

Mutually Exclusive Neuronal Expression of Peptides Encoded By the FMRFa Gene Underlies a Differential Control of Copulation in *Lymnaea**

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An innovative method, direct peptide profiling of small samples of nervous tissue by matrix-assisted laser desorption ionization mass spectrometry, in combination with peptide characterization, immunocytochemistry in conjunction with specific neuronal labeling by backfilling of the penis nerve, and bioassay of peptides was used to study the intrinsic neuronal expression patterns of distinct sets of related FMRFa peptides and their significance for the organization of male copulation behavior in the mollusk, *Lymnaea stagnalis*. Previous studies indicate that the sets of FMRFa-related and GDPFLRFa-related peptides are encoded by two alternatively spliced transcripts of the single FMRFa gene. Direct mass spectrometry revealed that both FMRFa-related and GDPFLRFa-related peptides are present in the penis nerve, the sole nerve that innervates the penis complex. Accordingly, authentic FMRFa, GDPFLRFa, and related peptides were purified from the penis complex. The loci of synthesis of FMRFa and related peptides could be traced to the right cerebral ventral lobe, those of GDPFLRFa and related peptides to the B group neurons in the right parietal ganglion and to a few unidentified neurons in the right pleural ganglion. Notwithstanding their related structures, the two sets of peptides have distinctly different actions on the penis retractor muscle.

Knowledge about the role of neuropeptides in the organization of behavior has grown tremendously over the past decade and is derived from diverse sources, including basic studies in various animal species and studies of human neuro- and psychopathology (e.g. Refs. 1–4). These studies suggest that complex strategies of peptide expression, synthesis, and action underlie the control of behavior. Bioactive peptides are commonly synthesized in the form of a larger precursor protein from which they are liberated by the action of prohormone convertases. Then the peptides are modified (e.g. amidated, acetylated and glycosylated) and sorted into dense core vesicles that are targeted to the appropriate neuronal compartments, where the peptides are released in response to depolarization. Often, several peptide genes are co-expressed, frequently to-

gether with classical transmitters (2, 3). It has recently been suggested (5) that functionally related and closely apposed neurons may produce overlapping yet distinct sets of peptides. This may be achieved by one or a combination of various molecular and cellular processes, e.g. differential gene expression (5), cell-specific processing (6), and sorting into distinct types of granules (7). As research progresses in this area, it is clear that the study of the significance of the intrinsic peptide patterns for behavior will require innovative techniques that allow examination of the entire peptide profile in defined small brain loci and even single neurons as well as target tissue. In the present study, we use the newly developed technique of direct mass spectrometry of nervous tissue in combination with conventional peptide chemistry, cDNA cloning data, and neurobiological techniques to examine the intrinsic patterns of peptide expression and targeting underlying behavior. We studied the mollusk *Lymnaea stagnalis*, a model neurobiological preparation that has been widely used for integrated molecular, physiological, and behavioral studies (e.g. Refs. 8–12).

We examined the penis putative motoneurons which occur as identifiable clusters in the right cerebral and pedal ganglia and as scattered neurons in the right pleural and parietal ganglia (12, 13). These neurons send their axons into the penis nerve, the sole nerve to innervate the penis complex (comprising of the preputium, penis, retractor muscles, and vas deferens). Several peptide messengers synthesized by the penis motoneurons in the cerebral ganglion have been elucidated, and their effects on various muscular systems of the penis complex have been studied (10, 11, 14, 15). However, by using conventional methods of peptide chemistry and molecular biology it is difficult to gain insight into the whole spectrum of peptide messengers sent by central neurons to the penis complex. Equally, peptide contents of the unidentified scattered motoneurons in the right pleural and parietal ganglia are inaccessible for biochemical analysis by conventional methodology. To circumvent these problems, we employ here an alternative strategy that makes use of the newly developed direct peptide profiling of nervous tissue by mass spectrometry (5, 16). The penis nerve was subjected to direct mass determinations by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS),¹ and the measured masses of all the peptides were scrutinized closely. We detected in the penis nerve two sets of identified peptides, the tetrapeptides FMRFa and FLRFa and the heptapeptides GDPFLRFa

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¹ The abbreviations used are: MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; DEILSR, the KQQVATDDSGELDDEILSR peptide; HPGPC, high performance gel permeation chromatography; HPLC, high performance liquid chromatography; rpHPLC, reversed phase HPLC; SEELY, the SEQPDVDDYLDRVV-LQSEELY peptide.

