delay, to BAL, EEZ or a 2:1 blend of these two components. Based on the firing rate, about 20% of the tested neurons gave different responses to BAL and EEZ; 30% distinguished EEZ from the mixture containing it, while only 15% distinguished BAL from a mixture (Figure 1). Similar results were obtained on the basis of response delay. When the two response parameters were combined, the percentage of neurons that showed a statistical difference in their responses to the three stimuli markedly increased, with 42% for EEZ vs. BAL, 51% for EEZ vs. mixture, and 22% for BAL vs. mixture, suggesting that protocerebral neurons may integrate more than one dimension of information to distinguish stimuli. Nevertheless about 80% of the tested neurons did not differ in their responses to BAL and the mixture, perhaps reflecting the prominent representation of BAL in the mixture.

The next tests aimed to determine whether the protocerebral neurons distinguished among mixtures with more subtle differences in composition. A neuron situated in the superior protocerebrum and arborizing unilaterally (Figure 2A) showed excitatory responses to EEZ (10 ng), BAL (20 ng), and a mixture of these components (EEZ 10 ng + BAL 20 ng), with the mixture evoking the strongest response (Figure 2B). Increasing or decreasing the proportion of either component in decadic steps resulted in a decline of response magnitude (Figure 2C). The soma of this neuron was located at the dorsal edge of superior protocerebrum with its primary neurite extending ventrolaterally toward the pedunculus of the ipsilateral mushroom body. Then the primary neurite bifurcated with one set of branches going medially but not passing the midline of the brain, thus innervating the superior-medial (SMPC) and inferior-medial protocerebrum (IMPC) (bottom left of panel a), and the other set of branches going laterally innervating the dorsal portion of inferior-lateral protocerebrum (ILPC) (bottom right of panel a). Both sets of branches appeared to lack varicosities. Another unilateral neuron with dense arborization in the ILPC and sparse fibers in the SLPC and SMPC (Figure 3A) gave indistinguishable responses to EEZ (10 ng), BAL (20 ng), and the mixture (EEZ 10 ng + BAL 20 ng; Figure 3B). Furthermore, increasing the proportion of either component 100-fold did not cause significant changes in this neuron's responses (Figure 3C), suggesting that such neurons do not contribute to coding of mixture proportions. The soma of this neuron was located near the lobula of the ipsilateral optic lobe with its primary neurite extending dorsomedially toward the calyx of the mushroom body (bottom right of panel a). Then the primary neurite branched to innervate broadly the ILPC, SLPC and SMPC (bottom left of panel a). Unlike the neuron in Figure 2, this neuron had varicosities in most of its branches.

To know if the labeled protocerebral neurons could be targeted by the MGC PNs, we examined the anatomical links between the MGC and the protocerebrum (Figure 4). The fluorescent dye that was electroporated into the MGC labeled all three subcompartments of the MGC, i.e. the Cumulus (C), Toroid (T) and Horse shoe (H) (Figure 4a). Three tracts --- the outer antennocerebral tract (OACT), medial antennocerebral tract (MACT) and inner antennocerebral tract (IACT) --- leaving the MGC ventromedially in parallel were clearly visible, along with the somata of the OACT and MACT neurons in the lateral cell cluster and the somata of IACT neurons at the medial cell cluster (Figure 4a). Shortly after exiting the AL posteriorly, the OACT fibers turned dorsolaterally and later posteriorly toward the pedunculus of the ipsilateral mushroom body. At a location anterior to the calyx, these fibers branched out forming a triangular isthmus, which was named as deltoid inferior-lateral protocerebrum (ILPC) in the silk moth *Bombyx mori* (Seki et al. 2005). The MACT fibers, which accompanied the IACT fibers after exiting the AL, left the IACT and turned dorsolaterally, bypassing the pedunculus and terminating at the lateral corner of the delta

region (Figure 4b). The IACT continued to travel posteriorly until reaching the calyx where it sent in collaterals forming a ring-shape arborization pattern. These axons exited the calyx and continued anteriolaterally and joined the MACT neurons at the lateral corner of the delta region (Figure 4c,d). The delta region therefore appeared to be an information-processing center for sex pheromone. This region could be defined as an area anterior to the calyx, inferior to the dorsal edge of the calyx and laterally adjacent to the pedunculus (Figure 4d,e). If we define a horizontal plain tangential to the dorsal edge of the calyx to separate the superior from the inferior protocerebrum, and define a sagittal plain cross the midline of the primary pedunculus to separate the medial from the lateral protocerebrum, then the delta region is located at the ILPC. Both neurons depicted in Figure 2 and 3 appeared to overlap with the delta region. For the purpose of clarity, we artificially copied these two neurons onto the image frame that showed the delta region, using the pedunculus as a landmark for alignment. Although we don't assume any synaptic contact in this artificial arrangement, these two neurons seem to overlap with the delta region dorsally and laterally (Figure 4f).

