The Journal of Experimental Biology **199**, 1095–1104 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0071

CHARACTERIZATION OF TWO MYOTROPIC NEUROPEPTIDES IN THE FMRFamide FAMILY FROM SEGMENTAL GANGLIA OF THE MOTH *MANDUCA SEXTA*: CANDIDATE NEUROHORMONES AND NEUROMODULATORS

TIMOTHY G. KINGAN[1,2,*], JEFFREY SHABANOWITZ[3], DONALD F. HUNT[3] AND JANE L. WITTEN[4]

[1]*USDA ARS Insect Neurobiology and Hormone Laboratory, BARC-East Building 306, Room 322, Beltsville, MD 20705, USA,* [2]*Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD 21228, USA,* [3]*Department of Chemistry, University of Virginia, Charlottesville, VA 22901, USA* and [4]*Department of Biological Sciences, Lapham Hall, PO Box 413, University of Wisconsin-Milwaukee, Milwaukee, WI 53201, USA*

Accepted 26 January 1996

Summary

We have characterized two new members of the FMRFamide family of neuropeptides from the segmental ganglia of the tobacco hornworm *Manduca sexta*. Levels of peptides in ganglia used for purification were enhanced by manipulating their exposure to the steroid molting hormones. Explants of ganglia were cultured in the low-level ecdysteroid environment of diapausing pupae shown previously to evoke accumulation of FMRFamide-like immunoreactivity (FLI). Sufficient material for sequencing was obtained from 180 explanted ganglia. Extracts of ganglia were fractionated using two reverse-phase liquid chromatography procedures, and the immunoreactive fractions were subjected to sequence analysis using electrospray mass spectrometry. The sequences of the two peptides were determined to be GNSFLRFamide and DPSFLRFamide. These peptides have been named MasFLRFamide II and MasFLRFamide III, respectively; the previously characterized *M. sexta* FLRFamide (*p*EDVVHSFLRFamide) has been renamed MasFLRFamide I. The three peptides show distinctive tissue and developmental distributions as determined from fractionated extracts of larval and adult central nervous system structures and neurohemal organs. In the retrocerebral corpora cardiaca/corpora allata, MasFLRFamide I was the predominant form, while in the segmental ganglia MasFLRFamides II and III predominated. Higher levels of MasFLRFamide I and II were found in the adult, whereas there was little apparent change in the level of MasFLRFamide III upon metamorphosis. Determinations of peptide levels in fractionated hemolymph of newly emerged moths revealed that levels of MasFLRFamide I and III could exceed 10 nmol l^{-1}. The actions of the three peptides were tested on the moth ileum. MasFLRFamides II and III were found to be stimulatory. At 1 nmol l^{-1}, these peptides induced robust increases in the rate of rhythmic longitudinal and peristaltic waves of contractions. In contrast, MasFLRFamide I was ineffective even at 20 nmol l^{-1}. Thus, while all three peptides have the characteristics of neurohormones in *M. sexta*, the physiological findings show that the heptapeptide FLRFamides have properties distinct from those of the decapeptide.

Key words: insect, central nervous system, ileum, moth, *Manduca sexta*, FLRFamide, neuropeptide.

Introduction

Neuropeptides are perhaps the most versatile class of chemical messengers in the animal kingdom, acting as endocrines, paracrines and neuromodulators. Amongst the insects, a great number of neuropeptides have been identified (Schoofs *et al.* 1993) during the 20 years following the characterization of proctolin (Brown and Starratt, 1975). In addition to carrying out physiological studies, some investigators have also documented the release of peptides from source tissues and/or the presence of peptides in target tissues, thus corroborating their presumptive roles as neurohormones or neuromodulators. Examples include proctolin, which appears to serve a neuromodulatory function on a slow skeletal muscle in the cockroach (Adams and O'Shea, 1983). Eclosion hormone apparently triggers secretion from Verson's glands in caterpillars *via* a hormonal action (Hewes and Truman, 1991).

*Author for correspondence at address 1.

Perhaps the most widely studied of neuropeptide families is the 'FMRFamides', members of which have been identified in all phyla of animals (Walker, 1992). Amongst the insects, FMRFamides have so far been identified in the flies *Drosophila melanogaster* (Nambu *et al.* 1988; Nichols, 1992) and *Calliphora vomitoria* (Duve *et al.* 1992). *D. melanogaster* also has an N-terminally extended FLRFamide, 10 amino acids in length (Nichols, 1992), which is not encoded in the FMRFamide gene (Nambu *et al.* 1988; Schneider and Taghert, 1988). In fact, representatives of a number of orders of insects have been shown to harbor similar decapeptide 'FLRFamides' (Holman *et al.* 1986; Robb *et al.* 1989; Kingan *et al.* 1990; Fonagy *et al.* 1992; Nichols, 1992). By virtue of the similarity of their N-terminal extensions, these peptides probably share a common ancestry. This provides evidence for the existence of distinct subfamilies of RFamide-containing peptides in insects.

The FLRFamides are thought to act as neurohormones or neuromodulators. For example, the decapeptide FLRFamides have been found to have a stimulatory action on skeletal muscles, such as the locust *Schistocerca gregaria* extensor tibiae (Robb *et al.* 1989) and the moth *Manduca sexta* dorsal longitudinal flight muscles (Kingan *et al.* 1990). However, the actions of this group have been found to be inhibitory in insect visceral muscle. Spontaneous contractions of the cockroach *Leucophaea maderae* and grey fleshfly *Neobellieria bullata* hindguts (Holman *et al.* 1986; Fonagy *et al.* 1992), the locust oviduct (Lange *et al.* 1991), the locust heart (Robb *et al.* 1989) and the moth *Agrius convolvuli* midgut (Fujisawa *et al.* 1993) are all suppressed. While the decapeptide FLRFamides are myoinhibitory in visceral muscle, an extended FLRFamide and an extended FIRFamide with myostimulatory actions on locust oviduct have recently been identified (Lange *et al.* 1994).

Immunohistochemical studies on the moth *Manduca sexta* have revealed FMRFamide-like immunoreactivity (FLI) in neurons of the central nervous system (CNS) and in neurohemal organs (Homberg *et al.* 1990; Witten and Truman, 1996). The source of the majority of immunoreactive material in the brain–neurohemal axis has previously been characterized and shown to be the decapeptide *p*EDVVHSFLRFamide (Kingan *et al.* 1990). Our more recent goal has been to identify the immunoreactive peptide(s) in the segmental ganglia of the ventral nerve cord. To facilitate obtaining adequate amounts of peptide for sequencing, we made use of a recent finding that culturing ganglia in the ecdysteroid-free environment of the diapausing pupa increases FLI (Witten and Truman, 1996). A preliminary account of these findings has appeared previously (Witten *et al.* 1993).

Materials and methods

Animals and culture of ganglia

Manduca sexta (L.) (Lepidoptera: Sphingidae) were reared at 26 °C on an artificial diet (modified from Bell and Joachim, 1976) in long-day (17 h:7 h L:D) and short-day (12 h:12 h L:D) photoperiods. Under these conditions, insects reared in long-day photoperiods initiate adult development upon pupation, while those reared under short-day photoperiods enter diapause as the result of an arrest in the production of the molting hormone ecdysone (Bowen *et al.* 1984).

Chains of ganglia from the thorax (T1–T3) or from the abdomen (A3–A5) were removed from fifth-stage caterpillars on the second day after molting and implanted into the abdomens of diapausing pupae. After 2 weeks in this low-ecdysteroid environment, during which time FMRFamide-like immunoreactivity accumulates in neurons (Witten and Truman, 1996), ganglia were removed and stored frozen at −80 °C. In total, 180 of these *in vivo* cultured ganglia were obtained.

