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Neuropeptidome of the Cephalopod *Sepia officinalis*: Identification, Tissue Mapping, and Expression Pattern of Neuropeptides and Neurohormones during Egg Laying

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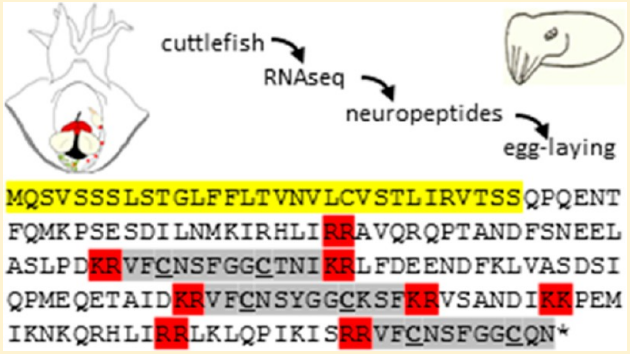
Supporting Information

ABSTRACT: Cephalopods exhibit a wide variety of behaviors such as prey capture, communication, camouflage, and reproduction thanks to a complex central nervous system (CNS) divided into several functional lobes that express a wide range of neuropeptides involved in the modulation of behaviors and physiological mechanisms associated with the main stages of their life cycle. This work focuses on the neuropeptidome expressed during egg-laying through de novo construction of the CNS transcriptome using an RNAseq approach (Illumina sequencing). Then, we completed the in silico analysis of the transcriptome by characterizing and tissue-mapping neuropeptides by mass spectrometry. To identify neuropeptides involved in the egg-laying process, we determined (1) the neuropeptide contents of the neurohemal area, hemolymph (blood), and nerve endings in mature females and (2) the expression levels of these peptides. Among the 38 neuropeptide families identified from 55 transcripts, 30 were described for the first time in *Sepia officinalis*, 5 were described for the first time in the animal kingdom, and 14 were strongly overexpressed in egg-laying females as compared with mature males. Mass spectrometry screening of hemolymph and nerve ending contents allowed us to clarify the status of many neuropeptides, that is, to determine whether they were neuromodulators or neurohormones.



KEYWORDS: *cuttlefish, brain, egg-laying, neuropeptidome, transcriptome, neurohormones*

1. INTRODUCTION

The successive reproduction steps of the cuttlefish *Sepia officinalis* in France are fully described. Mating is a stereotyped behavior during which males deposit spermatophores in a copulatory pouch prior to fertilization. The copulatory pouch is located below the females' beak and stores sperm. Just before fertilization, oocytes are embedded into two layers of egg-capsule proteins: The internal layer is secreted by the oviduct gland (OG) and the external layer by the main nidamental glands (MNGs).

When females are ready to start egg-laying, embedded oocytes that have released sperm-attracting peptides[1] are fertilized and then attached to seaweeds or some other material down on the sea bottom.

Egg masses are spawned in specific mating and spawning coastal areas where cuttlefish aggregate between April and June. Chemical communication probably plays an important role in these mechanisms. Boal and collaborators[2] demonstrated that crude ovary and female accessory sex gland (ASG) extracts had an attracting power during behavioral tests, while Enault and collaborators[3] recently characterized waterborne sex phero-

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mones. In the squid *Loligo pealeii*, the occurrence of a β-microseminoprotein-like pheromone inducing extreme aggression in males was demonstrated in the egg mass.[4] In cuttlefish, ovarian regulatory peptides with potential pheromonal action secreted by the oocytes are involved in the control of oocyte transport and egg capsule secretion.[5−8]

In cuttlefish, many papers show that regulation of the successive steps of egg-laying is monitored by neuropeptides, ovarian regulatory peptides, and sex pheromones; however, the neuropeptidome has not been fully investigated yet.

In other mollusks, the central nervous system (CNS) and neuropeptides play an important role in the control of reproduction, as described in gastropods.[9−13] The egg-laying hormone (ELH), a 36-amino-acid neuropeptide, is expressed by specific neuron clusters belonging to cerebral ganglia. It induced egg-laying and the associated behavior when it was injected to mature specimens.[14−18] Besides ELH, neuropeptides such as the APGWamide trigger penis erection by inhibiting the retractor muscle of the penis in gastropods[19] and inhibit the contraction of the oviduct in cuttlefish.[20] Moreover, FMRFamide-related peptides (FaRPs) stimulate the mechanical secretion of the egg capsule in cuttlefish.[21]

In this context, to gain a functional and structural overview of the cuttlefish neuropeptidome, we characterized neuropeptidome transcripts and expression products, using de novo ribonucleic acid sequencing (RNaseq) and mass spectrometry (MS), in mature males and egg-laying females (ELFs). Because the organization of the CNS appears to be more complex in cephalopods than in other mollusk classes, we distinguished between (1) the supraesophageal mass (SupEM) and the optic lobes (OLs), associated with the integration of neurosensorial information, and (2) the subesophageal mass (SubEM), linked to the single neurohemal area (NA) described in decapods like *S. officinalis*.[22−25] SubEM is suspected to be the main part of the CNS involved in neurohormone expression and secretion. Even so, the occurrence of a second NA is suspected but not yet demonstrated. Located between the optic gland, the subpedunculate nerve, and the pharyngo-ophthalmic vein, this putative NA has only been described in octopods.[26]

2. MATERIALS AND METHODS

2.1. Animal Handling and Collection of Cuttlefish Organs and Tissues

Two-year-old mature cuttlefish (*S. officinalis*) were trapped in the Bay of Seine in April, May, and June 2012 in an area that is neither privately owned nor protected in any way. They were maintained in aerated natural seawater in 1000 L outflow tanks at the Marine Station of Luc-sur-Mer (University of Caen, France).

The CNSs, ASGs, ovary, and posterior salivary glands were dissected from ethanol 3%-anesthetized ELFs ($N = 5$), frozen in liquid nitrogen, and stored at −80 °C until ribonucleic acid (RNA) isolation or peptide extraction. Central nervous systems (CNSs) and posterior salivary glands from ethanol 3%-anesthetized sexually mature males ($N = 5$) were treated as previously described.

No specific permits were required for these field studies, and the common cuttlefish is not an endangered or protected species.

2.2. Illumina Sequencing

Total RNA was extracted in TriReagent from each of the organs isolated from males and females to generate paired-end libraries for 2 × 150-bp paired ends. The total RNA concentration of each sample was quantified using a NanoDrop spectrophotometer (ThermoFisher) and RNA quality was checked using a bioanalyzer (Agilent Technologies). Complementary DNA (cDNA) libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, part no. 15008136 Rev. A) according to the manufacturer's protocol, from 4 μg of total RNA of each sample. In brief, poly(A)-mRNAs were purified with magnetic oligo dT beads and reverse-transcribed into first-strand cDNAs. Then, the second cDNA strands were synthesized. Double-stranded DNAs (dsDNAs) were cleaved into 300-bp fragments using Covaris S220 (Applied Biosystems) (duty cycle: 5%, intensity: 3, bursts per second: 200, duration: 80 s). Then, each sample was tagged, adapters were ligated to the dsDNA ends, and a polymerase chain reaction (PCR) step was performed to amplify the amounts of DNA in the library. Sample quality was assessed by Tape station 2200 (Agilent Technologies) and DNA concentrations were measured with a KAPA quantitative polymerase chain reaction (qPCR) Library Quantification kit (Kapabiosystems). Finally, cDNA libraries were loaded onto a flow cell (Illumina TruSeq PE Cluster Kit v2 - cBot - GA) channel at a total concentration of 2 pmol per lane. The sequencing of 150 paired ends was performed on an Illumina Genome Analyzer (GAIIX).

2.3. Sequence Cleaning

Each sample produced an average 35 million raw 150-bp paired reads. After removal of adaptor sequences and of ambiguous and low-quality reads as described in Cornet and collaborators,[27] RNA libraries yielded 460 million paired-end reads.

2.4. RNA-Seq Data Assembly

Assembly was performed using Trinity, the genome-independent transcriptome assembler. Data corresponded to the four different parts of the brain of both sexes: OLs, SubEM and supraesophageal mass (SupEM) were remapped to the global transcriptome to select transcripts with at least an FPKM (fragments per kilobase of exon per million fragments mapped) value above 1 and isoforms corresponding to >1% of the total gene count as described in Cornet and collaborators.[27] Annotation was performed using the trinotate pipeline (http://trinotate.github.io) and blastn against the nt database.

2.5. Mass Spectrometry: Identification and Localization of Neuropeptides

2.5.1. Recovery of Material from Tissues. Each tissue extraction was performed from $n = 3$ animals. The hemolymph was collected at the vena cava and gill hearts with a 10 mL syringe. Nerve endings were collected at the level of the female ASGs, along with glandular and conjunctive cells because it is impossible to separate these tissues. A previous study[21] showed that in MNGs and in the OG nerve endings surrounded glands associated with conjunctive tissue and also surrounded glandular lobes. At the level of the ovaries, ovarian follicles were manually removed to isolate the ovarian stroma, the innervated contractile structure that releases mature oocytes (smooth oocytes).

To optimize neuropeptide detection, we applied two complementary extraction protocols.

2.5.1.1. First Extraction. Tissues frozen and crushed in liquid nitrogen were extracted in 0.1% TFA with dithiothreitol (DTT) 50 mM at +4 °C (1 g of tissue for 10 mL of extraction medium). After a centrifugation of 20 min at 30 000*g* at +4 °C, supernatants were pooled and concentrated on C18 Sep-Pak cartridges (WATERS).

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2.5.1.2. Second Extraction. Tissues frozen and crushed in liquid nitrogen were extracted in cold methanol/water/acetic acid (90/9/1) adjusted to 50 mM DTT. The extract was centrifuged 20 min at 30 000*g* at +4 °C, and the supernatant was evaporated in a speed vac. Dry pellets were resuspended in 0.1% TFA and concentrated on C18 Sep-Pak cartridges. Finally, both extractions were aliquoted and evaporated. Three animals per sex were used for each tissue extraction.

In addition, alkylation of acidic samples followed by tryptic digestion was performed to recover larger peptides and alkylation alone to increase the dynamic range. Finally, the combination of two sample extractions (0.1% TFA versus methanol/water/acetic acid) with three sample preparations (acidic extract or alkylation or alkylation and digestion) allowed us to recover peptides covering a large MW and structural range.

2.5.2. NanoLC–MALDI-TOF/TOF. To deeply investigate the neuropeptide content of our tissue samples, we performed three distinct sample preparations from each extract obtained as previously described: coarse, reduced/alkylated, and reduced/alkylated followed by trypsin digestion. The peptide extracts were reduced with 100 mM DTT at 55 °C for 60 min, alkylated with 50 mM iodoacetamide at 55 °C for 45 min, and hydrolyzed with trypsin at 25 ng/μL at 37 °C overnight. The resulting peptides were analyzed by MS.

Dry peptide extract pellets were concentrated and desalted on OMIX-TIP C18 (10 μL, AGILENT), resuspended in 40 μL of 0.1% TFA in water. Thirty-five μL were preconcentrated and then loaded on a C18 150 mm × 75 μm capillary column and fractionated at a flow rate of 300 nL/min (nanoLC prominence, Shimadzu), with a 180 min acetonitrile (ACN) gradient generated from 2 to 90% leading to 380 28 s fractions mixed with the matrix and plated on the MALDI target (AccuSpot, Shimadzu). For each sample, the runs were performed using α-cyano-4-hydroxy cinnamic acid (CHCA) as a matrix (5 mg of CHCA in 1 mL of 1:1 ACN/0.1% TFA solution).

