MOLECULAR ANALYSIS OF A NOVEL NEUROPEPTIDE GENE: IMPLICATIONS FOR INVERTEBRATE BEHAVIOR

by

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A THESIS

Presented to the Department of Biology
and the Honors College of the University of Oregon
in partial fulfillment of the requirements
for the degree of Bachelor of Arts

June 1998

APPROVED: [redacted]

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An Abstract of the Thesis of

Patrick Erin Davol for the degree of Bachelor of Arts

in the Department of Biology to be taken June 1998

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Approved: [redacted]

Dr. Nathan Tublitz

The European cuttlefish *Sepia officinalis* is capable of rapid changes in body coloration, and can display complex body patterns associated with such behaviors as mating, camouflage, and self-defense. These changes in body coloration are regulated by members of the FMRFamide-related peptide family of neuropeptides (FaRPs). Previous work identified the presence of a FaRP gene in cuttlefish whose peptide products were found to cause chromatophore expansion *in vitro*. Furthermore, the mRNA for this FaRP gene was found to be expressed in the cell bodies of putative chromatophore motoneurons. However, some cells in the region of the cuttlefish brain believed to control chromatophore function were found to exhibit immunoreactivity with a FMRFamide antibody, but were not found to express the FaRP mRNA. This study reports the presence of a novel FaRP gene in cuttlefish, which encodes the peptides GNLFRFamide, NSLFRFamide, TIFRFamide, and a 24 residue-long peptide with no homology to FaRPs. The hexapeptides were found to affect chromatophore expansion *in vitro*, and were also found to react with the FMRFamide antibody. Thus, these two peptides are good candidates for the FaRPs believed to be expressed in the cell

bodies of the cuttlefish brain, and are therefore potential endogenous regulators of body patterning.

ACKNOWLEDGMENTS

The author expresses sincere appreciation to Professor Nathan Tublitz and Dr. Poh Kheng Loi for their support, patience, and insights over the past year. Their attitudes toward self-directed learning were greatly appreciated, as were their good-natured dispositions. I also thank the other members of the Tublitz lab for their camaraderie and support. I express gratitude to Professor Henry Alley for his assistance in the preparation of this manuscript. In addition, special thanks are due to various members of the Sprague, Postlethwait, and Stevens lab for their technical assistance on the molecular aspect of this project.

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INTRODUCTION

The neural basis of behavior, how the nervous system controls behavior, is a topic investigated at a number of different levels, from the molecular to physiological. The study of variations of a single behavior provides an opportunity to learn much about how a nervous system controls behavior because the variability provides a means by which researchers can assay the roles of controlling factors in the system.

Having the most highly developed nervous system of any of the invertebrates, cephalopods exhibit remarkably variable and complex behavior (Ruppert and Barnes, 1994). A class of the phylum Mollusca, Cephalopoda contains the nautili, cuttlefish, squids, and octopods. The latter three orders are capable of effecting changes in body coloration. These often rapid changes are associated with such diverse behaviors as camouflage (Fig. 1), mating (Fig. 2), and self-defense (Holmes, 1940).

At the physiological level, it is the chromatophore system that allows for such changes in body coloring. The fundamental coloration unit, the chromatophore, is a small organ ubiquitous in the dermal layer of the color-changing cephalopods. Each organ consists of a pigment cell, to which is attached several tiny chromatophore muscles innervated by neurons (Fig. 3). Contraction of the chromatophore muscles cause the pigment cell to expand, resulting in the heightened appearance of pigment. Recent experiments have demonstrated that each chromatophore muscle is innervated by 2-6 nerve axons, although it is not known whether the axons share the same cell body or multiple cell bodies

Fig. 1. The European cuttlefish *Sepia Officinalis* displaying a camouflage pattern (photo courtesy of John Forsythe).

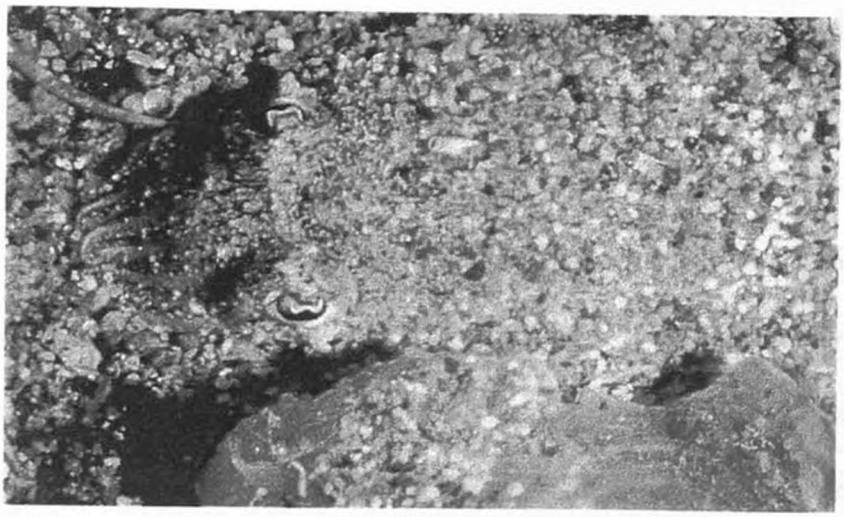
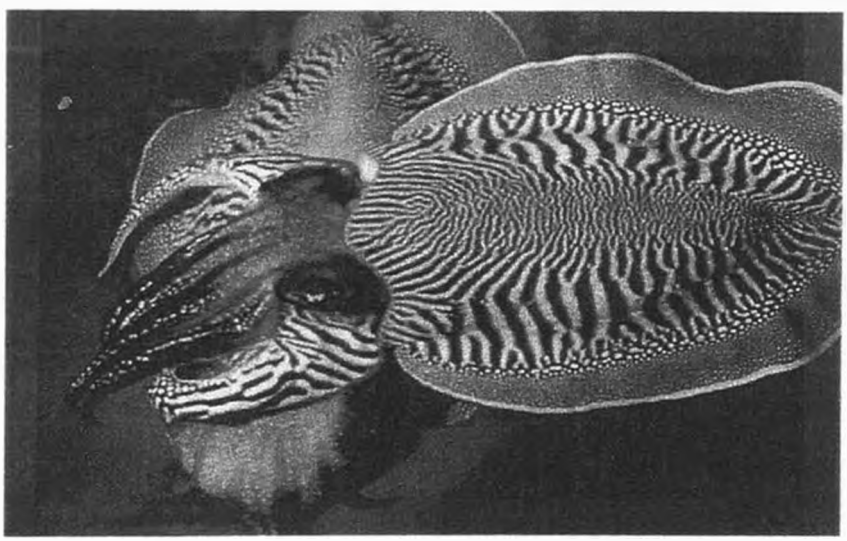


