Neuropeptide co-localisation in the lepidopteran frontal ganglion studied by confocal laser scanning microscopy

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Abstract A comparative study of the co-localisation of three different families of neuropeptides, viz. allatostatins of the Y/FXFGL-NH₂ type, Manduca sexta allatostatin (Mas-AS) and allatotropin, in the frontal ganglion of lepidopteran larvae has been carried out by means of immunocytochemistry and confocal laser scanning microscopy. The simultaneous application of three types of fluorochrome-conjugated antibodies reveals triple co-localisation in an anterodorsal pair of neurones in the frontal ganglion of the noctuids Heliothis virescens and Lacanobia oleracea. There is no evidence of differential axonal transport, since all parts of these neurones show complete co-localisation of all three peptides. Prominent axons of the ganglionic neurones project in the recurrent nerve to the foregut and stomodeal valve. Over the crop, lateral and sub-lateral branches follow the course of circular muscle fibres and terminate in varicosities. All three neuropeptides have previously been shown to be myoregulatory on the foregut; the Y/FXFGL-NH₂ allatostatins and Mas-AS are inhibitory, whereas allatotropin is excitatory. The morphological evidence of co-localisation of physiologically antagonistic peptides within the same terminals suggests that an extremely complex mechanism controls the contractile activities of the foregut. A posterodorsal pair of neurones in the frontal ganglion have prominent axons projecting via the frontal connectives to the brain and in the recurrent nerve to the stomodeal valve where extensive branching suggests control over the valve movements. Studies of another noctuid, Spodoptera frugiperda, and the sphingid, M. sexta, show interesting variations in the co-localisation phenomenon.

Keywords Immunocytochemistry · Co-localisation of neuropeptides · Allatostatins · Allatotropin · Confocal laser scanning microscopy · Frontal ganglion · Lepidopteran larvae · Heliothis virescens, Lacanobia oleracea, Spodoptera frugiperda, Manduca sexta (Insecta)

Introduction

The frontal ganglion plays a role in the feeding of lepidopteran larvae by regulating the contractile activity of the foregut. Evidence for this has come from electrophysiological studies of 5th instar larvae of the tobacco hornworm moth Manduca sexta (Miles and Booker 1994). It has been estimated that the ganglion contains about 35 neurones and, by means of cobalt backfills and/or simultaneous intracellular recordings, several motor neurones involved in movements of the foregut have been identified. These neurones (20–40 µm) have axonal projections to one, or several, of the nerve tracts associated with the frontal ganglion; the recurrent nerve posteriorly, frontal nerve anteriorly, and frontal connectives laterally. Other studies in which either the recurrent nerve has been cut (Timmins and Reynolds 1992) or the frontal ganglion removed (Bushman and Nelson 1990) have suggested a role for the frontal ganglion in crop emptying.

Despite its significance in key larval physiological activities, the frontal ganglion has received relatively little attention from an anatomical viewpoint. Early studies revealed two azan-positive cells in the frontal ganglion of the silkworm Bombyx mori (Bounhiol et al. 1953) and, in M. sexta, two neurosecretory cells were detected with paraldehyde fuchsin (Bell et al. 1974). With the advent of immunocytochemistry, it has been possible to identify some of the neuropeptides produced there and to correlate their presence with specific physiological activities.

Immunocytochemical studies of the frontal ganglion of the tortricid, Cydia pomonella, and the noctuids, Helicoverpa armigera and Lacanobia oleracea (Duve et al.
Approximately 30 larvae of each species were examined. The co-localisation of neuropeptides with contrasting changes in feeding that occur during larval development. Our primary objective has been to investigate the extent of co-localisation in the axons, varicosities and terminal arborisations of the neurones of the frontal ganglion. Are the aforementioned three types of peptides co-transported to their targets or are they routed differentially within the complex pathways traced by these neurones? A subsidiary question concerns the possible differences in peptidergic content of the neurones during the marked changes in feeding that occur during larval development. The co-localisation of neuropeptides with contrasting functions presents a challenge to physiologists analysing the peptidergic control of gut movements and this comparative morphological study is aimed at broadening our understanding of these complex events.