Purification of peptides

The ganglia were homogenized in methanol:water (50:50) containing 2 mmol l^{-1} HCl. Insoluble material was removed by centrifugation (10 000 ***g*** for 3 min) and the pellet was extracted with fresh solvent. The supernatants were pooled, diluted fivefold with water, and desalted on a Sep-Pac cartridge that had previously been wetted with methanol and equilibrated in 0.1 % trifluoroacetic acid (TFA). Peptides were eluted from the cartridge with 4 ml of 40 % acetonitrile in 0.1 % TFA.

Peptides were partially purified by two successive reverse-phase liquid chromatography (RPLC) protocols. These were carried out in C8 columns (Aquapore RP-300, Applied Biosystems, Foster City, CA, USA). The first step was in an analytical column (4.6 mm×25 cm) using a gradient of acetonitrile in 0.05 mol l^{-1} sodium phosphate, pH 6.0, while the second step was in a narrow-bore column (2.1 mm×25 cm) using a gradient of acetonitrile in 0.1 % TFA; details of these procedures have been described previously (Kingan *et al.* 1990). The distribution of peptides in the resulting chromatograms was determined by competitive enzyme-linked immunosorbent assay (ELISA; Kingan, 1989; Kingan *et al.* 1990) using an antiserum generously provided by Dr Eve Marder and Dr Ron Calabrese (Marder *et al.* 1987). The assay was calibrated using MasFLRFamide I (*p*EDVVHSFLRFamide), a standard solution of which was quantified by amino acid analysis. The amount of competitor peptide necessary to achieve a 15 % decrease in the maximum response, 5 fmol in 100 μl of assay volume, was defined as the detection limit.

Sequence determinations

Masses of peptides in RPLC fractions were determined by liquid secondary ion mass spectrometry (LSIMS; Hunt *et al.* 1987). Microcapillary RPLC was used to introduce samples into the instrument, a model TSQ-70 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) equipped with an electrospray (ES) source (Hunt *et al.* 1991). Sequencing of peptides was then carried out by collision-activated dissociation MS (CAD MS; Hunt *et al.* 1986). Samples of fractions were also converted to their methyl esters for determination of the presence of carboxyl groups (Hunt *et al.* 1986).

Distribution of peptides in tissues

For quantitation of specific FLRFamides, we extracted (as described above) between 54 and 73 ventral nerve cords (VNCs, abdominal and thoracic ganglia; here defined as the 'segmental' ganglia), brains + suboesophageal ganglia (Br/SOG; the 'cephalic' ganglia) and combined corpora cardiaca/corpora allata (CC/CA). In each case, tissue was collected from feeding fifth-instar larvae and from pharate adults. Extracts were desalted in Sep-Pac cartridges as described above. To fractionate these six extracts, we used an analytical (4.6 mm diameter) C4 column (Vydac, The Separations Group, Hesperia, CA, USA) with a gradient of acetonitrile in 0.1 % TFA. Fractions were collected at 1 min intervals except between 25 and 30 min, during which time fractions were collected at 30 s intervals. To each tube was added 25 μl of 1.5 mol l^{-1} NaCl and 10 μg of bovine serum albumin (BSA) in 10 μl of water. Samples were frozen at −80 °C and then dried by vacuum centrifugation. Samples were resuspended in 250 μl of 0.01 mol l^{-1} sodium phosphate, pH 7.4, containing 1 % normal goat serum and then assayed for FLI using ELISA.

Hemolymph was obtained from male moths 10 min after emergence. A puncture was made in the intersegmental cuticle of the posterior segments, and hemolymph collected from individual moths was diluted into a 20-fold excess of ice-cold 50 % ethanol. Precipitating material was removed by centrifugation (10 000 ***g*** for 5 min at 4 °C) and the pellet was extracted with fresh solvent; the supernatants were pooled and dried by vacuum centrifugation. Extracts were then fractionated without prior desalting in a narrow-bore (2.1 mm diameter) C4 column (Vydac) using a gradient of acetonitrile in 0.1 % TFA. Fractions were collected at 1 min intervals spanning the zone containing MasFLRFamides I–III. 25 μg of BSA was added to the fractions, which were then frozen and dried by vacuum centrifugation. Determination of FLI was by ELISA.

Biological activity

Peptides were synthesized commercially (Immuno-Dynamics, Inc., La Jolla, CA, USA; Medical College of Wisconsin, Protein/Nucleic Acid Shared Facility, Wauwatosa, WI, USA). Stock solutions at 10^{-3} mol l^{-1} were prepared in 50 % methanol/2 mmol l^{-1} HCl and stored at −20 °C. Peptides were diluted into a physiological saline (4 mmol l^{-1} NaCl, 40 mmol l^{-1} KCl, 3 mmol l^{-1} $CaCl_2$, 18 mmol l^{-1} $MgCl_2$, pH 6.4; see Kataoka *et al.* 1989) containing 0.5 % glucose for use in bioassays. The volume of the perfusion chamber was approximately 10 ml and the flow rate of saline through the chamber was approximately 10 ml min^{-1}.

Activity of synthetic peptides was tested on the ilea of 1- to 2-day-old male moths. A 1 cm segment of the ileum just rostral to the rectum was removed. This segment was ligated distally with human hair and proximally with fine nylon monofilament, leaving a 10 cm length for attachment to an isotonic transducer (Blackburn *et al.* 1995*a*) which was counterbalanced with a 10 mg load. The transducer was fitted with a high-pass filter for correction of drift in baseline (Giebultowicz *et al.* 1996). Output from the transducer was amplified and then recorded (Gould EasyGraf, model TA240).

Results

Peptide purification and sequencing

Approximately 90 % of the quantifiable FLI from extracts of cultured ganglia was found in two adjacent fractions after RPLC in acetonitrile/sodium phosphate (data not shown). These fractions were pooled and rechromatographed in acetonitrile/TFA. Again, the majority of the FLI was found in two adjacent fractions, at 21 and 22 min (Fig. 1). When quantified by ELISA using MasFLRFamide I as standard, these fractions were found to contain 23 pmol and 46 pmol of FLI, respectively. 10 % of each fraction was set aside for use after sequencing, and the remaining material was analyzed by mass spectrometry.

Fraction 21 was found by LSIMS to contain material of 839.6 Da. Treatment of this material to convert acids to methyl esters failed to produce the new molecular ion $[(M+H)^+]$ expected for a molecule containing a free carboxyl moiety. Fraction 22 was found to contain three $(M+H)^+$, including one of molecular mass 880.6 Da (although not an 839.6 Da species). When a sample from fraction 22 was subjected to methyl esterification, the 880.6 Da material increased in mass to 894.7 Da, an increment to be expected if a single carboxyl group were present.

When subjected to ESMS with CAD, the 839.6 and 880.6 Da molecules were initially shown to yield fragments consistent with Arg-Phe-NH_2 at their C termini. The two peptides were then found to have an identical series of y ions (C-terminal fragments) ranging from y_2 to y_5 (321, 434, 581 and 668 Da, respectively). These ions were also reported in an earlier analysis of MasFLRFamide I (Kingan *et al.* 1990) using similar procedures and establish the C-terminal pentapeptide sequence as Ser-Phe-Leu(Ile)-Arg-Phe-NH_2. The remaining sequence of the two peptides could be established by analysis of the b ions (N-terminal fragments). The 839.6 Da peptide contained b_1–b_3 ions of 59, 172 and 259 Da. Together with sequence information deduced from the y ions, the sequence of this peptide is determined to be Gly-Asn-Ser-Phe-Leu(Ile)-Arg-Phe-NH_2. Leu and Ile cannot be distinguished in this process because of their identical masses. The 880.6 Da peptide exhibited b_1–b_3 ions of masses 114, 214 and 301 Da. With the y ions from this peptide, the sequence is established as Asp-Pro-Ser-Phe-Leu(Ile)-Arg-Phe-NH_2. The N-terminal Asp is consistent with the addition of a single methyl group during esterification (see above) and the existence of the C terminus of both peptides as amides.