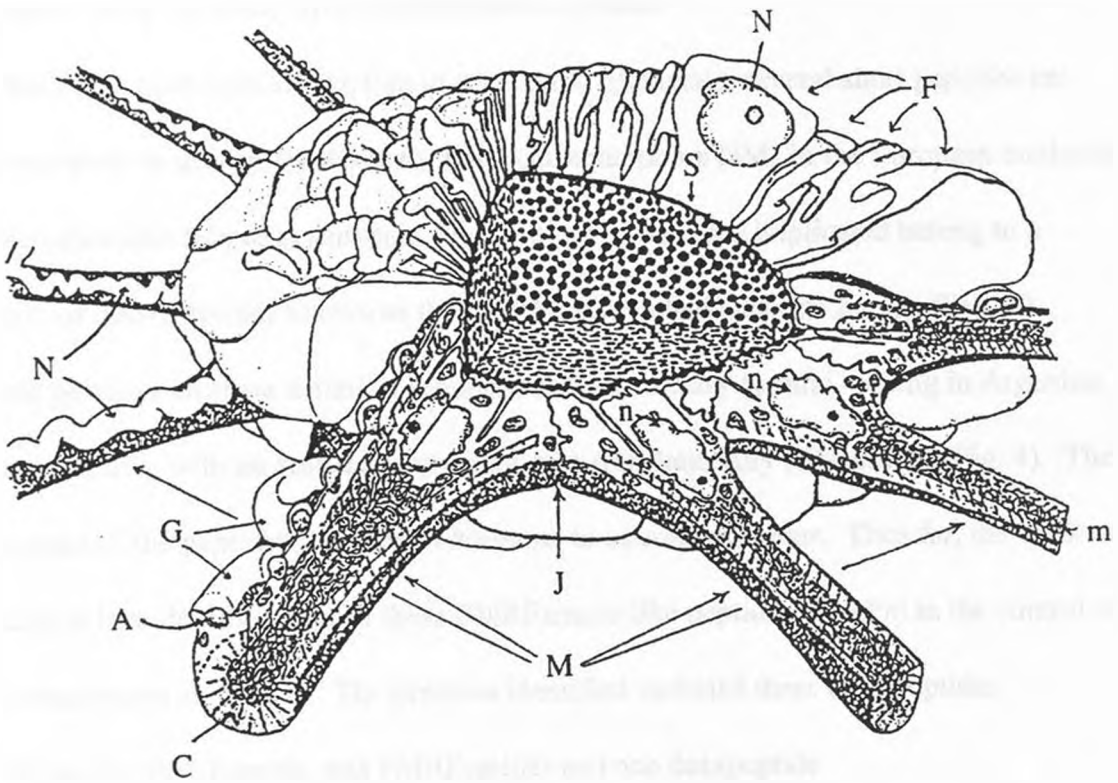
Fig. 2. Two cuttlefish displaying mating-associated body patterning (photo courtesy of John Forsythe).



(Messenger *et al*, 1997). The cell bodies of these terminals are believed to reside in the anterior and posterior chromatophore lobes of the cephalopod brain (Dubas *et al*, 1985; Boycott, 1961). The fact that chromatophores are under direct neuro-muscular control allows for the rapidity of color changing (Florey, 1966; Cloney and Florey, 1968; Florey and Kriebel, 1969; Reed, 1995). By transmitting signals to the muscles attached to the pigment cell, the chromatophore neurons mediate contraction of the chromatophore muscles and expansion of the pigment cell, and thus cause a change in body coloration.

How do chromatophore neurons signal the chromatophore muscles to contract and change body coloration? It has been known for some time that *L-glutamate* causes rapid expansion of chromatophores *in vitro* (Florey *et al* 1985; Cornwell and Messenger, 1995). However, only recently has it been demonstrated that L-glutamate is endogenous in the nerves innervating the chromatophore muscles of squid, suggesting that L-glutamate is indeed a true excitatory transmitter at the chromatophore *neuromuscular junction* (NMJ) (Messenger *et al*, 1997). Furthermore, serotonin has also been shown to be endogenous in some chromatophore motoneurons, where it putatively acts to aid in the relaxation of the chromatophore muscles (Messenger *et al*, 1997). Thus, the chromatophore system of squid uses at least two different neurotransmitters to control its body coloring, allowing for nuance in the control of color patterning.

Fig. 3. A diagramatic sketch of a cephalopod chromatophore. A retracted chromatophore with A, axon; C, contractile cortex of muscle fiber; F, folds of cell membrane of chromatophore; G, glial cell; N, nerve terminals; n, nucleus of muscle cell; M, muscle fibers; m, mitochondia; J, junction between adjacent muscle fibers; S, elastic sacculus. The sheath cells that cover the chromatophore and the muscle fibers are not shown (from Loi et al, 1996).



While squid are capable of rapid changes in body coloration, and are capable of producing different color patterns, the repertoire of body patterns employed by cuttlefish is far more complex. Not only are cuttlefish capable of creating the transient patterns employed by squid; they are capable of creating any number of complex body patterns which can be held for hours at a time. How is this remarkable difference in behavior achieved using the same basic chromatophore system?

Recent studies have shown that in addition to glutamate, several short peptides are also putative excitatory transmitters at the chromatophore NMJ in the European cuttlefish *Sepia officinalis* (*Sepia* is shown in Figs. 1-2). The peptides implicated belong to a family of neuropeptides known as the FMRFamide-related peptide family (FaRPs). These peptides all share a similar sequence at their carboxy termini, ending in Argenine, Phenylalanine, with an amide group added post-translationally (-RFamide) (Fig. 4). The functions of the peptides range from *hormone* to *neurotransmitter*. Thus far, the work on cuttlefish has identified four of these FMRFamide-like peptides (FaRPs) in the control of chromatophore expansion. The peptides identified included three tetra peptides (FIRFamide, FLRFamide, and FMRFamide) and one decapeptide (ALSGDAFLRFamide) (Loi and Tublitz, 1997). All four of these peptides are coded for on a single mRNA and thus are products from a single gene. Several lines of evidence, including immunocytochemical studies, bioassays, as well as a molecular analysis provide convincing evidence that these peptides are indeed used by cuttlefish to effect chromatophore expansion (Loi *et al.* 1996; Loi and Tublitz, 1997). Recent *in situ*

hybridization experiments have shown definitively that these compounds are expressed in some of the neurons in the posterior chromatophore lobe, further supporting the hypothesis that cuttlefish use these compounds to produce chromatophore expansion (Loi, personal communication).