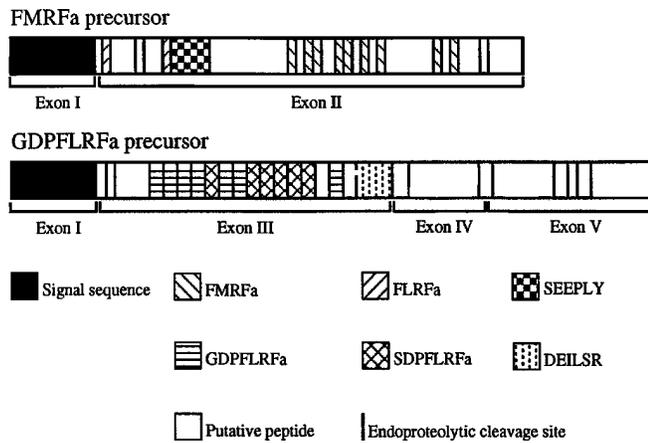


FIG. 1. The precursor structures as generated by alternative splicing of the FMRFa gene of *Lymnaea*. The signal sequence expressed by exon I of the FMRFa gene can be spliced to the sequence expressed by exon II, which encodes the FMRFa precursor from which the peptides FMRFa, FLRFa, and the SEEPLY peptide with other putative peptides can be derived. Alternatively, the signal sequence can also be spliced to the sequence expressed by exon III and exon IV/V, which encode the GDPFLRFa precursor from which the peptides GDPFLRFa, SDPFLRFa, and the DEILSR peptide together with other putative peptides can be derived (20).

and SDPFLRFa. Notwithstanding extensive studies of their site of synthesis (17–19), the heptapeptides in *Lymnaea*, in contrast to the tetrapeptides (18), have never been implicated in the control of the activities of the penis.

In *Lymnaea*, the FMRFa gene is alternatively spliced into two transcripts (Fig. 1), the FMRFa transcript encoding the tetrapeptides and the GDPFLRFa transcript encoding the heptapeptides (20). In view of our finding that both sets of peptides are targeted to the penis complex, we hypothesized that in principle different aspects of the complex copulation behavior may be controlled by these different sets of peptides through mutually exclusive expression in different penis motoneurons. Indeed, we could confirm the presence of the peptides in the penis complex by peptide purification and characterization. Moreover, we were able to identify the central neurons that synthesize the peptides by backfilling the penis nerve in conjunction with immunocytochemistry using two antibodies that specifically recognize end products of the tetrapeptide and heptapeptide transcripts, respectively. Finally, we could show by using *in vitro* bioassays that both sets of peptides have distinctly different effects on the penis retractor muscle.

MATERIALS AND METHODS

MALDI-MS of the Penis Nerve—Small pieces of penis nerve were dissected from mature laboratory-bred *L. stagnalis* under a microscope and transferred to 1- μ l drops of matrix solution (2,5-dihydroxybenzoic acid) on a stainless steel target. Each sample was ruptured by a pair of tiny hooks to release the peptides in the matrix. The solution was dried within minutes by a gentle stream of cool air, and the target was inserted into the mass spectrometer immediately afterwards. The acidic nature of the matrix (pH 2.0) inhibits the activities of enzymes that might be released during cell lysis. MALDI-MS was performed on a Finnigan MAT Vision 2000 laser desorption time of flight mass spectrometer equipped with a pulse nitrogen laser. An external standard peptide, renin, was used for calibration. Usually, 30 individual spectra were accumulated to increase the signal:noise ratio.

Electrospray Ionization Mass Spectrometry—Fractions obtained by reversed phase high performance liquid chromatography (rpHPLC) were dried in a Speed Vac and redissolved in 100 μ l of 7.0 mM trifluoroacetic acid in 60% acetonitrile. Mass spectrometry was performed as described (21) with minor modifications. 8 μ l of each fraction were injected via a 10- μ l loop into a Fisons BioQ triple-quadrupole mass spectrometer equipped with an electrospray atmospheric pressure ionization source. The mobile phase was 50% acetonitrile in 7.0 mM trifluoroacetic acid; the flow rate was 10 μ l/min.

Peptide Purification—The penis complex (consisting of the preputium, penis, penis retractor muscles, and vas deferens) was used as the starting material for purification of tetra- and heptapeptides. 750 penis complexes were dissected, collected on dry ice, and stored at -20°C until used. Extracts were made as described previously (14, 15) and subjected to high performance gel permeation chromatography (HPGPC) using an I-125 Protein Pak column (7.8×300 mm) and an I-300 Protein Pak column (7.5×300 mm; both from Waters Associates) connected in series, with a liquid chromatograph system (Waters Associates). The running solvent was 7.0 mM trifluoroacetic acid in 30% acetonitrile, the flow rate was 1 ml/min, and 1-min fractions were collected and dried in a Speed Vac. The small peptides were pooled and further resolved by sequential rpHPLC, using a Gynkotek 480 G HPLC system (21). Two different columns were used, a wide-pore 5- μ m C18 column (4.6×250 mm; Nucleosil) at a flow-rate of 1 ml/min, and a wide-pore 7- μ m C18 column (2.1×220 mm; Brownlee), at a flow rate of 400 μ l/min. The conditions for the different rpHPLC steps are depicted in Table I. In the first rpHPLC purification step, 1-min fractions were collected, and, in the following rpHPLC steps, 0.5-min fractions were collected. rpHPLC fractions were dried in a Speed Vac, redissolved in 50 μ l of distilled water, and 1- μ l aliquots were used in the dot-immunobinding assay.

Dot-Immunobinding Assay—The assays were performed as described previously (14, 15). In brief, the samples were dot-blotted onto nitrocellulose paper and conjugated to a carrier protein precoated on the nitrocellulose paper. After drying, the nitrocellulose paper was incubated with anti-FMRFa antibodies. These antibodies recognize both the tetrapeptides and the heptapeptides (22). Subsequently, the nitrocellulose was incubated in the secondary antibodies and developed in diaminobenzidine solution containing 0.2% H_2O_2 .