MS experiments were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF/TOF ion optics and OptiBeam on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated before analysis with a mixture of des-Arg-Bradykinin, angiotensin I, Glu1-fibrinopeptide B, ACTH (18–39), ACTH (7–38), and mass precision was better than 50 ppm for the reflectron mode. A laser intensity of 3400 was typically applied for ionizing. MS spectra were acquired in the positive reflectron mode by summarizing 1000 single spectra (5 × 200) in the 700 to 4000 Da mass range. MS/MS spectra from the 20 most intense ions were acquired in the positive MS/MS reflectron 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 was 1 kV, and air was used as the collision gas. Gas pressure medium was selected as settings.

2.5.3. NanoLC–ESI–LTQ-Orbitrap. Aliquots from the two types of extracts described above were pooled and reduced-alkylated, desalted on OMIX-TIP C18 (10 μL, AGILENT), and dried. The dried extracted peptides were resuspended in 5% ACN and 0.1% trifluoroacetic acid (TFA) and analyzed by online nanoLC–MS/MS (Ultimate 3000 and LTQ-Orbitrap Velos pro, Thermo Fischer Scientific). Peptides were sampled on a 300 μm × 5 mm PepMap C18 precolumn and separated on a 75 μm × 250 mm C18 column (PepMap, Dionex). The nanoLC method consisted of a 120 min gradient at a flow rate of 300 nL/min, ranging from 2 to 60% acetronitrile in 0.1% formic acid for 114 min, followed by 72% acetronitrile in 0.1% formic acid for the last 6 min. MS and MS/MS data were acquired using Xcalibur (Thermo Fischer Scientific). Spray voltage was set at 1.4 kV; capillary temperature was adjusted to 200 °C. Survey full-scan MS spectra (m/z = 400–2000) were acquired in the Orbitrap with a resolution of 60 000 after accumulation of 10^6 ions (maximum filling time 500 ms). The 20 most intense ions, including 1+ ions, from the preview survey scan delivered by the Orbitrap were fragmented by collision-induced dissociation (collision energy 35%) in the LTQ after accumulation of 10^4 ions (maximum filling time 100 ms).

Dynamic exclusion was enabled with the following setting: (Repeat Count: 1, Repeat Duration: 30 s, Exclusion List Size: 500, Exclusion Duration: 60 s, Exclusion mass width: relative to mass (10 ppm for low and high), and Early Expiration: disabled).

2.5.4. Peptide Sequencing and Identification. Database searching was performed using the Mascot 2.4.0 program (Matrix Science). Two databases were used: a homemade list of well-known contaminants (keratins and trypsin; 21 entries) and a homemade *Sepia officinalis* expressed sequence tag (EST) database (including 61 960 entries). The variable modifications allowed were as follows: C-Carbamidomethyl, N-terminal acetylation, methionine oxidation and dioxidation, C-terminal amidation, and N-terminal pyroglutamate. "No enzyme" was selected. For MALDI-TOF/TOF analysis, mass accuracy was set to 200 ppm and 0.6 Da for the MS and MS/MS modes, respectively. For Orbitrap analysis, these parameters were set to 20 ppm and 0.6 Da, respectively. Mascot data were then transferred to a developed validation software program for data filtering and elimination of protein redundancy on the basis of proteins being evidenced by the same set or a subset of peptides. This software program is freely available.[28] Each peptide sequence was checked manually to confirm or contradict the Mascot assignment. Sequences corresponding to irrelevant identifications were discarded.

2.6. In Silico Analysis of the CNS Trancsriptome of Egg-Laying Females

2.6.1. Peptraq. Peptraq is a homemade software program developed to perform in silico analyses of large batches of transcriptomic, proteomic, and peptidomic data. The three main strategies we used to investigate cuttlefish transcriptomes are summarized in Figure S-1.

The search for precursors or peptides through Peptraq can be achieved using one or several structural criteria such as precursor or peptide size, the occurrence of some amino acids, the number or percentage of some amino acids, the occurrence of consensus sequences, the occurrence of a glycine followed by a mono or a dibasic cleavage site for C-terminal amidation, the occurrence of a glutamine after a mono or a dibasic cleavage site for N-terminal pyroglutamate, the net electrical charge of precursors or peptides, and so on.

Except for a small batch of neuropeptide precursors that was already annotated (APGWamide, Pedal Peptide, FaRPs, etc.), identifications were performed using three main structural criteria: (1) a glycine followed by a mono- or a dibasic convertase cleavage site (C-terminal amidation), (2) a glutamine following a dibasic cleavage site (N-terminal pyroglutamate), and (3) tag repeats as observed for many neuropeptide precursors releasing many copies of similar neuropeptides (e.g., GWG for APGWamide-related peptides (APGWa-RPs), RFG for FaRPs, GSRG for tachykinins).

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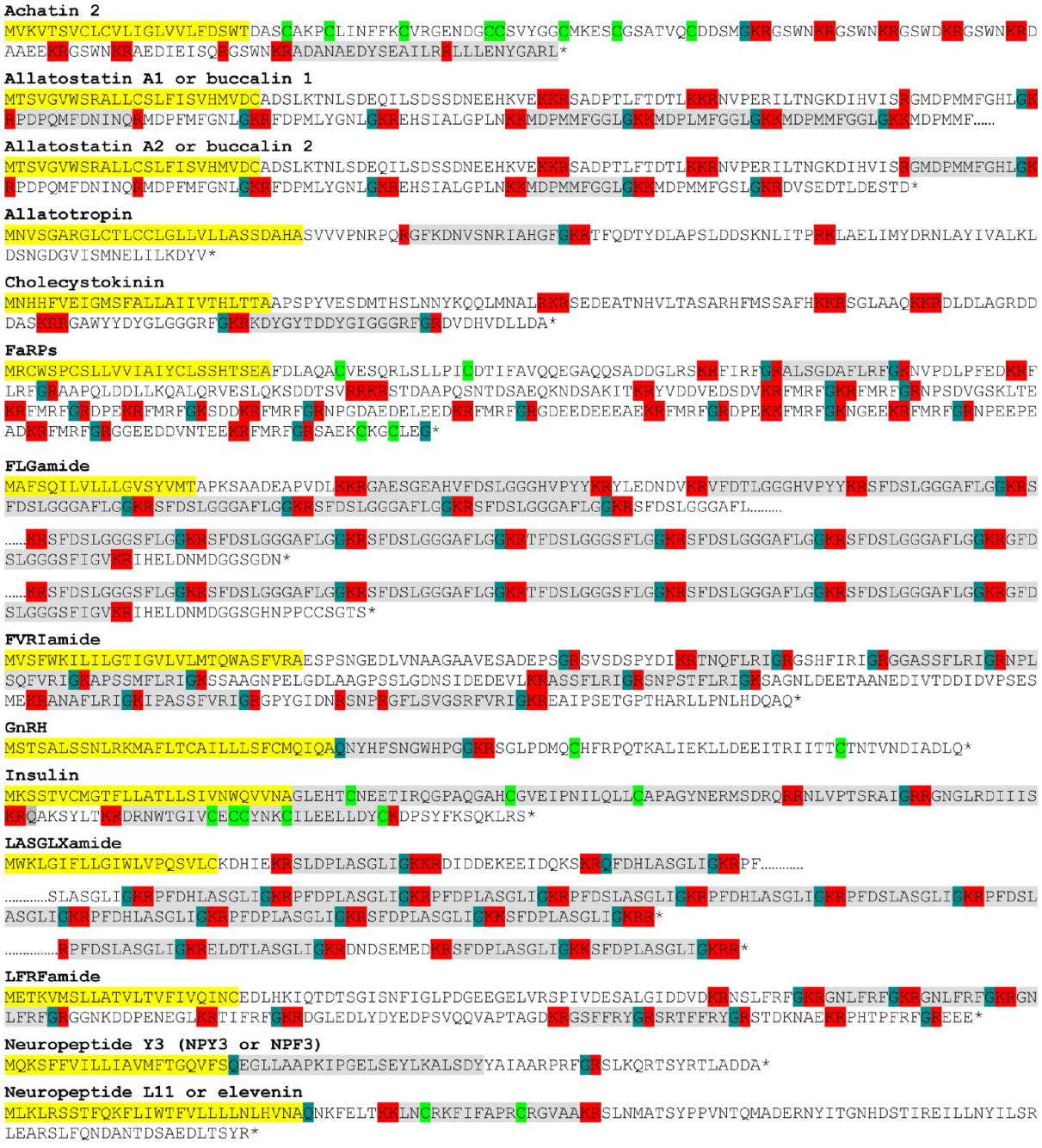

Figure 1. Predicted preprohormones of achatin 2, allatostatins A or buccalins, allatotropin, cholecystokinin, FaRPs, FLGamide, FVRIamide, GnRH, insulin, LASGLXamide, LFRFamide, NPY3, and neuropeptide L11, and encoding neurohormones detected in the hemolymph or in the neurohemal area of *S. officinalis* egg-laying females. Predicted signal peptides are highlighted in yellow, predicted convertase cleavage sites in red, cysteines in green, glycine residues predicted to be converted into C-terminal amides in dark gray, as well as glutamine N-terminal residues predicted to be converted into pyroglutamic acid. Neuropeptides detected by mass spectrometry are highlighted in light gray, and the stop codons at the end of the coding sequences are indicated by an asterisk.

Conserved sequences of well-known neuropeptide families were also used to screen the cuttlefish database.

Peptraq was also used to investigate mature expression products detected by MS in cuttlefish tissues. New neuropeptides cleaved from unknown precursors were identified that way. The search strategy is summarized in the Figure S-2.

2.6.2. Blast. The protein precursors selected by Peptraq as described in 6.1 and annotated "unknown" were submitted to a blast P (protein blast, http://blast.ncbi.nlm.nih.gov/). Neuropeptide precursors need particular attention because they usually generate poor *E* values. Their cryptic and signal peptides are barely conserved or not conserved at all, and their conserved tags are often only partially conserved and very short as compared with full-size neuropeptides. Thus, automated annotation works only for neuropeptide precursor identification when tags are repeated and well-conserved.

2.6.3. Protein Precursor Analysis. Secretion levels and classical post-translational modifications associated with regulatory peptides were checked. Signal peptides were predicted using PrediSi (http://www.predisi.de/) as well as convertase

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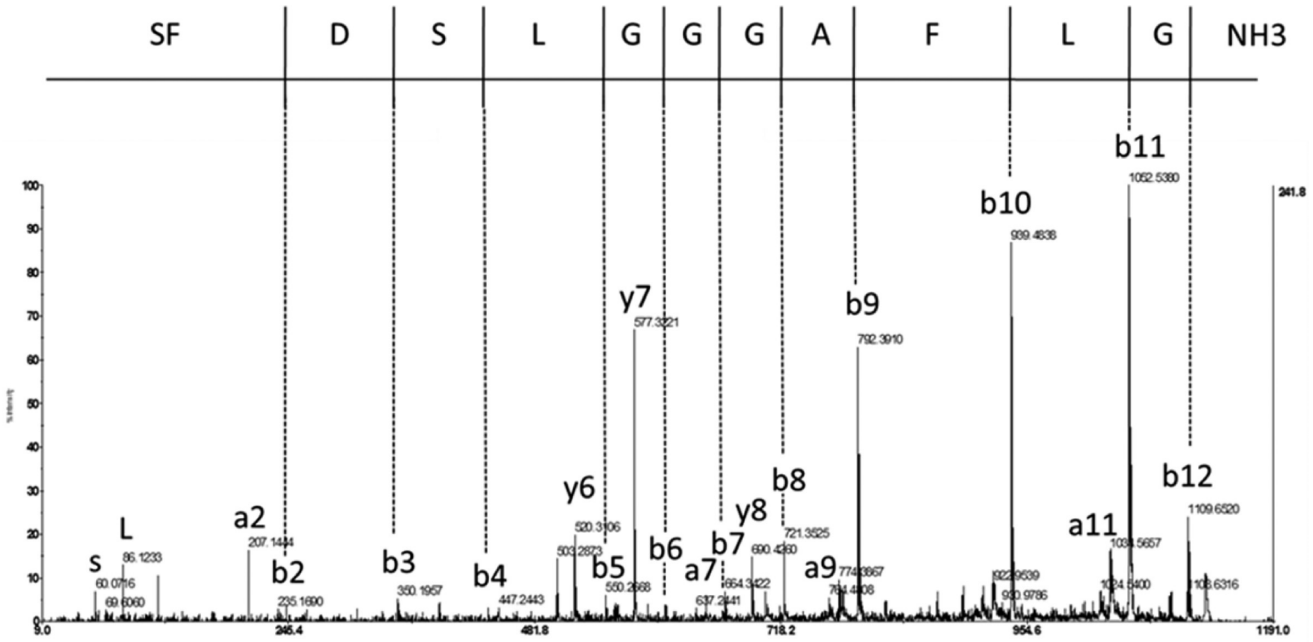

Figure 2. Off-line nLC-MALDI tandem MS analysis of *Sepia officinalis* subesophagal mass connected to the neurohemal area of the vena cava and dissected out during spawning. MS/MS spectrum of the neuropeptide SFDSLGGGAFLGamide, m/z 1126.50. Detected immonium, a, b, and y ions are indicated.

cleavage sites and C-terminal and N-terminal modifications. Sequence alignment was performed using CLC Main Workbench 6 (http://www.clcbio.com/).