Thirteen of the protocerebral neurons were tested with single pheromone components, mixtures with different BAL:EEZ ratios and undiluted hibiscus oil (Figure 5a,b). These protocerebral neurons exhibited a wide range of tuning properties or selectivity to VOC stimuli. When measured by firing rate during the response window (Figure 5a), some neurons responded to just a few stimuli – for example, neurons #2, #5 and #10 – whereas other neurons responded to the entire panel of stimuli – for example, neurons #1, #3, #7 and #12. Although hibiscus oil was at a high concentration, neuron #2, #5 and #8 were not affected by it. These neurons could thus be considered as pheromone specialists. Moreover, within the behaviorally effective range of BAL:EEZ ratios (Martin et al. 2013), 4 neurons (about 30% of the sample) gave strongest responses (white asterisks in Figure 5a) in comparison with their responses to other ratios or the two single components. Also noticeable was that about 50% of the sampled neurons exhibited response to the solvent control. The tuning characteristics of these neurons were less discernible when responses were measured by their response delay (Figure 5b). However, this quantification still revealed that four neurons displayed strongest response within the behaviorally effective range of BAL:EEZ ratios. These were different neurons from those in Figure 5a that were also tuned to the behaviorally effective ratios.

To gain more insight on how addition of a second pheromone component could affect the response of protocerebral neurons to the first component, we analyzed the responses by holding one component constant while increasing the proportion of the other component in decadic steps (Figure 5c–f). All data were normalized to the response evoked by BAL or EEZ. A large variation was observed in this analysis. When the proportion of BAL in the mixture was increased, 3 neurons showed a gradual decrease of response delay (open arrow heads; shown as an increasing trend of the inverse of the response delay) (Figure 5c); 4 protocerebral neurons (indicated by filled arrowheads in Figure 5c) showed the shortest response delay to the natural 2:1 ratio. The tendency was less clear when the proportion of EEZ in the mixture was varied, but nonetheless the same neurons displayed shortest response delay (Figure 5d). Similar results were observed when the firing rate of responses was examined (Figure 5e,f). One neuron showed a particularly strong response to the natural 2:1 ratio.

In addition to mixture-ratio coding, protocerebral neurons may also encode multimodal stimuli. In our experiments, about 50% of the neurons were responsive to the solvent control but their responses to odor stimuli were usually stronger (Figure 5a,b), suggesting that these neurons may integrate information from mechanical as well as olfactory stimuli. One such neuron had its soma in the ventromedial protocerebrum with varicose arbors in the

ipsilateral lateral accessory lobe and ventral protocerebrum neuropil (LAL-VPC). Posterior to the AL and ventrolateral to the α -lobe of the mushroom body and the central body complex, the pair of LAL-VPC neuropils flank the oesophageal foramen serving as the premotor centers in the ventral protocerebrum (Kanzaki et al. 1994; Iwano et al. 2010). Exiting the LAL-VPC neuropil, the main neurite of this neuron crossed the midline via the LAL commissure and projected dorsoposteriorly and entered the contralateral inferior protocerebrum (Figure 6a top panel). This neuron was strongly excited by EEZ (10ng), BAL (20ng) and the mixture of these components (30ng) with peak firing frequency of about 200 Hz; however, it also showed excitatory response to the solvent control albeit the peak frequency evoked by the control stimulus was only about 100 Hz (Figure 6a bottom panel). On the other hand, the neuron shown in Figure 6b exhibited strong excitatory responses to the pheromonal stimuli but no response at all to the solvent control (Figure 6b, bottom panel). This neuron had its soma on the dorsal region of the oesophageal foramen. Its primary neurite extended laterally to the VPC where it ramified into smooth branches. The primary neurite continued laterally and entered the optic lobe. Although not tested with any light stimulus, this neuron may be able to integrate optical and olfactory information, based on its anatomy.