Amino Acid Sequencing—Amino acid sequencing was done on an Applied Biosystems model 473 pulse liquid Sequencer, using the sequencing program recommended by the company.

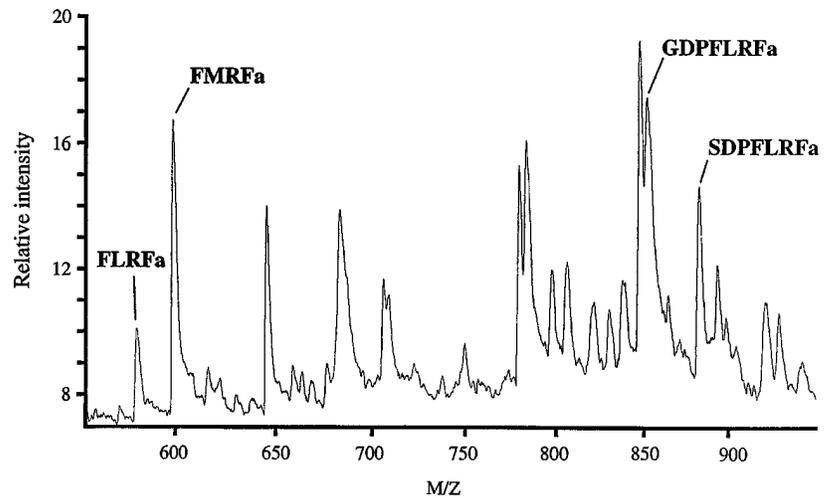
Backfilling and Immunocytochemistry—Central ganglia of the *Lymnaea* brain were dissected in Hepes-buffered saline, and, after removal of connective tissue, the penis nerve was cut and drawn into a glass micropipette using a mild vacuum. The saline in the pipette was then replaced by nickel-lysine (1.79 g of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 3.5 g of L-lysine free base in 20 ml of H_2O), and the preparation was left at room temperature for 18 h. The backfilled neurons were visualized by immersing the brain in 2 ml of fresh Hepes-buffered saline to which 5 drops of a saturated ethanolic rubenic acid solution had been added for 10–20 min. Back-filled neurons displayed a blue to gray color. Then, the tissue was fixed in an ascending series of paraformaldehyde in Tris-buffered saline (1%, 0.5 h; 2%, 0.5 h; 4%, 3–18 h), dehydrated, and embedded in paraffin. Alternating sections were placed onto sets of two slides: one was used for immunocytochemistry, the other was dewaxed in xylene and mounted with a coverslip. For immunocytochemistry, slides were dewaxed, rehydrated, and incubated in Tris-buffered saline:gelatin for 20 min prior to primary antiserum incubation. Sections were incubated in a 1:300 dilution of antibodies against either the synthetic peptide KQQVATDDSGELDDEILSR (DEILSR) or the synthetic peptide SEQPDVD-DYLDRVVLQSEEPLY (SEEPLY) in Tris-buffered saline:gelatin at 4°C for 18 h. Both antibodies were a kind gift of Dr. N. Santama. After washing, the secondary antiserum (peroxidase-conjugated swine anti-rabbit; DAKO, 1:100 in Tris-buffered saline:gelatin) was applied at room temperature for 1 h. Immunoreactivity was visualized as a brown reaction product by addition of 0.5 mg/ml diaminobenzidine, 0.01% H_2O_2 in Tris-buffered saline, after which the slides were dehydrated and mounted with a coverslip. The brain section with the labeled cells as well as the backfilled counterparts in the alternate section were photographed on Kodak EPY 64 T slide film.

Bioassay—The bioassays were performed as described previously (14, 15). In brief, the penis retractor muscle was allowed to relax for 1.5 h before application of the samples. Immediately after the application, the flow of Ringer was stopped for 5 min. The mixing of sample was ensured by continuously bubbling the Ringer with a small stream of gas. Muscle contractions induced by the peptides were measured using an isotonic displacement transducer and recorded by a chart recorder (Kipp & Zonen). The following synthetic peptides were assayed: FMRFa, FLRFa, GDPFLRFa, and SDPFLRFa.

RESULTS

Direct Peptide Profiling of the Penis Nerve by MALDI-MS—Small pieces (~ 0.5 mm) of the penis nerve taken from its origin were ruptured and immediately subjected to MALDI-MS. The peptide patterns from several preparations were identical, and

FIG. 2. MALDI-MS spectrum of the penis nerve. The mass spectrum of a piece of the penis nerve at the origin is shown within the mass range of 550–950 Da. It includes the molecules with masses corresponding to FMRFa (calculated *versus* measured mass, 598.8 Da *versus* 598.3 Da), FLRFa (580.7 Da *versus* 580.4 Da), GDPFLRFa (850.0 Da *versus* 850.0 Da), and SDPFLRFa (880.0 Da *versus* 880.0 Da). *x* axis, *m/z*, mass to charge ratio; *y* axis, arbitrary units.



