A Multisensory Centrifugal Neuron in the Olfactory Pathway of Heliothine Moths

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ABSTRACT

We have characterized, by intracellular recording and staining, a unique type of centrifugal neuron in the brain olfactory center of two heliothine moth species; one in Heliothis virescens and one in Helicoverpa armigera. This unilateral neuron, which is not previously described in any moth, has fine processes in the dorso-medial region of the protocerebrum and extensive neuronal branches with blebby terminals in all glomeruli of the antennal lobe. Its soma is located dorsally of the central body close to the brain midline. Mass-fills of antennal-lobe connections with protocerebral regions showed that the centrifugal neuron is, in each brain hemisphere, one within a small group of neurons having their somata clustered. In both species the neuron was excited during application of non-odorant airborne signals, including transient sound pulses of broad bandwidth and air velocity changes. Additional responses to odors were recorded from the neuron in Heliothis virescens. The putative biological significance of the centrifugal antennal-lobe neuron is discussed with regard to its morphological and physiological properties. In particular, a possible role in multisensory processes underlying the moth’s ability to adapt its odor-guided behaviors according to the sound of an echo-locating bat is considered. J. Comp. Neurol. 521:152–168, 2013.

INDEXING TERMS: modulatory neuron; antennal lobe; olfactory pathway; sound sensitive; heliothine moth; multimodal responses

Odor-guided behaviors are particularly prominent in insects. Olfactory cues are essential for vital tasks such as finding food, seeking a mate, and localizing a suitable host plant for oviposition. In order to respond optimally for the survival of the individual and preservation of the species, the odor-induced behaviors need to be adapted to both endogenous and exogenous conditions. For instance mating status, age, and previous sensory experience are reported to affect olfactory neuronal responses (Gadenne et al., 2001; Anderson et al., 2007; Anton et al., 2007; Arenas et al., 2009; Denker et al., 2010). Chemosensory neurons also seem to change their sensitivities according to the circadian rhythm (Linn et al., 1996). At the behavioral level, associative learning mechanisms involving pairing of stimuli from different sensory modalities are documented to be of vital importance for establishing appropriate adaptations (Menzel, 2001). Thus, previous olfactory exposure associated with rewarding or aversive taste cues is reported to form memory for distinct odors (Fan et al., 1997; Daly et al., 2004; Jørgensen et al., 2007). Furthermore, the combination of visual and olfactory stimuli is shown to promote nectar feeding behavior (Raguso and Willis, 2002). Bees even intensify their foraging activity when being exposed to particular colors formerly associated with a conditioned odor (Giurfa et al., 1994).

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Modulation of the various odor-guided behaviors presumes activation of distinct mechanisms, which implies that the principle of plasticity has to be built in at the neuronal level. The central olfactory pathway of insects includes three main neuropils: the primary olfactory center, i.e., the antennal lobe, and two higher integration centers, the mushroom body calyces and the lateral protocerebrum (Boeckh et al., 1984; Homberg et al., 1988). The antennal lobe is the final destination of the numerous olfactory sensory neurons located mainly on the antennae. Here, the sensory axon terminals make synapses with antennal-lobe neurons in spherical structures called glomeruli. The second-order neurons comprise two categories, local interneurons, which are confined to the antennal lobe, and projection neurons carrying the olfactory information to the higher integration centers in the protocerebrum. An additional and relatively small group of antennal-lobe processing units is comprised of the centrifugal neurons, which are presumed to play a key role in modification of olfactory information (reviewed by Anton and Homberg, 1999; Galizia and Rössler, 2010).

The general morphology of this neuron category includes neuronal processes in various regions of the nervous system and a fiber projecting into the antennal lobe, often innervating all glomeruli. The centrifugal neurons seem to receive input from various sensory channels and to forward these signals to the antennal lobe, presumably for appropriate adjustment of the olfactory information (Homberg and Müller, 1999). In addition to comparable branching patterns, a common hallmark of this category of neurons is their release of biogenic amines (e.g., serotonin, octopamine, and dopamine) or neuropeptides (reviewed by Galizia and Rössler, 2010). A main portion of the modulatory neurons identified so far has therefore been revealed by immunocytochemical studies (reviewed by Homberg and Müller, 1999; Schachtner et al., 2005).

One centrifugal neuron that is particularly well described in several moth species, as well as other insects, is the so-called serotonin-immunoreactive (SI) antennal-lobe neuron (Kent et al., 1987; Salecker and Distler, 1990; Hill et al., 2002; Dacks et al., 2006; Zhao and Berg, 2009). In Lepidoptera species, the SI neuron has wide-field arborizations in both protocerebral hemispheres and extensive terminal projections in all glomeruli of the antennal lobe contralateral to that containing the soma (reviewed by Kloppenburg and Mercer, 2008). The localization of input synapses primarily in the protocerebrum and output synapses in the antennal lobe confirms the assumption that the neuron is a descending element (Sun et al., 1993). Concerning functional properties of the SI-neuron, pharmacological investigations have reported that serotonin increases pheromone responses of antennal-lobe neurons (Kloppenburg et al., 1999; Kloppenburg and Heinbockel, 2000; Hill et al., 2003). A role of the SI-neuron in adjusting pheromone-guided responses according to the circadian rhythm has been suggested (Gatellier et al., 2004). However, the main function of this widespread neuron type is not yet fully understood. Electrophysiological recordings from the neuron itself have been obtained a few times, and they include responses to mechanical and odor stimuli (Hill et al., 2003; Zhao and Berg, 2009).

