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Neuropeptides associated with the frontal ganglion of larval Lepidoptera

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Abstract

The occurrence of neuropeptides in the frontal ganglia of larvae of the tobacco hawkmoth, *Manduca sexta*, the tomato moth, *Lacanobia oleracea* and the cotton leafworm, *Spodoptera littoralis* was investigated using reversed-phase high performance liquid chromatography (RP-HPLC), matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) and enzyme-linked immunosorbent assay (ELISA). Only three types of peptides could be identified or assigned from frontal ganglion extracts; *M. sexta* allatostatin (Manse-AS), *M. sexta* allatotropin (Manse-AT), and F/YXFGL-NH_2 allatostatins. The peptide profiles of frontal ganglion of *L. oleracea* and *S. littoralis* were similar, with ten identical $[M+H]^+$ ions, seven of which could be assigned to known lepidopteran peptides (Manse-AT, cydiastatin 2, 3, 4 and helicostatin 1, 5, 9). In addition, mass ions corresponding to helicostatin 7 (which was confirmed by MALDI-post source decay analysis) and Manse-AS were present in frontal ganglia of *L. oleracea* and helicostatin 6 in frontal ganglia of *S. littoralis*. Only four mass ions from *M. sexta* frontal ganglia corresponded to known peptides, cydiastatin 3 and 4, helicostatin 1, and Manse-AT. The only difference between the profiles of frontal ganglia from different stages of *L. oleracea* were mass ions which could not be assigned, and no differences were observed in the allatoregulatory peptides present. In HPLC fractions of *M. sexta* frontal ganglia, F/YXFGL-NH_2 allatostatin-like immunoreactivity was widespread suggesting that more allatostatins were present than were identified.

Keywords: Peptidomics; Insect; Lepidoptera; Allatostatin; Allatotropin

1. Introduction

The stomatogastric nervous system that innervates the gut has been reported to be involved in a variety of functions including feeding, crop emptying, and defecation [34]. Central to this system is the frontal ganglion, which is situated on the anterior wall of the esophagus and is linked to the brain by a pair of frontal connectives. This ganglion innervates parts of the stomodaeum, anteriorly via the frontal nerve, and posteriorly through the recurrent nerve.

In larvae of the tobacco hawkmoth, *Manduca sexta*, the frontal ganglion has been implicated in the regulation of feeding. Using a combination of electrophysiological recordings and severance of the recurrent nerve, Miles and Booker [33] showed that foregut contractions were controlled by the frontal ganglion. In adult *Periplaneta americana* [41], nymphal and adult *Schistocerca gregaria* [26], and in adult *Heliothis zea* [7], removal of the frontal ganglion results in the accumulation of food in the foregut. A similar effect was observed by cutting the recurrent nerve in larval *M. sexta* [38].

Using immunocytochemical techniques, the allatoregulatory peptides *M. sexta* allatostatin (Manse-AS), *M. sexta* allatotropin (Manse-AT) and allatostatins of the Y/FXFGL-NH_2 family have been localized in large neurosecretory cells of the frontal ganglia of various lepidopteran larvae [16,18,19,21]. These peptides are also present in the recurrent nerve, and branches of this nerve supplying the muscles of the crop and the region of the stomodeal valve [19,21]. *Manduca sexta* allatotropin has also been detected in the frontal ganglia of larval *M. sexta* using both immunohistochemistry and in situ hybridization [6]. This tissue localization implies that these peptides are myoactive on the foregut. Spontaneous contractions of the foregut of larval *Cydia pomonella*, *Helicoverpa armigera* and *L. oleracea* are inhibited by Y/FXFGL-NH_2 allatostatins [16,18,19]. *Manduca sexta*-AS also inhibits foregut peristalsis in larval *L. oleracea* [19]; whereas, Manse-

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AT has been shown to have a stimulatory effect on the foregut of larval *H. armigera* and *L. oleracea* [18,19].

Other peptides have also been detected in the frontal ganglion and/or stomatogastric nervous system of lepidopterans using immunochemical techniques. Zitnan et al. [47] reported FMRF-NH_2-like immunoreactivity in the ganglia (including frontal ganglion) and nerves of the stomatogastric system of the waxmoth, *Galleria mellonella*, and Golubeva et al. [24] reported pheromone biosynthesis activating neuropeptide (PBAN)-like immunoreactivity in the esophageal nerve and anterior midgut of the Gypsy moth, *Lymantria dispar*. Immunoreactivity to adipokinetic hormone and eclosion hormone have been detected in the recurrent nerve of pharate adult *M. sexta* [27] and pheromonotropic melanizing peptide (PMP)-like immunoreactivity has been detected in the frontal ganglion and esophageal nerve of larval *Helicoverpa zea* [37].