an example of a mass spectrum is given in Fig. 2. This spectrum revealed that a large number of peptides is present in the nerve and that two of the peptides have masses corresponding to those of FMRFa (calculated *versus* measured mass, 598.8 Da *versus* 598.3 Da) and FLRFa (580.7 Da *versus* 580.4 Da). The peak height of the putative FMRFa peak is several times higher than that of the putative FLRFa peak, suggesting that more FMRFa than FLRFa is present, which agrees with the number of copies, 9 and 2, respectively, in the precursor (*cf.* Fig. 1). Interestingly, two prominent peaks correspond exactly to the masses of GDPFLRFa (calculated mass *versus* measured mass, 850.0 Da *versus* 850.0 Da) and SDPFLRFa (880.0 Da *versus* 880.0 Da) that are represented by 7 and 6 copies, respectively, in the precursor. Again, the signal intensity reflects the relative number of peptides contained in the precursor. These results suggest that, in addition to neurons containing tetrapeptides, also central neurons expressing the alternative GDPFLRFa transcript encoding the heptapeptides innervate the penis complex.

Peptide Purification and Characterization—FMRFa and GDPFLRFa and SDPFLRFa have been previously isolated from the central nervous system of *Lymnaea* (221). Based on our MALDI-MS data, we propose that these peptides are transported from the central nervous system to the penis complex via the penis nerve. To further substantiate this suggestion, we decided to demonstrate unequivocally the presence of the peptides in the penis complex. To this end, 750 penis complexes were extracted and the supernatant was size-fractionated by HPGPC. Fractions 26 to 32 containing molecules <1 kDa were further separated by rpHPLC using trifluoroacetic acid as a counterion (Step 1; Table I). All fractions were dotted to nitrocellulose paper and screened for immunoreactivity by the anti-FMRFa antibodies. Fig. 3A reveals that four groups of immunoreactive fractions were present, which were designated A, B, C, and D. These fractions were resolved separately using HCl as a counterion (Fig. 3, B–E). The resulting immunoreactive fractions from the groups B–D (Fig. 3, C–E) appeared to be pure and were subjected to chemical characterization (Table II), which revealed the presence of FMRFa (group B), FLRFa (group C), and GDPFLRFa and SDPFLRFa which are both contained in the same HPLC fraction (group D). The immunoreactive material from group A was purified further using a third step of rpHPLC with trifluoroacetic acid as a counterion (Step 3, Table I; Fig. 3F). The immunoreactive material was subjected to Edman degradation, which yielded an identical amino acid sequence as found in group B (Table II). However, mass measurement revealed that this peptide is 16 Da heavier than the intact FMRFa. This suggests that the peptide is

oxidized at the methionine residue, presumably during the extraction procedure (23, 24).

Distribution and Identification of Immunopositive Neurons—Because the tetrapeptides FMRFa and FLRFa and the heptapeptides GDPFLRFa and SDPFLRFa are structurally similar, the antibodies raised against FMRFa also recognize the other peptides (see above). To be able to selectively detect the presence of products from each one of the two precursors, antibodies raised against the peptides SEEPLY and DEILSR were used that are contained by the FMRFa precursor and the GDPFLRFa precursor, respectively (see Fig. 1). In previous experiments (18, 19), the specificity of these antibodies has been rigorously tested: anti-SEEPLY antibodies recognize only neurons in the nervous system that contain the peptides encoded by the FMRFa transcript, whereas anti-DEILSR antibodies specifically recognize neurons that contain the peptides encoded by the GDPFLRFa transcript. DEILSR immunoreactive fibers could be detected in the penis nerve and the penis complex. No immunoreactive cell bodies could be detected in the penis complex (data not shown). As previous studies did not give any cue of the identities of the central penis motoneurons that express the GDPFLRFa transcript, we decided to re-examine this issue. Backfilling of the penis nerve combined with immunostaining revealed that there are several neurons in the right pleural and parietal ganglia that send their axons to the penis complex (Fig. 4, A and C) and that are immunopositive with the DEILSR antibodies (Fig. 4, B and D). These cells therefore form the origin of the heptapeptides present in the penis nerve and penis complex. The immunoreactive penis motoneurons in the right parietal ganglion appear to be part of the identified cluster of B cells (8). The antibodies against SEEPLY immunostained a number of neurons in the right cerebral ventral lobe (see below). In accordance with the innervation pattern of these neurons (13), immunoreactive fibers were found in the penis nerve and throughout the penis complex. In addition, backfilling of the penis nerve with nickel lysine followed by staining with antibodies against SEEPLY confirmed that the immunopositive anti-SEEPLY neurons of the right cerebral ventral lobe indeed project down the penis nerve (Fig. 4E).

Determination of the Effects of Tetrapeptides and Heptapeptides on the Penis Retractor Muscle—The effects of application of various concentrations of the synthetic peptides FMRFa, FLRFa, GDPFLRFa, and SDPFLRFa were examined using the penis retractor muscle as an *in vitro* bioassay preparation. When tetrapeptides were applied, the muscle exhibited a fast contraction reaching a plateau within seconds, then returned to basal levels during extensive washing (Fig. 5, A and B).

