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LOCALIZATION AND RELEASE OF FMRFamide-LIKE IMMUNOREACTIVITY IN THE CEREBRAL NEUROENDOCRINE SYSTEM OF *MANDUCA SEXTA*

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SUMMARY

The distribution of FMRFamide-like immunoreactivity (FLI) in the cerebral neuroendocrine system of the moth, *Manduca sexta*, is described and evidence is provided for calcium-dependent release of FLI from the neurohaemal organs.

1. FLI was detected by indirect immunofluorescence in approximately 25 bilaterally symmetrical pairs of somata in the pupal protocerebrum. In addition, FLI was observed in neurites in the brain, as well as in axons of the nervi corporis cardiaci and nervi corporis allati, and in terminals in the neurohaemal corpora cardiaca (CC) and corpora allata (CA). All immunocytochemical staining was blocked by preabsorption of the anti-FMRFamide antiserum with synthetic FMRFamide.

2. We localized FLI to identified protocerebral neurosecretory cells (NSCs) by combining intracellular injection of Lucifer Yellow and indirect immunofluorescence. Among the NSCs in each hemisphere, FLI was observed in both group IIa (lateral) cells, in most group IIb (lateral) cells, and in two cells of group Ib (medial).

3. FLI was extracted from the brain and neurohaemal organs and measured using radioimmunoassay (RIA).

4. Calcium-dependent release of FLI was evoked from isolated CC–CA by high potassium depolarization *in vitro* and was quantified by RIA of the bathing medium.

These results suggest that FLI may have a neurohormonal or neurotransmitter function in *Manduca*.

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INTRODUCTION

Neuropeptides secreted by insect cerebral neurosecretory cells (NSCs) regulate a wide range of processes including physiology, development and behaviour. In a few cases, the source NSCs for these insect neurohormones have been identified (Agui, Granger, Gilbert & Bollenbacher, 1979; Copenhaver & Truman, 1985). Recently, insect NSCs and neurohaemal organs have been shown to be immunoreactive to antisera directed against vertebrate and invertebrate neuropeptides (reviewed by Kramer, 1985). Thus, the insect homologues of these neuropeptides may serve a neurohormonal function. A possible neuroendocrine role in insects for the tetrapeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) (Price & Greenberg, 1977*a*,*b*) or analogues thereof was suggested by immunocytochemical staining of neurohaemal corpora cardiaca (Boer, Schot, Veenstra & Reichelt, 1980; Veenstra & Schooneveld, 1984). In addition, NSCs in the suboesophageal ganglion of the Colorado potato beetle contain FMRFamide-like immunoreactivity (FLI) (Veenstra, 1984; Veenstra & Schooneveld, 1984). The current study was undertaken to further clarify the role of FLI in insect neuroendocrine systems.

The protocerebral neuroendocrine system of the moth, *Manduca sexta*, has been the subject of anatomical, biochemical and physiological studies. This neuroendocrine system comprises two bilaterally symmetrical clusters of identified NSCs, one in the pars intercerebralis and the other in the pars lateralis (Fig. 1). The axons of the NSCs leave each brain hemisphere *via* the fused nervi corporis cardiaci I and II (NCC I+II) and terminate in either of the two cerebral neurohaemal organs, the corpus cardiacum (CC) or the corpus allatum (CA) (Nijhout, 1975; Carrow, Calabrese & Williams, 1984). The cells in each cluster have been classified according to the size and location of their somata as well as the architecture of their neurites, axons and terminal ramifications in the neurohaemal organs (Nijhout, 1975; Buys & Gibbs, 1981; Carrow *et al.* 1984; Copenhaver & Truman, 1984).

We now describe the distribution of FLI in the pupal brain and cerebral neurohaemal organs of *Manduca*. FLI was further localized to identified cerebral NSCs by combining intracellular dye injection and indirect immunofluorescence. Finally, calcium-dependent release of FLI was evoked from isolated neurohaemal organs with high potassium depolarization. A preliminary report of these findings has been presented (Carroll & Carrow, 1985).

MATERIALS AND METHODS

Animals

Larvae of *Manduca sexta* were reared at 25°C on an artificial diet under a 17-h light/7-h dark photoperiod (Bell & Joachim, 1976). Tissues from pupae, 1 or 2 days after ecdysis, were used for immunocytochemical analysis because of the facility with which the brains could be desheathed. Tissues from premetamorphic larvae, 3–4 days prior to pupal ecdysis, were used for extraction and release of FLI since the lower fragility of tissues at this stage facilitated collection and manipulation.