Another intensively studied category of centrifugal antennal-lobe neurons is the so-called midline neurons of the subesophageal ganglion (reviewed by Anton and Homberg, 1999). This kind of octopaminergic antennal-lobe neuron has been identified in the fruit fly (Stocker et al., 1990), the locust (Bräunig, 1991), and the honeybee (Hammer et al., 1993). In a sequence of well-designed experiments carried out in the last mentioned species, Martin Hammer (1993) showed that one particular neuron of this category, the VUMmx1 neuron, is sensitive to sucrose and mediates information about the unconditioned taste stimulus in associative olfactory learning. The morphology of the VUMmx1 neuron, having dendritic arborizations in the subesophageal ganglion, i.e., the primary gustatory center, and extensive projections in the antennal lobe and the calyces of both brain hemispheres, corroborates its function. Octopaminergic projections in the antennal lobe, possibly originating from the same type of neuron, have also been reported in the moth (Dacks et al., 2005).

Because most centrifugal neurons have been identified through immunocytochemical rather than electrophysiological methods, our understanding of their functions and encoding characteristics is, in general, still limited. The intracellular recording and staining technique, which provides first-hand information from the individual processing units, is suitable for obtaining knowledge about physiological and morphological features characterizing these neuron categories—even though the data materials may be quantitatively restricted. Based on the current methodological approach, we here present the morphology and, in addition, physiological properties of a unique category of antennal-lobe centrifugal neurons in the moth brain that has previously not been described.

**MATERIALS AND METHODS**

The current analyses are based on intracellular recording and staining of two neurons, plus data from mass staining experiments. Even though the number of recorded neurons is small, the morphological similarity between the successful stainings in two heliothine species confirms the presence of this hitherto undiscovered neuron type in moth brains.
Insects and preparation

Pupae of *Heliothis virescens* and *Helicoverpa armigera* were imported from laboratory cultures (*H. virescens* from Syngenta, Basel, Switzerland; and *H. armigera* from Henan University of Science and Technology, Henan, China). Male and female pupae were separated and kept in climate chambers on reversed photoperiod light-dark 14:10 hours at 22°C. The adults were fed a 5% sucrose solution. Experiments were performed on adult males 2–5 days after ecdysis, as previously described by Zhao and Berg (2009). The moth was restrained inside a plastic tube with the head and antennae exposed. The head was immobilized with wax (Kerr, Romulus, MI), and the antennae were lifted up by needles. The brain was exposed by opening the head capsule and removing the mouth parts, the muscle tissue, and major trachea. The sheath of the antennal lobe was removed by fine forceps in order to facilitate microelectrode insertion into the tissue. Once the head capsule was opened, the brain was supplied with Ringer’s solution (in mM: 150 NaCl, 3 CaCl₂, 3 KCl, 25 sucrose, and 10 N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid, pH 6.9). The whole preparation was positioned so that the antennal lobes were facing upward and were thus accessible for intracellular recordings.

Intracellular recording and staining

The intracellular recordings from the antennal-lobe neurons were carried out as previously described (Zhao and Berg, 2009). Recording electrodes were made by pulling glass capillaries (borosilicate glass capillaries; Hilgenberg, Malsfeld, Germany; OD: 1 mm, ID: 0.75 mm) on a horizontal puller (P97; Sutter Instruments, Novarto, CA). The tip was filled with a fluorescent dye (4% tetramethylrhodamine dextran with biotin; Micro-Ruby, Molecular Probes) picked up by the fine tip of a micro needle were then applied into the antennal lobe by hand. The brain was subsequently supplied with Ringer’s solution and kept for 2 hours at room temperature for transportation of the dye. The following procedure was as described above.

Odor and air puff stimulation

The odor delivery system for the intracellular recording consisted of two glass cartridges (ID 0.4 cm) placed side by side, both pointing toward the antenna at a distance of 2 cm. One replaceable cartridge contained a piece of filter paper onto which a particular odor stimulus was applied. The other cartridge contained a clean filter paper. An air flow (500 ml/min) led through the odorless cartridge was continuously blown over the antenna. During each stimulus period, which lasted for 400 ms, the air flow was switched by a valve system from the odorless to the odor-bearing cartridge. As olfactory stimuli we used the two-component pheromone blends of the two species, i.e., *cis*-11-hexadecenal (Z11–16:AL) and *cis*-9-hexadecenal (Z9–14:AL) in a ratio of 94:6 for *H. virescens* and Z11–16:AL and cis-9-hexadecenol (Z9–16:AL) in a ratio of 95:5 for *H. armigera* (pheromone chemicals, Plant Research International, Pherobank, Wageningen, Netherlands), plus the plant oil ylang-ylang (Dragoco, Totowa, NJ). Odor compounds diluted in hexane were applied onto a small filter paper. The hexane was allowed to evaporate before the filter paper was wrapped up and placed in the cartridge. All stimuli were prepared so that the filter paper contained 10 ng of the binary pheromone blend, and 100 µg of the plant oil. A cartridge containing a clean filter paper was used as control. The odor stimuli were regularly renewed during the experimental period.
Sound stimulation