The protocerebral neurons in this study may have been able to discriminate stimuli more effectively as a population than as individual neurons, as suggested by other studies in which interactions among neurons in a network were found to encode specific stimuli (Chapin and Nicolelis 1999; Sakurai 1999). In our data set, we observed a large diversity of response patterns evoked by identical stimuli across neurons, which suggests a rich reservoir for population coding. Figure 7 shows examples of 8 different protocerebral neurons, each responding to BAL (20 ng) in a consistent but apparently different manner, summarized here as monophasic (tonic excitation or suppression, bursting excitation), biphasic (excitation followed by a period of inhibition or inhibition followed by excitation), and triphasic responses (excitation followed by a period of inhibition and then a second excitation). More examples showing diverse morphological and physiological properties of protocerebral neurons are included in Supplemental Figures 2–4. Trial-to-trial consistency permitted accurate description of a neuron's overall responsiveness by means of PSTHs, in which principal-component analysis was performed across all protocerebral neurons to produce principal axes that integrate information from all neurons (Chapin and Nicolelis 1999). The first principal axis (Figure 8a), responsible for about 65% of the variation embedded in the data set, closely resembled the averaged responses to EEZ, BAL, and the mixture across all neurons (Figure 8b). The three curves in Figure 8A largely overlap during the entire time course, indicating that the population of protocerebral neurons did not distinguish among stimuli in their summed responses. The second principal axis (Figure 8c), however, revealed more differences among the stimuli, especially around 0.3 sec. To examine further the differences at higher-order statistics, we calculated the Euclidean distance between pairwise comparisons among EEZ, BAL, and the mixture, again on the basis of PSTHs across all neurons (Figure 8d). The higher the distance value, the more different the comparison is. In Figure 8d, the distance curve for BAL vs. mixture (black curve) was the lowest, indicating that the representation of these two stimuli by the protocerebral neurons was most similar. The largest difference was found between EEZ vs. BAL (magenta curve) and EEZ vs. mixture (cyan curve), indicating that EEZ was mapped equidistant from BAL and the mixture in the constructed multidimensional olfactory space. Examination of the time evolution of the distance between stimuli indicated that the stimuli were best separated at around 0.3 sec, before the peak population response was reached, indicated by the vertical dashed line in Figure 8 b,d.

Discussion

Given the demonstrated importance of mixture information in sex-pheromonal communication among moths (e.g. Tumlinson et al. 1989,1994; Vickers et al. 2003; Domingue et al. 2007, 2008; Kanno et al. 2010; El-Sayed et al. 2011; Leary et al. 2012; Martin et al. 2013), our finding that 24% of the sampled, pheromone-responsive protocerebral neurons in this study did not distinguish among the tested stimuli (supplemental Table 1) is surprising. Pairwise comparisons of responses to different pheromonal stimuli (EEZ vs. BAL, EEZ vs. mixture, BAL vs. mixture) showed that 50–80% of neurons exhibited no significant differences in their responses to these stimulus pairs (Figure 1, bottom). Thus, it appears that the behavioral significance of the pheromone mixture is not reflected in mixture-selectivity among many of the responsive protocerebral neurons. Although our sample size is limited (n=34), mixture-selective neurons appear to be uncommon at this level of processing. Only a few neurons selectively discriminated the natural mixture of BAL and EEZ at the 2:1 ratio from other related stimuli (Figure 2, 5).

