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Deep-tissue confocal imaging of the central projections of ovipositor sensory afferents in the Egyptian cotton leafworm, *Spodoptera littoralis*

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ABSTRACT

The pre-ovipositon behavior of moths is largely dependent upon the cues that a gravid female perceives while assessing potential oviposition sites. Assessment of such sites is accomplished, at least in part, by mechanosensory and gustatory sensilla located on the ovipositor whose sensory neurons project into the terminal abdominal ganglion (TAG). Using anterograde backfill staining, confocal laser scanning microscopy, and three dimensional reconstruction, we traced and analyzed the central projections of the sensory neurons housed in the sensilla located on the ovipositor papillae and explored the neuropilar composition of the TAG in the Egyptian cotton leafworm, *Spodoptera littoralis*. The TAG consists of three fused neuromeres (6–8th Ner) associated with the 6–8th abdominal segments. Within the TAG, and specifically in the 8th neuromere, four unstructured neuropilar compartments are present; the dorso-ipsilateral motor neuropil (MN), the medio-ipsilateral mechanosensory neuropil (MchN), the medio-ipsilateral small gustatory neuropil (GN), and the medio-contralateral posterior ovipositor glomerulus (Og). The Og appears quite compact, with a hollow core free of terminal arborizations. The MchN is further subdivided into 4 unstructured glomeruli in the 8th neuromere, whose afferents are subsequently extended into 3 glomeruli in the 7th and 6th neuromeres. Few neurites of the Og are populated with large dense varicosities reminiscent of neurosecretory vesicles. Given that all ovipositor nerves converge into a common ganglionic center, the TAG, we assume that this ganglion may be a center for coordination of oviposition behaviors, including movements of the ovipositor during assessment of oviposition substrates and egg laying in *S. littoralis*.

1. Introduction

Insect ovipositors function primarily as a tool for laying eggs onto suitable ovipositon substrates. This task, however, requires coordination of multiple sensory inputs (Ahmed et al., 2013). Sensory hairs, sensilla, located on this organ contain mechano- and chemo-sensory neurons responsible for receiving inputs from the oviposition site (Anderson and Hallberg, 1990; Tousson and Huster, 2000; Banga et al., 2003). Depending on context, stimulation of the ovipositor sensory receptors could trigger different motor patterns, such as the assessment of oviposition substrates before and during oviposition, egg deposition, geometrical arrangement of eggs within the egg patch, and sweeping behavior to cover the egg patch with the scales (Banga et al., 2003; Kumashiro and Sakai, 2001; Wallace et al., 2004; Baba et al., 2010).

In *Spodoptera littoralis*, the ovipositor is composed of two papillae densely packed with a vast number of mechanosensory and few gustatory sensilla (Seada et al., 2015). The mechanosensory sensilla are classified into three types (I–III) based on differences in distribution, length and morphology. The gustatory sensilla are scattered between type III mechanosenory sensilla at the distal surface of the ovipositor papillae (Seada et al., 2015). Mechanosensory hairs are generally innervated by a single mechanosensory neuron, whereas gustatory sensilla are innervated by multiple neurons, containing

Abbreviations: AC, anterior connective; AG, abdominal ganglion; aMN, anterior motor neuropil; CI–III, commissures I–III; Cont. L, contralateral; GN, gustatory neuropil; Ipsi. L, ipsilateral; Mchg, mechanosensory glomerulus; Mchmg, mechanosensory microglomeruli; MchN, mechanosensory neuropil; Mchn, mechanosensory neuritis; MN, motor neuropil; Mn, motor neurons; mON, motor ovipositor nerve; Ner, neuromere; Nsv, neurosecretory varicosities; Og, ovipositor glomerulus; ON, ovipositor nerve; OP, ovipositor papilla; TAG, terminal abdominal ganglion; TAGN, terminal abdominal ganglion neuropil; sON, sensory ovipositor nerve.

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one mechanosensory and several chemosensory neurons (Newland et al., 2000).

The central nervous system of insects is composed of a supraoesophageal ganglion (brain), suboesophageal ganglion, and thoracic and abdominal ganglia (see Gullan and Cranston, 2005). While detailed information on the lepidopteran brain and its linkages is already available (Kvello et al., 2009; El Jundi et al., 2009), almost nothing is known about the central projections of the ovipositor sensory input and the neuroanatomical features of the abdominal ganglia of female *S. littoralis*, including the terminal abdominal ganglion (TAG), which is closely associated with the ovipositor sensory components and sex pheromone glands. Central projections of the sensory axons of the ovipositor sensilla occur within the TAG, where they synapse with local and ascending giant interneurons to swiftly transmit information to command units located in the superior ganglia (Palka et al., 1977; Gnatzy and Tautz, 1980; Edwards and Williams, 1981; Boyan and Ball, 1989; Tousson and Huster, 2000; Insausti et al., 2008). Therefore, a necessary prerequisite for the study of neural integration mechanisms underlying ovipositon behaviour is the comprehension of how the physical and chemical characteristics of the oviposition sites are perceived, coded, and assessed by the TAG.