TABLE I
rpHPLC steps for the purification of FMRFa-amide-related peptides

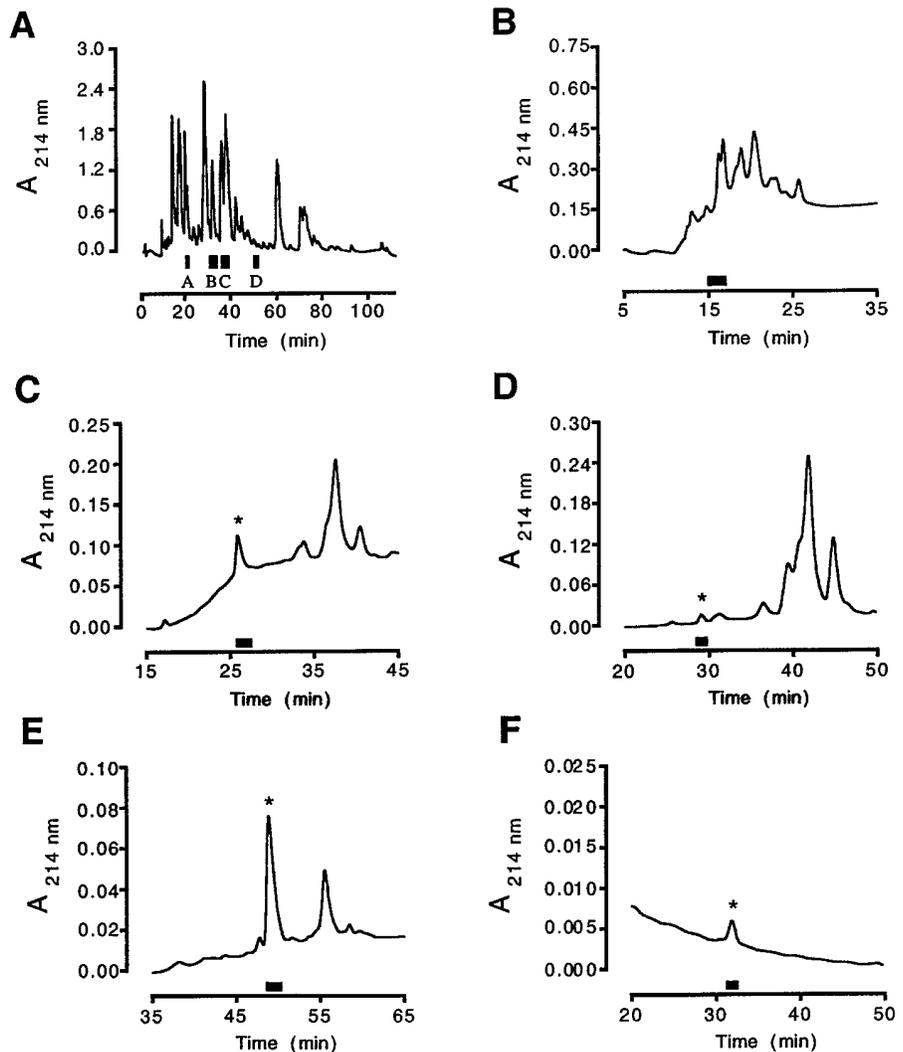
The abbreviations used are: ACN, acetonitrile; HCl, hydrochloric acid; TFA, trifluoroacetic acid.

Step	Column ^a	Solvent A	Solvent B	Gradient ^b	
1	C18 Nucleosil	7.0 mM TFA	7.0 mM TFA in 60% ACN	%	min
				0–20	10
				20–60	80
2	C18 Brownlee	0.05% HCl	0.05% HCl in 60% ACN	0–5	5
				5–40	70
				40–100	10
3	C18 Brownlee	7.0 mM TFA	7.0 mM TFA in 60% ACN	0–5	5
				5–30	80
				30–100	10

^a Two rpHPLC columns were used; the 5- μ m C18 (4.6 \times 250 mm; Nucleosil) and the 7- μ m C18 (2.1 \times 220; Brownlee)

^b Indicated is the linear increase of Solvent B (%) in time (min).

FIG. 3. Purification of FMRFa-related peptides from the penis complex. After HPGPC, FMRFa-related peptides were purified using three subsequent rpHPLC steps. Fractions were screened using anti-FMRFa antibodies. The immunoreactivity is indicated by bars. *A*, HPGPC fractions containing small molecules were further purified by rpHPLC using a 5- μ m C18 column (see Step 1, Table I). *B*, the fractions from group A (see *A*) were purified further by rpHPLC using a 7- μ m C18 column (see Step 2, Table I). *C*, the fractions from group B (see *A*) were purified further by rpHPLC using a 7- μ m C18 column (see Step 2, Table I). The fractions corresponding to the peak indicated by the asterisk were used for chemical characterization. *D*, the fractions from group C (see *A*) were purified further by rpHPLC using a 7- μ m C18 column (see Step 2, Table I). The fractions corresponding to the peak indicated by the asterisk were used for chemical characterization. *E*, the fractions from group D (see *A*) were purified further by rpHPLC using a 7- μ m C18 column (see Step 2, Table I). The fractions corresponding to the peak indicated by the asterisk were used for chemical characterization. *F*, the immunoreactive fractions from group A after a second rpHPLC step (see *B*) were purified further by rpHPLC using a 7- μ m C18 column (see Step 3, Table I). The fractions corresponding to the peak indicated by the asterisk were used for chemical characterization.