Interestingly, when FMRFamide was tested on the skin of the squid *Alloteuthis subulata* and *Loligo vulgaris*, no chromatophore expansion was observed (Messenger *et al*, 1997). A likely explanation for these results stems from the behavioral differences between cuttlefish and squid. As mentioned previously, in addition to using quick-changing coloration patterns, cuttlefish are capable of creating highly complex color patterns that can be held for hours at a time. Squid, on the other hand, have a much

Fig. 4. The FMRFamide-Related Peptide Family (from P.K. Loi, personal communication).

F M R Famide
Phe Met Arg Phe

FMRFamide Related Peptides (FaRPs)

1. FMRFamide
2. N-terminally extended FMRFamide e.g. PDNFMRFamide
3. FLRFamide
4. N-terminally extended LRFamide e.g. TFLRFamide, AVPGVLRFamide
5. N-terminally extended FRFamide e.g. TIFRFamide, GSLFRFamide
6. N-terminally extended IRFamide e.g. FIRFamide, AGPRFIRFamide
7. Some N-terminally extended RIamide e.g. EFLRIamide, LSSFVRIamide

simpler battery of body patterns, and often leave their chromatophores retracted as a means of becoming transparent (Hanlon and Messenger, 1996). Thus, a reasonable

hypothesis is that FMRFamide and other FaRPs are acting in cuttlefish to elicit the tonic muscle contractions that allow for sustained body patterning. This is remarkable because it suggests that a single population of neurons (the chromatophore motoneurons) utilize multiple neurotransmitters, providing the nuance necessary to turn this structurally fixed system into one capable of expressing a wide variety of complex behaviors.

Furthermore, variability in transmitter phenotype is likely even more complicated: FaRPs other than those identified by Loi may be playing a role in the control of chromatophore activity. Double-staining experiments, in which chromatophore motoneurons were stained with both an *antibody* recognizing the general FaRP motif (-RFamide) and an *in situ* probe specific for the *mRNA* transcript coding for the previously-identified FaRPs, revealed cells that stained for the antibody but not the *in situ* probe (Loi, personal communication). This strongly suggests that other FaRPs are expressed in the cell bodies of the chromatophore motoneurons, and therefore might be playing a role in the control of chromatophore expansion.

Indeed, it is likely that the cuttlefish genome contains another FaRP gene based on molecular data from squid. A partial cDNA that encodes several FaRPs different than those identified in *Sepia* has been isolated from the squid *Loligo opalescens* (David Price, personal communication). This partial cDNA codes for at least two hexapeptides, GNLFRFamide and NSLFRFamide, and a pentapeptide, TIFRFamide (interestingly, the function of these peptides in squid is not known) (D. A. Price, unpublished). These data raise the possibility that cuttlefish have another FaRP cDNA encoding other FaRPs.

To determine which (if any) other FaRPs are involved in the regulation of chromatophore expansion, the following study was conducted to first isolate a new FaRP cDNA from cuttlefish and then determine a possible role for the encoded peptides in chromatophore activity. The findings from this investigation include the cloning and sequencing of a novel FaRP cDNA which contains coding regions for the hexapeptides GNLFRF and NSLFRF, the pentapeptide TIFRF, and a 24 residue-long peptide with no homology to FaRPs. The hexapeptides were found to effect chromatophore expansion when tested *in vitro*, and were immunoreactive in a dot-blot assay against a FMRFamide antibody.

EXPLANATION OF MATERIALS AND METHODS

cDNA Libraries

This study involves the use of a cDNA library, a population of *plasmids* containing cDNA copies of the mRNA present in a given tissue. Such libraries are constructed by isolating mRNA from the cells of interest (in this case from the brain cells of *Sepia*), turning the mRNA into DNA using a special enzyme called *reverse transcriptase*, and then inserting these DNA fragments into plasmids (Fig. 5). The plasmids are put into host phage, allowing for ease of amplification and manipulation. In this study, a cDNA library is screened for FaRP-coding sequences. Because cDNA is mRNA in DNA form, it represents actual coding sequences, and is thus useful for determining what genes are being expressed in a given tissue. Therefore, the discovery of a FaRP cDNA in a library constructed from brain cells indicates that the FaRP gene is being expressed in those cells.

Polymerase Chain Reaction (PCR)

The cDNA library used in this study was initially screened using the polymerase chain reaction (PCR). PCR is an extremely powerful tool of molecular biology because it provides a means of obtaining vast quantities of DNA from a relatively small starting

quantity if some sequence information is known. Combined as reactants in PCR are template DNA (the DNA to be amplified), short DNA sequences called *primers* (which are complementary to each strand of DNA flanking the region to be amplified), a heat-stable *DNA polymerase*, free nucleotides (dNTPs), buffer, salt, and water. The reactants are run through tightly controlled heating and cooling cycles, which leads to the geometric amplification of the target sequence (Fig 6).

Fig. 5. Construction of cDNA. A complementary DNA is produced from mRNA using reverse transcriptase. A short oligonucleotide primer annealed to the 3' end of the mRNA facilitates this synthesis. The DNA/RNA helix is treated with alkali, which degrades only the mRNA. The remaining single-stranded cDNA is made into double-stranded DNA using a DNA polymerase (adapted from Alberts *et al*, 1994).