In Lepidoptera, there are around fifty characterized neuropeptides with molecular masses of less than 2500 Da. Of these, 16 are F/YXFGL-NH_2 allatostatins and approximately, 20 others are peptides that are also myoactive on the gut, including myokinins, myotropins, and myoinhibitory peptides [23]. Some of these peptides may be active on the foregut and may also be present in the frontal ganglion.

The aim of this study was to profile and compare the neuropeptides in the frontal ganglion of three lepidopteran species, *L. oleracea*, *M. sexta*, and *Spodoptera littoralis* using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS).

2. Materials and methods

2.1. Experimental animals

Lacanobia oleracea and *Spodoptera littoralis* were reared at 20 °C and 65% relative humidity, under a 16-h light: 8-h dark photoperiod as described by Corbitt et al. [8]. Larvae were fed on a maize-flour based noctuid artificial diet (Bio-Serv, Frenchtown, NJ, USA). *Manduca sexta* were reared from eggs supplied by Prof. S. Reynolds, University of Bath, using methods described by Yamamoto [45].

2.2. Tissue extraction and liquid chromatography

For single tissue extraction, frontal ganglia were dissected from last day fourth instar larval *M. sexta* or last day fifth instar larval *L. oleracea* and *S. littoralis* (same physiological age) and placed into Eppendorf tubes containing 10 μl of 0.1% (v/v) trifluoroacetic acid (TFA). For separation by reversed-phase high performance liquid chromatography (RP-HPLC), 170 frontal ganglia from fifth stadium larval *M. sexta* or 230 frontal ganglia from sixth stadium larval *L. oleracea* were dissected into 500 μl of 0.1% TFA. The tubes were agitated at room temperature for 15 min, and then centrifuged at 10,000 × *g* for 5 min. For single tissue extracts 0.5 μl of supernatant was applied directly to the MALDI sample plate (as described below); whereas, the supernatant for HPLC separation was diluted with 0.1% TFA and loaded, via a Rheodyne loop injector, onto a Jupiter C_{18} 10 μm 300 Å analytical column (250 mm × 2.1 mm i.d.; Phenomenex, Macclesfield, UK) fitted with a guard column (30 mm × 2.1 mm i.d.) of similar packing material. The column was eluted with a linear gradient of 5–60% acetonitrile/0.1% TFA, over 55 min at a flow rate of 0.2 ml/min, and elution monitored at 214 nm, using a Beckman 32 Karat chromatographic system (Beckman Coulter Ltd., UK), comprising a dual pump programmable solvent module 126 and a UV detector module 166. Fractions (1 min, 0.2 ml) were collected and concentrated to *c*.10 μl by centrifugal evaporation for mass analysis.

The elution positions of Manse-AS, Manse-AT, and cydiastatin 4 were determined using the same HPLC conditions.

2.3. Mass analysis

Mass spectra were acquired on a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK). The matrix, α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) was prepared at a concentration of 10 mg/ml in 50% acetonitrile/0.05% (v/v) TFA. Samples (0.5 μl) were added to the MALDI sample plate followed by 0.5 μl of matrix, and dried at room temperature. Standards (bradykinin, angiotensin, somatostatin, and adrenocorticotropic hormone; Sigma-Aldrich, UK) were added adjacent to samples. Spectra represent the resolved monoisotopic $[M+H]^+$ masses in reflector mode within the mass range *m/z*, 500–5000. Spectra are the accumulation of 5 × 50 shots. The measured monoisotopic masses $[M+H]^+$ were compared to the calculated monoisotopic masses $[M+H]^+$ of known peptides. Monoisotopic masses were calculated using protein prospector (University of California, San Francisco). Analyses by MALDI-PSD were performed on the same instrument and samples, using angiotensin as the standard. A PSD spectrum was produced from 7–8 spectral segments and stitched together using the Voyager software.

2.4. ELISA

Indirect ELISAs for Manse-AS, Manse-AT, and cydiastatin 5 were used to measure immunoreactivity in HPLC fractions, using methods reported by Audsley et al. [1]. Briefly, HPLC fractions and synthetic peptides were dried onto multiwell plates (Sigma-Aldrich, UK; Cat. No. M4034) at 37 °C then incubated overnight at 4 °C with 100 μl of 0.1 M bicarbonate (coating) buffer (pH 9.6). Plates were washed three times with 150 μl of 10 mmol/l phosphate buffer/0.1% TWEEN-20 (PBS), blocking solution (150 μl; 2% non-fat milk in PBS) was added, and the plates incubated for 90 min at 37 °C. After a further PBS wash, 100 μl of primary antiserum (dilutions: 1:3000, Manse-AS; 1:5000, Manse-AT; 1:5000, cydiastatin 5) were added to each well and the plates incubated for another 90 min at 37 °C. One hundred micro-