GDPFLRFa induced a slow relaxing effect at 3×10^{-7} M, which was often not fully reversible after extensive washing (Fig. 5C). At a high concentration of 10^{-5} M, however, GDPFLRFa induced a small tonic contraction, which was often followed by relaxation (Fig. 5D). The effect of SDPFLRFa was similar to that of GDPFLRFa. The dose-response curves of FMRFa and FLRFa were similar (Fig. 6), with a threshold dose for both peptides at 3×10^{-8} M, and a maximum effect at 3×10^{-7} M. The EC_{50} values are 1.07×10^{-7} M and 1.10×10^{-7} M for FMRFa and FLRFa, respectively. At a high concentration of 10^{-5} M, however, FLRFa induced the muscle to contract at a

lower level of about 75% of the maximum level. The dose-response relationships of GDPFLRFa and SDPFLRFa were profoundly different from those of FMRFa and FLRFa. GDPFLRFa showed a biphasic curve with a minimum effective dose of 10^{-7} M that induced a relaxing effect. This relaxing effect increased with higher concentrations, but at 10^{-5} M showed a (minor) contractile effect. The effect of SDPFLRFa was similar to that of GDPFLRFa: at lower concentrations (3×10^{-8} – 3×10^{-7} M) SDPFLRFa induced a small relaxing effect, and at higher concentrations (10^{-6} – 10^{-5} M) a small tonic contraction (Fig. 6).

TABLE II
Automated Edman degradation of the FMRFa-amide-related peptides purified from the penis complex

Cycle number	Group A		Group B		Group C		Group D	
	PTH-derivative	Yield	PTH-derivative	Yield	PTH-derivative	Yield	PTH-derivative	Yield
		<i>pmol</i>		<i>pmol</i>		<i>pmol</i>		<i>pmol</i>
1	Phe	122	Phe	924	Phe	166	Gly	774
2	Met	86	Met	719	Leu	96	Ser	113
3	Arg	12	Arg	441	Arg	65	Asp	860
4	Phe	54	Phe	69	Phe	14	Pro	741
5							Phe	921
6							Leu	694
7							Arg	418
							Phe	178

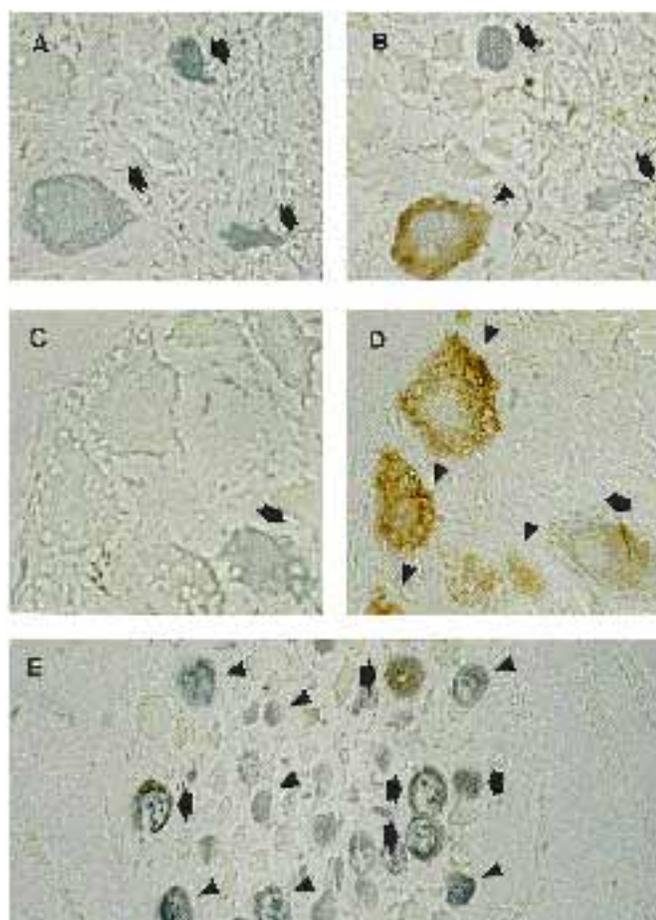


FIG. 4. Identification of central neurons involved in male copulation behavior by a combination of backfills and immunocytochemistry. Neurons backfilled via the penis nerve are recognized by their blue/gray color; the immunoreactive neurons are brown, whereas neurons that are stained by both nickel lysine and antibodies have an intermediate color. A, three backfilled neurons (arrows) in the right parietal ganglion are shown. B, on the alternate section of A, one backfilled neuron (arrowhead) was also immunoreactive to the anti-DEILSR antibodies, whereas the others were not (arrows). The location and the immunoreactivity of the cell suggest that it is a member of the B group cells. C, here, only one (arrow) of several backfilled neurons present in the right pleural ganglion is shown. D, on the alternate section of C, the backfilled neuron appeared immunoreactive also after staining with anti-DEILSR antibodies (arrow). Other immunoreactive neurons are also present (arrowheads). E, by applying anti-SEEPLY antibodies to a cross-section of the ventral lobe of the right cerebral ganglion, it could be confirmed that a number of the backfilled neurons contain peptides derived from the FMRFa precursor, which are double-labeled with blue/gray and brown (arrows). Non-immunoreactive back-filled neurons are also present (arrowheads).