To determine the identity of the fifth amino acid, both the Leu- and Ile-containing peptides were synthesized. The portion of the isolated pooled material from fractions 21 and 22 (see above) that was set aside prior to sequencing was spiked with 20 pmol each of the Leu-containing peptides. A chromatogram of the Leu-containing peptides is shown in Fig. 2A; note the

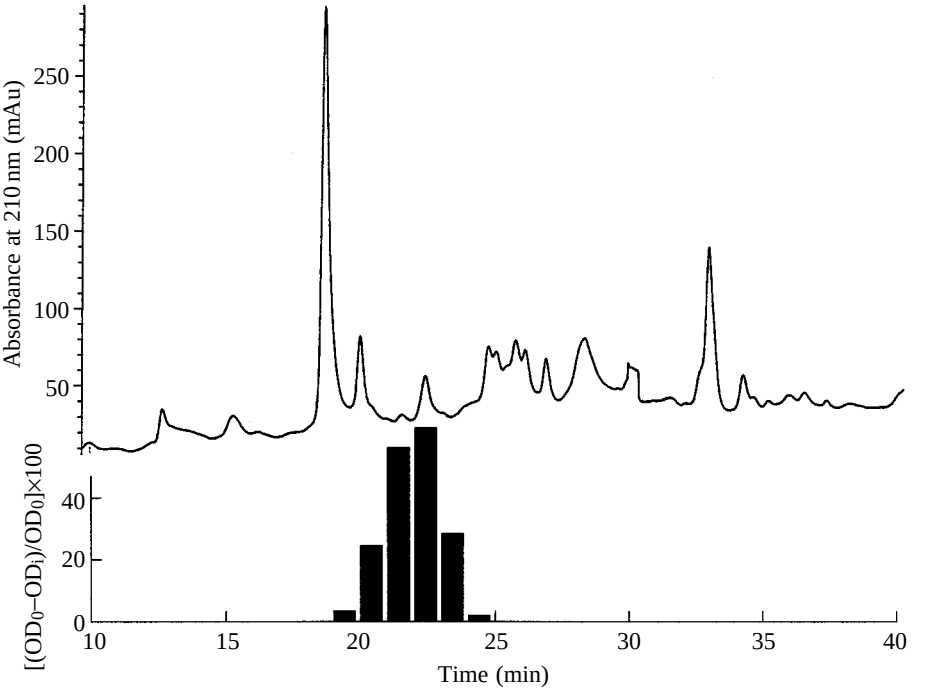

Fig. 1. Second step of fractionation of FMRFamide-like immunoreactivity (FLI) from ganglion extracts using reverse-phase liquid chromatography. Crude extracts were fractionated first in sodium phosphate, pH 6.0, using an acetonitrile gradient (not shown); two adjacent active fractions were then pooled and fractionated in 0.1 % trifluoroacetic acid using a similar gradient of acetonitrile (see Materials and methods). Shown in the upper trace is the absorbance at 210 nm; mAu, milli-absorbance units. The response in ELISA is shown in the lower trace; OD_0, optical density in the presence of no competitor peptide; OD_i, optical density obtained with samples from each fraction. FLI in fractions 21 and 22 was then separately subjected to sequence analysis by electrospray mass spectrometry with collision-activated dissociation.

peaks with retention times (R_t) of approximately 32 min (GNSFLRFamide) and 34 min (DPSFLRFamide). The spiked sample was chromatographed under identical conditions; the result is shown in Fig. 2B. The peaks for the Leu-containing peptides both increased in height, indicating their identity with the endogenous peptides. This chromatogram also shows that fraction 21 and/or 22 contained material in addition to the heptapeptides, probably reflected in the additional species found during ESMS (data not shown). To determine whether the Ile-containing peptides could also have been present in fractions 21 and 22, we chromatographed the Leu- and Ile-containing peptides together in conditions identical to those shown in Fig. 2. The Ile-containing peptides eluted approximately 1.5 min earlier than their Leu-containing counterparts and all four peptides were resolved (data not shown). Comparing this result with those in Fig. 2B we find that the Ile-containing peptides were not present in amounts sufficient to be detected in the absorbance trace at 210 nm, approximately 1 pmol in Fig. 2B. To corroborate this conclusion, and to complete the characterization of ventral nerve cord FMRFamides, we are now isolating sufficient material for sequencing from the three additional immunoreactive peptides found in extracts of 'control' ventral nerve cords (see below and Fig. 4). Thus, we have identified two new *Manduca* FLRFamides. In accordance with the previously identified decapeptide (Kingan *et al.* 1990), we name these peptides MasFLRFamide II (GNSFLRFamide) and

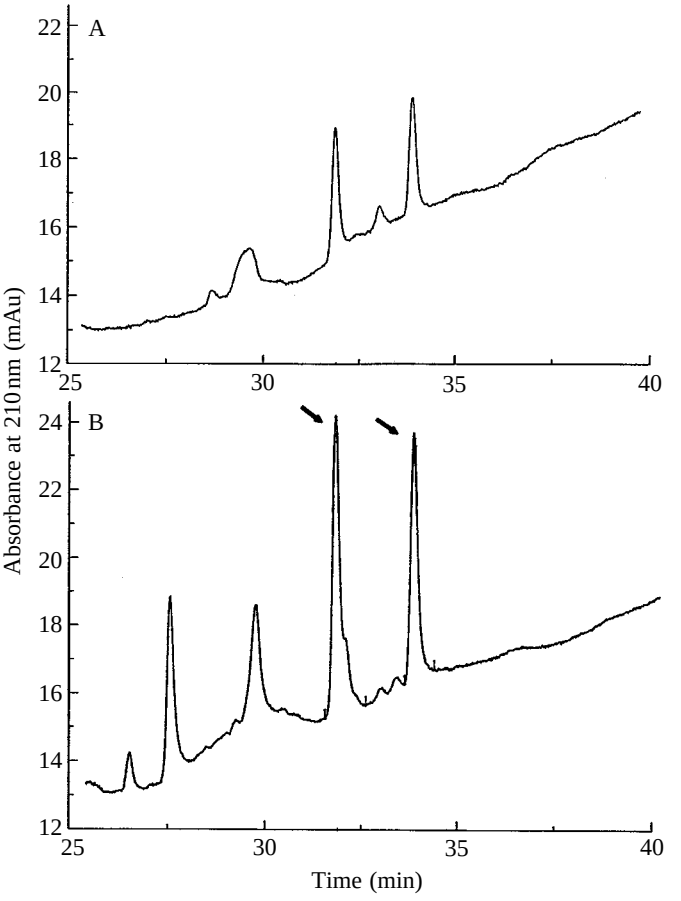

Fig. 2. Fractionation of synthetic GNSFLRFamide and DPSFLRFamide and their co-elution with peptides found in material subjected to amino acid sequencing by mass spectrometry. (A) 20 pmol of each synthetic peptide in an acetonitrile gradient containing 0.1 % TFA (see Materials and methods). Retention times are 31.9 min for GNSFLRFamide and 33.9 min for DPSFLRFamide. (B) 10 % of fractions 21 and 22 (see Fig. 1) combined with 20 pmol of GNSFLRFamide and DPSFLRFamide. The symmetrical increase in the peak heights of the synthetic peptides (arrows) indicates that they are identical with the tissue peptides.

