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Molecular analysis of a novel FMRFamide-related peptide gene ($SOFaRP_2$) and its expression pattern in the brain of the European cuttlefish Sepia officinalis

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ABSTRACT

FMRFamide-related peptides (FaRPs) are among several neurotransmitters known to regulate the chromatophore function in the European cuttlefish Sepia officinalis. Here we report the cloning and sequencing of a novel S. officinalis FaRP gene ($SOFaRP_2$). The complete 835-base pair cDNA sequence of the $SOFaRP_2$ gene contains an open reading frame of 567 base pairs encoding 188 amino acids and four putative FaRPs, NSLFRFamide, GNLFRFamide, TIFRFamide and PHTPFRFamide. All except TIFRFamide cause chromatophore expansion when assayed in an in vitro chromatophore bioassay. To investigate the expression pattern of $SOFaRP_2$ gene in the cuttlefish brain, in situ hybridization was performed using a full length RNA probe. The $SOFaRP_2$ gene was expressed primarily in the posterior chromatophore, anterior chromatophore, lateral basal and optic lobes among other brain locations. The $SOFaRP_2$ gene appears to be expressed in all brain regions involved in chromatophore regulation. These data suggests that some or all of the four FaRPs encoded by $SOFaRP_2$ might be involved in controlling chromatophore activity in cuttlefish.

1. Introduction

Cephalopods have an extraordinary ability to rapidly change their skin color and pattern to conceal from predators and to communicate with each other or other species [12,13]. The primary cellular basis for this unique, body patterning behavior is hundreds of thousands of chromatophore organs in the skin of cephalopods [4]. Each chromatophore organ is composed of a central chromatophore cell with a cytoelastic sacculus containing black, reddish brown or yellow pigment granules [12]. Surrounding every chromatophore cell are 10–25 striated radial muscles directly innervated by chromatophore motoneurons located in the subesophageal mass of the cuttlefish brain [9,11,17]. Unlike vertebrate striated muscles, these radial muscles are poly-innervated by both excitatory and inhibitory motoneurons [8,10,11,17].

Chromatophore muscles receive both fast and slow excitatory innervation at the chromatophore neuromuscular junction (NMJ) and these 2 types of inputs underlie the generation of transient and sustained body patterns, respectively [20]. Glutamate has been shown to be the fast excitatory neurotransmitter at the chromatophore NMJ [1,17,21] whereas the activities of the slow excitatory motoneurons are mediated by several members of the FMRFamide-related peptide family [17].

To further investigate the involvement of FaRPs in the regulation of chromatophore function, Loi and Tublitz cloned the first FaRP gene, $SOFaRP_1$, from cuttlefish Sepia officinalis [18]. The only mRNA from the $SOFaRP_1$ gene is 1850 base pairs long and encodes a single precursor protein containing four FaRPs: ALSGDAFLRFamide, FIRFamide, FLRFamide and FMRFamide. Each of these FaRPs caused chromatophore expansion when applied to chromatophores in an in vitro bioassay [18]. Standard in situ hybridization methods revealed that the $SOFaRP_1$ gene was expressed in the posterior chromatophore lobe (PCL), fin lobe (FL), anterior chromatophore lobe (ACL) and other brain areas known to contain the somata of chromatophore muscle motoneurons [20]. The $SOFaRP_1$ gene expression pattern was confirmed using immunohistochemical methods with an anti-FMRFamide antibody [20].

To obtain additional insights into the role(s) of FaRPs in chromatophore activity and other possible functions in cephalopods, we have cloned and sequenced a second FaRP gene, $SOFaRP_2$, and examined its mRNA expression pattern in the S. officinalis brain using in situ hybridization. The deduced sequence of the $SOFaRP_2$ gene indicates that it encodes a single mRNA that, when translated and post-translationally processed, generates four additional FaRPs: NSLFRFamide, GNLFRFamide, TIFRFamide and PHTPFRFamide. Three of the 4 FaRPs coded on the $SOFaRP_2$ gene cause chromatophore expansion on a newly developed in vitro chromatophore bioassay. The extensive expression pattern of $SOFaRP_2$ gene in the cuttlefish brain indicates a possible role in chromatophore regulation.

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2. Materials and methods

2.1. Animals

Sepia officinalis were purchased from the National Resource Center for Cephalopod (Marine Biological Institute, Galveston, TX) and housed in our facility for periods of between several days and three months using animal husbandry techniques described elsewhere [19]. Three adult cuttlefish were used to prepare brain sections for in situ hybridization results and small pieces of fins excised from several other animals were used for the chromatophore bioassays.

2.2. Cloning and sequencing of the SOFaRP$_2$ gene

Polymerase chain reaction (PCR) was performed with degenerate primers using the extracted DNA from a Sepia officinalis λ Zap cDNA library as the template. Degenerate oligonucleotide primers were designed according to the partial FaRP-encoding cDNA sequence from the squid Loligo opalescens [18]. The sense primer 5′-CAAYAGYCTNTTYCGNTTYG-3′ was derived from the sequence encoding the hexapeptide NSLFRF while the antisense primer 5′-NCCRAAYCTRAADATNGTGC-3′ was complementary to the sequence encoding the pentapeptide TIFRF. Synthetic oligonucleotides used as primers in PCR and sequencing were synthesized by DNA Express and Integrated DNA Technologies.