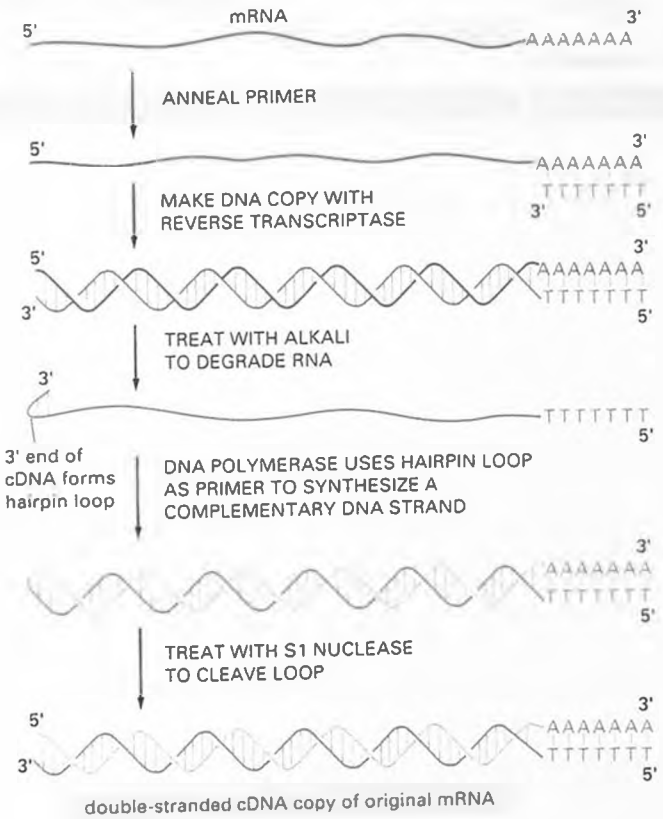
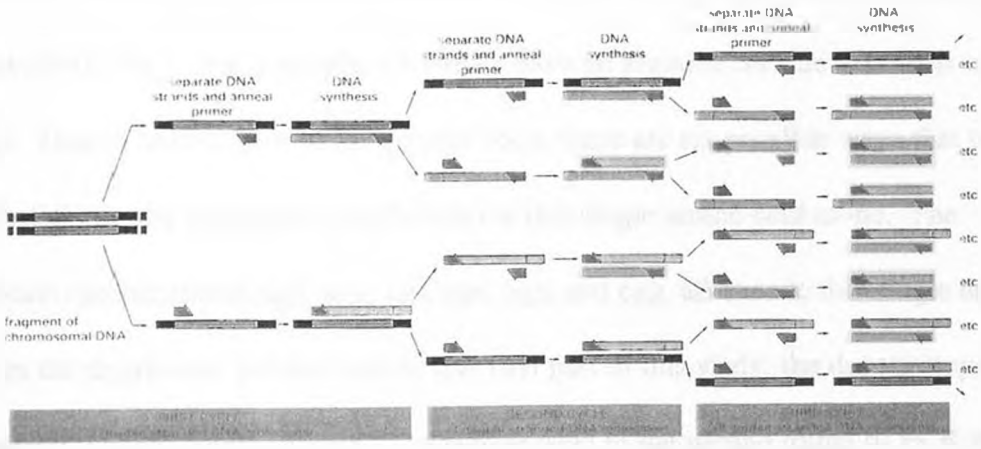


Fig. 6. The Polymerase Chain Reaction. With each cycle of denaturing, annealing, and elongating, the number of target-length strands doubles (from Alberts *et al*, 1994).



PCR Primers

This study uses primers complementary to FaRP sequences of interest. In theory, using PCR primers coding for FaRPs results in the specific amplification of FaRP DNA only. The primer initially used is degenerate, that is it includes all of the different nucleotide combinations that the cDNA could potentially use to encode the FaRP of interest (GNLFRF). For example, all FaRPs have an arginine residue near their carboxy termini. Due to redundancy in the genetic code, there are six possible ways that the RNA in cuttlefish (or any organism) could code for this single amino acid alone. The nucleotide combinations, aga, agg, cgt, cga, cgc, and cgg, all encode this single amino acid. In the degenerate primer used in this first part of this study, the degeneracy runs on the order of 16,000 combinations. The primer used in the second round of PCR was constructed to recognize a region of sequence obtained from the first screen, and is therefore exactly complementary.

Isolation of Amplified DNA

To isolate amplified DNA, PCR products are run on polyacrylamide or agarose gels that separate DNA fragments according to size, a procedure called gel electrophoresis. DNA is loaded into one end of a gel, and an electric field is applied. DNA, being negatively charged due to its phosphate backbone, migrates to the positive end of the

field. Fragments of different sizes migrate at different speeds, and after a given time the smallest fragments will be farthest from the origin. The gel is then bathed in ethidium bromide (or ethidium bromide is added to the gel mixture before electrophoresis), a substance that binds DNA and fluoresces under ultraviolet light. This provides a means by which to visualize and remove specific PCR products from the gel.

Sub-Cloning and Transformation

Once the PCR product of interest is isolated and purified, it can be sequenced. For storage and amplification purposes, it is convenient to first sub-clone the product into a different expression plasmid. The plasmid can be amplified and purified in the same manner as the cDNA plasmids. The process of sub-cloning is easy to do when DNA is isolated from a low-melting agarose gel. The gel slice can be added directly to a special cloning *vector* with insert sites specifically tailored to accept PCR products. Once products are inserted into new vectors, the vectors are put into bacterial cells that have been treated so as to be especially prone to infection. The infected cells are then grown in a medium conducive to growth and allowed to divide, amplifying the plasmid DNA as they do so. Bacterial cells are plated onto a special medium containing antibiotics lethal to wild-type bacteria. Only those bacteria infected with the cloning plasmids survive because the plasmids contain genes which confer resistance to the antibiotics. To determine which bacterial colonies contain the transformed vector (and not one without

the PCR product insert), the PCR products are inserted into a region of the vector which normally codes for the enzyme beta-galactosidase. The antibiotic medium onto which the cells are plated also contains a substance called X-Gal that turns blue when cleaved by beta-galactosidase. Therefore, colonies of cells in which the transformation was unsuccessful (i.e. no DNA inserted to interrupt the enzyme-coding region) will turn blue, while colonies in which the transformation reaction was successful will remain white because they cannot produce the enzyme to cleave X-Gal. Individual white colonies are selected and again put in a medium conducive to growth. After growth, the cells are lysed, and the plasmid DNA isolated and purified. The purified DNA can then sequenced.

DNA Sequencing

DNA sequencing is now an automated process. The in-house sequencing facility at the University of Oregon has a fluorescence sequencer, allowing for convenient and accurate DNA sequencing. This sequencer carries out a single PCR reaction using as a template the DNA to be sequenced. In addition to dNTPs, a relatively small amount of special nucleotides are used. These nucleotides share the property of stopping chain elongation when incorporated into the growing strand by a DNA polymerase, and in addition are covalently linked to a fluorescent dye, a differently colored dye for each nucleotide. The products of this PCR are then run on a gel. The sequence is determined

as a sensor detects the color of each band of DNA eluted from the bottom of the gel. For example, if A happened to be the first nucleotide in the sequence, the shortest PCR product would end in A and reach the sensor first. The A-specific label would be read by the sensor, indicating that the product did indeed end in A.

Chromatophore Bioassay

The sequence information was used to determine which peptides had the potential to be transmitters at the chromatophore NMJ. To test whether or not the encoded peptides could cause chromatophore expansion, a bioassay was conducted. This bioassay consisted of applying various concentrations of synthetic peptides to a piece of cuttlefish fin from which the muscle and dermal layers (the layers above and below the chromatophore layer) had been removed. The skin was placed into a chamber mounted on a microscope. An instrument (called a photodiode) capable of detecting light intensity was placed between the light source and the optics. The photodiode was used to quantify the expansion of chromatophores observed in the assay. When chromatophores expanded, the amount of light passing through the sample decreased, this difference in light intensity being detected by the photodiode. The signal obtained from the photodiode was recorded on a chart to give a visual record of the assay.