Behavioral studies have demonstrated the importance of mixture proportions in sex pheromones of moths. In the grape berry moth *Paralobesia viteana*, doubling single components in an attractive blend—thus altering the mixture proportions of components—caused significant reduction of its attractiveness for male moths (Cha et al. 2011). Closely related moth species are often found to use information about mixture proportions to distinguish olfactory cues from different emitters, but very little is known about how an insect's olfactory system encodes these stimuli. Martin et al. (2013, this issue) found that synchronous firing of PNs is involved in representing the natural ratio of the female's sex-pheromone mixture (BAL:EEZ=2:1) in the AL of adult male *M. sexta*. In short, the pheromone-responsive PNs produce more synchronized spiking activity at millisecond precision when a pheromone mixture at the natural ratio of key components is presented. Synchronized spikes are believed to exert stronger impact on postsynaptic neurons (Aertsen et al. 1996; Kisley and Gerstein 1999; Rudolph and Destexhe 2003; Person and Raman 2012), and we hypothesized that postsynaptic neurons in the lateral protocerebrum, which is innervated by AL PNs, would show stronger responses to the mixture at the natural ratio than to mixtures at abnormal ratios. The only neuron in our sample that displayed such a physiological trait and was visualized by intracellular staining innervates an area overlapping with the dorsal side of the delta region of the ILPC, as well as the SMPC and the IMPC (Figure 2, 4). The labeling data from mass-staining revealed that the delta region is indeed an olfactory center processing sex-pheromonal information transmitted from all three major antennocerebral tracts (Figure 4). This finding is consistent with earlier work in male silkmoths *Bombyx mori* (Kanzaki et al. 2003) and in male hawk moths *M. sexta* (Homberg et al. 1988). Other branches of the neuron in Figure 2 innervate the SMPC and IMPC outside the delta region. Nevertheless, neurons that link the lateral and the superior protocerebrum have been reported previously (Lei et al. 2001), and data show that multiglomerular PNs terminate in the superior protocerebrum in female *Heliothis virescens* moths (Løfaldli et al. 2012) and likely so in male *M. sexta* moths (Homberg et al. 1988). These results suggest that the mixture-ratio selectivity documented in Figure 2 may reflect synchrony detection. The neuron depicted in Figure 3, although overlapping with the delta region laterally, does not encode mixture-ratios, however. In fact, out of 13 neurons that were tested with different mixture ratios, only 4 of them showed the strongest response to the behaviorally effective mixture ratios (Figure 5). In addition to neurons that exhibited selective responses to the range of behaviorally effective ratios, about 50% of the sampled protocerebral neurons responded to the solvent control and thus likely to mechanical stimulus such as air movement (Figure 5 and 6). The neuron shown in Figure 6a clearly gave an excitatory response to the control, although the responses to pheromonal stimuli were

much stronger. This neuron arborized in the LAL-VPC neuropil, which is believed to produce neural patterns that control the characteristic zig-zagging odor-tracking behaviors in moths (Kanzaki et al. 1994; Iwano et al. 2010). Therefore, the neuron in Figure 6a may function as a multimodal sensory integrator for both olfactory and mechanic stimuli. The source of the mechanical component of the response could be from the antennal mechanosensory motor center (AMMC), which is located ventroposteriorly to the AL in the deutocerebrum (Homberg et al. 1988), via unknown pathways; or from some AL PNs that are shown to respond to mechanical stimuli (Han et al. 2005). Similarly, the neuron shown in Figure 6b also arborized in the LAL-VPC neuropil. A difference is that this neuron may integrate olfactory and visual information because its neurite extends to the contralateral optic lobe. Visual information has been shown to play an important role in odor-source seeking behavior in insects (Goyret et al. 2007; Brévault and Quilici 2010; Chow et al. 2011). Based on our limited data, the LAL-VPC neuropil may be a multimodal sensory center in the protocerebrum in addition to its role as a pattern generator for controlling zig-zagging flight behavior.

Our results reveal diverse response patterns evoked by identical stimuli among protocerebral olfactory neurons (Figure 5). Such physiological diversity might enhance information coding at the population level, as suggested by a modeling study of visual cortical neurons (Chelaru and Dragoi 2008). Our findings provide two lines of evidence for this notion. First, taking account of two response parameters together, firing rate and response latency, yielded a higher percentage of protocerebral neurons that distinguished among stimuli than with either parameter alone (Figure 1). Some neurons discriminated stimuli based on firing rate and others did so based on response latency, but the population of neurons discriminates among the stimuli better than individual neurons. Second, the principal-component analysis (PCA) of neural responses (Chapin and Nicolelis 1999) provided distinguishable response curves, especially on the second principal axis (Figure 8C). The response curves on the first principal axis, which closely resemble the averaged PSTHs across all neurons, however, showed less difference among the stimuli (Figure 8A, B). This result may be related to the high-order computation of the olfactory system, where vital stimulus information is extracted in independent principal axes, the first correlating with the summed neural activity and the second reflecting overall quality of stimuli (Haddad et al., 2010). In our case, the second principal axis may capture the salience of the stimulus, i.e. pheromone mixture is more “meaningful” than single components. The PCA in this study is based on the averaged PSTHs across multiple trials. Therefore the variations potentially produced by the same stimulus across multiple trials are not extracted. However, such within-neuron variations to the same stimulus seem minimal in comparison with the variations across different neurons in our dataset (Figure 7; Supplemental figure 1). Such within-neuron stability coexisting with between-neuron variability may be advantageous in a pheromonal communication system that requires precision and complexity at the same time. Our findings also suggest that protocerebral neurons adopt elemental rules to process the stimulus mixture. That is, responses evoked by the mixture resemble those elicited by its constituents (Kay et al. 2003; Lei and Vickers 2008). For example, calcium imaging study of the moth *Spodoptera littoralis* AL showed that mixture of plant volatiles evoked a response pattern that appeared to be the sum of responses of individual compound (Carlsson et al. 2007). In the AL of male *M. sexta*, the pheromone mixture evokes excitatory responses in both of the main glomeruli (the cumulus and toroid I) of the male-specific macroglomerular complex (MGC), as do cognate inputs from either EEZ or BAL stimulation, thus reflecting the elemental rule of mixture processing (Lei and Vickers 2008). This rule appears to be maintained at the level of protocerebrum but with a bias toward BAL, the most prominent component of the pheromone mixture. Shown in Figure 1, parametric analysis revealed that the percentage of protocerebral neurons showing significantly different responses to BAL vs. the mixture was