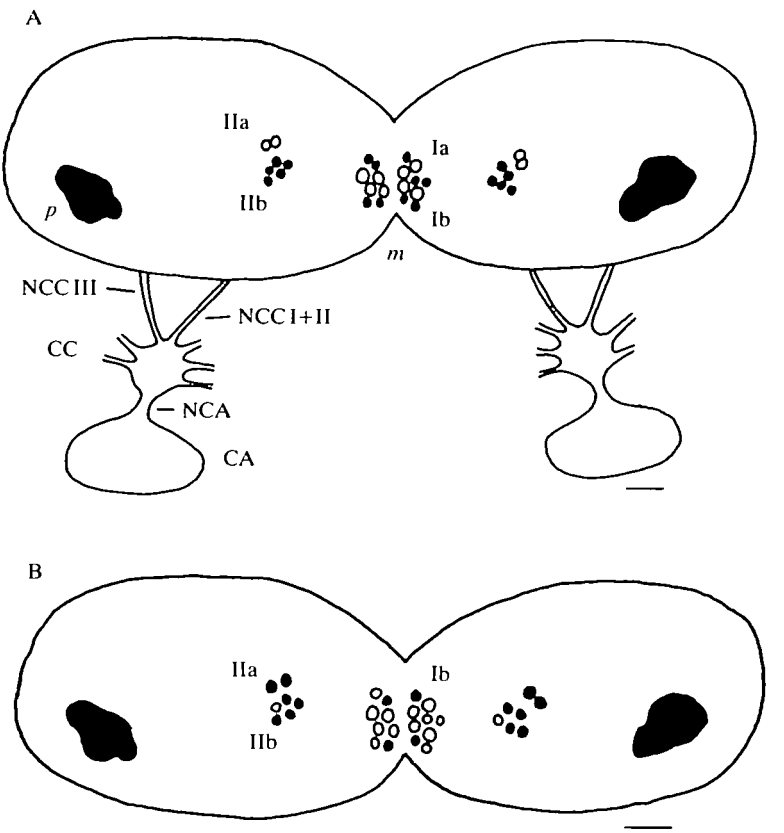

Fig. 1. (A) The cerebral neuroendocrine system of pupal *Manduca sexta*. *Camera lucida* drawing of a dorsal view of the brain–retrocerebral complex showing the location of cell bodies of protocerebral neurosecretory cells (NSCs); anterior is upwards. Four groups of NSCs are indicated: two, Ia (open) and Ib (solid), in the pars intercerebralis and two, IIa (open) and IIb (solid), in the pars lateralis. The neurohaemal corpora cardiaca (CC) and corpora allata (CA) lie posterior to the brain. (B) NSCs showing FMRFamide-like immunoreactivity (FLI). Same diagram of protocerebrum as in A except that FLI-positive (solid) and FLI-negative (open) NSCs are indicated. *m*, midline of the brain; NCA, nervus corporis allati; NCC, nervus corporis cardiaci; *p*, pigment granules in the optic lobe. Scale bars, 100 μm. (Modified from Carrow, Calabrese & Williams, 1984.)

Experimental salines

Compositions of the experimental salines as modified from Carrow (1983) are given in Table 1. The osmolarity and pH were the same for all salines.

Dissection of tissues

For immunocytochemistry, brain–retrocerebral complexes (brain with attached CC and CA) along with attached tissue and tracheae were dissected from unanaesthetized pupae. The tissues were pinned to a Sylgard-coated Petri dish under

Table 1. *Compositions of experimental salines used in release experiments*

Experimental salines*	NaCl ($mmol\,l^{-1}$)	KCl ($mmol\,l^{-1}$)	$CaCl_2$ ($mmol\,l^{-1}$)
Normal	140	—	5
High potassium, calcium-containing	47	93	5
High potassium, calcium-free	54	93	—
Calcium-free	147	—	—

* All salines contained 5 $mmol\,l^{-1}$ PIPES (piperazinediethanesulphonic acid), 7 $mmol\,l^{-1}$ KOH, 1 $mmol\,l^{-1}$ $MgCl_2$, 5 $mmol\,l^{-1}$ trehalose, 5 $mmol\,l^{-1}$ glucose, 10 $mmol\,l^{-1}$ sucrose, pH 6·7.

normal saline. Brains were then desheathed (Carrow *et al.* 1984) to facilitate the entry of antisera.

Organs intended for extraction or release of FLI were dissected from unanaesthetized larvae immersed in normal saline.