Gly-Asn-Ser-Phe-Leu-Arg-Phe-NH_2	MasFLRFamide II
Asp-Pro-Ser-Phe-Leu-Arg-Phe-NH_2	MasFLRFamide III
*p*Glu-Asp-Val-Val-His-Ser-Phe-Leu-Arg-Phe-NH_2	MasFLRFamide I

Fig. 3. The sequences of the new heptapeptides, named MasFLRFamide II and III, and the previously characterized decapeptide, MasFLRFamide I. All peptides have the same C-terminal five amino acids.

MasFLRFamide III (DPSFLRFamide) and rename the decapeptide MasFLRFamide I. The sequences of the new peptides and their homology to MasFLRFamide I are shown in Fig. 3.

Tissue and developmental distribution of MasFLRFamides

Three observations suggested that this family of peptides might show distinctive tissue- and stage-specific distributions. First, high levels of only one FLRFamide, MasFLRFamide I, were initially found in extracts of neurohemal structures from pharate adults (Kingan *et al*. 1990). Second, FLI was found to be widely distributed in motoneurons in the segmental ganglia of larvae, but not in adults (Witten and Truman, 1996). Third, only the heptapeptides were isolated from the cultured ganglia. Therefore, we wanted to determine the distribution of the identifiable peptides in the cephalic ganglia and neurohemal structures as well as in the segmental ganglia of normal caterpillars and moths.

A chromatogram of the extract prepared from the larval ventral nerve cord (VNC) with the distribution of FLI is shown in Fig. 4; the fractionation of MasFLRFamides I, II and III under identical conditions is shown in the inset. Six peaks of FLI were detected in the tissue extract. The greatest amount of FLI was found in fractions that correspond to MasFLRFamide II and MasFLRFamide III, here with peak R_t values of approximately 26.5 and 28.8 min, respectively. The actual quantities of FLI found in the six immunoreactive peaks for the larva and pharate adult VNCs are shown in Fig. 5A. It is evident that, while FLI could be detected in additional fractions, these peaks are minor components in the VNCs of larvae with respect to those corresponding to MasFLRFamide II and III (Fig. 5A). Thus, it appears that the effect of culture of implanted ganglia in the diapausing pupa is to evoke the additional accumulation of the neuropeptides already in greatest abundance in fresh tissue. It should be noted, however, that MasFLRFamide III predominated in cultured ganglia, while slightly greater amounts of MasFLRFamide II were found in fresh ganglia.

By the time the adult emerges, the quantity of MasFLRFamides I and II in the VNC has increased substantially, while that in the MasFLRFamide III peak remains nearly unchanged (Fig. 5A). In addition, FLI in fractions 20 and 24 (see Fig. 4) increases dramatically. The identities of the immunoreactive peptides found in fractions 20, 24 and 30 have not yet been determined. With respect to the possible presence of Ile-containing peptides (see above), it should be noted that, from RPLC of synthetic material, fraction 24 has a retention time close to that expected of GNSFIRFamide.

The relative amounts of FLI in the Br/SOG of larvae and pharate adults is shown in Fig. 5B. Similar and relatively low levels of all three identified MasFLRFamides are found in the

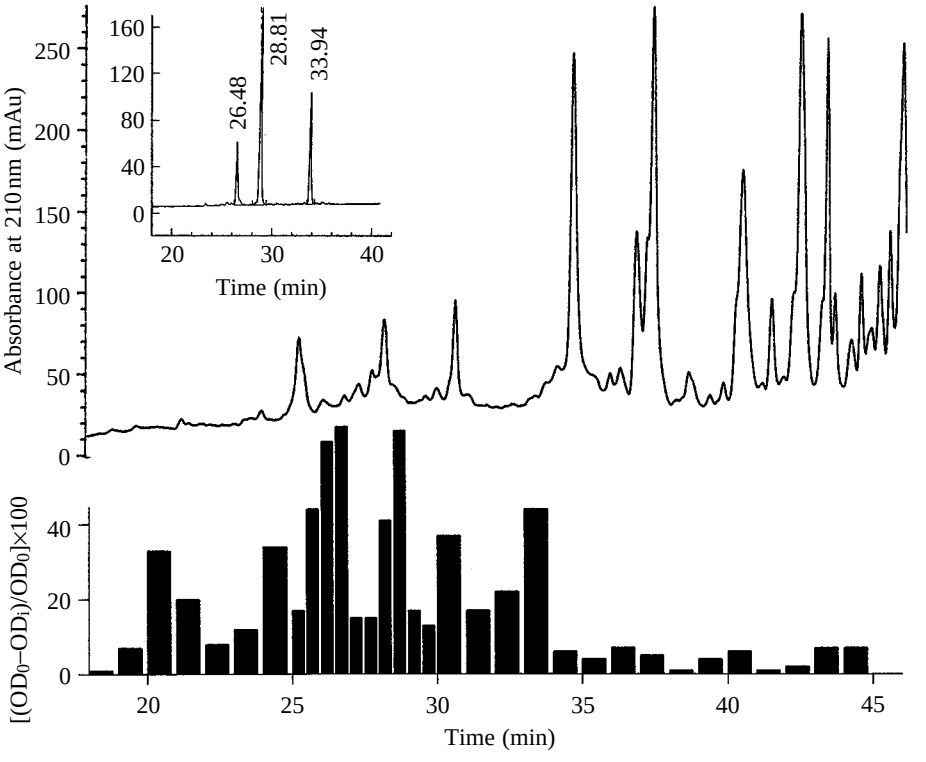

Fig. 4. FLI in larval ventral nerve cords (VNCs). The extract from 30 VNCs was fractionated by RPLC (see Materials and methods) and samples of fractions were assayed using ELISA. Note that 1 min fractions were collected, except between 25 and 30 min, during which time 0.5 min fractions were collected. Inset: the fractionation of MasFLRFamides I–III under conditions identical to those used for the tissue extract. Peak retention times are approximately 33.9 (I), 26.5 (II) and 28.8 min (III). The units shown are as defined in the legend of Fig. 1; the units for the *y*-axis of the inset are in mAu at 210 nm.