Dot-Blot Immunoassay

Previous work demonstrated that neurons in the chromatophore lobes and neurons surrounding the chromatophores are reactive against a FMRFamide antibody, and therefore contain FaRPs. To determine whether or not the hexapeptides identified in this study could have been detected in the earlier work, they were tested for immunoreactivity with the FMRFamide antibody. The test is relatively straightforward: various concentrations of the two peptides were applied to a membrane and fixed. The samples were then treated with the FaRP antibody, which binds anything that looks like a FaRP (ends with –RFamide). A secondary antibody, which binds primary antibody, was then added. Next, a compound with a *peroxidase* was added, the peroxidase binding to a compound attached to the secondary antibody. Finally, the membrane was placed in substrate, a substance cleaved by peroxidase to yield a blue-colored dye. Thus, spots on the membrane containing FaRPs recognized by the primary antibody turned blue after completion of the assay, and were said to be recognized by the antibody. The intensity of the blue spot indicated how well the primary antibody bound to the test substance.

MATERIALS AND METHODS

PCR Screening of a Sepia officinalis *cDNA Library*

First Screen

A lambda zap cDNA library constructed from *Sepia* brain cells was screened with PCR using a degenerate oligonucleotide primer obtained from GibcoBRL, and the vector primer M13 reverse (Promega). The degenerate oligonucleotide primer was constructed to recognize the antisense strand of the FaRP cDNA encoding the hexapeptide GNLFRF. The sequence of this primer was:

5'd[CC(AG)AAIC(GT)(GA)AAIA(GA)(GA)TTICCIC(GT)]- 3' ("I" represents inosine). The following reactants were combined in the PCR reaction: 0.5 ng of template, 50 picomoles of the M13 reverse primer, 200 picomoles of the degenerate oligonucleotide primer, 1.5 units of Taq DNA polymerase (Promega), 5 µl of 10X reaction buffer (Promega), 8 µl of 25 mM $MgCl_2$ (Promega), 1 µl each of 10 mM dNTPs (dATP, dCTP, dGTP, dTTP; Promega), and enough sterile water to bring the reaction volume to 50 µl. The reaction was run through the following program in a Peltier PTC-200 Thermocycler (MJ research): 2 min at 94°C; 2 min at 60°C; 2 min at 72°C. These steps were repeated for 35 cycles. PCR products were visualized on 5% polyacrylamide gel in TBE buffer stained with ethidium bromide.

Second Screen

The second screen utilized both a primer complementary to a region of the sense strand upstream from the NSLFRF region obtained in the first screen and a primer complementary to a section of the poly(A) tail and the first several base pairs of the pBluescript plasmid. The sequences of the two primers were:

5' d[GCTCGGCATTGACGATGTCG] 3' = sense primer

5' d[GGCCCCCCCTCGAGTTTTTT] 3' = antisense primer

The second reaction used similar concentrations of reactants with the following adjustments: 100 picomoles of the antisense primer, 50 picomoles of the sense primer, and 5 ng of DNA were used in lieu of the original amounts. The same PCR program was used for the second screen, and products were visualized on a 5% polyacrylamide gel in TBE buffer.

Sub-cloning and Transformation

The products from each screen were sub-cloned in two different cloning reactions. Amplification was repeated for the products of each screen using four reactions identical to the ones described above in order to obtain enough DNA for sub-cloning. To insure proper ligation of PCR products to the cloning vector, the aforementioned program was adjusted so as to include a final 10 min elongation step (furthermore, to reduce the time

of the program, the annealing and elongation steps were reduced to 1 min each). To reduce the volume of the solution containing the PCR products to a volume small enough to run on a single gel lane, the four reaction products were concentrated using a Microcon-100 spin column (Amicon). The following adjustments were made to the manufacture's protocol: 200 µl of PCR product were added to one column, and sterile water was added to bring the total volume to 500 µl. In addition, the column was spun for only 12 min on the first spin. The concentrated product was run on a 1% SeaPlaque low-melt agarose gel (FMC Bioproducts) in "E" buffer (48 g trizma base and 7.4 g disodium EDTA dissolved in 600 ml water; glacial acetic acid is added until pH reaches 8), and the appropriate band was excised. The excised PCR product was cloned into a pCR 2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen) following the kit protocol.

Sequencing of Cloned PCR Products

A Wizard miniprep kit (Promega) was used to extract and purify the cloned PCR product. A restriction enzyme digest using EcoR1 was done to confirm the presence of the correct insert. DNA from the purified sample was then sent for sequencing at the in-house facilities at the University of Oregon. The vector primer T7 was used in the first sequencing reaction, while M13 reverse was used in the second.

In vitro *Chromatophore Bioassay*

To test the peptides encoded in the cDNA for bioactivity, an *in vitro* bioassay was performed. Synthetic NSLFRF and GNLFRF peptides (Research Genetics) were applied to a patch of skin excised from the fin of an adult cuttlefish. A piece of fin approxiamately 1 cm^2 was removed from the fin of a *Sepia officinalis* specimen, and the dermal and muscle layers surgically removed from the ventral chromatophore layer. The sample was pinned in a perfusion chamber and immersed in artificial seawater (ASW). The chamber was mounted onto the stage of an Olympus CK-2 inverted microscope, and a single, brown chromatophore was brought into the center of the field of view. A photocell placed in the optical pathway was used to quantify chromatophore expansion. Chromatophore expansion resulted in the reduced transmission of light through the optics and thus reduced detection of light by the photocell. Photocell output was recorded on a chart recorder (Gould 2200s).

The test included the application of various concentrations of the NSLFRF and GNLFRF peptides. Stock solutions were diluted in ASW and tested in ranges of 10^{-7} through 10^{-4} M. Starting with the lowest concentration, each application began by stopping the flow of ASW to the chamber and applying 300 µl of test solution to flush the chamber. After this volume had flowed from the chamber, outflow was halted, and 100 µl of the same test substance was applied for 5 min. After this time, the substance was washed out of the chamber for 10 min by re-establishing the flow of ASW.

Dot-Blot Immunoassay

A 1.5 cm x .5 cm strip of NitroBind nitrocellulose membrane (Micro Separations Inc.) was blotted with NSLFRFamide, GNLFRFamide, and FMRFamide peptides at concentrations ranging from 10^{-7} to 10^{-4} M. Each blot consisted of 0.3 µl of peptide solution. After blotting, the membrane was allowed to dry, and then heat-fixed for 25 min at 95°C. The membrane was then washed in buffer I (0.1M Tris, 0.05% Tween 20, pH 7.4) for 10 min. Next, the membrane was placed in blocking solution (3% BSA w/v in 0.1M Tris, pH 7.4) for 1 hr. The blocking solution was then replaced by primary antibody at a 1: 40,000 concentration and left overnight. After treatment with the primary antibody, the membrane was washed three times with buffer I at 5 min per wash. Secondary antibody (rabbit IgG, Vectastain) was then applied at a concentration of 1: 500 for 30 min. This was followed by three washes with buffer I (5 min each), and an application of ABC (avidin/biotin complex at 1:100 concentration in buffer I; Vectastain) for 30 min. This was followed by three more washes in buffer I (5 min each). The membrane was then transferred to a peroxidase-free well and treated with TMB Membrane Peroxidase Substrate for 12 min (Kirkegaard and Perry Laboratories). A final 5 min wash in double-distilled water was then carried out. Staining was analyzed after allowing the membrane to dry.