The sound stimuli originated from the solenoid valve system that controls the air flow during odor stimulation. The two valves (2-way Direct Lift Solenoid Valves, 01540; Cole-Parmer, Vernon Hills, IL) are mounted on one of the walls of the room, 2 m away from the insect. Valve 1 (type normally open) regulates the continuous airflow and valve 2 (type normally closed) the odor/air puff. When the odor stimulation system is activated, the two valves produce four separate sound pulses; the first pulse is caused by valve 1 being closed (first event); the second pulse, which appears 1 second later, by valve 2 being opened (second event); the third pulse occurs 0.4 second thereafter by valve 2 being closed (third event); and the forth pulse, which appears after another 1 second, by valve 1 being reopened (fourth event; Fig. 1). The second and the third event thus correspond with the odor stimulus’s onset and offset, respectively. Activation of the two valves was recorded in the Spike program via separate channels.

We recorded the sound pulses created by opening and closing the valves using a 1/4-inch microphone and preamplifier (types 4138 and 4939; Bruel & Kjær, Nærum, Denmark), plus a front end amplifier (type 1201/30517; Norsonic, Lierskogen, Norway); the pulses were then digitized (sample frequency 192 kHz), and the signals

![Figure 1](image-url)
were stored on a signal analyzer, WinMLS. The sounds were measured with and without air flow both at the position of the insect’s ear in air as well as inside the plastic tube wherein the moth was restrained. The recorded signals were analyzed by using commercial software, BatSound Pro (Pettersson Elektronik, Uppsala, Sweden) (Fig. 1). The pulses were broad band with energy from audible up to ultrasonic frequencies of 40–50 kHz. The main effect of the plastic was to dampen frequencies above ~25 kHz (Fig. 1: compare spectrogram of “click 1” with “click 1 in plastic”). Inside the plastic the spectra of all clicks had energy up to above 20 kHz (Fig. 1, lower panel). Thus the frequency range of the clicks overlapped with the frequency range of 10–60 kHz, to which noctuid moths are most sensitive. The initial pulse amplitude was high, falling off quickly. Due to the decreasing amplitude it was difficult to measure pulse length accurately. The duration for which the amplitude was above 50% of the maximum was 30–40 ms for all pulses. Pulse durations measured as the time containing 95% of the total energy were ~100 ms (Fig. 1). The sound pressures of the four pulses were between 71 and 89 dB SPL (peak–peak) which is well above typical moth hearing thresholds of 35–40 dB around 15–30 kHz.

Measurement of air flow

We measured the changes in air flow during the time period when the odor stimulation system was activated by using an air velocity meter (VelociCalc model 8355WS; TSI, Shoreview, MN). The air flow was measured at the site of the insect antenna over an interval of 11 second, starting 4 seconds before the closing of valve 1 and ending 4.6 seconds after the reopening of valve 1. The length of the Teflon tubes (ID 2 mm) transporting the air from the valves to the glass cartridges is approximately 4 m. The changes in air flow during the relevant time period are shown in Figure 1; the initial event, the closing of valve 1, caused a relatively rapid decrease in air flow. The two subsequent events, opening and closing of valve 2, induced a transient air flow increase and decrease, respectively. The fourth air flow change, caused by valve 1 being reopened, differed from the three preceding ones by a pronounced slower time course. As the exact onsets of the air-flow changes at the position of the insect could not be plotted via the Spike2 program, the curve describing the air flow is aligned with the time signal of the sound pulses according to the minimum delay possible (Fig. 1). In order to simplify the terminology, the rapid increase in air velocity caused by valve 2 being opened is termed air puff stimulation in the subsequent text.

Immunocytochemistry

Antibody characterization

In order to identify antennal-lobe glomeruli and protocerebral neuropil structures, immunostaining with antibodies marking synaptic regions was performed on the labeled brains. Two monoclonal antibodies shown to detect proteins associated with synaptic terminals were used, both developed in mouse (Table 1). The anti-SYNORF1 was raised against fusion proteins composed of glutathione-S-transferase and the \textit{Drosophila} SYN1 protein (SYNORF1; Klagges et al., 1996). The specificity of the anti-SYNORF1 has been described previously; in \textit{Drosophila} at least four, probably five, isoforms were recognized by the monoclonal antibody in Western blots of head homogenates (three bands at 70, 74, and 143 kDa; Klagges et al., 1996). In Western blots of \textit{H. virescens} brains, the current antibody labels five bands, two at 74 kDa and three at 55–60 kDa (Rø et al., 2007). The antibody, nc46, which was raised against homogenized \textit{Drosophila} heads (Hofbauer et al., 2009), detects an epitope of a protein that is connected with synaptic vesicles, the so-called synapse-associated protein of 47 kDa (SAP47; Hofbauer et al., 2009; Saumweber et al., 2011). In Western blots of \textit{Drosophila} brains, the antibody labels five bands, two at 74 kDa and three at 55–60 kDa (Rø et al., 2007). The anti SYNORF1 was delivered by Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) (Table 1). The other antibody, nc46, which was raised against homogenized \textit{Drosophila} heads (Hofbauer et al., 2009), detects an epitope of a protein that is connected with synaptic vesicles, the so-called synapse-associated protein of 47 kDa (SAP47; Hofbauer et al., 2009; Saumweber et al., 2011). In Western blots of homogenates from isolated \textit{Drosophila} brains, the antibody is reported to label a prominent band at 47 kDa (Reichmuth et al., 1995). The nc46 antibody was kindly donated by E. Buchner (Table 1).