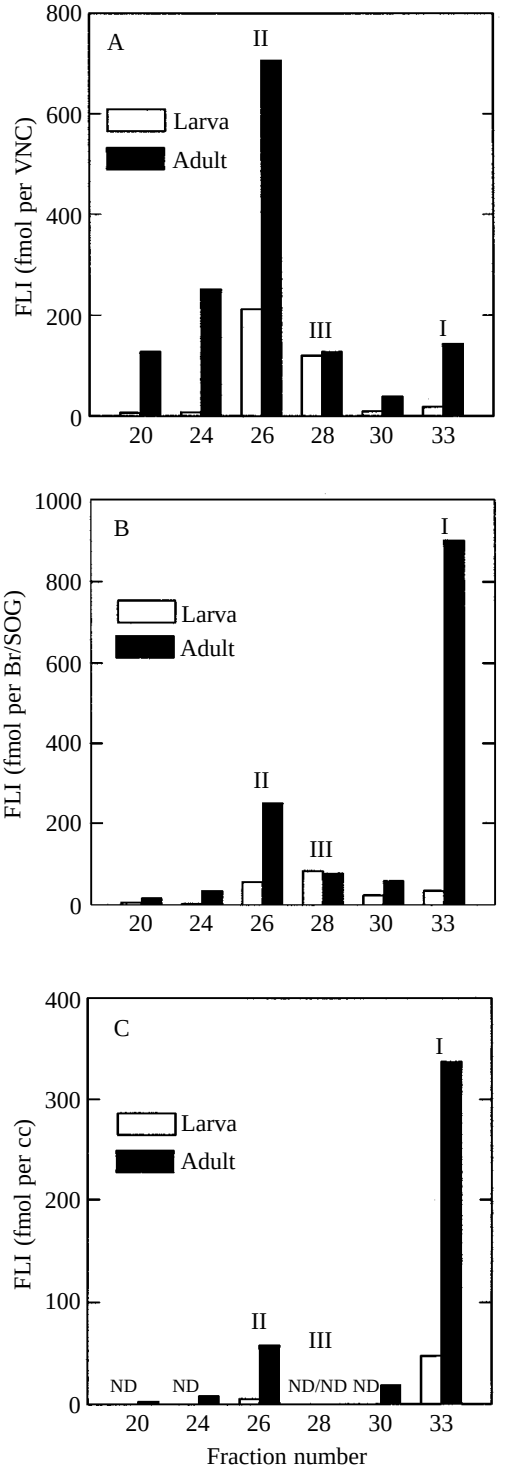

Fig. 5. FMRFamide-like immunoreactivity (FLI) in the central nervous system and neurohemal structures from fifth-instar larvae and pharate adults. Tissue extracts were fractionated and all fractions were assayed. Only those fractions containing quantifiable amounts of FLI are shown; three of the six immunoreactive peaks could be assigned on the basis of matched retention times to the three identified FLRFamides. RPLC was carried out on tissue from 23–56 insects. (A) FLI in the ventral nerve cord (VNC); (B) FLI in the brain/suboesophageal ganglion (Br/SOG); (C) FLI in the paired corpora cardiaca/corpora allata (cc). ND, not detected; the detection limit in each case was approximately 1 fmol per pair of corpora cardiaca/corpora allata.

larval Br/SOG. In the adult, however, MasFLRFamide I predominates in the Br/SOG, and MasFLRFamide II levels are enriched relative to MasFLRFamide III levels. In the combined neurohemal corpora cardiaca/corpora allata of the adult, MasFLRFamide I is found at high levels, >300 fmol per pair. In addition, we found approximately 60 fmol of FLI for the MasFLRFamide II peak, while MasFLRFamide III was undetectable (Fig. 5C). The combined amount of MasFLRFamides I and II in the corpora cardiaca of feeding fifth-instar larvae, 31 fmol, is greater than that previously reported for total FLI in the corpora cardiaca of premetamorphic larvae (4 fmol; Carroll *et al.* 1986). Thus, MasFLRFamide II along with MasFLRFamide I (Kingan *et al.* 1990) must be considered as candidate neurohormones of cephalic origin. MasFLRFamide III may play a different, possibly local neuromodulatory, role in the brain of both stages.

A number of neuropeptides are thought to be hormonally released in the minutes following eclosion (e.g. see Tublitz and Evans, 1986). Thus, as a more direct test of which FLRFamides may act as neurohormones, we fractionated hemolymph extracts from newly emerged moths and quantified FLI in those fractions co-migrating with the three FLRFamides (Fig. 6). The principal components in this profile had retention times corresponding to MasFLRFamides I and III (peak R_t values in this RPLC system for I, II and III were 36.7, 28.8 and 31.2 min, respectively); levels of I reached 35–40 nmol l^{-1} and levels of III reached nearly 20 nmol l^{-1} in some individuals. While not evident in Fig. 6, consistent differences between FLI found in fraction 28 and the adjacent fractions suggest that MasFLRFamide II is also present in the hemolymph on emergence, although in concentrations at or below 1 nmol l^{-1}. In addition to these three peaks, FLI was also consistently found in fraction 34; the identity of this material has not been determined, but its R_t corresponds to that expected for the peptide found in tissue extracts which elutes between MasFLRFamides III and I (30 min, Fig. 4). Thus, the major FLRFamide neurohormone of ventral nerve cord origin appears to be MasFLRFamide III, while MasFLRFamide I is the major neurohormone of cephalic structures.

Biological activity

A number of insect neuropeptides, including the FLRFamides, are active in modulating the frequency of spontaneous contractions of visceral muscle. The cockroach homolog of MasFLRFamide I (leucomyosuppressin) is, in fact, a potent inhibitor of spontaneous contractions in the hindgut (Holman *et al.* 1986). MasFLRFamide I has a similar effect in the midgut of the sphingid moth *Agrius convolvuli* (Fujisawa *et al.* 1993). Therefore, we investigated the effects of MasFLRFamides I, II and III on visceral muscle in *M. sexta*.

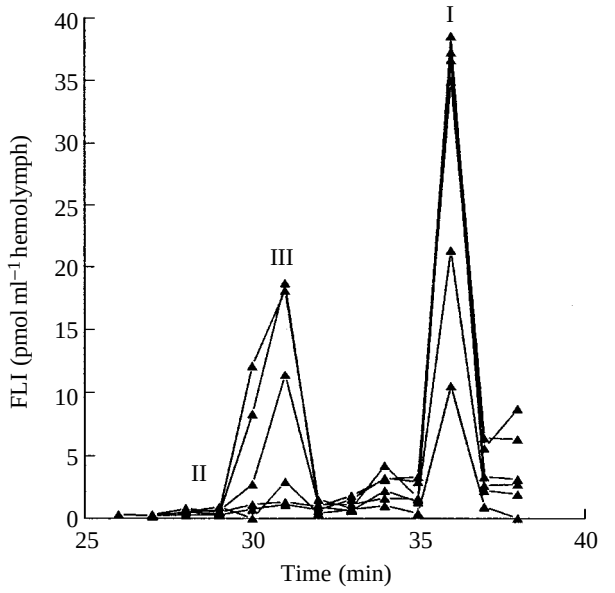

Fig. 6. FMRFamide-like immunoreactivity (FLI) in fractionated hemolymph of *Manduca sexta* collected approximately 10 min after emergence. The order of elution in the narrow-bore column (see Materials and methods) of the three peptides was conserved from that in the analytical column. The peak retention times in this RPLC system for the MasFLRFamides were 36.7 (I), 28.8 (II) and 31.2 min (III). Under the conditions shown, MasFLRFamide III elutes in fraction 30 as well as in fraction 31. Each trace represents the profile of immunoreactivity from one moth (N=6).

The ileum of adult *M. sexta* is spontaneously contractile; under our conditions, the rate was approximately 1 contraction min^{-1}. When MasFLRFamide I was applied at 20 nmol l^{-1} (data not shown) or at 1 nmol l^{-1}, little or no change in the frequency of spontaneous contractions ensued (Fig. 7A). In contrast, when 1 nmol l^{-1} MasFLRFamide II was applied, a robust increase in the rate of contraction occurred. The latency for this increase was 1 min or less, the uncertainty being due to the time required to exchange the buffer completely in the transfusion chamber. The increase in contraction frequency was reversible with a saline wash; the time course of the reversal was approximately equal to that of the onset.

Interestingly, MasFLRFamide III, while eliciting an increase in the rate of contraction, was consistently less active than MasFLRFamide II (Fig. 7B). Fig. 7B also reveals an additional aspect of the response generally observed with >1 nmol l^{-1} MasFLRFamide II application: the onset of the increase in rate was accompanied by an increase in basal tension (shortening) of the muscle, as indicated by the rise from baseline in the trace (Fig. 7B). The apparent relaxation that follows is attributable to the action of the filter in the transducer (see Materials and methods).

Discussion

We have isolated two new heptapeptide FLRFamides from the larval segmental ganglia of *M. sexta*. To accomplish this, we used a novel method to enrich levels of these peptides, thus permitting their complete characterization from only 180 original ganglia. This method exploited a recent observation that FLI is revealed or enhanced in many motoneurons of larval segmental ganglia when steroid hormone titers are maintained at low levels by either ligation or transplant and culture in diapausing pupae (Witten and Truman, 1996).