Immunocytochemistry

Immunocytochemistry was performed on whole-mount preparations (Li & Calabrese, 1985). Tissues were fixed at 10°C for 18–24 h in 4 % paraformaldehyde in 120 $mmol\,l^{-1}$ sodium phosphate buffer, pH 7·4 or for 4 h in 1 % paraformaldehyde in 150 $mmol\,l^{-1}$ sodium cacodylate buffer and 5 $mmol\,l^{-1}$ $CaCl_2$, pH 7·4. The rabbit polyclonal antiserum used was raised against synthetic FMRFamide conjugated to succinylated bovine thyroglobulin (no. 232, O'Donohue *et al.* 1984) and was a gift from Thomas O'Donohue. This primary antiserum was used at a dilution of between 1:300 and 1:500. Binding of the anti-FMRFamide antiserum was visualized with rhodamine conjugated to goat anti-rabbit IgG (Cappel) at a final dilution of 1:100.

A check for specificity of immunolabelling was made by incubating tissues with anti-FMRFamide antiserum that had been preabsorbed for 2–4 h with 100 $\mu g\,ml^{-1}$ of synthetic FMRFamide (Peninsula Laboratories).

Double labelling

To identify immunoreactive NSCs, intracellular injection of the dye Lucifer Yellow was combined with indirect immunofluorescence (Reaves & Hayward, 1979). NSC somata can be observed under a dissecting microscope due to their reflective opalescence. Thus, NSCs were identified on the basis of soma size and position and were iontophoretically injected with Lucifer Yellow CH (Sigma) using hyperpolarizing current (3–5 nA) for 10–30 s (Carrow *et al.* 1984). Brains with injected cells were subsequently incubated in saline at 10°C for 1–4 h. Finally, tissues were fixed and processed for immunocytochemistry. Labelled cells were visualized with a fluorescence photomicroscope (Zeiss or Leitz).

Tissue extracts

Brains or neurohaemal organs were collected from premetamorphic larvae and washed three times in calcium-free saline; the number of organs in each sample

varied in accordance with the organ type (see Table 2). The organs were homogenized in 100 μl of an acidified methanol solution (90 % methanol, 9 % acetic acid, 1 % water). Homogenates were sonicated (20 kHz) and then centrifuged (15 000 ***g***) for 1 min. The supernatant was withdrawn and the pellet was resuspended in 100 μl of acidified methanol and centrifuged again for 1 min. This step was repeated. The supernatants were pooled, dried in a Savant Speed Vac concentrator, and stored at −20°C until assayed.

Release of FLI

For each experiment, approximately 30 isolated retrocerebral complexes from premetamorphic larvae were washed three times in calcium-free saline. The organs were transferred to a Linbro culture well and incubated for 1–2 h at 25 °C in 50 μl of either high potassium, calcium-free saline or normal saline. After this preincubation, tissues were transferred to another culture well and incubated in high potassium, calcium-containing saline for 1–2 h. In two experiments, the preincubation period was omitted and organs were incubated immediately in high potassium, calcium-containing saline. After incubation, tissues were discarded and the incubation saline was transferred to a microcentrifuge tube. Samples were then sonicated and extracted as described above.

Radioimmunoassay

The amount of released or extracted FLI was measured using the radioimmunoassay (RIA) protocol of Price (1982) as modified by Li (1985). The tracer used for RIA was ^{125}I-Tyr-Met-Arg-Phe-NH_2, iodinated by a modified Chloramine T method (Greenwood, Hunter & Glover, 1963). The anti-FMRFamide antiserum was the same as that used for immunocytochemistry. Each assay tube contained synthetic FMRFamide or sample diluted in 100 μl of RIA buffer (0·1 % bovine serum albumin and 0·02 % sodium azide in 0·1 mol l^{-1} sodium phosphate buffer, pH 7·5). The antiserum (final dilution between 1:50 000 and 1:100 000) and the tracer (6000 c.p.m.; specific activity 50–150 Ci $mmol^{-1}$), each diluted in 50 μl of RIA buffer, were added to the assay tubes. After vortexing, the tubes were incubated at 4°C for 18–24 h. Unbound peptides were then precipitated by the addition of 100 μl of a 2 % dextran-coated charcoal solution (5 % goat serum, 0·02 % sodium azide, in 0·1 mol l^{-1} sodium phosphate buffer, pH 7·5). Finally, the assay tubes were vortexed and centrifuged (15 000 ***g***) for 10 min. Pellet and supernatant fractions were counted in a Packard gamma counter.

The sensitivity range of the RIA was between 10 and 300 fmol of FMRFamide. The logit transformed binding curve for synthetic FMRFamide was log linear ($r \geq 0{\cdot}97$) and was used to quantify FLI. Salts did not alter the slope of the standard curve; however, they decreased the sensitivity of the RIA, resulting in possible underestimations of FLI.