<table>
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<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Manufacturer, species antibody was raised in, mono- vs. polyclonal, cat. no.</th>
<th>Dilution</th>
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<tr>
<td>Synapsin</td>
<td>Fusion protein of glutathione-S-transferase (GST) and the \textit{Drosophila} SYN1 protein</td>
<td>Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA; developed by Erich Buchner, Würzburg University, Würzburg, Germany), mouse; monoclonal; 3C11</td>
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<td>Synapse-associated protein of 47 kDa (SAP47)</td>
<td>Homogenized \textit{Drosophila} heads</td>
<td>Erich Buchner, Würzburg University, Würzburg, Germany</td>
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**Immunostaining for identification of glomeruli and brain structures**

After analysis of the iontophoretically stained neuron by confocal laser scanning microscopy, the brain was rehydrated through a decreasing ethanol series (10 minutes each) and rinsed in PBS. To minimize nonspecific staining, the brain was submerged in 5% normal goat serum (NGS; Sigma, St. Louis, MO) in PBS containing 0.5% Triton X-100 (PBSX; 0.1 M, pH 7.4) for 3 hours at room temperature. The preparation was then incubated for 2 days at 4°C in the primary antibodies, anti-SYNORF1 and nc46 (dilutions 1:10 and 1:40 in PBSX containing 5% NGS, respectively; Table 1). After rinsing in PBS 6 × 20 minutes at room temperature, the brain was incubated with Cy2-conjugated anti-mouse secondary antibody (Invitrogen, Eugene, OR; dilution 1:500 in PBSX) for 2 days at 4°C. Finally, we rinsed, dehydrated, cleared, and mounted the brain in methylsalicylate.

**Confocal image acquisition**

We created serial optical images by using a confocal laser scanning microscope (LSM 510, META Zeiss, Jena, Germany) with a 10× (C-Achromat 10×/0.45 W), 20× (Plan-Neofluar 20×/0.5), and 40× objective (C-Achroplan 40×/0.8W). The intracellular staining, obtained from the fluorescence of rhodamine/Cy3 (Ex max 550 nm, Em max 570 nm), was excited by the 543-nm line of a HeNe laser and the immunostaining, obtained from the Cy2 (Ex max 490 nm, Em max 508 nm), was excited by the 488-nm line of an Argon laser. The distance between each section was 8 µm for the 10× objective and 2 µm for the 20× and 40× objectives. The pinhole size was 1 and the resolution 1,024 × 1,024 pixels. Optical sections from the confocal stacks were reconstructed by means of the LSM 510 projection tool.

**Digital 3D reconstructions**

The centrifugal neuron and its surrounding brain structures were manually reconstructed in subsequent confocal slices by means of the visualization software AMIRA 4.1 (Visage Imaging, Fürth, Germany); the neuron was reconstructed by using the skeleton module of the software, and the brain structures by using the segmentation editor. Thus, the neuron was traced so that a surface model built by cylinders of particular lengths and thicknesses was created, whereas each brain structure was outlined based on its gray value according to the background so that a polygonal surface model could be created. The neuronal structures and the neuropil regions were reconstructed from the same confocal image stacks.

**Data analyses and image processing**

The electrophysiological recordings were stored and analyzed by using the Spike2 software. We counted the numbers of spikes within intervals of 100 ms during a total period of 5 seconds. This period started 1 second before the onset of the odor/air stimulus, close to the onset of the first auditory pulse, and ended 2.5 seconds after the onset of the last auditory pulse. Based on the spike frequencies, we made histograms to visualize the neuronal activity during the sequence of sound, air velocity, and odor/air puff stimuli for each recording. The images were adjusted in Photoshop CS5 (Adobe Systems, San Jose, CA) by means of the auto-contrast tool before the final figures were edited in Adobe Illustrator CS5. The orientation of all brain structures is indicated relative to the body axis of the insect, as in Homberg et al. (1988).

**RESULTS**

In males of both species we identified a unique protocerebral neuron with extensive processes in the antennal lobe, which was comparable not only morphologically but also physiologically, one in an *H. virescens* male (Fig. 2) and one in an *H. armigera* male (Fig. 3). For simplicity, these neurons are named the vir neuron and the arm neuron, respectively.