Sequences and physiology

The sequences of the new peptides, GNSFLRFamide (MasFLRFamide II) and DPSFLRFamide (MasFLRFamide III), resemble a decapeptide (*p*EDVVHSFLRFamide) previously characterized from cephalic CNS tissue of adult *M. sexta* (Kingan *et al.* 1990). All characterized peptides in this family from moths contain leucine. In fact, among the insects, FMRFamides have been found only in dipterans (Nambu *et al.* 1988; Duve *et al.* 1992; Nichols, 1992). The sequences of the heptapeptides differ from that of the decapeptide, however, in that their N-terminal extensions consist primarily of small hydrophilic amino acids. In this regard, they are similar to, for instance, the FLRFamides isolated from the pulmonate snails *Helix aspersa* (SDPFLRFamide, NDPFLRFamide; Price *et al.*

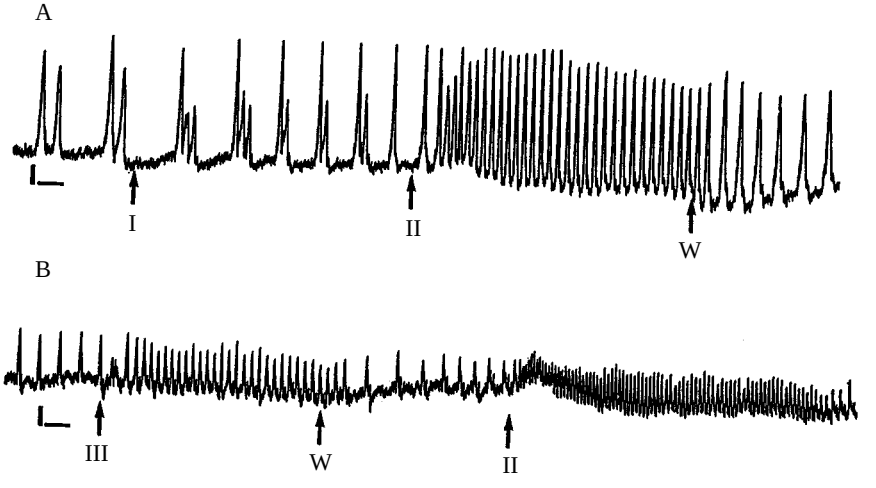

Fig. 7. Action of the three characterized MasFLRFamides on the moth ileum. Concentrations of peptides were all 1 nmol l^{-1}. I, MasFLRFamide I (*p*EDVVHSFLRFamide); II, MasFLRFamide II (GNSFLRFamide); III, MasFLRFamide III (DPSFLRFamide); W, saline wash. Scale bars, 150 μm (vertical); 1 min (horizontal). (A) Treatment with MasFLRFamide I was followed by treatment with MasFLRFamide II. Only MasFLRFamide II showed a significant effect. (B) Treatment with MasFLRFamide III was followed by treatment with MasFLRFamide II with an intervening wash.

1985) and *Lymnaea stagnalis* (SDPFLRFamide, GDPFLRFamide; Ebberink *et al.* 1987) and the lobster *Homarus americanus* (SDRNFLRFamide, TNRNFLRFamide; Trimmer *et al.* 1987).

In addition to differing in their N-terminal extensions, the decapeptide and the heptapeptides are also distinguished in their action on the moth ileum. While the decapeptide was inactive even at 20 nmol l^{-1}, the heptapeptides at 1 nmol l^{-1} evoked robust increases in twitch frequency in the ileum. This difference was unexpected, given the identities of the five amino acids at the C termini of the three peptides. In locusts, two N-terminally extended peptides (one FLRFamide and one FIRFamide) with myostimulatory action on the oviduct have recently been isolated from VNCs of *Locusta migratoria* (Lange *et al.* 1994). Information on the basis for the differing action of the moth FLRFamides is not yet available, but it is possible that MasFLRFamides II and III are able to adopt a receptor-active conformation that is energetically disallowed for MasFLRFamide I. Nevertheless, the physiological findings show that the heptapeptides can be considered to be in a subfamily of FLRFamides distinct from the decapeptides.

In addition to the FLRFamides, it was recently shown that a family of moth kinins (Blackburn *et al.* 1995*b*), homologues of the family of peptides originally found in the cockroach *Leucophaea maderae* (Schoofs *et al.* 1993), is stimulatory to the moth ileum (M. B. Blackburn, personal communication). The same investigators have also shown that spontaneous contractions in this tissue are inhibited by a recently discovered family of myoinhibitory peptides (Blackburn *et al.* 1995*a*). The mechanisms for the stimulatory or inhibitory actions of the three classes of neuropeptides now known to act on the spontaneously contractile ileum have not yet been investigated.

The significance of neuropeptide modulation of activity in the ileum of the moth is uncertain at present. The ileum serves to conduct the nitrogenous wastes of metamorphosis to the rectum for storage, from which they are voided upon emergence. Thus, activity in this portion of the alimentary canal may be of more significance to the insect during development. It has been pointed out that, in cecropia moths, this portion of the hindgut is spontaneously active, beginning in the second week of adult development (Judy and Gilbert, 1969).

Regional distribution of peptides and changes with development

Determining the levels of MasFLRFamides in the central ganglia, in their neurohemal structures and in the hemolymph may be an aid in the search for additional physiological processes evoked or subserved by these peptides. Three types of comparisons in our quantitative determinations from tissue extracts show that the FLRFamides may be differentially regulated in their expression and have different functions. First, MasFLRFamide I predominates in cephalic structures, while MasFLRFamide II predominates in the segmental ganglia; the exception to this generalization is in the larval Br/SOG, which contains similarly low levels of all three identified FLRFamides. Interestingly, while MasFLRFamide II is the second most abundant FLRFamide in the adult corpora cardiaca/corpora allata, MasFLRFamide III was undetectable in this tissue; thus, its presence in the brain and SOG suggests a function other than as a hormone.

In the second comparison, we found that metamorphosis brings about dramatic changes in the total content and profile of MasFLRFamides I and II, but not III, in the VNC and the Br/SOG and its associated neurohemal structure, the corpora cardiaca/corpora allata (Fig. 5). For instance, MasFLRFamide II levels increase approximately 2.5-fold in the VNC; moreover, levels of the as yet unidentified FMRFamides found in fractions 20 and 24 (Figs 4, 5) increase 10- to 20-fold in the adult VNC. Since the number of motoneurons exhibiting FLI declines dramatically during metamorphosis (Witten and Truman, 1996), we suggest that the increased peptide levels of the adult VNC reflect the content of neurosecretory cells. One likely source is cardioacceleratory peptide neurons in the abdominal ganglia (Tublitz and Truman, 1985). FLI begins to appear in these neurons during the larval–pupal transformation (Witten and Truman, 1996) and continues to be expressed in the adult. In the thoracic ganglia, there are 2–3 pairs of large ventrolateral neurosecretory cells that consistently express FLI. The location of their somata and axonal projections is similar to that described for a pair of FLI-containing neurosecretory cells in the embryo, the CF neurons (Wall and Taghert, 1991*a*). The axons of these neurons descend into the next posterior perivisceral organ *via* the median nerve (J. L. Witten, unpublished observation). FLI-containing neurosecretory cells in the brain and, for instance, on the ventral midline of the SOG arborize in cephalic neurohemal structures (Homberg *et al.* 1990) and are likely to contribute to the increase in the content of MasFLRFamides I and II during metamorphosis. In addition, during metamorphosis, the adult brain acquires a number of FLI-containing interneurons in the sensory neuropil and the associative centers (Homberg *et al.* 1990).