RESULTS

Immunocytochemical staining

Approximately 25 bilaterally symmetrical pairs of somata in the pupal protocerebrum showed FLI (Fig. 2). A subset of these cells was located in the two NSC

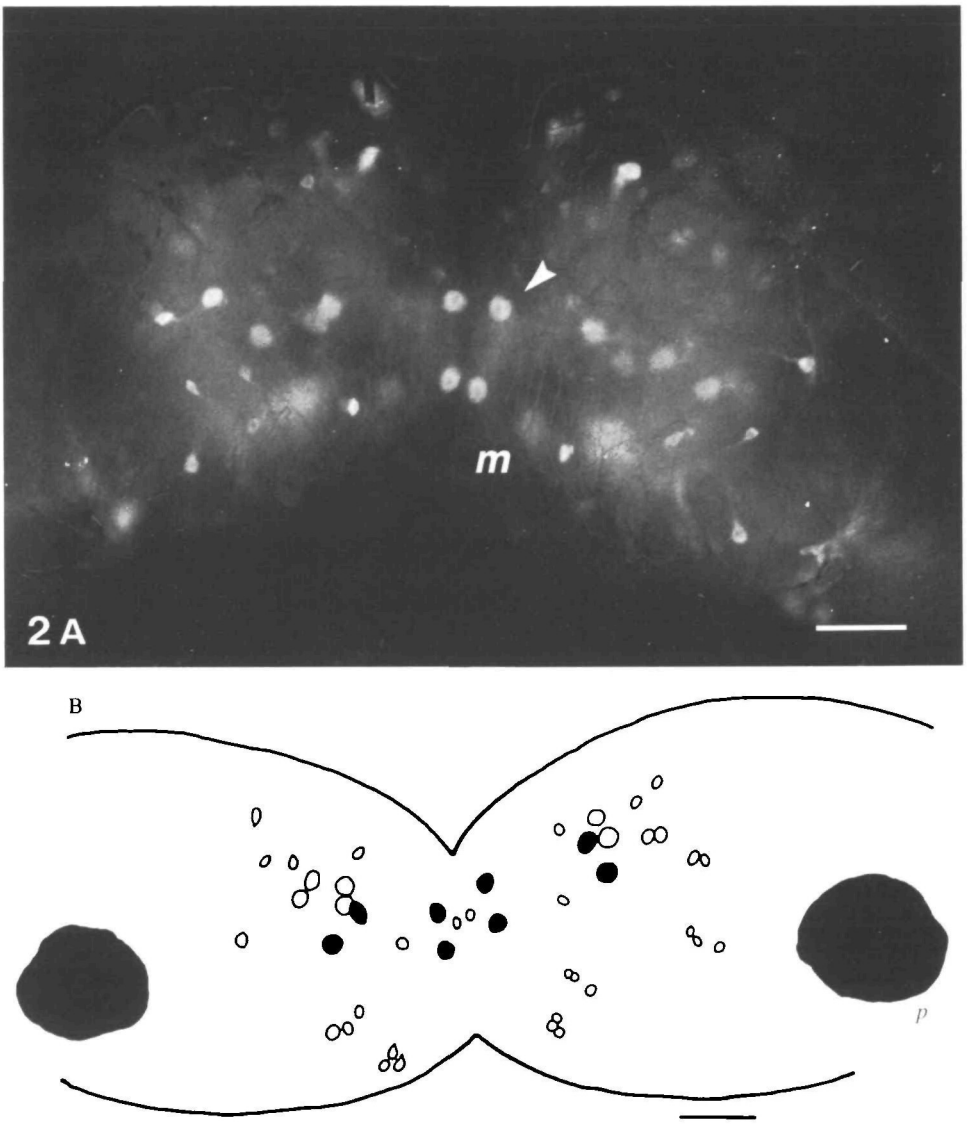

Fig. 2. The distribution of FMRFamide-like immunoreactivity in the pupal brain visualized with a rhodamine-conjugated secondary antiserum. (A) Fluorescence photomicrograph showing the two pairs of bilaterally symmetrical and intensely immunoreactive medial neurosecretory cells (NSCs) (arrowhead); the intensely immunoreactive lateral NSCs are out of the plane of focus. (B) Composite *camera lucida* drawing of the immunoreactive neurones in the protocerebrum; neurones in all planes of focus are shown in one plane; the solid cells are the intensely immunoreactive NSCs. Abbreviations and orientation as in Fig. 1. Scale bars, 100 μm.

clusters: in the pars intercerebralis, two pairs of bilaterally symmetrical cells stained intensely; in the pars lateralis, 4–7 pairs of bilaterally symmetrical cells showed FLI, and two of these pairs stained intensely (Figs 1B, 2). Immunoreactive neurones in the protocerebrum outside these two clusters are likely to be non-endocrine since they do not stain with paraldehyde fuchsin (Nijhout, 1975).