lower than the other two comparisons, indicating that fewer neurons could discriminate the mixture from BAL. In other words, the neural representation of the mixture at this level of processing is more similar to the representation of BAL than to that of EEZ. This observation is further supported by population data. In Figure 8D, the distance curve computed from the comparison of BAL vs. mixture (black) across all protocerebral neurons is lower than the other two distance curves, indicating a higher similarity between representations of BAL and the mixture than between other representations. One explanation for the more prominent role of BAL representation could lie in the evolutionary history of the pheromone production. In the female's sex-pheromone gland, the 16-carbon dienals such as BAL are produced from hexadecanoate through desaturation and isomerization; the 16-carbon trienals such as EEZ result from further desaturation of corresponding dienals (Fang et al. 1995; Matoušková et al. 2007). The time sequence of dienal and trienal production suggests that BAL might be an ancestral pheromone component of *Manduca* spp, and therefore more robustly represented by protocerebral neurons.

Notably, the peak population response did not correspond with the largest difference among population responses (Figure 8B, D, vertical dash line). Instead, the stimulus pairs were best discriminated before the population response reached its peak. The early differentiation is also reflected on the second principal axis, where the response curves overlap least at around 0.3 sec, before the peak response (Figure 8C). Because MGC PNs reach maximal synchronous firing before their firing rate reaches maximum (Lei et al. 2002; Martin et al. 2013), we have hypothesized that spiking synchrony, rather than firing rate, might carry the most meaningful information in terms for discriminating the quality of olfactory stimuli. The MGC PNs can fire more synchronously while keeping their firing rate unchanged, possibly due to the modulation of local interneurons (Lei et al. 2002; Martin et al. 2013). Some postsynaptic neurons in the protocerebrum may be more sensitive to the changes of synchronized input from AL PNs than to the unsynchronized input. At the level of the AL, firing rate might solely encode concentration information while the synchronous spiking might encode the behavioral significance of the stimulus. At the level of the protocerebrum, the second principal axis, which is believed to capture the overall quality of the stimulus (Haddad et al. 2010), might be more sensitive to synchronized input.

In summary, these studies of a limited number of protocerebral olfactory neurons that responded to sex pheromone showed that some neurons of this class are tuned to the natural mixture ratio of the key sex-pheromone components, responding most strongly to BAL:EEZ 2:1. These neurons may be critical players in recognizing the behaviorally optimal mixture. Other, more numerous neurons in our sample exhibited diverse response patterns, suggesting considerable complexity of higher-order representation of olfactory stimuli. These protocerebral olfactory neurons appear to adopt the elemental principle of mixture processing, but the most abundant pheromone component, BAL, seems to be more prominent in the representation of the mixture. In a constructed multidimensional space, the second principal axis captures more differences among the stimuli, and the largest difference occurs before the population response reaches its peak, suggesting that the system may use information contained in the early phase of the response of AL output neurons—possibly synchronized spiking—to distinguish the quality of stimuli.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Statistical comparison between pairs of stimuli (EEZ vs BAL, EEZ vs Mixture and BAL vs Mixture) based on two response parameters (Response delay and Firing rate). The results for all 34 protocerebral neurons (top panel) are vertically aligned, with orange indicating $p \leq 0.05$ and green indicating $p > 0.05$. The '+' or '-' sign in the matrices annotates the polarity of a comparison. For example, the '-' sign of the top left cell of the first matrix indicates the response delay shown by neuron #1 to EEZ stimulation is significantly shorter than that to BAL stimulation. P values were obtained from the Kruskal-Wallis test followed by Tukey-Kramer multicomparison procedure. The matrix on the right is the combination of the first two matrices. The bar graphs at the bottom panel show percentage of neurons that statistically discriminated the corresponding stimulus pairs when measured by the response delay, firing rate or the two parameters combined. As indicated by the arithmetic signs, mixture did not always evoke stronger response.