2.6.4. Expression Levels of Neuropeptide Transcripts. Expression can be quantified using FPKM values. The FPKM values of a given transcript from several tissues can be compared to establish an expression pattern. In this study, FPKM values can be equated to average expression in the five animals used to determine each transcriptome.

3. RESULTS AND DISCUSSION

3.1. RNaseq/Assembly/Annotation

Reads were assembled to a final transcriptome of 61 960 transcripts ranging from 201 to 22 702 bp lengths, with an average length of 1080.91 bp. 177 37 sequences were annotated either by trinotate analysis or by blastn against an nt database, with E values below 10^{-15}.

3.2. Neuropeptide Identification

3.2.1. Neurohormones. Table S-1 gives an alphabetical list of the neuropeptides. Neurohormones were represented by 26 transcripts from 20 protein families identified in *S. officinalis* ELFs. The neurohormone status was established from MS analyses performed at the level of the hemolymph and the NA of the vena cava. 59 neurohormones were detected out of 97 predicted by the preprohormones presented in Figures 3 and 6. The Mascot scores of the detected neuropeptides are summarized in Table S-2.

In addition, 13 neuropeptide families were detected in the hemolymph but not in the NA of the vena cava, suggesting the occurrence of at least a second, as yet unknown NA responsible for the secretion of a large number of neurohormones in the hemolymph.

3.2.1.1. Achatin. Achatin is a GdFAD tetrapeptide with a D-conformation Phe residue, first identified in the gastropod *Achatina fulica*.[29,30] Achatin-I acts as a neuromodulator in *Achatina* giant neurons.[31] In *Aplysia californica*, achatin (GdFFD) is thought to be involved in the feeding circuit.[32] In *S. officinalis*, we identified Achatin 1 and Achatin 2. Achatin 2 encoded five copies of GSWN, one copy of GSWD, and two mature products were detected in the hemolymph (Figure 1).

3.2.1.2. Allatostatins. Allatostatins (ASTs) are arthropod neurohormones involved in the inhibition of juvenile hormone synthesis[33] and feeding.[34] They were first identified from *Aplysia* by Cropper and collaborators and are also called buccalins.[35] Many molluscan ESTs have been reported in the following species:[36] *L. gigantea*, *C. gigas*, *B. glabrata*, *L. stagnalis*, *H. asinina*, *V. philippinarum*, *M. galloprovincialis*, and *M. californianus*. In *S. officinalis*, we identified three allatostatin A preprohormones and recovered three mature products cleaved from hemolymph ASTs A1 and A2.

3.2.1.3. Allatotropin. Allatotropins are insect neuropeptides that stimulate the synthesis of juvenile hormones[37] and the secretion of saliva.[38] The preprohormone of allatotropins encodes a single neuropeptide of 13 amino acids in arthropods versus 14 in mollusks.[36] In *S. officinalis* (Figure 1), the mature product detected in SupEM, SubEM, OLs, and hemolymph was a 14-amino-acid neuropeptide similar to *Idiosepius* allatotropin.

3.2.1.4. Cholecystokinin/Sulfakinin. In vertebrates, cholecystokinin/sulfakinins (CCKs) are involved in the regulation of pancreatic secretion. In opistobranch mollusks, CCK-8 is a neurohormone that regulates feeding behavior by inhibiting motoneurons implied in prey capture.[39] One of the two CCKs encoded by the *S. officinalis* preprohormone was detected in the CNS, the NA, and the hemolymph (Figure 1).

3.2.1.5. FMRFamide-Related Peptides. FMRFamide-related peptides (FaRPs) are widely distributed in the animal kingdom. In mollusks, they are involved in many physiological regulation processes such as heart activity,[40] amylase secretion,[41] feeding,[42] and reproduction.[43] In *S. officinalis*, the FaRP preprohormone encodes 11 copies of FMRFamide, 1 of FLRFamide, 1 of FIRFamide, and 1 of ALSGDAFLRFamide. They regulate egg-

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Figure 3. Sequence alignment of precursors of insulin of the mollusks *Aplysia californica* (NP_001191615), *Lymnaea stagnalis* (P80090), and *Sepia officinalis*. Signal peptide in yellow, B-chain in light blue, A-chain in blue, Cb peptide in brown (gastropods), Cb1 peptide in pink (cuttlefish), Cb2 peptide in light pink (cuttlefish), Ca peptide in green, D peptide in purple (Aplysia and Sepia), and convertase cleavage sites in red (dibasic) and orange (monobasic).

capsule secretion and oocyte transport in the oviduct[21] and also chromatophore control pathways.[44] In this study, the decapeptide ALSGDAFLRFamide was detected in the hemolymph and in the NA as well as in each CNS part.

3.2.1.6. FLGamide. We called FLGamide a novel neuropeptide family never described so far in the animal kingdom. We identified two incomplete preprohormones from the CNS transcriptome, probably resulting from alternative splicing (Figure 1). We detected all FLGamide neuropeptides and two PYY neuropeptides predicted by the two preprohormones in all parts of the CNS (Figure 2) and in the hemolymph. Blastn revealed a similar precursor annotated "uncharacterized protein" (ELU03112) in the polychaete worm *C. teleta*.[45]

3.2.1.7. FVRIamide. In gastropods, the FVRIamide neuropeptides identified in *L. stagnalis*[46] may regulate the buccal system involved in feeding[47] and also the penial complex during copulation by inhibiting the spontaneous contraction/relaxation cycle of the vas deferens.[46] In *S. officinalis*, nine mature products were detected both in the CNS and in the hemolymph and only eight in the NA.

3.2.1.8. GnRH. The gonadotropin-releasing hormone (GnRH) is common to a large number of invertebrate species, such as *O. vulgaris*,[48] *C. gigas*,[49] or *A. californica*.[50] In *S. officinalis*, the encoded GnRH, an N-elongated neuropeptide of 12 amino acids totally identical to *Octopus* GnRH, was detected in all parts of the CNS as well as in the hemolymph and the NA. The involvement of GnRH in reproduction is not yet clearly established in mollusks.

3.2.1.9. Insulin. Numerous insulin-like neuropeptides are known in mollusks, especially in gastropods,[36,51−56] and also in bivalves.[57] In *S. officinalis*, B-chain and A-chain insulin and Cβ1 and Cβ2 peptides, all encoded by a single insulin gene, were detected in the hemolymph or in the NA (Figure 3).

3.2.1.10. LASVGLXamide. The LASVGLXamide neuropeptides were first identified in *L. gigantea* and have been recovered in many mollusks since.[36] The preprohormone isolated from cuttlefish CNS was not full length but predicted at least seven different amino acid neuropeptides with a conserved C-terminal LASGSLIamide end. The seven neuropeptides were detected in the CNS, and four of them in the hemolymph. In *L. gigantea* and *C. gigas*, two similar C-terminal ends, LASGSLVamide and LASGSLIamide, were recovered,[36] but their function remains known.

3.2.1.11. LFRFamide. First identified in *L. stagnalis*,[58] LFRFamide appears to be involved in the control of various steps of the feeding behavior such as biting, swallowing, and rejection, and it also has antagonistic activity, along with 5-HT, on *Aplysia* radula muscle.[59−61] In *S. officinalis*, the hexapeptide GNLFRFamide displays myotropic activity at the level of the rectum.[62] Mature products encoded by the LFRFamide precursor are LXRFamide and FRYamide neuropeptides. Three of them were detected in the hemolymph or in the NA: GNLFRFamide, GSFFRYamide, and SRTFFRYamide.

3.2.1.12. Neuropeptide Y3 (NPY3 or NPF3). Neuropeptides Y of mollusks appear to be well-conserved among the three main classes of mollusks and to share structural similarity with NPY of vertebrates (Figure 4). Nevertheless, in mollusks and in

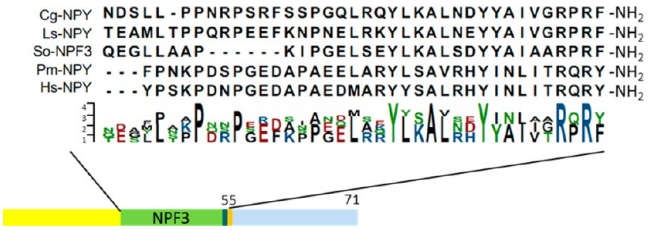

Figure 4. Sequence alignment of precursors of NPY/NPF of the mollusks *Crassostrea gigas* (XP_011448178), *Lymnaea stagnalis* (CAB63265), and *Sepia officinalis*, the leech *Petromyzon marinus* (57231270), and *Homo sapiens* (AAA59944). Signal peptide in yellow, monobasic convertase cleavage sites in orange, and glycine residues predicted to be converted into C-terminal amides in gray. As shown by mass spectrometry analyses, the N-terminal glutamine is converted into pyroglutamic acid in *Sepia officinalis*.

arthropods, the C-terminal tyrosine is replaced by a phenylalanine. In *S. officinalis*, five distinct preprohormones were identified by in silico analysis and detected by MS/MS. Similar diversity has been described in the worm *Helobdella robusta*.[63] We detected a C-terminal truncated form of the neuropeptide Y3 in the hemolymph of ELFs. We can suspect that the receptor-activating C-terminal sequence, YAIAARPRFamide, had been released and is the bioactive circulating neuropeptide, but it went undetected by MS. It is interesting to observe that a similar cleavage was recovered for NPY1 with the following C-terminal sequence, YAIVARPRFamide, detected in the CNS. The release of both full-length neuropeptide and short receptor-activating C-terminal sequences should modify the affinity for the receptor and thereby modulate the biological activity.

3.2.1.13. Neuropeptide L11 or elevenin. The preprohormone was identified in *A. californica* by Taussig and collaborators.[64] The mature product has never been identified, but Veenstra[36] noted that the best conserved region is after the signal peptide where a disulfide bond could be formed (Figure 5). The results obtained from *S. officinalis* confirmed this hypothesis: We detected the mature product LNCRKFIFAPRCRGVAA in the hemolymph by MS/MS. Biological activity is not yet elucidated.