**Morphology of the centrifugal antennal-lobe neurons**

The morphologies of the two neurons, characterized by processes in the medial protocerebrum of one hemisphere and extensive innervations in the ipsilateral antennal lobe, showed a high degree of similarity. The soma, which had a diameter of 20 µm, was located dorsally of the central body and close to the brain midline (Figs. 2, 3). From the soma, the primary neurite projected anteriorly along the medial margin of the brain and bifurcated adjacent to an area where the mushroom body lobes merge. One thin branch projected further anteriorly along the medial edge and entered into the antennal lobe. Here it gave off relatively solid branches with blebby terminals apparently in all glomeruli (Figs. 2, 3). The fiber projected outside the antennocerebral tracts. The other branch, being considerably thicker, ran laterally for a short distance and then turned posteriorly along the medial border of the mushroom body lobes, where numerous fine arborizations extended in a restricted region of the medial protocerebrum (Figs. 2B, 3C). This area was located anterior to the median calyx, dorsolateral to the central body, dorso medial to the pedunculus, ventromedial to the x-lobe (vertical lobe), and posterior to the β-lobe (medial lobe). One small distinction between the neurons in the two preparations was observed; whereas the arm neuron...
targeted the mushroom body lobes via a few protocerebral branches (Fig. 3D), the vir neuron did not innervate this structure (Fig. 2C). Whereas the centrifugal neuron was the only neuron stained in *H. virescens*, an additional antennal-lobe local interneuron was stained in the *H. armigera* preparation. Figure 3B thus includes branches of this neuron. Its soma, however, is not visible in the current confocal stack.

Mass staining experiments (*n* = 5), including application of dye into one or both antennal lobes of *H. virescens*, showed (in four of the five preparations) a cluster of two or three adjacently located cell bodies positioned close to the brain midline in each hemisphere, dorsal to the central body (Fig. 4). This site precisely corresponds with the location of labeled cell bodies in the two iontophoretically stained preparations. The soma visualized via mass stainings had diameters identical to those labeled by iontophoresis, i.e., 20 μm. The characteristic branching pattern in the dorsomedial protocerebrum obtained by mass staining of the antennal lobe also corresponds well with that of the neuron type labeled by the single cell technique (Figs. 2–4).

The stained centrifugal neuron in the *H. virescens* preparation (the vir neuron) permitted detailed analysis of the glomerular branching pattern. Eight sub-branches were given off from the main axon in the antennal lobe, each targeting a group of adjacently located glomeruli. This is illustrated in Figure 5, in which reconstructed glomeruli are given distinct colors according to the sub-branch they are connected to. The number of glomeruli targeted by each branch varied in a range from 2 to 19. The projection patterns within the glomeruli differed slightly, some having more dense innervations than others (Fig. 5E,F). Whereas most glomeruli were targeted by one sub-branch only, some received terminals from two (Fig. 5G). One of the sub-branches targeted the macroglomerular complex (MGC) and some of the ordinary glomeruli located adjacent (Fig. 5A,B). By adding up the numbers of glomeruli innervated by each sub-branch, we counted 71 units. This number includes four posterior glomeruli that were not incorporated in the previous anatomical study by Berg et al. (2002). Taking into account the fact that a few units also received projections from different sub-branches, the number of innervated glomeruli seems to correspond...
Physiological properties of the centrifugal antennal-lobe neurons

The two neurons also showed notable similarities regarding their physiological properties. Both displayed spikes with amplitudes of approximately 40 mV and spike durations of ~4 ms. Furthermore, their spontaneous activities were similar at the beginning of the recordings, showing a frequency of approximately 20 Hz (Figs. 6, 7). The vir neuron, tested for stimuli several more times than the arm neuron, showed a decreased spontaneous activity during the time course of recording, however (Fig. 6). Both neurons displayed excitatory responses to distinct non-odorant stimuli produced by the valve system (Figs. 6, 7). Also, the characteristic transient time courses of these responses were similar. The non-odor activations of the two neurons occurred at different time points of the stimulation sequence, however. The vir neuron responded consistently during the last event (Fig. 6), which involved an acoustic pulse and a slow increase in air velocity (Fig. 1). The arm neuron, on the other hand, repeatedly showed an increased firing rate during the first event (Fig. 7), which involved an acoustic pulse and a decrease in air velocity (Fig. 1). Odor stimuli were delivered with long pauses, so adaptation during the testing series seems unlikely, but the repetition rate of non-odorant stimuli within each sequence may explain the clear response only during the first event in the arm neuron. As shown in Figure 7, the response in the arm neuron occurred before the change in air flow. It is therefore reasonable to assume that the excitation was induced by the sound pulse specifically. In contrast, the stimulus giving rise to the non-odorant response in the vir neuron is not obvious; as shown in Figure 6, both the sound pulse and the air flow change occurred before the onset of the response.