Figure 2.

A protocerebral neuron showing selective response to the pheromone mixture composing of BAL and EEZ in 2:1 ratio. Intracellular staining reveals it is a unilateral neuron arborizing both the superior and inferior protocerebrum (a, top panel). Zoom-in view shows the neuron lacks varicosities in its branches (a, bottom panels). Peristimulus time histograms derived from 5 odor pulses show this neuron's excitatory responses to the single pheromone components and their mixture (b). The black bar below each histogram indicates the odor stimulation. This neuron responded most strongly to the mixture of BAL and EEZ in 2:1 ratio, but increasing the proportion of either component by 10 folds did not result in significant changes (c). Different letters above the box plots indicate statistical significance ($p < 0.05$, Kruskal-Wallis test followed by Tukey-Kramer multicomparison procedure). SMC: superior-medial protocerebrum; IMPC: inferior-medial protocerebrum; SLPC: superior-lateral protocerebrum; ILPC: inferior-lateral protocerebrum; SOG: suboesophageal ganglion; D: dorsal; V: ventral; L: lateral; M: medial; EEZ: *E10-E12-Z14-hexadecatrienal*; BAL: bombykal, i.e. *E10-Z12-hexadecadienal*.

Figure 3.

A protocerebral neuron showing no statistical difference in its responses to the pheromone components and mixtures composing of BAL and EEZ in various ratios. Intracellular staining reveals it is a unilateral neuron arborizing the superior and inferior protocerebrum (a, top panel). Zoom-in view shows the varicosities of the neuron's branches (a, bottom panels). Peristimulus time histograms derived from 5 odor pulses show this neuron's excitatory responses to the single pheromone components and the mixture of the two components (b). The black bar below each histogram indicates olfactory stimulation. This neuron demonstrated excitatory responses of equal magnitude to the mixture composing of BAL and EEZ in 2:1 ratio and other mixtures deviating from this ratio (c). Different letters above the box plots represent statistical significance ($p < 0.05$, Kruskal-Wallis test followed by Tukey-Kramer multi-comparison procedure). SMPC: superior-medial protocerebrum; IMPC: inferior-medial protocerebrum; SLPC: superior-lateral protocerebrum; ILPC: inferior-lateral protocerebrum; SOG: suboesophageal ganglion; D: dorsal; V: ventral; L: lateral; M: medial; EEZ: *E10-E12-Z14*-hexadecatrienal; BAL: bombykal, i.e. *E10-Z12*-hexadecadienal.

Figure 4.

Mass-staining showing the antennocerebral tracts that link the macroglomerular complex (MGC) to the protocerebrum. Microruby fluorescent dye was electroporated into the MGC area, staining projection neurons from all three MGC subcompartments – the Cumulus (C), the Toroid (T) and the Horseshoe (H); the axons from C, T and H exit the MGC in three visible tracts – the Inner Antennocerebral Tract (IACT), the Medial Antennocerebral Tract (MACT) and the Outer Antennocerebral Tract (OACT) (a). The OACT fibers turn dorsoposteriorly shortly after exiting the antennal lobe and ramified into a triangular isthmus (delta region) just lateral to the pedunculus and anterior to the calyx; the MACT fibers accompany the IACT posteriorly until the mid-point of the pedunculus where they turn dorsomedially and send terminals to the lateral corner of the delta region (b). The IACT fibers continue posteriorly, reaching the calyx of the and collaterally innervating the calyx before exiting from it laterally; these fibers then turn anteriomedially, joining the MACT neurons and innervating the lateral corner of the delta region (c). The spatial relationship of the delta region, the three antennocerebral tracts and the calyx is further depicted with computer-generated sagittal and frontal views of the preparation, showing the delta region is anterior to the calyx and lateral to the pedunculus, thus situated at the inferior-lateral protocerebrum (d, e). The two neurons from Figure 2 (solid line) and Figure 3 (dash line) are artificially mapped onto the image frame where the delta region is visible using the pedunculus for alignment, showing a potential overlapping between these two neurons and the delta region (f); the image frame is flipped to be aligned with the two neurons. MCB: medial cell body cluster; LCB: lateral cell body cluster; D: dorsal; V: ventral; L: lateral; M: medial; A: anterior; P: posterior; MBC: mushroom body cluster; SMPC: superior-medial protocerebrum; IMPC: inferior-medial protocerebrum; SLPC: superior-lateral protocerebrum; ILPC: inferior-lateral protocerebrum.