3.2.1.14. Pedal Peptide 2. Pedal peptide nomenclature appears to be complex and unclear. The single and incomplete

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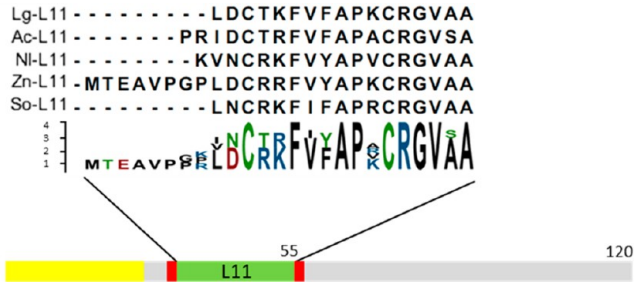

Figure 5. Sequence alignment of precursors of neuropeptide L11 (11in) of the mollusks *Lottia gigantea* (XP_009066893), *Aplysia californica* (NP_001191479), and *Sepia officinalis* and the insects *Nilaparvata lugens* (BAO00952) and *Zootermopsis nevadensis* (KDR13438). Signal peptide in yellow, dibasic convertase cleavage sites in red.

pedal peptide precursor identified in *S. officinalis* encoded neuropeptides with the GSGLI C-terminal end, very similar to GSSFI of *A. californica* pedal peptide 2[65] and to GSGFI of the echinoderm *S. purpuratus*.[66] 13 different neuropeptides encoded by the precursor were detected in the CNS, and 2 were detected in the hemolymph (Figure 6).

3.2.1.15. Pro-Sepiatocin. The precursors of the invertebrates' oxytocin/vasopressin (OT/VP) family are very similar to the vertebrates'. Nevertheless, in mollusks, the nature of the amino acid in position 8 does not induce a functional difference.[67] In gastropods, OT/VP peptides are similar to vertebrate vasopressins, whereas in cephalopods and bivalves OT/VPs are oxytocin-like peptides.[57,68−70] As observed by Henry and collaborators,[69] Pro-sepiatocin (CFFRNCPPGamide), one of the two members identified in cuttlefish, shows strong homology with oxytocin from *C. gigas* (CFIRNCPPGamide),[57] *Daphnia pulex* (CFITNCPPGa),[71] and New World monkeys (CYIQNCPPGa),[72] with a proline in position 8.

The neurophysin domain of the two protein precursors coding the two OT/VPs in cuttlefish is totally conserved, so the precursors are probably issued from alternative splicing.[69]

Finally, only Pro-sepiatocin was detected both in the SubEM and the hemolymph.

3.2.1.16. PRQFVamide. The pentapeptide PRQFVamide was first identified from *A. californica* using a bioassay on gut contraction.[73] In *S. officinalis*, although assembly did not yield full-length transcripts, the incomplete PRQFVamide prepro-hormones appeared as very large neuropeptide precursors. Two sequences starting by two distinct signal peptides and displaying two C-terminal stop codons let us suspect the occurrence of at least two transcripts probably resulting from alternative splicing. Five mature products were recovered in the hemolymph, among them the pentapeptide PMEFLamide.

3.2.1.17. Samide. Samide is a novel neuropeptide family first identified by MS/MS that could release a mature neuropeptide of 37 amino acids with an N-terminal pyroglutamate, C-terminal amidation, and one disulfide bond between cysteines 24 and 29. The neuropeptide detected in the hemolymph is an N-terminus truncated form of the putative 37-amino-acid peptide previously described, with only 24 amino acids, one disulfide bond, and C-terminal amidation. This neuropeptide exhibits strong homology with *Platinereis dumerilii* QSamide (AHB62379) that is not functionally characterized and with two uncharacterized proteins from *L. gigantea* (XP_009051664)[45] and *C. gigas* (EKC20562)[74] (Figure 7). Samide is a novel neurohormone family recovered in the three main classes of mollusks and in Annelids that still has to be functionally characterized.

3.2.1.18. TAamide. A novel neuropeptide encoded by a large 608-amino-acid precursor was identified by MS screening in the CNS and the hemolymph. It is a C-terminally amidated neuropeptide with the following primary sequence: RVTIKG-LDLTAamide.

3.2.1.19. Tachykinin. Tachykinins are one of the largest neuropeptide families expressed in both vertebrates and invertebrates. A single precursor was identified in *S. officinalis* as in *O. vulgaris* (BAD26598), encoding 9 C-terminally amidated neuropeptides (Figure 6) recovered in all parts of the CNS. A similar C-terminal end, GFMGSRamide, was recovered in both *Sepia* tachykinin and *Lottia* tachykinin 2.[36] Five tachykinin neuropeptides were detected in cuttlefish hemolymph.

3.2.2. Neuropeptides Likely Acting as Neuromodulators within the CNS and Neuropeptides Undetected by Mass Spectrometry. 111 neuropeptides encoded by 33 protein precursors belonging to 20 neuropeptide families are listed in Table S-1. The neuromodulator status was established by MS analyses of CNS extracts. The protein precursors of neuropeptides undetected by MS screening were exclusively deduced by in silico screening based on structural criteria. Seven protein precursors able to release 11 neuropeptides belonged to that category.

43 neuropeptides were detected out of 111 predicted by the preprohormones presented in Figures 10, 12, 14, and 15. The Mascot scores of the detected neuropeptides are summarized in Table S-2.

3.2.2.1. Achatin 1. Achatins have been previously described. Achatin 1 preprohormone putatively encoded 13 copies of the tetrapeptide GFGD, one copy of YFLD, and one of an N-terminal elongated form AFDDDTGFDD. None of these mature products was detected by MS/MS analysis, but a 27 amino acid C-terminally amidated peptide following signal peptide was detected in the SubEM as well as a 11 amino acid C-terminal truncated peptide ALEESFKSDGAamide (Figure 8).

3.2.2.2. Allatostatin A3 (AST A3) or Buccalin 3. In insects, FGLamide allatostatins (ASTs), also called allatostatins A or buccalins, are involved in the inhibition of juvenile hormone biosynthesis by the corpora allata in the inhibition of vitellogenesis and visceral muscle contraction[75] and in feeding decisions interacting with AKH and insulin-like peptides.[76] One of the two expected mature products (Figure 7) was detected in cuttlefish SupEM and SubEM.

3.2.2.3. APGWamide. The tetrapeptide APGWamide was first identified in the gastropod *Fusinus ferrugineus* by Kuroki and collaborators.[77] It regulates penis erection in the pond snail *Lymnaea stagnalis*.[78] In *S. officinalis*, the dipeptide GWamide is cleaved in the CNS from the tetrapeptide by a dipeptidyl aminopeptidase; it stimulates oviduct contractions during spawning.[20] In *Lottia*, GWamide is encoded as a single copy among nine copies of the tetrapeptide. In cuttlefish, the preprohormone encoded 14 copies of the tetrapeptide detected in the CNS (Figure 8).

3.2.2.4. Bursicons A and B. Bursicons are cuticle tanning hormones coexpressed with CCAP in the CNS of arthropods.[79,80] They were structurally characterized in cockroach by Dewey and collaborators[81] and in *Lottia gigantea* by Veenstra.[36] Bursicons are also expressed in bivalves, like bursicon B in *C. gigas*.[57] Bursicons occur as homo- or heterodimer hormone isoforms. They are suspected to be involved in immunopro-

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Figure 6. Predicted preprohormones pedal peptide 2, Pro-Sepiatocin, PRQFVamide, Samide, TAamide, and tachykinin, encoding neurohormones detected in the hemolymph or in the neurohemal area of *S. officinalis* egg-laying females. Predicted signal peptides are highlighted in yellow, predicted convertase cleavage sites in red, cysteines in green, glycine residues predicted to be converted into C-terminal amides in dark gray, as well as glutamine N-terminal residues predicted to be converted into pyroglutamic acid. Neuropeptides detected by mass spectrometry are highlighted in light gray, and the stop codons at the end of the coding sequences are indicated by an asterisk.

tective defense mechanisms of arthropods.[82] Bursicons A and B preprohormones were identified in *S. officinalis* CNS by in silico analysis. It was not full length, but the cysteine pattern appeared to be conserved (Figure 9). MS analyses did not detect mature products in the CNS or in the hemolymph.

3.2.2.5. Cerebrin. Cerebrin was first identified from *A. californica* cerebral ganglia by Li and Floyd.[83] It is involved in feeding behavior. In *S. officinalis* as in gastropods, cerebrin is a C-terminally amidated peptide encoded as a single copy from the C-terminal end of a small precursor (Figure 10). Cuttlefish cerebrin was detected in SubEM.

3.2.2.6. Clionin. Clionin was first identified in the gastropod *Tritonia diomedea* (I1SKI5) (unpublished data). It is a large cysteine-rich neuropeptide encoded as a single copy from a small precursor. In *S. officinalis*, Clionin is a 57-amino-acid, C-terminally amidated peptide with three putative disulfide bonds (Figure 10), detected in SubEM. Clionin is a novel neuropeptide without any function described so far. Blast analysis revealed a similar precursor in *C. gigas* (EKC23167) and in *A. californica* (XP_005099829) (Figure 11).

3.2.2.7. Crustacean Cardioactive Peptides. Initially described in crustaceans, crustacean cardioactive peptides (CCAPs) were first identified from the gastropod *H. pomatia*.[84]

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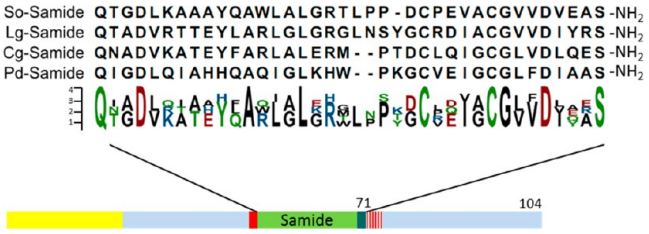

Figure 7. Sequence alignment of precursors of Samide neuropeptides of the mollusks *Sepia officinalis*, *Lottia gigantea* (XP_009051664), *Crassostrea gigas* (EKC20562), and the annelid *Platynereis dumerilii* (AHB62379). Signal peptide in yellow, dibasic convertase cleavage sites in red, tetrabasic convertase cleavage sites in striped red, and glycine residues predicted to be converted into C-terminal amides in gray. The N-terminal glutamine could be converted into pyroglutamic acid.

Whereas CCAPs are usually C-terminally amidated neuropeptides in arthropods[85−89] as in mollusks,[53,77,78,56] the *S. officinalis* preprohormone predicted three nonamidated peptides detected in SupEM, SubEM, and OLs (Figure 10).

3.2.2.8. FFamide. FFamide was first identified in *Lymnaea stagnalis*,[90] in *Lottia gigantea*,[36] and in *Theba pisana*.[56] It is involved in the transport of semen in the vas deferens. In *S. officinalis*, the preprohormone predicted three C-terminally amidated peptides (Figure 10). Two of them (GLNPNVNSLFFamide and VYAPTRGQQNPHSYamide) were detected in the CNS.

3.2.2.9. GGNG (GGNamide). Initially characterized from the earthworms *Eisenia fetida* and *Pheretima vittata*,[91] GGNGs were also found in the worms *Capitella teleta* and *Helobdella robusta*.[63] C-terminally amidated GGNG-like peptides were identified in the mollusks *Thais clavigera*[92] and *Lottia gigantea*.[36] In *S. officinalis*, the preprohormone predicted a single GGNamide (Figure 12) that was detected in SupEM.

3.2.2.10. Leucokinins. Leucokinins (LKs) were identified in several mollusks such as *Lymnaea stagnalis* and *Lottia gigantea*.[36,93] In *S. officinalis*, LK is encoded by two genes that we called leucokinins 1 and 2. The preprohormones of leucokinin 1 and 2 predicted, respectively, 3 and 4 neuropeptides (Figure 12). The heptapeptide SRFSPWHamide was the single LK-like peptide detected in cuttlefish CNS.

3.2.2.11. Luqin or Cardioexcitatory Peptide. Luqin was first identified from *A. californica*.[94,95] In cuttlefish, the preprohormone predicted a single 13-amino-acid peptide, C-terminally amidated immediately after the signal peptide, as described in other mollusk classes (Figure 12). The neuropeptide was detected only in SubEM.

3.2.2.12. Myomodulin. Myomodulins are encoded by two precursors in *A. californica* and *L. gigantea*. They were initially identified in *A. californica* and *L. stagnalis*[96,97] and have been recovered in many gastropod and bivalves mollusks. In cuttlefish, one single precursor, not full length, encodes at least 10 distinct neuropeptides with at least 20 copies of the AVSMLRLamide sequence (Figure 12). Only three neuropeptides were detected in the *S. officinalis* CNS.