FLI was observed throughout the retrocerebral complexes as shown in Fig. 3. Immunoreactivity was visible in terminals in the CC and CA as well as in axons in the nervi corporis cardiaci and nervi corporis allati. FLI was also evident in neurites in

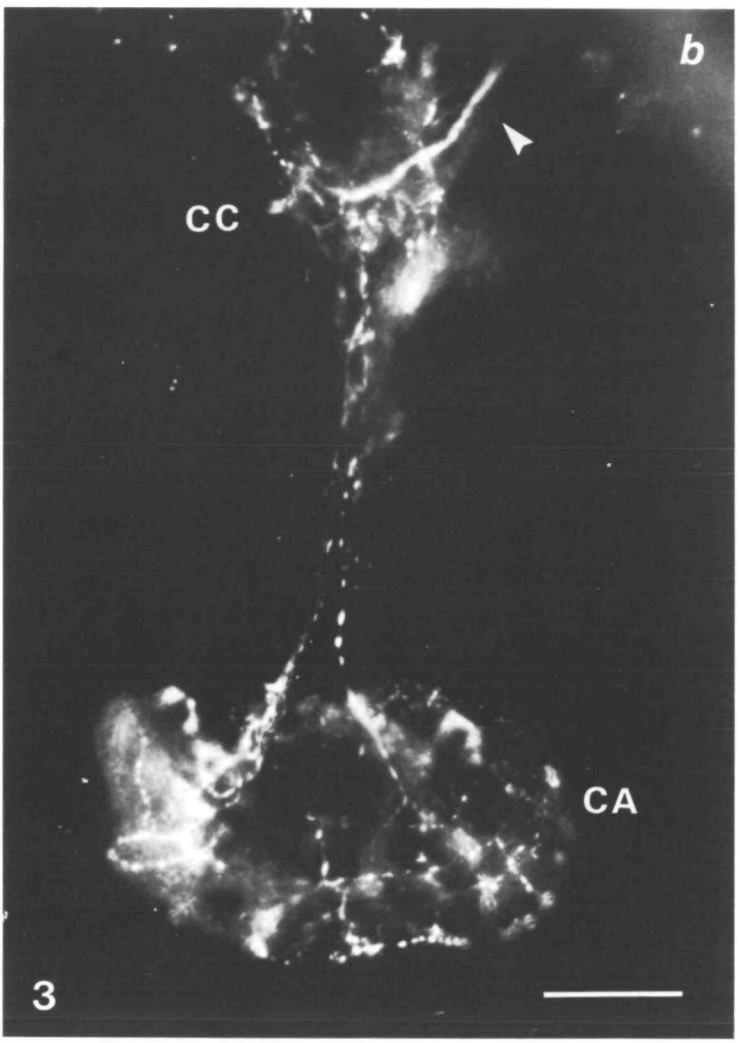

Fig. 3. The distribution of FMRFamide-like immunoreactivity in the pupal neurohaemal organs visualized as in Fig. 2. The arrowhead denotes an immunostained nervus corporis cardiaci (NCC I+II). *b*, brain; other abbreviations and orientation as in Fig. 1. Scale bar, 100 μm.

the protocerebrum and tritocerebrum (Fig. 4B,D,F). Similar immunolabelling was observed in the brain–retrocerebral complexes of premetamorphic larvae.

Preabsorption of the anti-FMRFamide antiserum with synthetic FMRFamide eliminated immunoreactive staining in the brain–retrocerebral complexes of pupae and larvae.

