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Characterization of GnRH-related peptides from the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Gonadotropin-releasing hormone (GnRH), a key neuropeptide regulating reproduction in vertebrates has now been characterized in a number of non-vertebrate species. Despite the demonstration of its ancestral origin, the structure and the function of this family of peptides remain poorly known in species as distant as lophotrochozoans. In this study, two GnRH-related peptides (Cg-GnRH-a and CgGnRH-G) were characterized by mass spectrometry from extracts of the visceral ganglia of the Pacific oyster *Crassostrea gigas*. These peptides showed a high degree of sequence identity with GnRHs of other mollusks and annelids and to a lesser extent with those of vertebrates or with AKH and corazonins of insects. Both the mature peptides and the transcript encoding the precursor protein were exclusively expressed in the visceral ganglia. Significant differences in transcriptional activity of Cg-GnRH encoding gene were recorded in the ganglia along the reproductive cycle and according to trophic conditions with a higher level in fed animals compared to starved animals. This suggests the involvement of Cg-GnRHs as synchronizers of nutritional status with energy requirements during reproduction in oyster. Evidence for a role of Cg-GnRHs as neuroregulators and as neuroendocrine factors in bivalve is discussed.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a hypothalamic neuropeptide that plays a key role in the regulation of reproduction in vertebrates [11]. This neurohormone induces the release of the pituitary gonadotropins LH and FSH that stimulate gonad steroidogenesis and promote gametogenesis. With the identification of more than fourteen structural variants and three different forms of GnRH across various species of protochordates and vertebrates, it has become clear that GnRH peptides regulate various functions with neuroendocrine, paracrine, autocrine and neurotransmitter/neuromodulatory activities [27]. This wide distribution in the chordate lineage suggests that GnRH peptides and signaling pathways with conserved reproductive functions probably arose before the divergence between Deuterostoma and Protostoma [16] (for review). This early evolutionary origin was formerly proposed after the finding that yeast α-mating factor shows some structural and functional similarity to GnRH [19].

Although the ancestral origin of GnRH-like peptides was clearly suggested by immunochemical and biological studies carried-out essentially in mollusks and cnidarians [10,25,35,37], evidence came with the isolation and structural characterization of the first protostomian GnRH-like peptide from the cephalopod mollusk *Octopus vulgaris* [15]. This oct-GnRH is a dodecapeptide that deviates from the decapeptide motif possessed by all chordate GnRHs but displays the features characteristic to all GnRH molecules [34]. More recently, cDNAs encoding GnRH-like peptides were identified from the gastropod mollusks *Aplysia californica* [38] and *Lottia gigantea* as well as from the annelid *Capitella* [36]. Firm identification of GnRH-like peptides from lophotrochozoa stresses the singular lack of GnRH peptides in Ecdysozoa, the second large clade of Protostoma including arthropods and nematodes, two phyla with considerable accessible genomic resources. The likelihood that GnRH would have been lost in Ecdysozoa was thus logically put forward [34]. Interestingly, a *Caenorhabditis elegans* peptide related to insect adipokinetic hormone (AKH) whose precursor displays similarities at the structural level with the AKH prepropeptides of arthropods and with the GnRH precursors of vertebrates [18] was found. Like its insect

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counterparts [31] this AKH-GnRH peptide binds a GnRH receptor ortholog. Interestingly, silencing of the genes encoding this peptide or its receptor induces a delay in the egg laying process in *C. elegans* [18]. This significant result supports the view of a role of the GnRH signaling system in the physiological control of reproduction in Protostoma, a point which remains a matter of debate [34].

Management of fertility is of crucial economical relevance for aquacultural species [26]. This is especially the case for the oyster *Crassostrea gigas* whose reproductive process has a major impact on several physiological functions, generating phenotypic and genetic trade-offs with growth and survival [7,12]. In this species, alternative splicing of a single gene generates multiple forms of gonadotropin-releasing hormone receptor orthologs expressed either specifically in the gonad or in a variety of tissues [29,30]. In line with the demonstration of co-evolution of ligand–receptor pairs [24]; one would expect some of these receptors to bind a GnRH-like peptide. Due to the recent development of genomic resources in *C. gigas* [8], we have identified two forms of GnRH-like neuropeptides (Cg-GnRH) from visceral ganglia of this bivalve mollusk using mass spectrometry. In addition, pattern of expression of Cg-GnRH neuropeptides and their transcript was investigated using mass spectrometry or immunocytochemistry and RT-qPCR respectively.