3.2.2.13. NKY. Neuropeptide KYs (NKYs) were first identified in *A. californica* and called bradykinin-like neuropeptides.[98] A bradykinin-like neuropeptide precursor gene is expressed in neuron L5 of *Aplysia californica*. The related neuropeptides have been renamed neuropeptides KY because the N-terminal amino acid is predicted to be a lysine and the C-terminal amino acid is predicted to be a Yamide.[99,100]

In *S. officinalis*, three precursors of NKK were identified. NKY1 encoded a single 34-amino-acid, C-terminally amidated neuropeptide detected by MS in the CNS, together with a truncated form composed of 17 amino acids cleaved at the level of the arginine located at position 17. NKY2 encoded a single 24-amino-acid, C-terminally amidated neuropeptide detected in the CNS (Figure 12). NKY3 predicted a 29-amino-acid LLamide peptide and the expected 38-amino-acid neuropeptide FRYamide (Figure 12). Finally, two N-terminal truncated FRYamide neuropeptides were detected in the CNS: one composed of 26 amino acids and one composed of 22 amino acids.

3.2.2.14. Neuropeptide Y (NPY or NPF). While NPY3 was detected in the hemolymph, NPY1, 2, 4, and 5 were detected

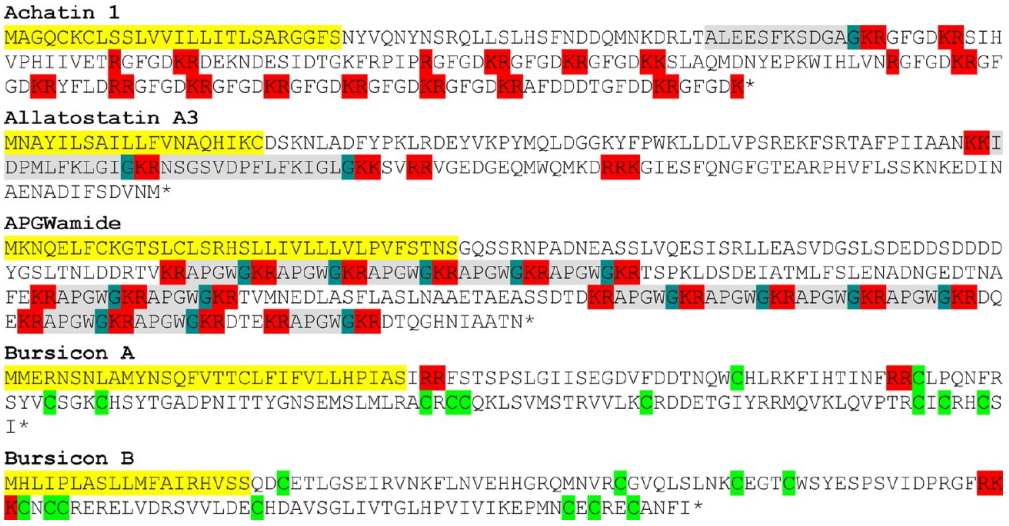

Figure 8. Predicted preprohormones of achatin 1, allatostatin, APGWamide, and bursicons A and B, encoding neuropeptides detected in the nerve endings or in the CNS of *S. officinalis* egg-laying females or by in silico data mining. Predicted signal peptides are highlighted in yellow, convertase cleavage sites in red, cysteines in green, glycine residues predicted to be converted to C-terminal amides in dark gray, as well as glutamine residues predicted to be converted into pyroglutamate. Neuropeptides detected by mass spectrometry are highlighted in light gray, and the stop codons at the end of the coding sequences are indicated by an asterisk.

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Figure 9. (A) Sequence alignment of precursors of bursicon A of the insects *Nilaparvata lugens* (BAO00937), *Drosophila melanogaster* (AAF55915.1), and *Zootermopsis nevadensis* (KDR13886), the crustacean *Carcinus maenas* (ABX55995), and the mollusk *Sepia officinalis*. (B) Sequence alignment of precursors of bursicon B of the insects *Nilaparvata lugens* (BAO00938), *Drosophila melanogaster* (Q9VJS7), and *Zootermopsis nevadensis* (KDR13885), the crustacean *Homarus gammarus* (ADI86243), and the mollusk *Sepia officinalis*. Signal peptide in yellow.

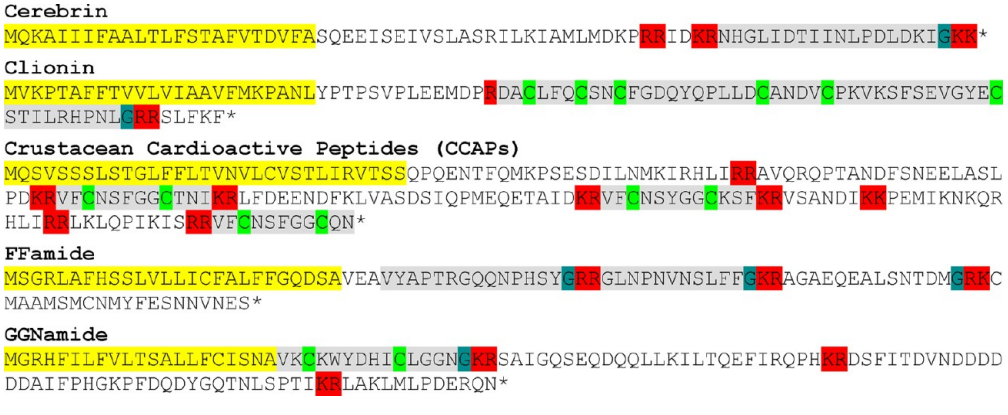

Figure 10. Predicted preprohormones of cerebrin, clionin, CCAPs, FFamide, and GGNamide, encoding neuropeptides detected in the nerve endings or in the CNS of *S. officinalis* egg-laying females or by in silico data mining. Predicted signal peptides are highlighted in yellow, convertase cleavage sites in red, cysteines in green, glycine residues predicted to be converted into C-terminal amides in dark gray, as well as glutamine residues predicted to be converted into pyroglutamate. Neuropeptides detected by mass spectrometry are highlighted in light gray, and the stop codons at the end of the coding sequences are indicated by an asterisk.

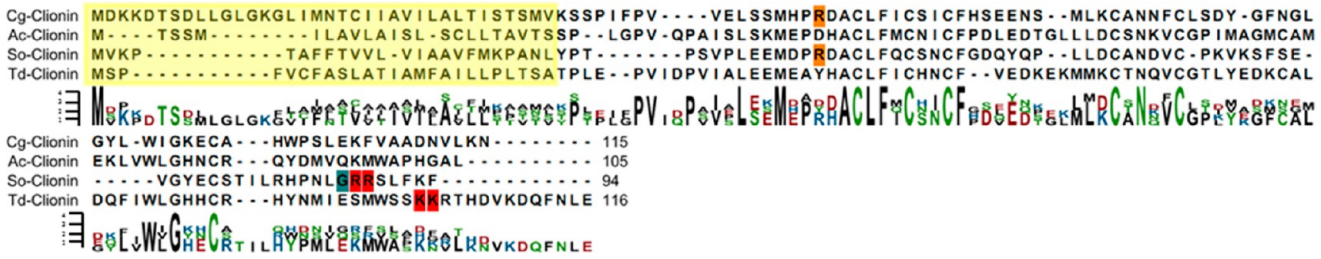

Figure 11. Sequence alignment of precursors of clionin of the mollusks *Crassostrea gigas* (EKC23167), *Aplysia californica* (XP_005099829), *Sepia officinalis*, and *Tritonia diomedea* (I1SKI5). Signal peptide in yellow, monobasic convertase cleavage sites in orange, dibasic convertase cleavage sites in red, and glycine residues predicted to be converted into C-terminal amides in gray. Convertase cleavage sites were deduced from *S. officinalis* mature clionin detected in the CNS by mass spectrometry.

only in SupEM and SubEM, suggesting they could act as neuromodulators (Figure 13).

3.2.2.15. NdWFamide. NdWFamide is a D-tryptophan-containing neuropeptide first identified in *A. kurodai*.[101] In *S. officinalis*, the NdWFamide preprohormone encoded a single neuropeptide N(d?)YYamide following signal peptide (Figure 13). With only 80 amino acids, the precursor is shorter than the two precursors isolated by Morishita and collaborators[102] in *A.*

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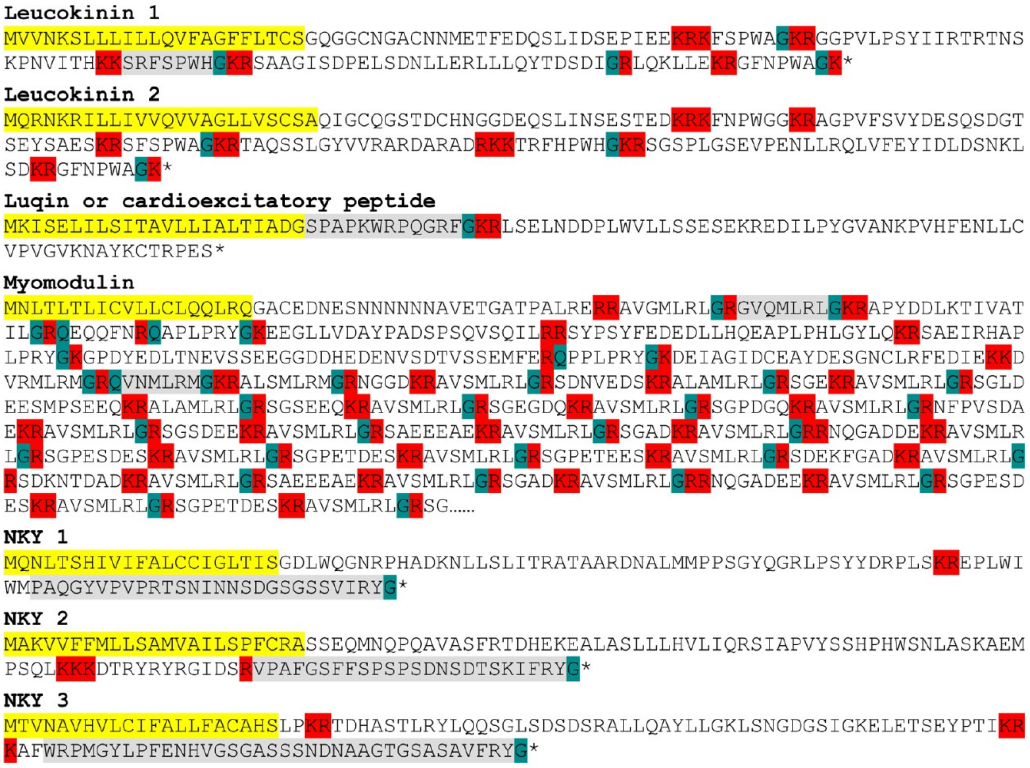

Figure 12. Predicted preprohormones leucokinins, luqin, myomodulin, and NKY 1, 2, and 3, encoding neuropeptides detected in the nerve endings a or in the CNS of *S. officinalis* egg-laying females or by in silico data mining. Predicted signal peptides are highlighted in yellow, convertase cleavage sites in red, cysteines in green, glycine residues predicted to be converted into C-terminal amides in dark gray, as well as glutamine residues predicted to be converted into pyroglutamate. Neuropeptides detected by mass spectrometry are highlighted in light gray, and the stop codons at the end of the coding sequences are indicated by an asterisk.

kurodaï, with lengths of 87 and 90 amino acids, respectively. Their function remains unknown.