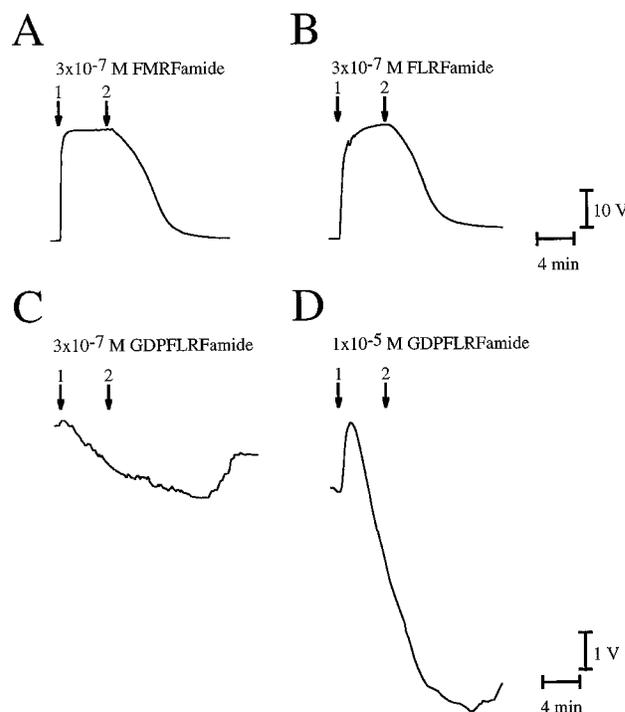


FIG. 5. Physiological effects of the tetra- and heptapeptides on the penis retractor muscle *in vitro*. At the first arrow, the peptides were applied and the flow of Ringer was stopped, and, at the second arrow, the flow of Ringer was started again. A, effect of 3×10^{-7} M FMRFa on the penis retractor muscle. B, effect of 3×10^{-7} M FLRFa on the penis retractor muscle. C, effect of 3×10^{-7} M GDPFLRFa on the penis retractor muscle. D, effect of 10^{-5} M GDPFLRFa on the penis retractor muscle.

DISCUSSION

By using direct MALDI-MS determinations of peptides in the penis nerve we were able to gain insight into the diversity of candidate peptides that may be involved in the control of male copulation in *Lymnaea*. We focused on two sets of closely related peptides that belong to the FMRFa family and that are derived from the alternatively spliced transcripts of the FMRFa gene. We hypothesized that these two sets of peptides are synthesized by different central neurons to coordinate the complex and flexible copulation behavior of *Lymnaea* (12, 13). In the following we will present arguments that lead to the conclusions that the sets of tetra- and heptapeptides encoded by the alternative FMRFa and GDPFLRFa transcripts, respectively, indeed are expressed in a mutually exclusive way in different penis motoneurons and, in addition, have different effects on their peripheral targets, *i.e.* the penis retractor muscles. The outcome of these experiments demonstrates a novel and attractive principle of molecular and cellular regulation of different aspects of a complex behavior by one peptide gene.

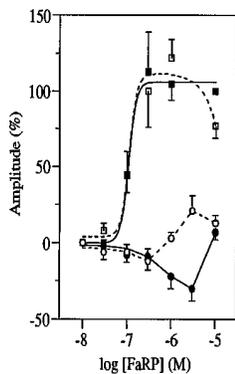


FIG. 6. Dose-response relationships of tetra- and heptapeptides on the penis retractor muscle. In each experiment, the effects of the peptides are indicated relative to the effect of 10^{-5} M FMRFa, which is set at 100%. Each data point indicates the average of four independent experiments. Closed squares, FMRFa; open squares, FLRFa; closed circles, GDPFLRFa; open circles, SDPFLRFa.

More generally, this finding may have important implications for theories and models of the control of behavior and physiological processes by bioactive peptides.

Tetrapeptides and Heptapeptides Derived from Two Alternatively Spliced Transcripts of the FMRFa Gene Are Neuropeptides Involved in the Control of Penis Functions—Neuropeptides are an important class of messengers used by the nervous system for cell-to-cell communication. Generally, their characterization is a first step toward the understanding of the molecular basis of the functioning of a neuronal circuit that governs a particular behavior (25). Here we focus on the peptides that are contained by the penis nerve. Because the penis nerve is the sole nerve that relates the neuronal information from the central ganglia to the penis complex and vice versa, the peptides present in this nerve must be involved in copulation. As conventional methodology lacks the ability to envision the whole peptide profile present in a given tissue, we applied the novel MALDI-MS method to a single penis nerve and demonstrated that multiple peptides are contained by the nerve. We then focused on two sets of neuropeptides, the tetrapeptides and heptapeptides of the *Lymnaea* FMRFa family of bioactive peptides. Previous peptide chemical (22) and recombinant DNA studies (17–19, 26) have revealed the presence of these peptides in the *Lymnaea* nervous system. Our mass spectrometric data strongly suggest that the peptides are also present in the penis nerve because, firstly, the measured masses of the peptides are in full agreement with the calculated masses of the peptides, and, secondly, the ratios of the tetrapeptides FMRFa and FLRFa and the heptapeptides GDPFLRFa and SDPFLRFa as determined in the mass spectra also reflect the molar ratios that are predicted based on the cDNA cloning studies. It follows then that the peptides must be present in the penis complex, which was confirmed by their purification from this organ.