RESULTS

Molecular Analysis

PCR using the degenerate primer recognizing the putative anti-sense sequence to the GNLFRF coding region yielded what appeared to be several tightly-associated bands at approximately 500-550 base pairs in length (Fig. 7). As the partial cDNA obtained by Price showed three GNLFRF coding regions close together, the presence of several tightly associated bands was expected (Price, personal communication). The largest band was isolated and amplified again in multiple, identical reactions. The products from these reactions were sub-cloned into a new expression vector (TA TOPO), and a vector containing one of the larger inserts was sequenced on the automated fluorescence sequencer at the in-house sequencing facilities at the University of Oregon. This sequence was found to encode one copy of the NSLFRFamide peptide, and two copies of the GNLFRFamide peptide. This information was used to construct a new primer, which was exactly complementary to the sense strand of a region of cDNA upstream of the NSLFRFamide coding region. However, amplification using this primer and the commercially available primers T7 and M13 forward failed. To circumvent the problem, a new vector primer was constructed. This primer was complementary to a portion of the poly(A) tail and several basepairs of the arm of the pBluescript vector, elements found in all cDNAs in the library. PCR with the new primers yielded the rest of the cDNA

sequence (Fig. 8), which included a coding region for the pentapeptide TIFRFamide, along with a putative 24 residue long peptide with no homology to the FaRPs. Each of the putative peptides within the larger propeptide are flanked by a pair of dibasic residues (K and R), which serve as proteolytic cleavage sites during post-translational processing (Loh and Gainer, 1983). All of the FaRPs were found to have a glycine residue at their carboxy termini. These glycines are presumably converted to amides after translation (Loh and Gainer, 1983; Eipper and Mains, 1988). The entire cDNA is 808 base pairs long, with a start codon at nucleotides 97-99 and a stop codon at nucleotides 634-636. The region between the stop codon and the poly(A) tail contains two AAUAAA polyadenylation signals. Despite careful amplification and sequencing efforts, there is a putative base pair deletion at position 208. An A has been added to the figure to put the sequence in the proper reading frame. The mutation likely arose because Taq polymerase was used in the amplification process, and as a "sloppy" polymerase it tends to make mistakes every so often.

Fig. 7. Results of the first screen. A picture of a polyacrylamide gel showing the presence of several tightly-associated bands in the 500-550 base pair region. The DNA ladder is shown in lane 2, while the amplified product of screen 1 is shown in lane 1. The brightest ladder band is 700 base pairs.

The presence of this new mRNA demonstrates that cuttlefish have the capacity to produce other FaRPs, namely GNLFRFamide, NSLFRFamide, and TIFRFamide, and that these may be used in the control of chromatophore expansion.

In vitro Chromatophore Bioassay

To determine whether any of the three peptides identified in the molecular study were capable of causing chromatophore expansion, a bioassay was conducted. Synthetic NSLFRFamide and GNLFRFamide peptides were found to cause chromatophore expansion when applied to a patch of cuttlefish fin. The application of either NSLFRFamide or GNLFRFamide at a concentration of 10^{-4} M caused expansion of brown chromatophores (Fig. 9). The application of TIFRFamide at concentrations as high as 10^{-4} M did not produce observable expansion (however, only one test was conducted due to a shortage of animals; further testing of this peptide should be done before any conclusions concerning its bioactivity are drawn). That chromatophores responded to at least two of the peptides demonstrates that the FaRP receptors present in the chromatophore recognize these peptides. However, the concentration of peptides needed to effect expansion was significantly higher that found by Loi for FMRFamide and the other previously identified FaRPs. For example, the threshold concentration of FMRFamide needed to effect chromatophore expansion is 10^{-7} M.

Fig. 8. *Sepia officinalis* LFRFamide cDNA. The non-coding base pairs are in lower-case letters, while coding residues are represented in upper-case letters. The amino acid coded for by each codon is given below the codon. Cut sites residues are colored blue, while FaRP residues are colored red. The two putative polyadenylation sites are underlined. The A added to the position of the putative deletion mutation is colored purple.

gcacgagccgaatcgcgtttctcgtacagaatacaaagcatttcttagaa 50
cctgggcttaacaaatacagcgaaccggccaacggtcggatcaaat 96

