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RESEARCH ARTICLE

Functional characterization of a short neuropeptide F-related receptor in a lophotrochozoan, the mollusk *Crassostrea gigas*

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ABSTRACT

Members of the short neuropeptide F (sNPF) family of peptides and their cognate receptors play key roles in a variety of physiological processes in arthropods. *In silico* screening of GigasDatabase, a specific expressed sequence tag database from the Pacific oyster *Crassostrea gigas*, resulted in the identification of a receptor (*Cg*-sNPFR-like) phylogenetically closely related to sNPF receptors (sNPFRs) of insects. A reverse endocrinology approach was undertaken to identify the peptide ligand(s) of this orphan receptor. Though structurally distinct from insect sNPFs, three RFamide peptides derived from the same precursor, i.e. GSLFRFamide, SSLFRFamide and GALFRFamide, specifically activate the receptor in a dose-dependent manner, with respective EC_{50} values (half-maximal effective concentrations) of 1.1, 2.1 and 4.1 µmol l^{-1}. We found that both *Cg*-sNPFR-like receptor and LFRFamide encoding transcripts are expressed in the oyster central nervous system and in other tissues as well, albeit at lower levels. Mass spectrometry analysis confirmed the wide distribution of LFRFamide mature peptides in several central and peripheral tissues. The *Cg*-sNPFR-like receptor was more abundantly expressed in ganglia of females than of males, and upregulated in starved oysters. In the gonad area, highest receptor gene expression occurred at the start of gametogenesis, when storage activity is maximal. Our results suggest that signaling of LFRFamide peptides through the *Cg*-sNPFR-like receptor might play a role in the coordination of nutrition, energy storage and metabolism in *C. gigas*, possibly by promoting storage at the expense of reproduction.

KEY WORDS: Oyster, *Crassostrea gigas*, sNPF, Energy metabolism, Reproduction, Starvation

INTRODUCTION

Peptides of the short neuropeptide F (sNPF) family have been identified in a broad range of arthropod taxa, and constitute an important family of neuroregulators with key roles in a variety of physiological processes such as feeding behavior (Lee et al., 2004) (Brockmann et al., 2009), osmotic and metabolic stresses (Kahsai et al., 2010a), regulation of locomotion (Kahsai et al., 2010b), control of hormone release (Nässel et al., 2008) and reproduction (Cerstiaens et al., 1999; De Loof et al., 2001; Dillen et al., 2013) (for a review, see Nässel and Wegener, 2011). Originally, these peptides were named because of the RxRFamide carboxyterminal sequence they share with invertebrate neuropeptide F (NPF). Despite this minor structural similarity, sNPF-encoding genes from insects appear phylogenetically distinct from NPF genes (Nässel and Wegener, 2011). Interestingly, NPF genes are well conserved across bilaterian phyla, whereas sNPF-encoding genes have only been characterized in arthropods and might constitute an evolutionary innovation restricted to this phylum. These separate families of peptides specifically bind distinct G protein-coupled receptors (GPCRs) that, however, display structural similarities (Feng et al., 2003; Mertens et al., 2002). Based on the theory that interacting proteins co-evolve (van Kesteren et al., 1996), a search for orthologs of sNPF receptors (sNPFRs) might represent a means of providing insight into the evolution of the sNPF signaling system outside the arthropod phylum. Interestingly, recent studies suggest the origin of this signaling system in the common ancestor of protostomes (Mirabeau and Joly, 2013) and even the ancestral bilaterian (Jékely, 2013).

Because of the availability of extended transcriptome resources (Fleury et al., 2009) and the recent availability of its genome sequence (Zhang et al., 2012), the Pacific oyster *Crassostrea gigas* (Thunberg 1793) has become an interesting model to investigate the evolution of regulatory pathways in Lophotrochozoa, one of the three major clades of bilaterian animals. In the present study, we identified a GPCR from *C. gigas* that is phylogenetically closely related to sNPFRs, and found its specific activation by a distinct family of RFamide-related peptides with potential involvement in the control of feeding, energy metabolism and reproduction.