The vir neuron also showed a strong excitatory response at the onset of odor/air puff stimulation, which overlapped in time with the second acoustic pulse (Fig. 6). The different strengths of the responses elicited...
during pheromone, plant odor, and pure air puff stimulation, and also the relatively longer response durations compared with those occurring at the last event, at least during the initial stimulation sequences, indicate the presence of odor responses from the current neuron. The restricted repetitions of each test stimulus and the change in spontaneous spiking activity make statistical calculations difficult, but the mean responses of 79.74 spikes per second to the pheromone mixture (n = 2, SD = 7.05), 43.61 spikes per second to the plant odor (n = 2, SD = 8.81), and 35.72 spikes per second to the air puff (n = 3, SD = 12.29) suggest the presence of specific odor responses as well as an additional and somewhat weaker air puff response. The arm neuron showed no increase in spike firing rate at the time of the air/odor puff (Fig. 7). The durations of the excitatory responses seemed to depend on the modality. On the whole, the responses elicited during the second event, which included application of odor/air puff stimuli, had longer durations, i.e., up to 200 ms, compared with those elicited by the non-odor stimuli, which lasted about 50–100 ms. The response delays varied considerably; in the vir neuron the delay of the odor/air puff responses was measured to 130 ms whereas the subsequent response, occurring at the fourth event, started approximately 90 ms after the onset of the air flow change and 270 ms after the sound pulse. In the arm neuron, the response started 100 ms after the sound pulse. The sequence of recordings as listed in Figures 6 and 7 corresponds to the order of stimuli applied during the experiments.

The recording from the arm neuron, which was stained together with a local interneuron, probably originated from the centrifugal neuron and not from the local one; in addition to comparable response properties, the duration and amplitude of the spikes were similar in the two recordings, as mentioned above, and therefore indicate that they originated from the same neuron category.

**DISCUSSION**

The main result of our study is the identification of a central neuron type in the moth brain, which responded regularly to distinct non-odorant airborne stimuli comprising transient broad band sound pulses and air velocity changes, as well as in some cases to odors. Its morphology, characterized by extensive ramifications in the protocerebrum and the antennal lobe, plus a cell body located close to the protocerebral branches, suggests a function in modulating odor information. Furthermore, we found that the soma of the neuron is, in each brain hemisphere, located within a small cell cluster including one or two additional somata of antennal-lobe neurons.

**Morphological properties**

The morphology of the two neurons, which is characterized by fine branches in the protocerebrum and considerably thicker and partly blebby structures in the antennal lobe, plus a cell body located in the protocerebrum, indicates the presence of a particular type of centrifugal antennal-lobe neuron. This kind of neuron has not been found previously in any moth species or other insects, although there might be one exception. A particular centrifugal neuron type formerly identified in the honeybee is to some extent morphologically comparable to the category presented here (Iwama and Shibuya, 1998; Kirschner et al., 2006). This type, which was called the feedback neuron 1 (ALF-1) by Kirschner and colleagues, also connects parts of one protocerebral hemisphere to the ipsilateral antennal lobe. Furthermore, the smooth neuronal branches in the protocerebrum and terminals with blebble structures in the antennal lobe match our findings in the heliothine moths. Differences, however, include the distinct target regions of the protocerebral processes and their branching pattern, which is considerably less extensive in the honeybee than in the moth. The position of the honeybee’s cell body adjacent to the α-lobe (vertical lobe) of the mushroom bodies also differs from that of the moth. Another distinction is that the ALF-1 is reported to

**Figure 4.** Labeling of somata and neural branches in the protocerebrum (PC) obtained by mass staining of the antennal lobes (AL) of a *H. virescens* male. The labeled processes occupying the dorsomedial protocerebrum (PC; arrows) are in each hemisphere connected to two somata located close to the brain midline (arrowheads in the magnified image). Ca, calyces; P, posterior; A, anterior, Scale bar = 100 μm; 50 μm in inset.
Figure 5. Glomerular innervation pattern of the centrifugal neuron in *H. virescens* (digital model of the *vir* neuron made by the AMIRA software). A: Frontal view of the eight neuronal sub-branches extending from the main axon in the antennal lobe, each of which is given a distinct color. B,C: Frontal and posterior view, respectively, of the glomerular clusters targeted by the eight sub-branches. Each cluster is given a color corresponding to that of the innervating sub-branch. D: Neuronal tree showing the branching patterns of the eight axonal extensions and, in addition, one example of glomerular innervation from each category. E-G: Three examples of glomerular innervation patterns, two showing the typical pattern, i.e., terminals from one sub-branch only, one with relatively dense innervations (E) and the other with scarce innervations (F) whereas the third example shows the unusual innervation pattern, i.e., terminals originating from two different sub-branches (G). Scale bar = 50 μm in A–C.
Figure 6. Physiological characteristics of the neuron found in the *H. virescens* male, i.e., the so-called vir neuron. A: The initial spontaneous activity of the neuron (~20 Hz) decreased considerably during the sequence of recording. The spiking activity demonstrates excitatory responses at the last event that included a sound pulse plus an air velocity increase and at the second event implying the presence of an odor in addition to the non-odorant stimuli. An odor-specific response is indicated by the higher spike frequency rate appearing during pheromone and plant odor application compared with that displayed when the pure air puff was delivered. The occurrences of acoustic signals are, at all four events, indicated by a closed arrowhead below the final recording, whereas the estimated start of air flow changes is shown at the first and last event by an open arrowhead. The onset/offset of the odor/air puff stimulus is indicated by a horizontal bar below each recording. Vertical bar, 10 mV; horizontal bar, 400 ms. B: Histograms made by counting the number of spikes every 100 ms. Significant responses are indicated by stars. Horizontal bar, 400 ms; vertical bar, 30 Hz.