ATG GAA ACA AAA GTA ATG AGT TTG TTG GCG ACT GTG CTA ACC CGG 141
M E T K V M S L L A T V L T R

TTC ATT GTG CAG ATA AAT TGC GAA GAC CTA CAC AAA ATA CAA ACA 186
F I V Q I N C E D L H K I Q T

GAC ACT TTC CGG ATT TCT AAT ATT ATC GGT CTA CCA GAT GGA GAA 231
D T F R I S N I I G L P D G E

GAA GGC GAA CTA GTG AGA TCC CCG ATT GTT GAC GAA TCA GCG CTC 276
E G E L V R S P I V D E S A L

GGC ATT GAC GAT GTC GAC AAA CGC AAC AGC CTC TTC CGA TTC GGC 321
G I D D V D K R N S L F R F G

AAG CGT GGA AAC CTC TTC CGG TTT GGT AAA CGT GGA AAC CTC TTC 366
K R G N L F R F G K R G N L F

CGT TTT GGA CGC GGT GGC AAC AAG GAC GAC CCC GAA AAC GAA GGA 411
R F G R G G N K D D P E N E G

CTC AAA CGC ACC ATC TTT AGA TTC GGT AAA AGA GAC GGA CTC GAA 456
L K R T I F R F G K R D G L E

GAC CTT TAC GAC TAC GAG GAT CCC TCA GTA CAA CAG GTG GCA CCA 401
D L Y D Y E D P S V Q Q V A P

ACA GCT GGA GAC AAG CGC GGA TCA TTC TTC CGG TAT GGA CGA AGC 546
T A G D K R G S F F R Y G R S

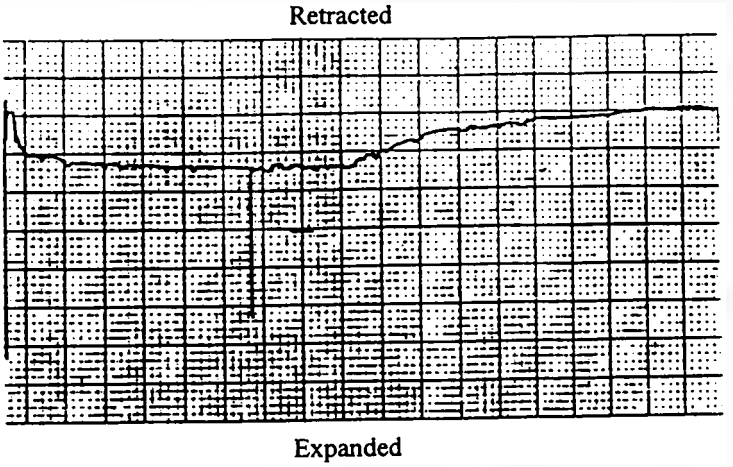
CGC ACT TTC TTT CGA TAC GGT AGA AGC ACT GAT AAG AAC GCC GAG 591
R T F F R Y G R S T D K N A E

AAG AGG CCA CAC ACA CCC TCC CGA TTT GGA AGA GAA GAA GAG TAA 636
K R P H T P S R F G R E E E STOP

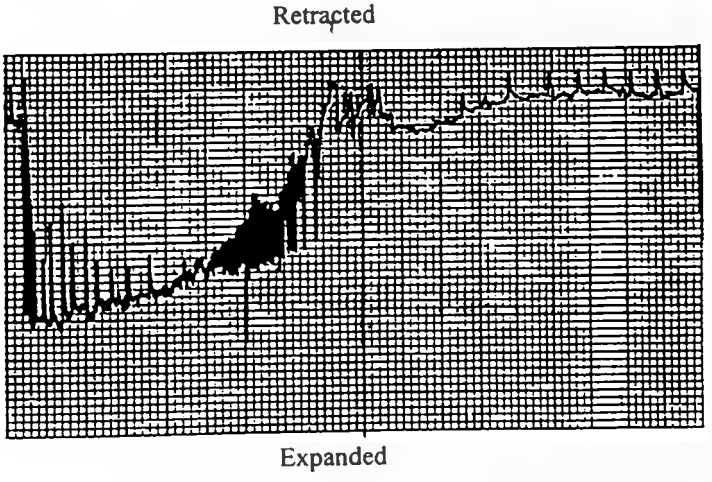
aataatgactccatagaattttaccttaaaaagattaaagagagacaagaaggagagaaaatatacaaagaagacttgagttgaca 722
aagcacgggaaagcaaaataacaaagaattggaaaaaaattataaataataaaatgaattttattaataaaaaaaaaaaaaaaaa 808

Fig. 9. Bioassay Results. The top figure gives the results of a bioassay using 10^{-4}M GNLFRFamide. The bottom figure gives the results of a bioassay using 10^{-4}M NSLFRFamide. The lower trace position signifies an expanded state, the upper postion a retracted state. Each test was done on a different piece of skin, thus the differences between the two traces are due to differences in individual animals and preparations. A single chromatophore could not be used for both tests because treatment with such high levels of peptide left the chromatophores in an unnatural state, even after prolonged washout.

GNLFRFamide, 10^{-4}M



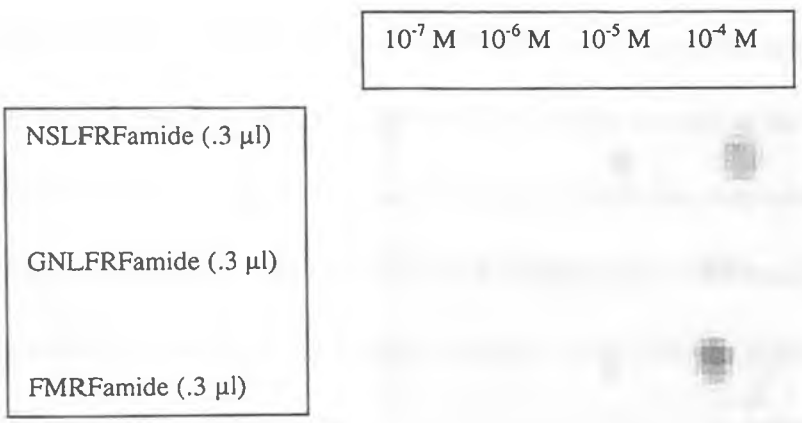
NSLFRFamide, 10^{-4} M



Dot-Blot Immunoassay

To determine whether or not a FMRFamide antibody could recognize NSLFRFamide or GNLFRFamide, and thus demonstrate whether or not these peptides could be those recognized by the FMRFamide antibody (but not the *in situ* probe) in the double-staining experiments, a dot-blot immunoassay was conducted. The hexapeptides were tested for immunoreactivity with an FMRFamide antibody at concentrations ranging from 10^{-7} M to 10^{-4} M. As a control, FMRFamide was also tested, at the same concentrations as the hexapeptides. Staining was observed for all peptides (Fig. 10). Both NSLFRFamide and FMRFamide showed light staining at 10^{-5} M and heavy staining at 10^{-4} M, while GNLFRFamide showed only light staining at 10^{-4} M. These results suggest that the FMRFamide antibody recognizes these peptides, and is therefore binding peptides with an –RFamide motif. These data also suggest that previous FMRFamide immunostaining work by Loi may have detected the hexapeptides in the chromatophore lobes and/or neurons surrounding the chromatophores. While this does not prove that such was the case, it is consistent with the hypothesis that these are indeed endogenous neurotransmitters.

Fig. 10. Immunostaining of GNLFRFamide, NSLFRFamide, and FMRFamide by a FMRFamide antibody in a dot-blot assay. The membrane depicted below shows staining for GNLFRFamide, NSLFRFamide, and FMRFamide. NSLFRFamide samples are in the top row, followed by GNLFRFamide and FMRFamide. Each spot consists of .3 µl of solution. From left to right, the concentration of each spot is 10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M.



DISCUSSION

This study sought to test the hypothesis that cuttlefish contain other FaRPs, and use these FaRPs in conjunction with other transmitters to control their body patterning. The results obtained from the molecular analysis clearly show that cuttlefish possess a gene coding for previously uncharacterized FaRPs and a novel peptide totally devoid of homology to FaRPs. The sequence obtained was fairly similar to the partial sequence isolated by Price. However, the *Sepia* sequence contains one less coding region for GNLFRFamide, and the *Sepia* cDNA did not encode a GSLFRFamide peptide as the Price sequence did. Because the Price sequence ended with this putative FaRP-coding region, it is conceivable that what was really present was the beginning of the novel 24 residue-long peptide, which begins at the same relative position as the GSLFRFamide peptide in the Price sequence. Furthermore, the first few residues of the longer peptide are similar to GSLFRF.