The Egyptian cotton leafworm, *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) is a serious herbivorous pest of cultivated plants including cotton and many other economically important crops. The host plant acceptance (or avoidance) behavior is mediated by a combination of mainly tactile, olfactory, and gustatory stimuli that a gravid female perceives (Renwick and Chew, 1994; Thöming et al., 2013). The perception of these stimuli is accomplished mainly by the mechanosensory and gustatory sensilla located on the insect ovipositor (Calatayud et al., 2006), whose sensory neurons project into the TAG.

Although the organization of sensory afferents and local interneurons into the TAG, the first center for the integration of the ovipositor mechanosensory and chemosensory signals, are available in detail for other insect species, e.g crickets *Acheta, Gryllus*, *Gryllodes*, the cockroach *Periplaneta*, and locusts (Edwards and Palka, 1974; Boyan et al., 1989; Matsura and Kanou, 1998; Tousson and Huster, 2000; Kanou et al., 2006), a detailed description of the organization of the central neuropil of the TAG in *S. littoralis* has not yet been published. Hence, the current study strives to present the canonical central projections of the ovipositor sensory inputs and to describe the organization and nomenclature of the major unstructured neuropils of the TAG in the female *S. littoralis*.

2. Materials and methods

2.1. Insects

Female *S. littoralis* used in the experiments originated from a laboratory culture with wild-caught moths from cotton fields in the delta region of Egypt. Larvae were reared on fresh castor bean leaves, *Ricinus communis* L. (Malpighiales: Euphorbiaceae). All developmental stages of the moths were kept at 25 °C, 70% R.H. and LD 16:8 h. Pupae were collected, sexed, and then kept separately until they emerged and were used for experiments.

3. Histological procedures

3.1. Anterograde neurobiotin backfills

In order to investigate the projection patterns of the sensory neurons and motor output neurons of the ovipositor in the TAG, staining using anterograde-neurobiotin backfills were performed as follows:

A virgin female 1–2 days old was restrained in a holder made of a 1 ml disposable plastic pipette tip, with its ovipositor papillae protruding from the tip of the pipette. To prevent movement of the abdomen of the moth, the exposed terminal abdominal segment was covered with wax except for the ovipositor. The mounted insect was then fixed onto a Petri dish; the ovipositor was secured onto an elevated microscope slide with a piece of double-sided sticky tape and kept in a Petri dish with a humid paper tissue. One of protruding ovipositor papillae was then cut near the apical surface with a pair of fine scissors and capped with a glass microcapillary filled with distilled water for 5 min, which aided the next staining step. After removal of the capillary of water the ovipositor was dried with fine tissue paper and then a glass microelectrode filled with 2% neurobiotin (Molecular Probes, Carlsbad, CA, USA) in 0.25% M KCl, was placed over the cut ovipositor papilla (Fig. S1). Insects were kept overnight at 4 °C, then decapitated and the tergal cuticle of the abdomen was dissected to expose the abdominal ventral nerve cord, and subsequently fixed in 4% formaldehyde in 0.01 M PBS for 5 h at room temperature. The abdominal nerve cord was then dissected out in 0.01 M PBS, washed 4 × 10 min in 0.01 M PBS, and incubated overnight in 0.01 M PBS with 4% Triton-X. The preparation was then washed 3 × 10 min in 0.01 M PBS with 1% Triton-X and incubated for 2 days in Alexa Fluorescein Avidin 488, Alexa 546 Phalloidin (Molecular Probes, Carlsbad, CA, USA) and 0.01PBS with 1% Triton-X (5:3:200) at 4 °C. The samples were then rinsed 4 × 10 min in 0.1 M PBS and finally mounted in a mounting medium (Vectashield Hard Mount, Vector Laboratories, Burlingame, CA). To prevent the abdominal nerve cord from compression they were placed into spacer rings (Secure-seal TM imaging spacers, Sigma–Aldrich), 0.12 mm thick.

3.2. Confocal scanning microscopy

The preparations were examined using an Olympus FluoView 500 confocal laser-scanning microscope, equipped with Ar 488 nm and 514 nm, and HeNe 544 nm and 633 nm lasers. Stacks of optical sections were analyzed using ImageJ software. The ganglia were scanned with 0.9 μm optical sections for detailed imaging. Stacks of 50–200 confocal images were scanned and the images were stored at a resolution of 1024 × 1024 pixels. Confocal image stacks of at least 20 female moths were analyzed. Only seven preparations were randomly chosen for further illustrations.

3.3. Three-dimensional reconstruction

Confocal image stacks were loaded into the software AVIZO® for Windows (v.8.0.0, FEI Visualization Sciences Group, Hillsboro, OR, USA), which was used to visualize, demarcate, and analyze the 3D images of the parts of the interest. To segment different parts of the ganglia, anatomically relevant areas were assigned to specific materials. Segmentation was performed automatically using thresholds, and then the materials were manually revised by selecting individual voxels to be added or subtracted from the material. A three-dimensional model of the ganglia and their associated inputs was obtained by “surface generator” option.

4. Results

4.1. General description of the ovipositor

The external structure of *S. littoralis* ovipositor system is composed of 2 ovipositor papillae, each with 6–8 gustatory sensilla (Seada et al., 2015) and covered with a dense field of mechanosensory sensilla which differ in length, thickness, and distribution. The first destination of the ovipositor sensory projections is the TAG of