2. Materials and methods

2.1. Animals

Two years old adult oysters *C. gigas* purchased from a local oyster farm (Normandie, France) were used for peptide characterization, transcription analyses and immunohistochemistry. Stages of reproduction (stage 0: resting undifferentiated stage, stage I: gonial multiplication stage, stage II: maturation stage, stage III: sexual maturity) were determined by histological analysis of gonad sections as described in [29]. To study the influence of trophic conditions, one-year-old adult oysters were reared in water tanks either in the absence of food or with a 12% diet (12% of oyster dry weight in algal (mixture containing *Isochysis galbana* (clone T-ISO), *Chaetoceros calcitrans* and *Skelectonema costatum*) dry weight per day) in controlled experimental conditions during 3 weeks. Adult mussels were obtained from commercial mussel farms from west Cotentin (Normandy, France).

2.2. Extraction of tissues for peptide analysis

Twenty animal equivalents of each organ studied (visceral ganglia, gonadic area, labial palps, digestive gland, mantle, mantle edge, muscles, gills) were extracted in 0.1% trifluoroacetic acid (TFA) at 4 °C and centrifuged for 30 min at 35,000 × *g* at 4 °C. The supernatants were concentrated on Chromafix C18 solid phase extraction cartridges (Macherey-Nagel). Samples were evaporated for nano LC or HPLC purification.

2.3. Nano-LC purification of visceral ganglia extract

The chromatography step was performed on a nano-LC system (Prominence, Shimadzu). Peptides were concentrated on a Zorbax 5 mm × 0.3 mm C18 pre-column (Agilent) and separated onto a ACE 50 mm × 0.5 mm C18 column (AIT, France). Mobile phases consisted of 0.1% acetic acid, 99.9% water (v/v) (A) and 0.1% acetic acid, 20% water in 79.9% ACN (v/v/v) (B). The nanoflow rate was set at 800 nL/min, and the gradient profile was as follows: constant 5% B for 5 min, from 5 to 100% B in 75 min, constant 100% B for 20 min, and return to 10% B. The 800 nL/min volume of the peptide solution was mixed with 1.6 μL/min volume of a mix of 5 mg/mL CHCA matrix prepared in a diluting solution of 50% ACN with 0.1% TFA. Fifteen second fractions were spotted by an Accuspot spotter (Shimadzu) on a stainless steel Opti-TOF™ 384 targets. MS experiments were realized on an AB Sciex 5800 proteomics analyzer.

2.4. Mass spectrometry analysis

MS analysis were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF–TOF ion optics and an OptiBeam™ on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of des-Arg-Bradykinin, Angiotenin I, Glu1-Fibrinopeptide B, ACTH (18-39), ACTH (7-38) and mass precision was above 50 ppm. A 0.8 μL volume of the HPLC fraction was mixed with 1.6 μL volume of a suspension of CHCA matrix prepared in 50% ACN/0.1% TFA solvent. The mixture was spotted on a stainless steel Opti-TOF™ 384 targets; the droplet was allowed to evaporate before introducing the target into the mass spectrometer.

All acquisitions were taken in automatic mode. A laser intensity of 3000 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 600 to 4000 Da. MS/MS spectra were acquired in the positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 3900. For the tandem MS experiments, the acceleration voltage applied was 1 kV and air was used as the collision gas. Gas pressure medium was selected as settings. The fragmentation pattern was used to determine the sequence of the peptide. Database searching was performed using the Mascot 2.3.02 program (Matrix Science) from a GnRH database elaborated with the software AASeq 5-2 (Zanuttini and Henry, http://zanutti.perso.info.unicaen.fr/aaseq/). This GnRH random library is built on the basis of the tags identified from lophotrochozoan GnRH alignment (N-terminal pyroglutamate, amidation, consensus sequence S_7-N-G-W). The MS/MS spectrum of the peptide is then screened by Mascott using the focused sequence database. The results were confirmed by screening the latest version of *C. gigas* "GigasDatabase" [8] (including 1,013,570 entries) http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html. The variable modifications allowed were as follows: C-terminal amidation, N-terminal pyroglutamate, N-terminal acetylation, methionine oxidation and dioxidation. Mass accuracy was set to 100 ppm and 0.6 Da for MS and MS/MS mode respectively.

2.5. Mapping of GnRH by HPLC analysis

HPLC analysis was performed with the software LC-STAR VARIAN connected to a VARIAN 9012 solvent delivery system and a VARIAN 9050 wave length UV–VIS detector set at 214 nm. Tissue extracts were resuspended in 0.1% acetic acid and fractionated by HPLC onto a nucleodur C8 column (Macherey Nagel, 250 × 4.5 μm) with a 45-min acetonitrile linear gradient of 1.33% per minute in 0.1% acetic acid. HPLC fractions co-eluting with synthetic Cg-GnRH (GL Biochem, China) were evaporated for mass spectrometry analysis.

2.6. Reverse transcription and quantitative PCR

Real time RT-qPCR analysis was performed using the iCycler iQ© apparatus (Bio-Rad). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma–Aldrich) according to the manufacturer's instructions. Recovered RNA was then further purified on Nucleospin RNAII columns (Macherey-Nagel). After treatment during