Tetrapeptides and Heptapeptides Are Each Contained in Different Central Penis Motoneurons—We postulated that the tetrapeptides and heptapeptides are synthesized by central neurons and transported to the penis complex. Indeed, our immunocytochemical studies detect only immunoreactive fibers but not immunoreactive cells in the penis complex. By backfilling the penis nerve, we were able to localize the penis motoneurons, and, in combination with immunocytochemistry using highly specific antibodies, we could unequivocally show that tetrapeptides and heptapeptides are synthesized in a mutually exclusive fashion in different penis motoneurons. The penis motoneurons containing the tetrapeptides could be mapped to a subset of neurons in the right cerebral ventral lobe, a cellular location which has been proposed previously

(18). More intriguing, however, is the finding that heptapeptides are also present in the penis nerve and penis complex. The loci of synthesis of these peptides have as yet not been described. However, we demonstrate here that the penis motoneurons that contain the heptapeptides are the “scattered” neurons in the right pleural and parietal ganglia. The location and phenotype of the neurons in the right parietal ganglia strongly suggest that they constitute a subset of the previously described group of B neurons (8). Thus, our results show that the B neurons do not form a homogeneous group, instead they are neurons with different physiological (and behavioral) effects.

Tetrapeptides and Heptapeptides Have Distinct Effects on the Penis Retractor Muscle—The tetrapeptides FMRFa and FLRFa induce very similar fast contractions with typical plateau characteristics when applied to the penis retractor muscle suspended *in vitro*. The EC_{50} values obtained in the dose-response experiments indicate that both peptides bind to the receptors with very similar affinities. However, the dose-response studies indicate also that at higher concentrations FLRFa, but not FMRFa, elicits submaximal contractions. Because FLRFa and the heptapeptides SDPFLRFa and GDPFLRFa share the same carboxyl-terminal sequence (*i.e.* FLRFa), and because the heptapeptides have a relaxing effect on the penis retractor muscle (see below), this observation can be explained by assuming that at higher concentrations FLRFa binds also to the heptapeptide receptors.

Interestingly, at concentrations up to 10^{-6} M, the main effects of GDPFLRFa and SDPFLRFa are opposite to those of FMRFa and FLRFa. At these concentrations, the heptapeptides induce a similar slow relaxation of the penis retractor muscle. However, at higher concentrations, they induce a small contraction, suggesting that at higher concentrations the heptapeptides may in addition activate the tetrapeptide receptors. In conclusion, our results show that at physiological concentrations the tetrapeptides and heptapeptides have opposite effects on the penis retractor muscle.

Multiple Peptides Are Involved in the Modulation of Penis Complex Activities—Copulation as a male in *Lymnaea* involves a series of intricate movements of the penis complex, *i.e.* extrusion of the preputium and penis, probing for the vagina, intromission, and transfer of semen followed by retraction. It is apparent that the activities of the various parts of the penis complex need to be accurately coordinated. Moreover, in case of improper intromission, the behavior needs to be (partially) resumed (12, 13). These activities therefore call for extensive fine-tuning of the activities of muscles in order to generate the appropriate sequence of events underlying copulation. The present (see *e.g.* Fig. 2) and previous experiments (10, 14–16) suggest that a multitude of neuropeptides is involved in the control of the penis complex in *Lymnaea*. A number of these peptides has been structurally characterized, and their effects on individual parts of the penis complex have been examined in detail. These studies have revealed various different molecular and cellular strategies that underlie the peptidergic control of copulation behavior. Thus, conopressin and APGWa have opposite effects on the activity of the vas deferens (14), whereas different isoforms of the myomodulin family of peptides have overlapping yet distinct effects on the penis retractor muscle (15). Conopressin (27) and APGWa (11) are encoded by distinct genes that are expressed in penis motoneurons in the right cerebral anterior lobe, whereas the myomodulin isoforms (15) are encoded by a single gene that is expressed in motoneurons of the ventral lobe. The present study demonstrates another important principle of peptidergic control of a complex and flexible behavior by a single peptide gene: mutually exclusive

cellular expression of distinct sets of peptides encoded by the FMRFa gene that are all structurally related yet have antagonistic actions. Thus, in the context of the copulation behavior of *Lymnaea*, this ingenious molecular and cellular strategy allows for a finely attuned control of the same target, the penis retractor muscles, by distinct penis motoneurons that in principle can operate independently.

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Mutually Exclusive Neuronal Expression of Peptides Encoded By the FMRFa Gene Underlies a Differential Control of Copulation in *Lymnaea*

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