In the third comparison, it appears that the relative amounts of MasFLRFamide II and III in control ganglia from fifth-instar larvae are reversed with respect to those found in implanted ganglia. During the implantation procedure, connections to peripheral targets and other ganglia are severed. Thus, it is possible that peripheral targets or connections with the rest of the nervous system may influence the expression or stability of MasFLRFamides. Interestingly, target influences do not appear to play a role in regulating the onset of FLI expression in *Manduca sexta* embryos (Wall and Taghert, 1991*a*).

In order to establish a role for any of the FLRFamides as a neurohormone, it is essential to establish its presence in the hemolymph in concentrations known to regulate its target tissue(s). As a first step, we quantified the FLRFamides in hemolymph immediately after the emergence of the moth, a time when a number of physiological and developmental events may be evoked by neuropeptides. MasFLRFamide I was the most abundant FLRFamide in the hemolymph, consistent with its content in the corpora cardiaca/corpora allata.

MasFLRFamide III could also be detected and at much higher levels than MasFLRFamide II. This is surprising, in that it could not be detected in the corpora cardiaca/corpora allata and was much less abundant than MasFLRFamide II in the VNC. However, these findings show the need to extend quantitative studies of blood and tissue levels. Peptide levels should be measured in the VNC with perivisceral organs (PVOs) attached. In this study, the PVOs were not reliably included in the dissections of the adult VNC. It will also be important to assess the possibility that other sources of neuropeptides could contribute to a changing profile of hemolymph FLRFamides. The enteric nervous system includes a number of intrinsic neurons as well as endocrine cells on the midgut of the larva that produce FLI (Copenhaver and Taghert, 1989; Zitnan *et al.* 1995). The neuropeptide complement of these cells in the adult has not been determined. In the periphery, the L1 neurons have been shown to contain FLI (Wall and Taghert, 1991*b*); in addition, the recently discovered and segmentally distributed endocrine 'Inka' cells (Zitnan *et al.* 1996) also contain a number of immunoreactive FLRFamides (T. G. Kingan and D. Zitnan, unpublished observation).

From the quantities of FLRFamides found in hemolymph and from their efficacy in evoking a response in the ileum, we conclude that MasFLRFamides II and III function as neurohormones in *M. sexta.* This does not preclude, however, the possibility that peptidergic innervation of the ileum plays a role in the regulation of its activity. In *M. sexta*, the hindgut of feeding larvae is innervated by the proctodeal nerve (Reinecke *et al.* 1973). The proctodeal nerve is known to contain eclosion hormone (Truman and Copenhaver, 1989), and FLI has also been observed in this nerve (J. L. Witten, unpublished observation).

In summary, the observation that FLI accumulates in motoneurons of the VNC in an ecdysteroid-free environment has led us to the characterization of two new FLRFamides with stimulatory action on the moth ileum. In future studies, it will be important to determine the identities of the peptides whose accumulation is specific to motoneurons, particularly if the FLRFamides are found to have a modulatory action on skeletal muscles.

We thank P. A. Thomas-Laemont for valuable technical assistance and Dr A. K. Raina for his support of this work. This work was supported by US Department of Agriculture National Research Initiative Competitive Grant 93-37302-8968 (T.G.K.) and by funds from The Graduate School and Graduate School Research Committee Award, University of Wisconsin-Milwaukee (J.L.W.).

References

ADAMS, M. E. AND O'SHEA, M. (1983). Peptide cotransmitter at a neuromuscular junction. *Science* **221**, 286–289.

BELL, R. A. AND JOACHIM, F. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. ent. Soc. Am.* **69**, 365–373.

BLACKBURN, M. B., WAGNER, R. M., KOCHANSKY, J. P. AND RAINA, A. K. (1995*a*). The identification of two myoinhibitory peptides from the ventral nerve cord of *Manduca sexta* with similarities to the galanins. *Regul. Peptides* **57**, 213–219.

BLACKBURN, M. B., WAGNER, R. M., SHABANOWITZ, J., KOCHANSKY, J. P., HUNT, D. F. AND RAINA, A. K. (1995*b*). The isolation and identification of three diuretic kinins from the abdominal ventral nerve cord of adult *Helicoverpa zea*. *J. Insect Physiol.* **41**, 723–730.

BOWEN, M. F., BOLLENBACHER, W. E. AND GILBERT, L. I. (1984). *In vitro* studies on the role of the brain and prothoracic glands in the pupal diapause of *Manduca sexta*. *J. exp. Biol.* **108**, 9–24.

BROWN, B. E. AND STARRATT, A. N. (1975). Isolation of proctolin, a myotropic peptide from *Periplaneta americana*. *J. Insect Physiol.* **21**, 1879–1881.

CARROLL, L. S., CARROW, G. M. AND CALABRESE, R. L. (1986). Localization and release of FMRFamide-like immunoreactivity in the cerebral neuroendocrine system of *Manduca sexta*. *J. exp. Biol.* **126**, 1–14.

COPENHAVER, P. F. AND TAGHERT, P. H. (1989). Development of the enteric nervous system in the moth. I. Diversity of cell types and the embryonic expression of FMRFamide-related neuropeptides. *Dev. Biol.* **131**, 70–84.

DUVE, H., JOHNSEN, A. H., SEWELL, J. C., SCOTT, A. G., ORCHARD, I., REHFELD, J. F. AND THORPE, A. (1992). Isolation, structure and activity of -Phe-Met-Arg-Phe-NH_2 neuropeptides (designated calliFMRFamides) from the blowfly *Calliphora vomitoria*. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2326–2330.

EBBERINK, R. H. M., PRICE, D. A., VAN LOOENHONT, H., DABLE, K. E., REIHM, J. P., GERAERTS, W. P. M. AND GREENBERG, M. H. (1987). The brain of *Lymnaea* contains a family of FMRFamide-like peptides. *Peptides* **8**, 515–522.

FONAGY, A., SCHOOFS, L., PROOST, P., VAN DAMME, J., BUEDS, H. AND DE LOOF, A. (1992). Isolation, primary structure and synthesis of neomyosuppressin, a myoinhibiting neuropeptide from the grey fleshfly, *Neobellieria bullata*. *Comp. Biochem. Physiol.* **102**C, 239–245.

FUJISAWA, Y., SHIMODA, M., KIGUCHI, K., ICHIKAWA, T. AND FUJITA, N. (1993). The inhibitory effect of a neuropeptide, *Manduca*FLRFamide, on the midgut activity of the sphingid moth, *Agrius convolvuli*. *Zool. Sci.* **10**, 773–777.

GIEBULTOWICZ, J. M., BLACKBURN, M. B., THOMAS-LAEMONT, P. A., WEYDA, F. AND RAINA, A. K. (1996). Daily rhythm in myogenic contractions of vas deferens associated with sperm release cycle in a moth. *J. comp. Physiol.* A (in press).

HEWES, R. S. AND TRUMAN, J. W. (1991). The roles of central and peripheral eclosion hormone release in the control of ecdysis behavior in *Manduca sexta*. *J. comp. Physiol.* **168**, 697–707.

HOLMAN, G. M., COOK, B. J. AND NACHMAN, R. J. (1986). Isolation, primary structure and synthesis of leucomyosuppressin, an insect neuropeptide that inhibits spontaneous contractions of the cockroach hindgut. *Comp. Biochem. Physiol.* **85**C, 329–333.