be unique in each hemisphere whereas the centrifugal type of the moth seems to be one in a particular subcategory. It should be mentioned, however, that in spite of the finding of a small cell cluster in the heliohine moths, the antennal-lobe neurons to which they connect may have different morphological and physiological properties.
Physiological properties

The non-odor stimulations of the preparation was an unplanned side effect of the activated valve system, and was thus not studied as systematically as we would have liked. However, the results show that both neurons responded consistently to non-odor cues occurring at particular events during each stimulation sequence. These air-borne signals included two subsequent constituents, sound pulses followed by air velocity changes, both produced by the activated valves. Because the sound pulses contained ultrasound frequencies at a level that is above the threshold for the thoracic tympanal organ of the moth, as demonstrated in Figure 1, there is no doubt that the insect was exposed to audible sound.

From the data obtained, it is reasonable to assume that the arm neuron was activated by the sound pulse. The lack of synchrony between the change in air flow and the response in the arm neuron renders the possibility of an alternative mechanical response very unlikely (Fig. 7). The response from the vir neuron, in contrast, might have been a result of either stimulus, i.e., the sound pulse or the air flow change (Fig. 6). Mechanical responses to air puffs have previously been recorded from the moth brain, usually from the main categories of antennal-lobe neurons (Kanzaki and Shibuya, 1986; Han et al., 2005; Zhao and Berg, 2010), but in one case also from a centrifugal antennal-lobe neuron (Hill et al., 2005). Taking the response pattern of both neurons into account, it seems relatively unlikely that the two individuals of the unique neuron type presented here, showing such an extent of morphological similarity, were activated by totally different sensory channels.

Interestingly, we have repeatedly measured responses to the sound pulses produced by the solenoid valves from a population of morphologically identified ventral-cord neurons in various species of heliothine moths, *H. virescens* and *H. armigera* included. Figure 8 demonstrates one typical example from an *H. virescens* male—a ventral-cord neuron that responded not only in synchrony with all four events linked to the activated valves, but also when it was exposed to a well-known ultrasound source, namely, a bunch of jingling keys. The neuron had innervations in the ventrolateral protocerebrum and an axon projecting in the ventral cord (the location of the soma outside the brain indicates the presence of an ascending neuron). Unfortunately, we did not test the keys on the centrifugal neurons presented here. However, the characteristic transient responses during non-odor stimulation as shown in both Figures 6 and 7 are quite comparable to the responses of the ventral-cord neuron (Fig. 8).

Taking these data together, we can conclude that both centrifugal neurons were activated by non-odorant air-borne stimuli produced by the valve system. The sound response as displayed by the arm neuron, combined with the previous findings of sound responses from ventral-cord neurons in the heliothine species, which were
Figure 8. Response properties of a ventral-cord neuron innervating the brain. A: Four excitatory and pronounced transient responses occurring in synchrony with the four sound pulses produced by the valves can be seen. As shown, the neuron also responded strongly to a well-known ultrasound source, i.e., a bunch of jingling keys. Horizontal bar, 400 ms; vertical bar, 10 mV. B: Confocal reconstruction of the whole brain in a dorsal orientation showing the neuronal branches innervating the ventrolateral protocerebrum (PC) close to the border of the antennal lobe (arrow; AL). C: Higher magnitude image in a frontal view showing the neuronal processes in the ventral part of the lateral protocerebrum (arrow). D,E: AMIRA reconstructions of the neuronal branches in the brain and the subesophageal ganglion (SOG) in a dorsal (D) and sagittal (E) orientation. The preparation was cut below the SOG and the axonal connection to the ventral cord is demonstrated in the sagittal reconstruction (E). The location of the cell body outside the brain indicates the presence of an ascending ventral-cord neuron. CB, central body; Ca, calyces; OL, optic lobe; P, posterior; A, anterior; D, dorsal; M, medial; Scale bar = 100 μm in B–E.
induced by the identical valve system, suggests the general significance of acoustic stimuli related to this centrifugal neuron type. Based on the stimulus conditions for the non-odor response of the vir neuron, we cannot exclude the possibility that air flow changes may also influence the activity of the current neuron. However, no matter whether the vir neuron was excited by sound or by mechano-stimuli from air flow changes, the responses to different stimulus modalities, as shown in Figure 6, demonstrate for the first time a multimodal reaction from an identified antennal-lobe centrifugal neuron in a moth brain.

The fact that the two neurons responded to different events in the stimulus sequence is difficult to interpret, owing to the small dataset. As already mentioned, we find it rather unlikely that two morphologically similar centrifugal neurons would respond to completely dissimilar sensory modalities. However, we found two to three closely located somata, and the different physiological response patterns of the neurons can thus be due to the possibility that they are of distinct subtypes. Alternatively, the difference may reflect the fact that the two individuals possessing this neuron type belong to allopatric species living on different continents. The behavioral context in which the auditory sense has evolved has strongly determined the design and properties of the underlying neural pathways in various insect species (reviewed by Stumper and Helversen 2001). Some moths use ultrasound for intraspecific acoustic communication in courtship, for instance (Hoy and Robert, 1996; Miller and Surlykke, 2001), which is a newer evolutionary adaptation than that ensuring a defense mechanism against a predator. Regarding heliothine moths, it is not known whether *H. virescens* males produce sounds, but the sympatric species *Helicoverpa zea*, does (Kay 1969)—whereas *H. armigera* is silent (Nakano et al., 2009). Thus, the different responses may have to do with species-specific properties concerning threshold curves, adaption rates, or other physiological distinctions characterizing these centrifugal neurons. However, it might also be that the general response pattern depends on previous neuronal activity, because the spontaneous firing rate in the vir neuron changed dramatically during application of the various stimuli.