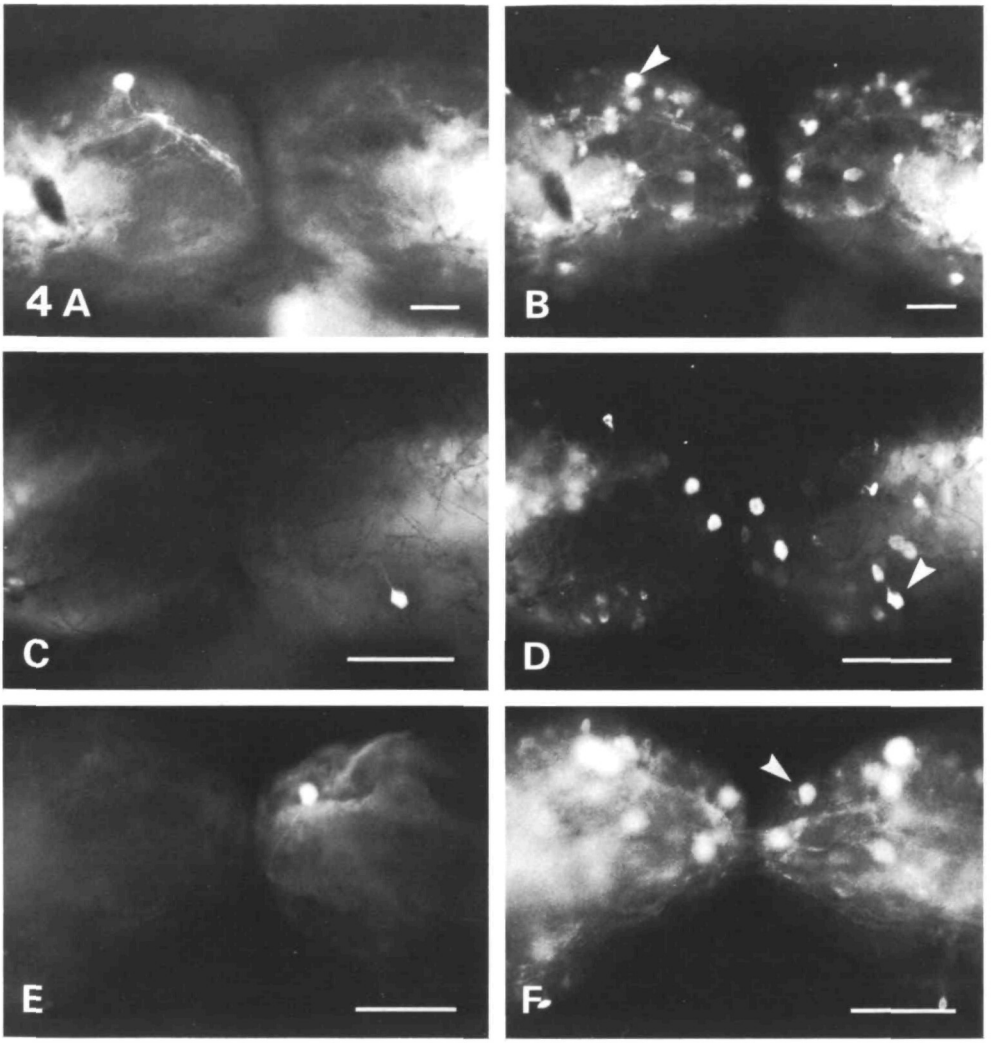

Fig. 4. Double labelling of representatives of the three groups of protocerebral neurosecretory cells (NSCs) showing FMRFamide-like immunoreactivity (FLI). NSCs injected with Lucifer Yellow are shown on the left (A,C,E), viewed with Lucifer Yellow optics. FLI in the same preparations is shown on the right (B,D,F), viewed with rhodamine optics; arrowheads denote double-labelled cells. (A,B) A group IIa cell showing identifying dendritic arborization in A; immunostained neurites are visible in B. (C, D) An intensely immunoreactive group IIb cell. (E,F) An intensely immunoreactive group Ib cell. Orientation as in Fig. 1. Scale bars, 100 μm.

Identification of immunoreactive neurones

FLI was localized to a subset of protocerebral NSCs by double labelling. In the pars lateralis, FLI was observed in groups IIa (Fig. 4A,B) and IIb (Fig. 4C,D). When both group IIa cells in one hemisphere were injected with Lucifer Yellow ($N = 4$), they both showed FLI, indicating that all cells of this group exhibit FLI. Of the 23 group IIb cells that were injected, 19 (83 %) showed FLI indicating that most, but not all, of the cells in this group are immunoreactive. Among the immunoreactive group IIb NSCs are the two intensely immunolabelled cells in the pars lateralis, one of which is shown double labelled in Fig. 4C,D. The intensely immunoreactive cells in the pars intercerebralis were group Ib cells (Fig. 4E,F). Thus, only two of the approximately five pairs of group Ib cells showed FLI. None of the group Ia cells that were injected with Lucifer Yellow ($N = 12$) exhibited FLI.

Cross-reactivity of antigens

To determine whether the RIA could be used to quantify FLI, an analysis was made of the cross-reactivity of brain extracts and synthetic FMRFamide. Tracer binding to antiserum was inhibited with serial dilutions of these antigens. The regression lines for the binding curves were parallel (the mean ratio of the slopes was $1{\cdot}03 \pm 0{\cdot}07$, $N = 3$) indicating complete cross-reactivity of the antigens with this antiserum.