3.2.2.16. Orcokinin B. The first orcokinin ever identified was isolated from the crayfish *Orconectes limosus*.[103] These neuropeptides are potent hindgut myotropic factors identified in many crustaceans (*Carcinus maenas*,[104] *Cancer borealis*[105]) and also in Arachnida (*Stegodyphus mimosarum*), in which a type-B orcokinin (KFM82953) was identified from the genome. In some insects, two isoforms of orcokinin B both expressed in the CNS and in the gut could be involved in diuresis and feeding.[106,107] *S. officinalis* orcokinin B is the first one ever identified in mollusks. Nevertheless, blast analysis revealed a similar precursor in *A. californica* (XP_005098921). Five orcokinins B out of seven predicted by the incomplete preprohormone were detected by MS in SupEM, SubEM, and OLs (Figures 13 and 14).

3.2.2.17. PKYMDT or Proctolin. Proctolin (RYPLT) was first identified in the cockroach *Periplaneta Americana* as a myotropic peptide regulating the contraction of the proctodeal muscle.[108] In mollusks, the PKYMDT hexapeptide is very well conserved across all species and could be a homologue of proctolin. In *S. officinalis*, the preprohormone was isolated by in silico screening (Figure 13). Two C-elongated forms of the expected PKYMDT hexapeptide were detected in the SupEM and in the SubEM.

3.2.2.18. PTSP-Like Peptide. We identified two incomplete preprohormones probably resulting from alternative splicing from the CNS transcriptome (Figure 13). These neuropeptides are already known in *A. californica*[65] and *L. gigantea*,[45] but their function is not yet elucidated. In cuttlefish, we detected neuropeptides in SupEM, SubEM, and OLs.

3.2.2.19. PXXXamide. A single 30-amino-acid, C-terminally amidated neuropeptide was detected by MS in *S. officinalis* SupEM and OLs. This neuropeptide was cleaved from a short unknown precursor never described in any animal species before (Figure 13).

It showed strong homology with several uncharacterized proteins from the polychaete worm *Capitella teleta*,[45] the owl limpet *L. gigantea*,[45] the insect *Acromyrmex echinator* (EGI60187), and the crustacean *Daphnia pulex*[70] (Figure 15).

A 14-amino-acid domain of the neuropeptide is conserved as well as the C-terminal amidation and the proline. PXXXamide is a novel neuropeptide family recovered in three protostomian phyla that remains to be functionally characterized.

3.2.2.20. Small Cardioactive Peptides (SCPs). A single precursor was identified is *S. officinalis*, encoding a single C-terminally amidated decapeptide (Figure 13) as described for octopus.[109] By contrast, gastropod precursors encode two nonapeptides called SCP A and SCP B and located after the signal peptide[110,111,56]. MS analyses did not detect the decapeptide in the CNS.

3.2.2.21. Sepiatocin. The OT/VP precursors of invertebrates and vertebrates are very similar. They encode a single neuropeptide that we did not detect in *S. officinalis* CNS (Figure 13).

3.2.2.22. SPamides 1 and 2. These neuropeptides were identified in *S. officinalis* using in silico data mining based on structural criteria, such as signal peptide prediction and glycines followed by putative convertase cleavage sites. Two precursors encoded previously unknown neuropeptides sharing a conserved C-terminal SPG end; the presence of a glycine made amidation likely (Figure 13). A first precursor encoded two

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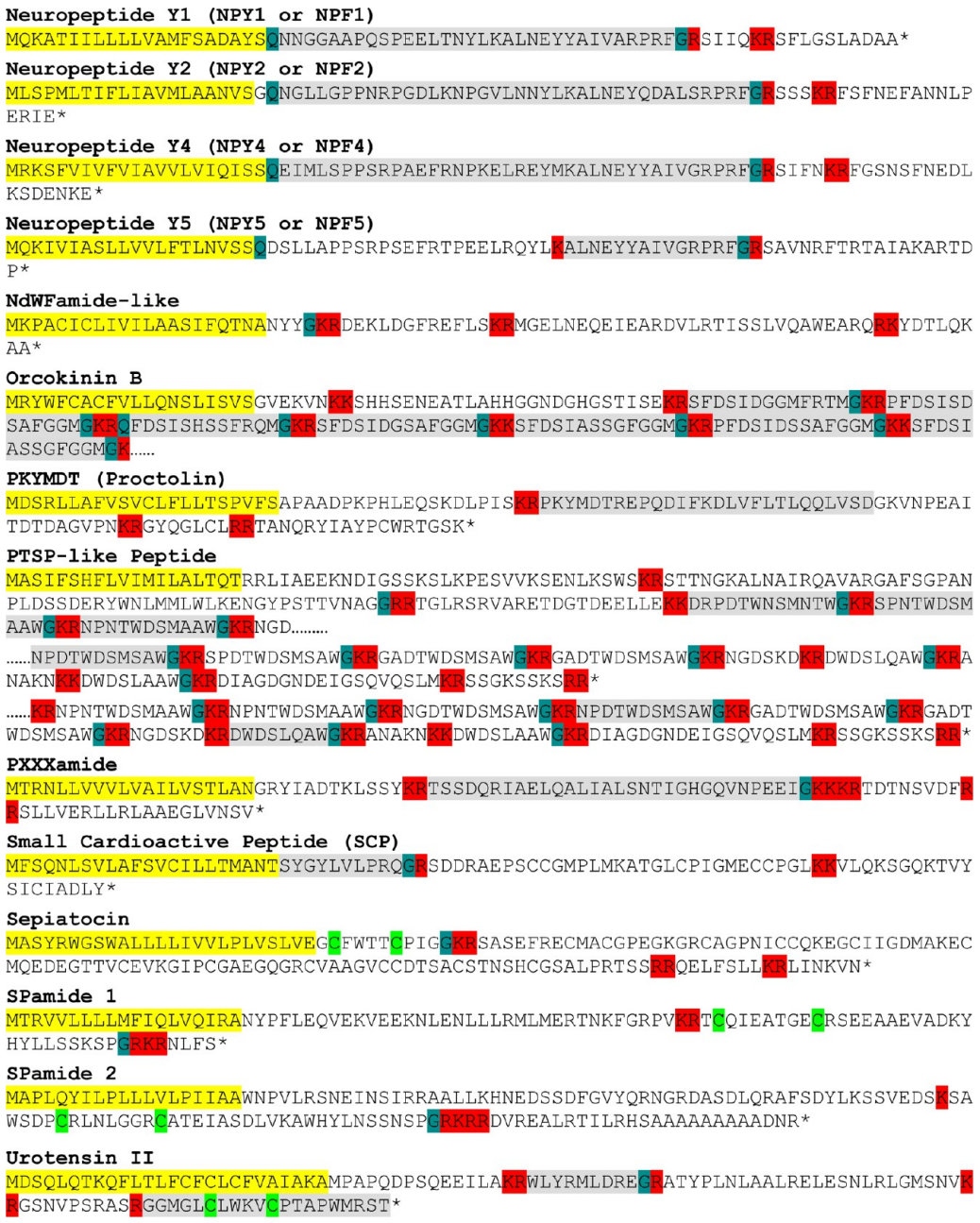

Figure 13. Predicted preprohormones of NPY 1, 2, 4, and 5, NdWFamide, orcokinin B, PKYMDT (proctolin), PTSP-like Peptide, PXXXamide, SCP, sepiatocin, SPamide 1 and 2, and urotensin II, encoding neuropeptides detected in the nerve endings or in the CNS of *S. officinalis* egg-laying females or by in silico data mining. Predicted signal peptides are highlighted in yellow, convertase cleavage sites in red, cysteines in green, glycine residues predicted to be converted into C-terminal amides in dark gray, as well as glutamine residues predicted to be converted into pyroglutamate. Neuropeptides detected by mass spectrometry are highlighted in light gray, and the stop codons at the end of the coding sequences are indicated by an asterisk.

neuropeptides: The first one was a putatively C-terminally amidated nonapeptide, and the second one was a 33-amino-acid neuropeptide with two cysteines at positions 2 and 10 that could form a disulfide bond. A C-terminal glycine could lead to C-terminal amidation. We called this neuropeptide SPamide 1. The second precursor encoded a single 37-amino-acid neuropeptide with a conserved C-terminal end (SPG) and two cysteines spaced as in SPamide 1. We called this second neuropeptide SPamide 2.

By screening NCBI databases, we established that this neuropeptide family had not been described in any species, whereas precursors from the gastropod *L. gigantea* (XP_009043607 and XP_009062318),[45] from the annelid *C. teleta* (ELT87883)[45] and from the arachnid *Stegodyphus mimosarum* (FM69182), encoded homologue neuropeptides. Sequence alignment showed strong conservation of the cysteines and a common C-terminal SPamide end. The precursor of *C. teleta*, *S. mimosarum*, and one of the two precursors of *L. gigantea* encode two SPamides. The structure of SPamide neuropeptides is conserved across three phyla (mollusks, annelids, arthropods) (Figure 16). This allows us to suspect that these new neuropeptides could be involved in the regulation of major physiological mechanisms.

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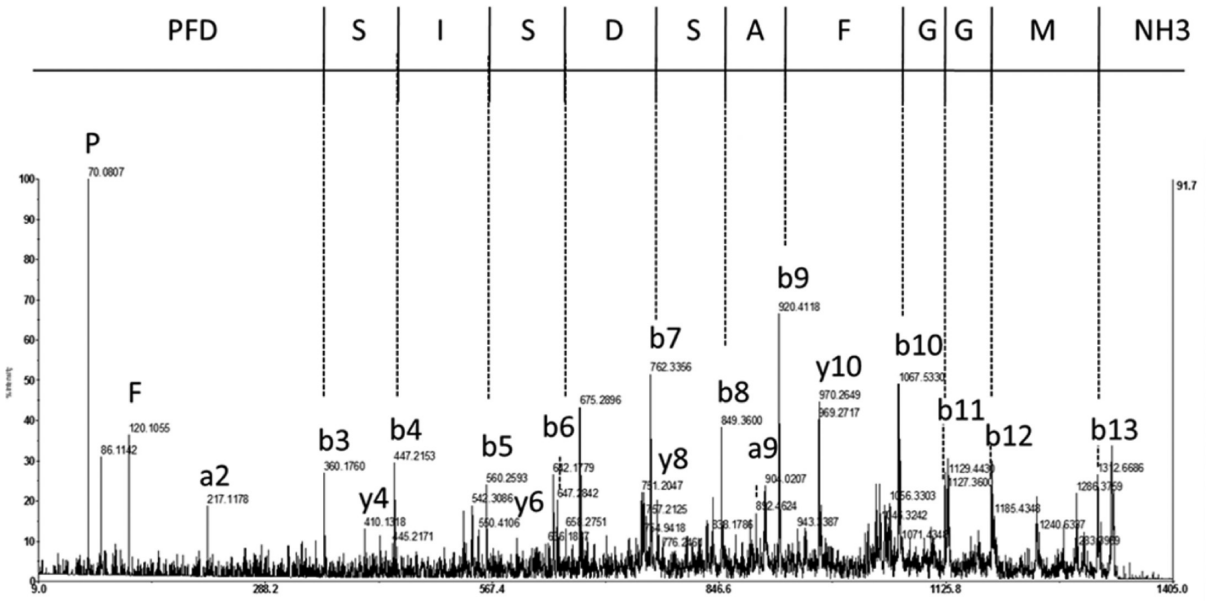

Figure 14. Off-line nLC-MALDI tandem MS analysis of *Sepia officinalis* SupEM collected during spawning. MS/MS spectrum of one of the seven neuropeptides (m/z 1329.51) predicted by the orcokinin B precursor. Immonium, a, b, and y ions detected are indicated.