HOMBERG, U., KINGAN, T. G. AND HILDEBRAND, J. G. (1990). Distribution of FMRFamide-like immunoreactivity in the brain and suboesophageal ganglion of the sphinx moth *Manduca sexta* and colocalization with SCP_b, BPP- and GABA-like immunoreactivity. *Cell Tissue Res.* **259**, 401–419.

HUNT, D. F., ALEXANDER, J. E., MCCORMACK, A. L., MARTINO, P. A., MICHEL, H., SHABANOWITZ, J., SHERMAN, N., MOSELEY, M. A., JORGENSON, J. W. AND TOMER, K. B. (1991). Mass spectrometric methods for protein and peptide sequence analysis. In *Techniques*

in Protein Chemistry II (ed. J. J. Villafranca), pp. 441–454. New York: Academic Press.

HUNT, D. F., SHABANOWITZ, J., YATES, J. R. I., ZHU, N.-Z., RUSSELL, D. H. AND CASTRO, M. E. (1987). Tandem quadrupole Fourier-transform mass spectrometry of oligopeptides and small proteins. *Proc. natn. Acad. Sci. U.S.A.* **84**, 620–623.

HUNT, D. F., YATES, J. R. I., SHABANOWITZ, J., WINSTON, S. AND HAUER, C. R. (1986). Protein sequencing by tandem mass spectrometry. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6233–6237.

JUDY, K. J. AND GILBERT, L. I. (1969). Morphology of the alimentary canal during the metamorphosis of *Hyalophora cecropia* (Lepidoptera:Saturniidae). *Ann. ent. Soc. Am.* **62**, 1438–1446.

KATAOKA, H., TROETSCHLER, R. G., LI, J. P., KRAMER, S. J., CARNEY, R. L. AND SCHOOLEY, D. A. (1989). Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2976–2980.

KINGAN, T. G. (1989). A competitive enzyme-linked immunosorbent assay: Applications in the assay of peptides, steroids and cyclic nucleotides. *Analyt. Biochem.* **183**, 283–289.

KINGAN, T. G., TEPLOW, D. B., PHILLIPS, J. M., RIEHM, J. P., RAO, K. R., HILDEBRAND, J. G., HOMBERG, U., KAMMER, A. E., JARDINE, I., GRIFFIN, P. R. AND HUNT, D. F. (1990). A new peptide in the FMRFamide family isolated from the CNS of the hawkmoth, *Manduca sexta*. *Peptides* **11**, 849–856.

LANGE, A. B., ORCHARD, I. AND TE BRUGGE, V. A. (1991). Evidence for the involvement of a SchistoFLRF-amide-like peptide in the neural control of locust oviduct. *J. comp. Physiol.* A **168**, 383–391.

LANGE, A. B., PEEFF, N. M. AND ORCHARD, I. (1994). Isolation, sequence and bioactivity of FMRFamide-related peptides from the locust ventral nerve cord. *Peptides* **15**, 1089–1094.

MARDER, E., CALABRESE, R. L., NUSBAUM, M. P. AND TRIMMER, B. (1987). Distribution and partial characterization of FMRFamide-like peptides in the stomatogastric nervous systems of the rock crab, *Cancer borealis* and the spiny lobster, *Panulirus interruptus*. *J. comp. Neurol.* **259**, 150–163.

NAMBU, J. R., MURPHY-ERDOSH, C., ANDREWS, P. C., FEISTNER, G. J. AND SCHELLER, R. H. (1988). Isolation and characterization of a *Drosophila* neuropeptide gene. *Neuron* **1**, 55–61.

NICHOLS, R. (1992). Isolation and structural characterization of *Drosophila* TDVDHVFLRFamide and FMRFamide-containing neural peptides. *J. molec. Neurosci.* **3**, 213–218.

PRICE, D. A., COTTRELL, G. A., DOBLE, K. E., GREENBERG, M. J., JORENBY, W., LEHMAN, H. K. AND RIEHM, J. P. (1985). A novel FMRFamide-related peptide in *Helix*: pQDPFLRFamide. *Biol. Bull. mar. biol. Lab., Woods Hole* **169**, 256–266.

REINECKE, J. P., COOK, B. J. AND ADAMS, T. S. (1973). Larval hindgut of *Manduca sexta* (L.) (Lepidoptera: Sphingidae). *Int. J. Insect Morph. Embryol.* **2**, 277–290.

ROBB, S., PACKMAN, L. C. AND EVANS, P. D. (1989). Isolation, primary structure and bioactivity of SchistoFLRF-amide, a FMRF-amide-like neuropeptide from the locust, *Schistocerca gregaria*. *Biochem. biophys. Res. Commun.* **160**, 850–856.

SCHNEIDER, L. E. AND TAGHERT, P. H. (1988). Isolation and characterization of a *Drosophila* gene that encodes multiple neuropeptides related to Phe-Met-Arg-Phe-NH_2 (FMRFamide). *Proc. natn. Acad. Sci. U.S.A.* **85**, 1993–1997.

SCHOOFS, L., VANDEN BROECK, J. AND DE LOOF, A. (1993). The myotropic peptides of *Locusta migratoria*: structures, distribution, functions and receptors. *Insect Biochem. molec. Biol.* **23**, 859–881.

TRIMMER, B. A., KOBIERSKI, L. A. AND KRAVITZ, E. A. (1987). Purification and characterization of FMRFamidelike immunoreactive substances from the lobster nervous system: isolation and sequence analysis of two closely related peptides. *J. comp. Neurol.* **266**, 16–26.

TRUMAN, J. W. AND COPENHAVER, P. F. (1989). The larval eclosion hormone neurones in *Manduca sexta*: identification of the brain–proctodeal neurosecretory system. *J. exp. Biol.* **147**, 457–470.

TUBLITZ, N. J. AND EVANS, P. D. (1986). Insect cardioactive peptides: cardioacceleratory peptide (CAP) activity is blocked *in vivo* and *in vitro* with a monoclonal antibody. *J. Neurosci.* **6**, 2451–2456.

TUBLITZ, N. J. AND TRUMAN, J. W. (1985). Identification of neurons containing the cardioacceleratory peptides (CAPs) in the ventral nerve cord of the tobacco hawkmoth, *Manduca sexta*. *J. exp. Biol.* **116**, 395–410.

WALKER, R. J. (1992). Neuroactive peptides with an RFamide or Famide carboxyl terminal. *Comp. Biochem. Physiol.* **102**C, 213–222.

WALL, J. B. AND TAGHERT, P. H. (1991*a*). The timing of initial neuropeptide expression by an identified insect neuron does not depend on interactions with its normal peripheral target. *J. Neurobiol.* **22**, 935–956.

WALL, J. B. AND TAGHERT, P. H. (1991*b*). Segment-specific modifications of a neuropeptide phenotype in embryonic neurons of the moth, *Manduca sexta*. *J. comp. Neurol.* **309**, 375–390.

WITTEN, J. L., KINGAN, T. G., SHABANOWITZ, J. AND HUNT, D. F. (1993). Isolation and sequencing of two new members of the *Manduca sexta* FLRFamide family of peptides. *Soc. Neurosci. Abstr.* **19**, 1274.

WITTEN, J. L. AND TRUMAN, J. W. (1996). Developmental plasticity of neuropeptide expression in motoneurons of the moth, *Manduca sexta*: steroid hormone regulation. *J. Neurobiol.* **29**, 99–114.

ZITNAN, D., KINGAN, T. G. AND BECKAGE, N. E. (1995). Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of *Manduca sexta*. *Insect Biochem. molec. Biol.* **25**, 669–678.

ZITNAN, D., KINGAN, T. G., HERMESMAN, J. L. AND ADAMS, M. E. (1996). Identification of ecdysis-triggering hormone from an epitracheal endocrine system. *Science* **271**, 88–91.