PCRs were carried out in a PerkinElmer 9600 thermal cycler. The PCR products were separated by electrophoresis and the most intensely stained band (170 bp) was cut out, purified and subcloned into a vector using Invitrogen TOPO TA Cloning Kit. The resulting plasmid purified by Zyppy Plasmid Miniprep Kit was sequenced at the DNA Sequencing and Genomics Facility of University of Oregon.

After confirming that the cDNA fragment encoded several new FaRPs, a new set of primers were designed according to the sequence results and were used with the primers from the vector of cDNA library to perform a second round of PCR. Two fragments, 350 and 400 bp, were obtained. The full-length cDNA sequence was constructed according to the sequence results of the first and second PCR and confirmed by the third round of PCR with primers derived from the 5′ end and 3′ end of the full-length sequence. The primer sequences are as follows: FaRP-F1, 5′-GAATCGCGTTTCTCGTACAG-3′; FaRP-R1, 5′-TTCCCGTGCTTTGTCAACTC-3′; FaRP-F2, 5′-AGTTTGTTGGCGACTGTGCT-3′; FaRP-R2, 5′-TTCTCTTCCAAATCGGGAGG-3′. The strategy of the cloning and sequencing of SOFaRP$_2$ is illustrated in Fig. 1.

2.3. In vitro chromatophore bioassay

Individual Sepia officinalis were anesthetized in 0.1% ethanol in artificial sea water (ASW) prior to the removal of a small piece of fin. The ventral dermal layer of the excised fin patch containing chromatophores was surgically separated from fin muscle and epidermis, pinned into a perfusion chamber and superfused with ASW. The four SOFaRP$_2$ neuropeptides were synthesized by GenScript Corporation (Piscataway, New Jersey USA). For each in vitro chromatophore bioassay, fin tissue was first washed with ASW for 1.5 min followed by application of the test neuropeptide for 5 min. The test neuropeptide was removed by an ASW wash for 3.5 min. Each of the four FaRPs was tested separately in a dilution series from 10^{-8} to 10^{-5} mol l^{-1}. The effects of the neuropeptides on chromatophores were captured digitally using a Prosilica GE1050C digital camera and analyzed by Matlab using the Image Processing Toolbox. Only black chromatophores were analyzed in this study.

Measurements of the neuropeptide-induced changes in the area of individual black chromatophores were obtained using a Matlab script. The script employed an RGB threshold test and polygon masking to isolate single chromatophores of interest and to convert them into binary images for area computation. Area was determined by computing the number of pixels contained within the chromatophore of interest. Pixel counts were converted into square millimeters by photographing a calibration slide under the same magnification. Two area measurements were collected for each chromatophore: 10 s prior neuropeptide application, and the peak area value during the 5 min neuropeptide application. Mean area data were obtained from 15 individual black chromatophores for each tested neuropeptide.

2.4. Statistics

For area change of each chromatophore, the area taken 10 s prior to treatment was subtracted from the peak area obtained during the 5 min peptide application or ASW washes. A one-way ANOVA was performed, testing for variance in the peptide treatments. If treatment had a significant effect, post hoc *t*-tests were used to compare ASW control area changes to peptide area changes. Statistical significance was ascertained if p values were less than 0.05 using SAS-JMP Pro v.9.

2.5. In situ hybridization

Sense and antisense RNA probes were prepared by in vitro transcription from the full-length SOFaRP$_2$ cDNA with DIG RNA Labeling Mix from Roche Applied Science and T_3/T_7 RNA Polymerase from Agilent Technologies following instructions from the manufacturers [14].

Individual animals were anesthetized with 0.1% ethanol for 5–10 min followed by rapid decapitation. Their brains were quickly removed and fixed in 4% paraformaldehyde for 2 days. After fixation, the surrounding muscles were trimmed and brains with intact cartilaginous cranium were immersed for a further 2 days in 30% sterile sucrose for cryoprotection. Following cryoprotection step, brains were rapidly frozen in O.C.T. (optimal cutting temperature) compound and sectioned in a cryostat at a thickness of 30 μm. Sections were mounted on Superfrost Plus microscope slides, dried and stored in −80 °C until needed for in situ hybridization.

For the in situ hybridization procedure, brain sections were defrosted, washed with PBS and permeabilized with 2 μg/ml RNase-free Proteinase K for 30 min. Pre-hybridization was performed with pre-hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 2×SSPE buffer, 1 mg/ml yeast t-RNA and 1 mg/ml sperm DNA) for 2 h at room temperature. After pre-hybridization, sense or antisense RNA probes in pre-hybridization buffer at a concentration of 0.2 μg/ml were added to the slides, which were incubated overnight in a humidified chamber at 50 °C. The following day saline-sodium citrate (SSC) buffer at 50 °C was used for washing as follows: two washes with 2×SSC for 15 min each, two washes with 1×SSC for 20 min each and two washes with 0.1×SSC for 20 min each. Following this series of washes, sections were incubated in blocking solution (100 mM Tris–HCl, 150 mM NaCl, 0.1% Triton X-100 and 2% normal goat serum) and then incubated overnight at room temperature with anti-DIG-alkaline phosphatase (Roche Applied Bioscience) at 1:5000 dilution. On the third day sections were stained with the alkaline phosphatase substrate Nitroblue tetrazolium chloride (NBT 5 μl/ml; Roche Applied Bioscience) and 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP 3.75 μl/ml; Roche Applied Bioscience) in a 10% polyvinyl alcohol solution [5] for 2 h at 37 °C in dark. After the completion of the staining step, slides were washed with PBS-0.1%Tween, dehydrated and mounted with Permount.