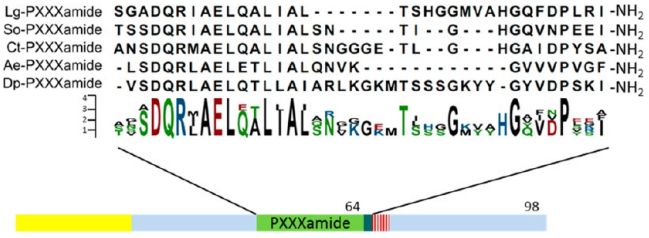

Figure 15. Sequence alignment of the neuropeptide PXXXamide of the mollusks *Lottia gigantea* (ESO97822) and *Sepia officinalis*, the annelid *Capitella tellata* (ELT91968), and the arthropods *Acromyrmes echinator* (EGI60187) and *Daphnia pulex* (EFX77601). Signal peptide in yellow, tetrabasic convertase cleavage sites in striped red, and glycine residues predicted to be converted into C-terminal amides in gray.

Figure 16. Sequence alignment of the neuropeptide SPamide of the mollusks *Sepia officinalis* and *Lottia gigantea* (XP_009043607 and XP_009062318), the annelid *Capitella tellata* (ELT87883), and the Arachnida *Stegodyphus mimosarum* (FM69182).

3.2.2.23. Urotensin II. Urotensin II (UII) is a cyclic peptide first isolated from the urophysis of the goby *Gillichthys mirabilis*.[112] It acts as a vasoactive effector. In invertebrates, UII was identified only in *A. californica*,[113] where it is thought to be involved in the modulation of feeding behavior. In *S. officinalis*, the UII precursor is a small precursor encoding a C-terminally amidated decapeptide and a 20-amino-acid UII-like peptide located at the C-terminal end (Figure 13). MS analyses confirmed in silico predictions: They detected the WLYRML-DREamide decapeptide and full-length UII in both SupEM and SubEM. Two C-terminally truncated forms of UII were also detected. Moreover, blasts performed against the NCBI database identified three novel UIIs in the mollusks *L. gigantea* (XP_009064146) and *C. gigas* (EKC42138) and in the polychaete *C. teleta* (ELU12290) (Figure 17).

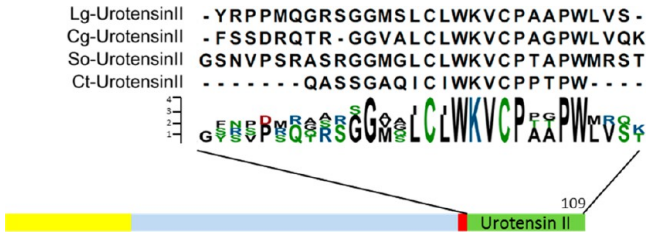

Figure 17. Sequence alignment of urotensin II of the mollusks *Lottia gigantea* (XP_009064146), *Crassostrea gigas* (EKC42138), and *Sepia officinalis* and the annelid *Capitella tellata* (ELU12290). Signal peptide in yellow and dibasic convertase cleavage sites in red.

3.2.2.24. Focus on Egg-Laying Neuropeptides. Two main criteria were initially retained to select neuropeptides putatively involved in egg-laying: (1) their occurrence in the nerve endings innervating female ASGs (OG, MNGs, and accessory nidamental glands (ANGs)) and ovaries in ELFs and (2) the neuropeptide expression pattern in the CNS of ELFs versus mature males. Moreover, we used a third criterion while examining expression patterns: the occurrence of neuropeptide mRNAs in ASGs.

3.3. Neuropeptidome Detected in the Nerve Endings Innervating Female Accessory Sex Glands

MS analysis revealed the occurrence of 29 mature neuropeptides cleaved from 9 precursors belonging to 8 families (see Table S-1). Twenty-eight neuropeptides were recovered at the level of the OG versus 10 in the MNGs and 3 in the ovarian stroma (OS). No neuropeptide was detected in the ANGs.

FLGamide was the only neuropeptide family detected in OG, MNGs, and OS. CCAPs (Figure 18), FaRPs, and myomodulins were detected in both OG and MNGs. We detected the

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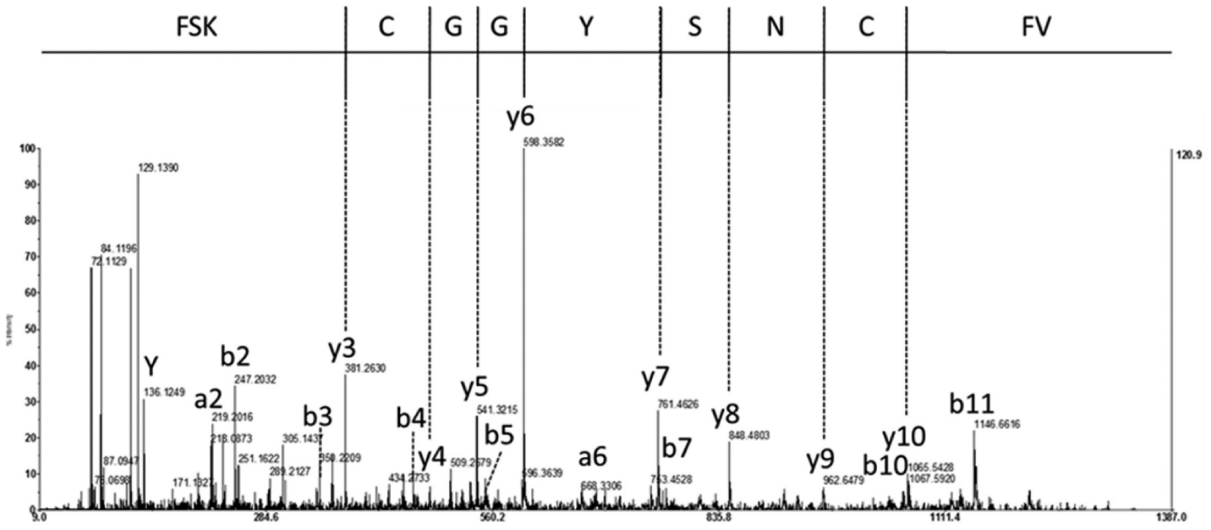

Figure 18. Off-line nLC-MALDI tandem MS analysis of *Sepia officinalis* oviduct gland collected during spawning. MS/MS spectrum of the neuropeptide VFCNSYGGCKSF (m/z 1311.53), one of the three CCAPs detected. Detected immonium, a, b, and y ions are indicated.

decapeptide cleaved from the FaRP precursor in OG and MNGs; this confirms the immunostaining results obtained by Henry and collaborators[21] in the ASGs of mature females. Seven PTSP-like peptides out of the 9 peptides predicted by the precursor were recovered from the OG.

3.4. Neuropeptidome Overexpressed during Egg-Laying

We sequenced the CNS transcriptome both in ELFs and in mature males to predict neuropeptidome but also to study and compare expression patterns between sexes.

Our data revealed that the neuropeptidome was globally overexpressed in ELFs versus mature males. Overexpression was mainly concentrated in the SubEM that is related to the single NA described in cuttlefish. It is also the CNS part that innervates the genital apparatus and the viscera. We calculated the average "ELF over mature male" ratios to illustrate overexpression in the three parts of the CNS. Ratios were, respectively, 22, 1.7, and 1.6 in the SubEM, the SupEM, and the OLs. In female SubEM, the expression of 14 neuropeptide families was at least 10 times higher than in male SupEM (Figure 19). These 14 neuropeptide families can release at least 36 neuropeptides that may be involved in the regulation of egg-laying or in physiological mechanisms stimulated by the beginning of egg-laying.

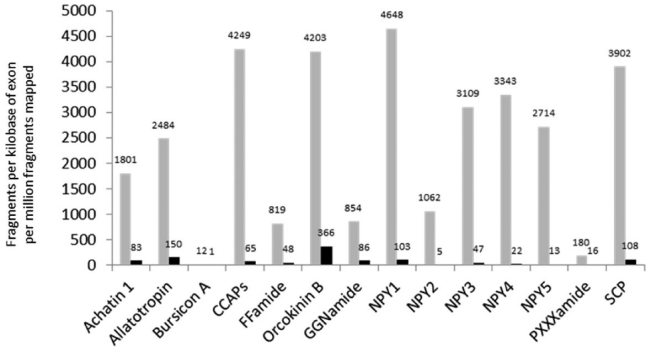

Figure 19. Expression pattern of 14 neuropeptide families overexpressed in the subesophageal mass of egg-laying females (gray) versus mature males (black).

3.5. Neuropeptide mRNAs Recovered from Female ASGs

The transcriptomes of ASGs and ovarian follicles (three stages) revealed the occurrence of high levels of neuropeptide transcripts. APGWamide transcripts were present in all ASGs, with a maximum value in ANGs, and also in the three ovarian follicle stages. CCK transcripts were recovered from ANGs and from two ovarian follicle stages (previtellogenic follicles and mature oocytes also called smooth oocytes). Clionin transcripts were restricted to mature oocytes, insulin transcripts were recovered from OG, accessory, and MNGs and three ovarian follicle stages, leucokinin 1 transcripts from MNGs and OG, myomodulin transcripts from MNGs and OG, orcokinin B transcripts from mature oocytes, accessory and MNGs and OG, sepiatocin transcripts from the three ASGs and from mature oocytes, SPamide 1 transcripts from ANGs and OG, and TAamide transcripts from MNGs and OG (Figures S-3 and S-4). We can hypothesize that neuropeptide mRNAs were located in the nerve endings of ASGs. Similar observations were reported in the pond snail *L. stagnalis* by van Minnen and Bergman.[114] High amounts of mRNAs encoding the egg-laying hormone were detected in the nerve terminals after a stimulus as well as polyribosomes, supporting that the translation of egg-laying hormone transcripts could occur in the axonal compartment.

In a recent paper, Cosker and collaborators[115] demonstrated that neurons could direct transcripts and that mRNA transport from the nucleus to the stimulated subcellular compartments was activity-dependent. In addition, Martin and Kim[116] used *Aplysia* as a model to show that netrin-1, already known to promote translation in axonal growth cones,[117] was able to increase translation of subcellular mRNAs localized at the level of dendrites or axons by binding the cytoplasmic domain of the netrin-1 receptor called DCC (for deleted colorectal cancer). Finally, localized secretory mechanisms at the level of the peripheral nervous system were established by ultrastructural studies revealing the occurrence of rough endoplasmic reticulum, smooth reticulum, and Golgi apparatus in the axonal compartment.[118] Thus, the translation of large amounts of neuropeptide mRNAs detected in female ASGs could allow for the mobilization of mature neuropeptides in response to local stimuli. Mating, which is immediately followed by the triggering

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of egg-laying, is one of the local stimuli that could induce the translation of axonal mRNAs to stimulate oocyte release in the mantle cavity and egg capsule secretion. The rapid reaction of female cuttlefish can be related to the state of readiness of the axons that innervate ASGs.

According to these data, we identified the netrin-1 precursor (not full length) and the netrin-1 DCC receptor from the *S.officinalis* transcriptome. Figure S-5 presents the alignment of Aplysia, cuttlefish, and human netrin-1 sequences.

Regarding the mRNAs recovered from the ovarian follicles (APGWamide, cholecystokinin (CCK), clionin, sepiatocin), we can suspect local transcription because no nerve endings are present. Functional interpretation of these data remains difficult. Moreover, detecting mature neuropeptides in the ovarian follicles by MS/MS analyses would definitively establish the occurrence of these neuropeptides in cuttlefish ovaries. Expression data are summarized in Figures S-4 and S-5.

3.6. Case of Accessory Nidamental Glands

Although we recovered many neuropeptide transcripts from accessory nidamental gland (ANGs) (APGWamide, CCK, FLGamide, Insulin, Sepiatocin, and SPamide), MS analyses did not detect any neuropeptides related to these transcripts. In fact, the subcellular localization of these mRNAs, probably at the level of terminal axons, suggests that ANGs dissected out from ELFs to perform RNaseq were waiting for stimuli to start translation. Therefore, the involvement of ANGs in egg-laying does not appear very clear regarding the data provided by this study.