Figure 5.

Responses, measured as firing rate within response window and response delay of a subset of protocerebral neurons to single pheromone components and mixtures of different ratios, are analyzed to examine the neurons' tuning properties (a–b) and the effect of systemically increasing the proportion of a second component in the mixtures (c–f). In a–b, all responses within a neuron are normalized to the strongest stimulus, thus revealing the neurons (marked with white asterisks) that produce strong response to the behaviorally effective range of pheromone ratios (black rectangle). The values of response delay are inversed so that the shortest delay is shown as the highest value (b). The white cells in matrix b are due to a lack of data. Neural activities associated with the control stimulus are sometimes indistinguishable from the background activities so that it is too difficult to determine “response onset”. In c–f, all values are normalized to the responses of the single component in each group. The stimulus labels are arranged in such a way that one component is kept constant while the proportion of other component changes in decadic step. The red curve in each panel represents the average across all neurons. While showing large variations, four neurons decreased their response latency, thus responding faster, to the mixture consisting of BAL and EEZ in 2:1 ratio (c–d, filled arrow heads). Three other neurons showed gradual decrease or increase of response delay with an increasing proportion of BAL, thus exhibiting an additive effect of BAL (open arrow heads) (c). Similar results are observed when systemically increasing the proportion of EEZ in the mixtures (d). The same analysis on the firing rate within response window reveals that a neuron was most strongly affected by the 2:1 ratio whereas others showed weaker or no tuning to the natural ratio (e–f).

Figure 6.

Multimodal protocerebral neurons. A neuron that arborizes the lateral accessory lobe and ventral protocerebrum (LAL-VPC) and crosses the midline of the brain via the LAL commissure (LAL Co), entering the inferior lateral protocerebrum (a, top panel), responded to the control stimuli with excitatory responses; its responses to the pheromone stimuli are, however, much stronger (b, bottom panel). The contralateral arbors are unavailable owing to an accidental loss of brain tissue during handling. A different neuron that innervated the ventral protocerebrum (VPC) sent its axon to the lobula of the ipsilateral optic lobe (O) (b, top panel). This neuron was unresponsive to the control stimuli but was strongly affected by the pheromone stimuli (b, bottom panel). The black bars below histograms indicate olfactory stimulation. SOG: suboesophageal ganglion; D: dorsal; V: ventral; L: lateral; M: medial.

Figure 7.

Representative traces from single trial of olfactory stimulation (left panels) and raster plots generated from 10 trials (right panels) demonstrating a large reservoir of different response patterns as well as the trial-to-trial consistency. Olfactory stimulation, indicated by the black bar at the bottom, was performed by delivering BAL (20ng) to all neurons.

Figure 8.

Multivariate analysis reveals discrimination of olfactory stimuli at a population level. The results from principal component analysis show that the population response projected onto the first principal axis (a) closely matches the arithmetic average of responses (b) across all neurons ($n=34$), displaying little difference among EEZ (red), BAL (blue) and the blend (green). The second principal axis (c), however, shows more differentiation among the stimuli. Moreover, analysis of Euclidean distance (d) on comparisons of stimulus pairs (EEZ vs. BAL, magenta; EEZ vs. Blend, cyan; BAL vs. Blend, black) shows the population representation of the blend is more similar to that of BAL than to EEZ, indicated by the lower distance curve (black). The peak distance occurs prior to the peak population response (vertical dash line). For all panels, the stimulus onset is at time zero, lasting for 100ms.