FLI in tissue extracts

The amount of FLI extracted from brains and neurohaemal organs is shown in Table 2. In order to obtain detectable quantities of FLI, organs were pooled for extraction and RIA. The paired neurohaemal organs contained 23 % of the immunoreactivity extracted from the entire brain–retrocerebral complex. Within the retrocerebral complex, the CC yielded 2·5 times the amount of FLI extracted from the CA.

Release of FLI

Localization of FLI to protocerebral NSCs and the neurohaemal organs in *Manduca* suggested that the immunoreactive substances might function as neurohormones or neurotransmitters. Therefore, we attempted to demonstrate release of

Table 2. *FMRFamide-like immunoreactivity extracted from the cerebral neuroendocrine system of* Manduca sexta *larvae as determined by radioimmunoassay*

Organ	Number of samples	Organs per sample	FMRFamide equivalents (fmol organ^{-1})*	FMRFamide equivalents (fmol mg^{-1}†)*
Brain	8	1	$28{\cdot}0 \pm 3{\cdot}8$	84 ± 14
CC	7	15	$2{\cdot}5 \pm 0{\cdot}2$	59 ± 16
CA	6	20	$0{\cdot}9 \pm 0{\cdot}1$	22 ± 6
CC–CA	5	10	$4{\cdot}1 \pm 0{\cdot}2$	48 ± 5

* Mean ± S.E.M.
† Organ wet weight.
CC, corpora cardiaca; CA, corpora allata.

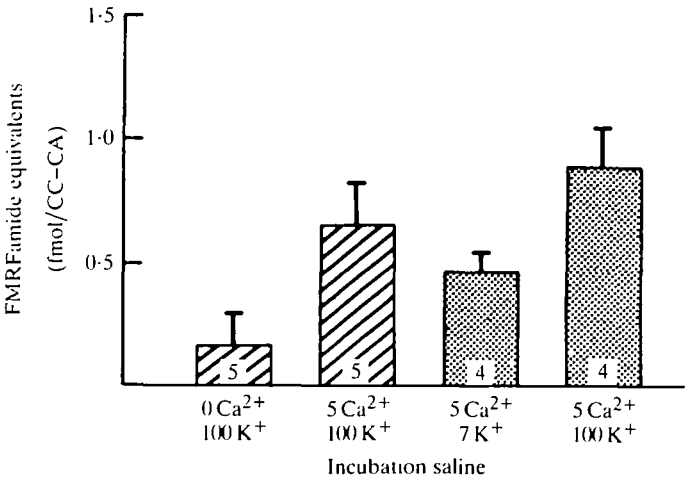

Fig. 5. Spontaneous and evoked release of FMRFamide-like immunoreactivity (FLI) from isolated retrocerebral complexes (combined corpora cardiaca, CC, and corpora allata, CA, from larvae) *in vitro* determined by radioimmunoassay. The means ± S.D. are plotted; the number in each bar denotes the number of trials. Striped bars: left bar indicates the amount of FLI released from retrocerebral complexes preincubated for 2 h in high potassium, calcium-free saline; right bar indicates the amount of FLI released from the same organs during a subsequent 2-h incubation in high potassium, calcium-containing saline. Stippled bars: left bar shows the amount of FLI released from retrocerebral complexes incubated in normal saline; right bar shows the amount of FLI released from the same organs during subsequent incubation in high potassium, calcium-containing saline. All concentrations are in mmol l^{-1}.

FLI. Release was evoked by high potassium depolarization of retrocerebral complexes *in vitro* and was measured by RIA of the bathing medium (Carrow, Calabrese & Williams, 1981).

As shown in Fig. 5, retrocerebral complexes preincubated in high potassium, calcium-free saline released little FLI. By contrast, there was more than three times as much FLI released upon transferring the same organs to high potassium, calcium-containing saline ($P < 0{\cdot}01$). Thus, an amount of FLI equivalent to about 16 % of that extracted from the retrocerebral complex was released in a calcium-dependent manner. When preincubated in normal saline, the neurohaemal organs spontaneously released a basal amount of FLI significantly greater than that released from organs preincubated in high potassium, calcium-free saline ($P < 0{\cdot}01$). Nevertheless, these same organs released twice the basal level when transferred from normal saline to high potassium, calcium-containing saline ($P < 0{\cdot}01$). Regardless of the preincubation treatment, or omission thereof, organs released a similar amount of FLI upon transfer to high potassium, calcium-containing saline ($P < 0{\cdot}01$).

DISCUSSION

FMRFamide-like immunoreactivity was localized to endocrine and non-endocrine neurones in the brain of *Manduca* as well as to terminals in the CC and CA.

Moreover, relative amounts of FLI in extracts of brain and neurohaemal organs were measured by RIA. Finally, calcium-dependent release of FLI was evoked from the retrocerebral complexes by high potassium depolarization *in vitro*.