3.7. Neuropeptides and Regulation of Egg-Laying

The results previously presented allowed us to discriminate neuropeptide families that had a high probability of being involved in the regulation of one or several steps of egg-laying.

CCAPs and SCP appeared to be both overexpressed in the SubEM and detected in OG nerve endings. CCAPs and SCP could regulate the secretion of the internal layer of the egg capsule and stimulate the biosynthesis of egg-capsule proteins and sex pheromones.[3] Because OG is closely associated with the distal oviduct, they could also modulate oocyte transport.

APGWamide transcripts recovered from whole female genital apparatus are clearly associated with egg-laying, as described by Henry and collaborators[119] and Bernay and collaborators[120] in *S. officinalis* and *C. gigas*, and in gastropod male behavior.[19] Using MS screening of genital products, Bernay and collaborators[120] showed that APGWamide was also present in the sperm of cuttlefish and oyster.

Insulin transcripts were abundant in ANGs. The role of these glands in egg-capsule elaboration is not clearly established, so we cannot discuss about the putative function of insulin in ASGs.

The localization of myomodulin transcripts in MNGs and OG led us to hypothesize that myomodulin might be involved in the secretion of the two egg-capsule layers, that is, the internal layer by OG and the external layer by MNGs. As previously described for CCAPs and SCP, myomodulin could be involved in the expression and the release of sex pheromones at the level of the OG.

With six neuropeptides detected in OG nerve endings, four in MNGs, and three in the ovarian stroma, FLGamide may be involved in the secretion of the two egg-capsule layers during egg-laying, in the stimulation of oocyte release in the genital coelom, and in the regulation of oocyte transport.

4. DISCUSSION AND CONCLUSIONS

For the first time in a cephalopod, the neuropeptidome was fully investigated using de novo RNAseq associated with a peptidomic approach. 38 families of neuropeptides expressed by 55 transcripts were recovered from the transcriptome of *S. officinalis*. Most of the mature expression products, neuropeptides, predicted from preproproteins were confirmed by MS analyses. A total of 170 neuropeptides were predicted from the transcriptome, out of which 131 were chemically characterized by MS. Among the neuropeptides detected by MS, 59 were present in the hemolymph or the NA.

In addition, five novel families of neuropeptides, FLGamide, PXXXamide, Samide, SPamide 1 and 2, and TAamide, were identified by in silico data mining or MS, and orthologous neuropeptides were also identified in gastropods, bivalves, annelids, and insects from transcripts annotated as "uncharacterized protein" or "predicted protein". This wide display across several protostomian phyla and the conservation of neuropeptide structure allow us to speculate about their putative involvement in the regulation of major physiological processes. These data show that a large investigation field remains open among protostomians to elucidate the involvement of newly discovered neuropeptides in the regulation of physiological processes.

Regarding the expression data released by RNAseq, the first important observation is the strong overall overexpression of the female neuropeptidome as compared with the male neuropeptidome, with an average ratio of 22, and up to 209 for NPF5. The physiological status of ELFs is probably the key to explain these data: Once the first gametogenesis stage has produced a first batch of oocytes, gametogenesis is resumed at the beginning of egg-laying, with the release of a second batch of mature oocytes in the genital coelom. Because oocytes contain a large quantity of vitellus for embryonic development, females have to maintain a high level of predation to synthesize yolk proteins. Among the neuropeptides overexpressed in females, we found the five NPY/NPF transcripts usually involved in feeding behavior that could stimulate predation. Moreover, the resumption of gametogenesis may also be associated with the overexpression of neuropeptides regulating vitellogenesis in ovarian follicles. Thus, a female can spawn several hundred eggs in two or three goes (or more) separated by rest periods allowing the production of a new batch of mature oocytes. By contrast, in males gametogenesis is ended 6 months before reproduction and produces a much smaller volume of gametes than in females. The energy consumed by male gametogenesis is probably very low compared with the energy consumed by female gametogenesis. To see whether peptide expression is related to sex or reproductive state, it would be very interesting to compare ELFs with nonreproductive (virgin) females. The neurotranscriptome of subadult males and females (one year-old animals) will soon be available for differential studies.

Tissue mapping performed by MS is the tool we used to discriminate neurohormones from neuromodulators by localizing them differentially in the hemolymph, the NA, nerve endings, or only in the CNS of ELFs.

Tissue mapping revealed the occurrence of a second NA. Until now, histological and immunocytological investigations had not localized such a structure in cephalopod decapods like cuttlefish and squid. Our MS/MS data showed that 13 neuropeptide families circulated but were not detected in the single NA described in cuttlefish. Searching for and localizing at

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least a second NA is now essential to identify the CNS part involved in neurohormone expression and secretion.

As previously explained, the selection of neuropeptides suspected to be involved in the regulation of egg-laying is also based on tissue localization, on the expression levels of transcripts in ELFs versus mature males, and on the occurrence of broad stocks of mRNA in nerve endings innervating female ASGs. Several neuropeptide families met one or more of these criteria: APGWamide, CCAPs, clionin, FLGamide, PTSP-like peptides, SCP, insulin, myomodulin, sepiatocin, and SPamide.

These data corroborate the observations of York and collaborators[121] about the involvement of APGWamide, insulin, and myomodulin in the regulation of egg-laying in the tropical abalone *Haliotis asinina*. In this gastropod, egg-laying is characterized by a dramatic increase in the expression of these three neuropeptides within 12 h of the spawning event. Expression strongly decreases 24 h after spawning, demonstrating that these neuropeptides have a regulatory role in the release of gametes.

Besides well-known neuropeptide families, FLGamide and SPamide belong to two novel families never described before and newly identified (FLGamide) or predicted (SPamide) in the present study. We detected FLGamide neuropeptides not only in the nerve endings of OG and MNGs but also in the nerve endings of the ovarian stroma, suggesting a strong involvement in the regulation of several processes associated with egg-laying. We detected it in every part of the CNS, in the hemolymph, but did not detect it in the NA. FLGamide appears to be a very interesting neuropeptide family to study the regulation of egg-laying by both neuromodulators and neurohormones and to elucidate the putative occurrence of a second NA.

We also detected significant amounts of mRNAs of the neuropeptides cited above in female ASGs. Their presence suggests that these neuropeptides are involved in the rapid response regulated by netrin-1. In the context of a netrin-1/DCC receptor system, the translation of axonal mRNAs of neuropeptides could be induced by external stimuli such as mating (mating is immediately followed by spawning) or detection of chemical messengers such as aquatic sex pheromones released by mates. When these stimuli are associated with a temperature and photoperiod threshold, they form a set of suitable conditions to induce egg-laying. In gastropods, environmental parameters trigger the expression and release of ELH, a 36-amino-acid neuropeptide able to induce egg-laying following a single intramuscular injection in the foot. Unfortunately, despite the depth of our de novo RNAseq of cuttlefish CNS, 38 neuropeptide families identified with a broad expression range, in silico data mining and MS did not identify neuropeptide homologues of gastropod ELH. Although we constructed our cDNA library from CNSs removed from mature females sacrificed during spawning, we failed to identify any ELH transcript. In addition, circulating ELH should be detected in the hemolymph. Considering that cephalopods are the only class among the three main mollusk classes in which no ELH was ever identified, we can suspect that the reason is the loss of the ELH gene or more probably a low level of structural conservation leading to a failure of the data mining experiment. Nevertheless, the present study shows that about 10 neuropeptide families met the criteria for displaying a regulatory activity during spawning. In many papers, three categories of regulatory peptides controlling egg-laying are described: the neuropeptides that trigger egg-laying by integrating environmental stimuli across a neurosensory network, the ovarian regulatory peptides synchronizing oocyte transport and egg-capsule secretion, and the waterborne sex pheromones involved in mating and reproduction behaviors by aggregating mates in egg-laying areas. These multiple regulatory layers can be correlated with the complexity of the successive steps of the egg-laying mechanism that involves the ovaries and ASGs and is performed thanks to a stereotyped behavior: (1) ovulation with the release of mature oocytes in the genital coelom, (2) oocyte transport by the oviduct, (3) secretion of the internal egg-capsule by the OG, (4) secretion of the external egg-capsule by the MNGs, (5) black pigmentation of the egg capsule by the ink bag, (6) fertilization of oocytes by the sperm stored in the female's copulatory pouch, and (7) attachment of eggs to something on the sea bottom to form an egg mass.

The present study can be considered as a crucial preliminary step with a view to reducing the number of neuropeptides screened by functional tests: MS analyses will allow us to focus onto specific tests according to neuropeptide localization.

The next step will now consist in checking the activity of the selected neuropeptides using in vitro bioassays at the level of cells and organs. This approach should lead to the identification of neuropeptides involved in the regulation of the successive steps of egg laying.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00463.

Figure S-1: In silico analysis using homemade software PEPTRAQ. Figure S-2: Search strategy to identify the overexpressed neuropeptidome in *S. officinalis* females during egg-laying. Mass spectrometry identifications were based on transcriptome data generated by a de novo RNaseq approach, and in silico data mining was performed using Peptraq and Predisi. Figure S-3: Expression pattern of neuropeptide transcripts recovered at the level of the ovary and the ASGs of egg-laying females. Figure S-4: Expression pattern of neuropeptide transcripts recovered at the level of the ovary and the ASGs of egg-laying females. Figure S-5: Sequence alignment of *S. officinalis*, *A. californica*, and human netrin-1. Cuttlefish netrin-1 is not full length. (PDF)

Table S-1: Overview of the neuropeptides predicted from the protein precursors identified by automated annotation performed on the transcriptome and by in silico data mining for the 72% of nonannotated transcripts. (XLSX)

Table S-2: Alphabetical list of the neuropeptides detected in each organ screened by mass spectrometry. In addition, values obtained from database searching using the Mascot 2.4.0 program (Matrix Science) are reported, and so are the mass spectrometers used to perform analyses. (XLSX)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. C.Z.-G. drew part of the figures, discussed the data and the manuscript with J.H., and played an important role

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in designing and performing the transcriptomic approach. V.C. helped to perform the transcriptomic approach, to collect and dissect animals, and to prepare samples for mass spectrometry. A.L. helped in the preparation of many samples for mass spectrometry. B.Z. is the computer scientist who built the PEPTRAQ software program in collaboration with J.H. E.C. and G.L.C. assembled and annotated the cuttlefish transcriptomes. B.B. performed MALDI-TOF/TOF analyses. J.G. discussed the results with J.H. A.K. and Y.C. performed the nanoLC−ESI−MS/MS analyses. J.H. performed some dissections and sample preparations, helped to perform the transcriptomic analysis, carried out in silico data mining with PEPTRAQ and analyses of mass spectrometry data, drafted the paper, and obtained funding for the experiments.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACN, acetonitrile; ANGs, accessory nidamental glands; APGWa-RPs, APGWamide-related peptides; ASGs, accessory sex glands; ASTs, allatostatins; cDNA, complementary DNA; CCKs, cholecystokinins; CHCA, α-cyano-4-hydroxy cinnamic acid; EST, expressed sequence tag; FaRPs, FMRFamide-related peptides; CNS, central nervous system; dsDNA, double-stranded DNA; DTT, dithiothreitol; ELF, egg-laying female; ELH, egg-laying hormone; FPKM, fragments per kilobase of exon per million fragments mapped; GnRH, gonadotropin-releasing hormone; LKs, leucokinins; MNGs, main nidamental glands; MALDI-TOF, matrix-assisted laser desorption/ionization−time-of-flight; MS, mass spectrometry; NA, neurohemal area; nanoLC, nanoliquid chromatography; NKY, neuropeptide KY; NPF, neuropeptide F; NPY, neuropeptide Y; OG, oviduct gland; OLs, optic lobes; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; RNAseq, ribonucleic acid sequencing; SubEM, subesophageal mass; SupEM, supraesophagal mass; SCPs, small cardioactive peptides; TFA, trifluoroacetic acid; UII, urotensin II

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