**Materials and methods**

**Insects and tissue preparation**

A variety of lepidopterans (L. olereacea, Heliothis virescens and M. sexta) were supplied by Dr. N. Audsley (Central Science Laboratory, Sand Hutton, York, UK). Specimens of Spodoptera frugiperda were a gift from Dr. R. H. Osborne (Department of Biological and Biomedical Sciences, University of the West of England, Bristol, UK). Larvae of two main types were used: (1) the last day first 1–3 days of the final instar (non-feeding, crop empty) and (2) the first 1–3 days of the final instar in their active feeding stage (crop full). They were dissected in phosphate-buffered saline (PBS; 139 mM NaCl, 10 mM sodium phosphate); tissues for whole-mount immunocytochemistry were fixed for 2 h in 4% paraformaldehyde in PBS. The brain was removed together with the frontal ganglion in several different species of lepidopterans. Our primary objective has been to investigate the extent of co-localisation in the axons, varicosities and terminal arborisations of the neurones of the frontal ganglion. Are the aforementioned three types of peptides co-transported to their targets or are they routed differentially within the complex pathways traced by these neurones? A subsidiary question concerns the possible differences in peptidergic content of the neurones during the marked changes in feeding that occur during larval development. The co-localisation of neuropeptides with contrasting functions presents a challenge to physiologists analysing the peptidergic control of gut movements and this comparative morphological study is aimed at broadening our understanding of these complex events.

**Antisera and immunocytochemistry**

An antiserum (code: R1–7) raised against ARGYDFGL-NH₂ (cydastatin 5 from the tortricid moth, C. pomonella), a representative of the Y/FXFGL-NH₂ superfamily of allatostatins, has been characterised in previous studies (Duve et al. 1997a, 1997b, 1999, 2000). The evidence is that this antiserum is specific for the conserved C-terminus of the allatostatin superfamily. Two further series of antisera against allatotropin, GFKNVEMMTARGF-NH₂, and a slightly modified form of M. sexta allatostatin, pEVFRQCYFPISCF-OH (Mas-AS), in which Gly replaced the N-terminal cysteine residue added and was then conjugated by using m-maleimidobenzoic acid N-hydroxysuccinimide ester, while Mas-AS was conjugated by using glutaraldehyde. All syntheses and conjugations of the peptides (including the subsequent fluorochrome conjugations) were carried out by Affiniti Research Products, Exeter, UK. For each peptide, polyclonal antisera were raised in Dutch rabbits. After collection of pre-immune sera, three rabbits per peptide-carrier protein-conjugate were immunised, first with antigen emulsified in Freund’s complete adjuvant and, subsequently, at intervals of 2 weeks, with antigen emulsified in Freund’s incomplete adjuvant. Aliquots of the conjugates (1 ml from a stock solution of 400 μg/ml in sterile physiological saline) were diluted by the addition of 1 ml saline and 2 ml Freund’s adjuvant. After emulsification, 1 ml immunogen (100 μg conjugate) was injected subcutaneously at four sites on the hindquarters of the rabbits. Bleeding from the marginal ear vein was carried out 1 week after each immunisation and tested by immunocytochemistry. Each of the three antisera for both Mas-AS and allatotropin, used in combination with a swine antirabbit fluorescein isothiocyanate (FITC)-conjugated secondary antiserum (code: F020520; DAKO, Glostrup, Denmark) revealed, in whole-mounts, the same populations of cells recorded in previous studies with different antisera against the same two peptides (Duve et al. 2000; Audsley et al. 2000). Specificities of the new antiserum were tested by liquid phase preabsorption with the parent antigen at concentrations of 10 nmol/ml diluted antiserum (1:1000) overnight at 4°C. Immunostaining was abolished following this treatment. In contrast, cross-absorption experiments in all combinations (e.g. Mas-AS preabsorbed with allatotropin) had no effect on the immunostaining. There was no immunostaining when pre-immune serum replaced the corresponding primary antiserum or when the primary antiserum was omitted.

The immunoglobulin fraction of a 4-ml sample of the ARGYDFGL-NH₂ antiserum (code: R1–7) was conjugated to tetramethyl-rhodamine isothiocyanate (TRITC) and a 4-ml sample of the newly produced allatotropin antiserum (code: AT 8.5–09LS) was conjugated to FITC. The pattern of neuronal labelling observed with these fluorochrome-tagged antisera was identical to that produced when the corresponding unlabelled primary antiserum were used in combination with a secondary goat anti-rabbit FITC-or AMCA (7-amino-4-methyl coumarin-3-acetic acid)-labelled antiserum. The third peptide in the co-localisation series (Mas-AS) was detected by using the newly-produced antiserum (code: MA 7.5–03LS in combination with a goat anti-rabbit AMCA-labelled antiserum (code: W047810; DAKO).

**Immunocytochemical protocol, microscopical observation and imaging**

Throughout the immunocytochemical procedure, tissues from the same individual were kept together in single Eppendorf tubes held on a rotator (22°C). After fixation, the tissues were rinsed in PBS (2×20 min) and permeabilised, first by placing them in 70% methanol (20 min), followed by absolute methanol (1 h) and finally 70% methanol (20 min). They were washed in PBS (3×20 min), followed by PBT (PBS + 0.2% BSA and 0.1% Triton X-100; 2×30 min). Tissues were incubated in the Mas-AS antiserum (1:1000) overnight, washed in PBT (3×20 min) and incubated in normal goat serum (X090710, DAKO; 1:10 in PBT) for 30 min.