Among the NSCs in each hemisphere, FLI was observed in both group IIa cells, most of the group IIb cells, and two cells of group Ib (see Fig. 1). Four of these NSCs showed intense immunoreactivity: the two in group Ib and two of those in group IIb. Although the location of these four cells resembled that of the FLI cells in the protocerebrum of the Colorado potato beetle (Veenstra & Schooneveld, 1984), in the latter case the neurones were not paraldehyde fuchsin positive and hence are probably not neurosecretory. The other FLI-containing NSCs in the pars lateralis stained less intensely. These included the pair of group IIa cells thought to be the principle source of prothoracicotropic hormone, a neuropeptide regulator of the prothoracic, or ecdysial, glands (Agui *et al.* 1979). Thus, the FLI detected here may be co-localized with other neuropeptides.

The FLI detected here may be distinct from peptides of the pancreatic polypeptide family. In some cases in insects, FLI cannot be distinguished from bovine pancreatic polypeptide (BPP)-like immunoreactivity; various anti-FMRFamide antisera cross-react with BPP and preabsorption of some anti-BPP antisera with FMRFamide blocks immunoreactive staining (Veenstra, 1984; Veenstra & Schooneveld, 1984; Myers & Evans, 1985*a*). However, other anti-FMRFamide antisera do not cross-react with BPP in immunocytochemistry (Myers & Evans, 1985*b*; Verhaert, Grimmelikhuijzen & De Loof, 1985). Furthermore, the anti-FMRFamide antiserum used in the present study does not significantly cross-react with BPP in RIA (O'Donohue *et al.* 1984). Finally, BPP-like immunoreactivity observed in the protocerebrum of adult *Manduca* was not located in the pars intercerebralis or pars lateralis (El-Salhy, Falkmer, Kramer & Speirs, 1983) whereas FLI has been observed in neurones in the pars intercerebralis of the adult brain (Homberg, Hoskins & Hildebrand, 1985). Consequently, FLI in *Manduca* may not be related to BPP-like substances.

Nagle (1981, 1982) previously provided evidence suggesting that FMRFamide is a neurotransmitter or neurohormone in the clam, *Macrocallista nimbosa*, by evoking calcium-dependent release of immunoreactive FMRFamide *in vitro*, extracting immunoreactivity from the haemolymph, and detecting high levels of immunoreactive FMRFamide in fractions of homogenized ganglia enriched for neurosecretory granules. Similarly, we evoked calcium-dependent release of FLI from retrocerebral complexes; the amount released was approximately 16% of the yield from extracts of complexes. For comparison, *in vitro* release of prothoracicotropic hormone is also calcium-dependent; the amount released from depolarized CA was equivalent to about 35% of the extracted bioactivity (Carrow *et al.* 1981).

The physiological role of FLI in *Manduca* remains to be investigated. FMRFamide was originally identified as a cardioexcitatory neuropeptide in molluscs (Price & Greenberg, 1977*a*,*b*). FLI could be cardioactive in *Manduca* since cardioactive peptides are found throughout the central nervous system, including the brain–retrocerebral complex (Platt & Reynolds, 1985; Tublitz & Truman, 1985).

However, the two cardioacceleratory peptides isolated from the CC of the cockroach, *Periplaneta americana*, bear no sequence homology with FMRFamide (Scarborough *et al.* 1984). A non-cardioactive function for FLI in *Manduca* is conceivable given the multifunctional nature of FLI in other species, including modulatory effects of FMRFamide on locust jump muscle (Walther, Schiebe & Voigt, 1984) and crab motor neurones (Hooper & Marder, 1984).

The data presented here show that FLI, as with other neurosecretory products, is stored in cerebral NSCs and can be released by depolarizing terminals in the neurohaemal organs. Therefore, the FLI detected here may have a neurohormonal or neuromodulatory function. In addition, the FLI might act as a neurotransmitter in the brain since neurites and non-endocrine neurones were also immunoreactive. In summary, the FMRFamide-like substances in the cerebral neuroendocrine system of *Manduca* may have multiple roles. The accessible system of identified FLI neurones described here should facilitate clarification of the functions of this class of neuropeptides in insects.

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NOTE ADDED IN PROOF

Recently, the sequence of a neuropeptide inhibitor of visceral muscle that was isolated from heads of the cockroach, *Leucophaea maderae*, was reported to be pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2 (Holman, Cook & Nachman, 1986). This is the first report of a purified neuropeptide in insects that has the C-terminal sequence recognized by the anti-FMRFamide antiserum used in the work presented here.

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