Functions of the centrifugal antennal-lobe neurons

The discovery of a centrifugal neuron type that responds to non-odorant air-borne stimuli and targets the brain olfactory center in two allopatric species of heliothines suggests its general significance for this subfamily of noctuid moths. The finding of distinct responses that are likely induced by acoustic stimuli is indeed interesting, particularly when taking into account the number of studies reporting that moths adjust their behavior according to sensory input through the two main modalities, odor and sound, in order to find a mate and to avoid echolocating bats (Baker and Cardé, 1977; Acharya and McNeil, 1998; Surlykke et al., 1999; Skals et al., 2003b, 2005; Svensson et al., 2007; Anton et al., 2011). Recently, Anton et al. (2011) reported data that may point to central neuronal mechanisms responsible for this kind of cross-modal integration. Their results showed, in addition to behavioral changes, long-term sensitization to olfactory stimuli of antennal-lobe projection neurons in the noctuid moth, *Spodoptera littoralis*, based on pre-exposure to pulsed ultrasonic bat calls.

This finding suggests that the sensitization depends on modulatory protocerebral neurons. Both olfactory subsystems, the plant odor and the pheromone system, were influenced by bat sounds in the study of Anton et al. (2011). Also, a previous study by Skals et al. (2003a), reporting the influence of ultrasound from an odor sprayer on the flight behavior of moths, points to a central integration of information about odor and sound. Even though the sound stimuli in our study were a side effect of the odor stimulus setup, their frequency range overlaps with the frequencies to which moth auditory organs are most sensitive. The simple noctuid moth ear contains two acoustic sensory neurons specialized for sensing echolocating bats, both responding to frequencies between 10 and 60 kHz (Roeder, 1966; Surlykke et al., 1999). The threshold for the most sensitive auditory sensory cell, A1, is around 30–35 dB SPL in this frequency range in most noctuid moths, with the other sensory cell, A2, being around 20 dB less sensitive (Surlykke and Miller, 1982, Surlykke et al., 1999). Thus, all four clicks from the valves very likely exceeded the threshold for both sensory cells in the ears of the moths.

It is also known from several moth species—*H. virescens* included—that both sensory neurons are connected to the brain, one directly and indirectly via interneurons, and the other via interneurons only (Surlykke and Miller, 1982; Boyan and Fullard, 1986; Boyan et al., 1990). The brain regions targeted by the afferent auditory projections are not yet mapped, however. The fact that the type of neuron we have identified here responds to non-odor air-borne stimuli, including sounds that are biologically relevant for the moth ear in terms of sound pressure as well as frequencies, in combination with its extensive projections into all glomeruli of the brain olfactory center, makes it a possible candidate for providing integration between the auditory and the olfactory system. Interestingly, an early synaptic level of the mammalian olfactory pathway, namely, the olfactory tubercle, has recently
been reported to receive auditory cross-modal information (Wesson and Wilson, 2010).

Various insect species with particular adaptations to distinct ecological niches have often served as suitable models for exploration of general neuronal principles. Their nervous system, comprising such unique and identifiable elements as the centrifugal neurons, offers the opportunity of revealing not only the functional circuits underlying innate response patterns but also more complex networks linked to learning and behavioral adaptations. The finding of the antennal-lobe centrifugal neuron presented here may be a key to understanding the neuronal basis for moth behavior in response to multimodal sensory stimulation. In order to determine in detail the distinct physiological properties of the neuron, an experimental approach similar to that employed here should be utilized, dedicated, however, to specifically disentangling the responses to different modalities.

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CONFLICT OF INTEREST STATEMENT

All authors confirm that there is no identified conflict of interest including any financial, personal, or other relationships with other people or organizations within three years of beginning the submitted work that can inappropriately influence, or be perceived to influence, their work.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. In addition, all authors have contributed significantly to the elaboration of the paper. Study concept and design: B.G. Berg and X.C. Zhao. Acquisition of data: X.C. Zhao and G. Pfuhl; Analysis and interpretation of data: X.C. Zhao, G. Pfuhl, A. Surlykke, J. Tro, and B.G. Berg; Drafting of the manuscript: B.G. Berg and X.C. Zhao; Critical revision of the manuscript for important intellectual content: B.G. Berg, A. Surlykke, and G. Pfuhl; Statistical analysis: X.C. Zhao; Obtained funding: B.G. Berg, A. Surlykke, and G. Pfuhl; Administrative, technical, and material support: B.G. Berg, A. Surlykke, and J. Tro; Study supervision: B.G. Berg.

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