The peptides discovered in this work have not been reported in any organism (save for the unpublished Price data): a PubMed QUERY on Medline revealed no hits for either GNLFRFamide, NSLFRFamide, or TIFRFamide. The only FMRFamide-related hexapeptides to be found were GSLFRFamide and SSLFRFamide, both of which were isolated from the ganglia of the prosobranch mollusc *Fusinus ferrugineus* (Kuroki *et al*, 1993). However, no function has of yet been determined for these FaRPs.

One possible function for the FaRPs isolated in this study, that they are involved in

modulating chromatophore activity, was tested. The results of the bioassay demonstrate that cuttlefish chromatophores are responsive to the hexapeptides, because application of each resulted in a visable expansion event. Interestingly, the threshold concentration needed to effect expansion was somewhat higher than that observed for FMRFamide and other peptides previously identified (Loi and Tublitz, 1997). It is conceivable that FaRP receptors are responsive to a host of different FaRPs, not just those endogenous in the chromatophore neurons. Studies in the snail *Helix aspera* have revealed that FaRP receptors in that system respond to a host of FMRFamide anologs (peptides that are constructed to be similar to FMRFamide in length and the –RFamide motif, but are not real FaRPs) (Chen *et al*, 1995). However, peptides with the N-terminal residues removed (e.g. MRFamide) elicited no response. It was thus concluded that the presence of four amino acids (ending in –RFamide) was sufficient to elicit a neuronal response *in vitro* (Chen *et al*, 1995). Thus, the bioassay used in this study, while demonstrating the potential for endogenous activity, is not a conclusive test in and of itself.

The hypothesis that the FaRPs isolated in this study are endogenous regulators of chromatophore activity was further supported by the dot-blot assay. The positive results raise the possibility that the previously published immunocytochemical work had detected the presence of GNLFRFamide and NSLFRFamide in the putative cell bodies of chromatophore motoneurons and in peripheral axons closely associated with the chromatophore muscles. Thus, these peptides are excellent candidates for regulators of chromatophore function based on the finding by Loi that some neurons in the

chromatophore lobes stained for the FaRP antibody but not the *in situ* probe specific for the FMRFamide mRNA. However, to prove that these peptides are indeed expressed in the chromatophore neurons, and to strengthen the hypothesis that they are regulators of chromatophore activity, *in situ* hybridization techniques should be utilized to demonstrate the presence of the newly identified mRNA in these neurons. The results presented here make possible such experiments because the results include sequence information for the entire mRNA, necessary information for constructing an *in situ* probe.

The ability of cephalopods, *Sepia* in particular, to effect rapid and complex changes in body coloration is truly remarkable. That a fixed chromatophore system can exhibit such incredible nuance and complexity is astounding, yet feasible if the system is using multiple neurotransmitters to elicit different types of responses. The evidence to date suggests that glutamate is used to control the phasic color changes, while FaRPs elicit tonic responses that allow for sustained body patterning. The use of multiple FaRPs in this control may provide the system with additional nuance. The next step in investigating how this system operates will be to elucidate how the use of different FaRPs and other transmitters is regulated, and ultimately how the cephalopod brain produces behavior of all kinds. The answer to these question and many others is left to future research. The investigation of such questions will ultimately provide insight into the neural basis of cephalopod -- if not all animal -- behavior.

GLOSSARY

Antibody: A protein made by the immune system that binds foreign molecules or invading organisms. The binding of the antibody either inactivates the foreign species or marks it for destruction. In this thesis, antibodies were used to mark the presence of specific proteins.

Bioassay: This is a general term for an experiment which tests a compound for biological activity. In this study, the bioassay is the application of LFRF-amide peptides to cuttlefish skin samples to see if they cause chromatophores to expand.

Complementary DNA (cDNA): Complementary DNA is DNA that represents the information stored in mRNA. As DNA is more stable than RNA, cDNA provides a reliable source of RNA information to work with. RNA from the cells of interest (in this case cells from the chromatophore lobes of cuttlefish) is isolated and turned into DNA using a special viral enzyme. These segments of DNA are then put into specially engineered bacterial plasmids (small, identical rings of DNA), the sum of these plasmids constituting a cDNA library.

DNA: Deoxyribonucleic acid, a polymer formed from deoxyribonucleic monomers, which carries genetic information.

DNA Polymerase: An enzyme that facilitates the replication of DNA.

Hormone: A long-distance signaling molecule, carried to its targets via the blood.

In Situ Hybridization: A powerful technique used to visual expression of RNA transcripts within fixed tissue samples. A labeled RNA (or DNA) probe which has a sequence complementary to that of the native RNA to be visualized is used to detect expression patterns by virtue of the probe's ability to bind the native RNA and mark it in a manner that makes it easy to detect.

Immunostaining: A technique in which labeled antibodies are used to visualize specific proteins within fixed tissue samples.

In Vitro: Occurring outside of the organism.

L-Glutamate: One of the twenty amino acids, this compound is a common neurotransmitter.

mRNA: Messenger RNA, an RNA molecule that carries the information for a specific amino acid sequence of a protein.

Neurotransmitter: A short-distance signaling molecule secreted by neurons at a chemical synapse. Many neuropeptides are classified as neurotransmitters.

Neuromuscular Junction: The synaptic contact between a nerve terminal and muscle fiber. This is the bridge between the nervous and muscular systems.

Peroxidase: An enzyme that catalyzes the reduction of an alkyl peroxide and a reductant to water and an alcohol.

Plasmid: A small, circular DNA molecule that replicates independently of genomic DNA. Plasmids are often used in molecular biology to carry a DNA fragment of interest into a recipient cell for the purpose of replicating, or cloning, that DNA fragment.

Polymerase Chain Reaction (PCR): PCR is a common molecular biology technique that provides a means of amplifying specific DNA regions if some sequence information is known. The reactants in a PCR consist of short DNA sequences called primers (which are complementary to a region of each DNA strand flanking the region to amplyfied), a heat-stable DNA polymerase (which acts to add nucleotides to the growing strand as amplification progresses), free nucleotides (dNTPs), buffer, salt, and water. The reaction is carried out in a thermocycler, a device which runs the reactants through a series of

tightly controlled heating and cooling cycles, leading to the geometric amplification of the target sequence (Fig. 6). By using primers complementary to the sequences which could potentially encode the FaRPs sought, PCR will result in amplication of the cDNA sequence of interest.

Reverse Transcriptase: An enzyme found in retroviruses. It converts single-stranded RNA to double-stranded DNA.

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