RESULTS

Characterization of a *Cg*-sNPFR-like receptor

A BLAST search in GigasDatabase using the *Anopheles gambiae* sNPFR sequence led to the identification of an expressed sequence tag (EST) (CU993362) encoding a partial GPCR. The full-length cDNA (1312 bp), generated by RACE, codes for a 404 amino acid protein exhibiting the hallmarks of a peptide GPCR with seven putative membrane spanning domains and a short extracellular domain. A search in the EMBL/GenBank database revealed maximum identity of this oyster GPCR with the *A. gambiae* sNPFR (46%) and was thus named *Cg*-sNPFR-like receptor. This receptor also displays identity with a set of functionally characterized receptors such as the gastropod mollusk *Lymnaea stagnalis* neuropeptide Y/F (NPY/F) receptor (36%) and both insect (*Drosophila melanogaster* 24%; *A. gambiae* 27.6%) and vertebrate NPY/F type 2 receptors (24.9% with human receptor). Maximum similarity between these GPCRs essentially concerns the regions encompassing the transmembrane domains, though an almost strictly conserved cluster of amino acids (A/GWM/LNDNFRKEF) of unknown function can be noticed in the cytosolic domain close to transmembrane domain 7 (Fig. 1). With the completion of the *C. gigas* genome sequence

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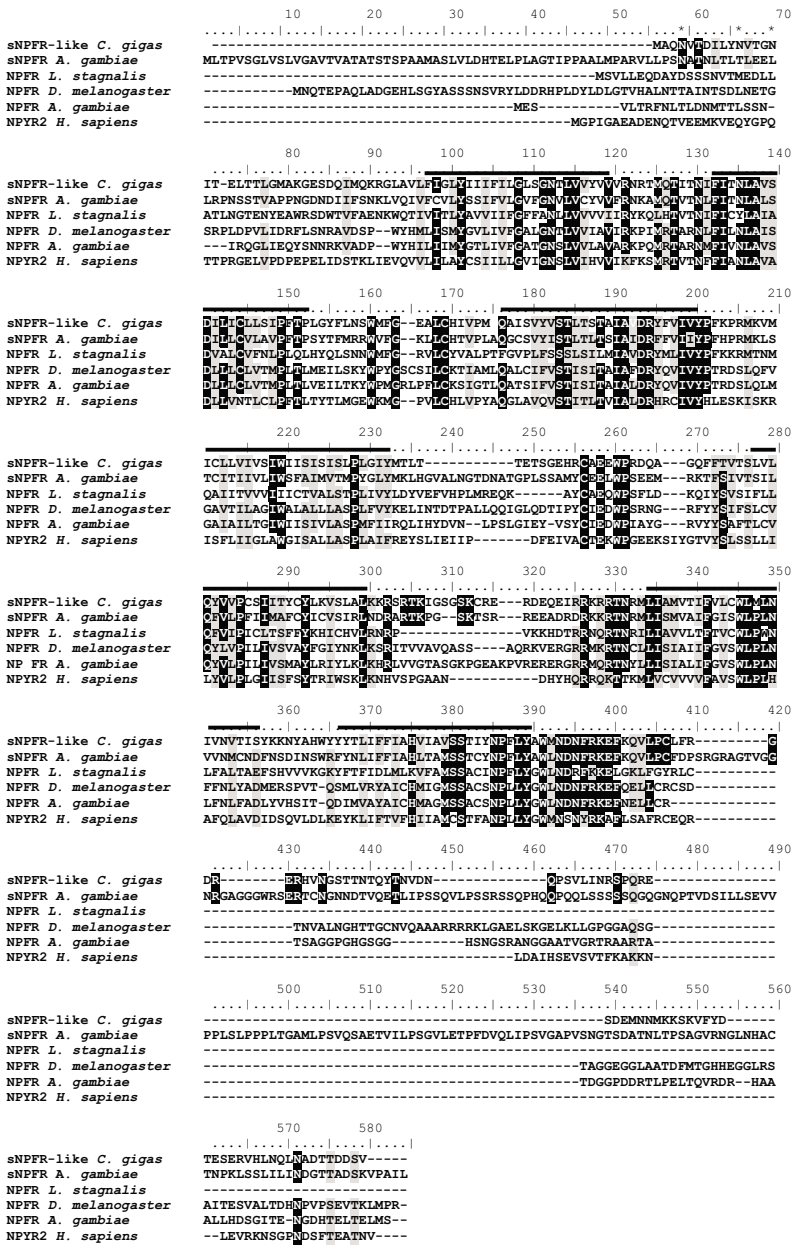

Fig. 1. Local alignment of a complete cDNA translation of the *Cg*-sNPFR-like receptor and the amino acid sequence of functionally characterized sNPF and NPY/F receptors. Accession numbers: short neuropeptide F receptor (sNPFR): *Anopheles gambiae* [ABD96049.1]; neuropeptide F receptors (NPFRs): *Anopheles gambiae* [AAT81602.1], *Drosophila melanogaster* [AAF51909.3] and *Lymnaea stagnalis* [CAA57620.1]; neuropeptide Y receptor (NPYR): *Homo sapiens* type 2 [NP_000901.1]. Transmembrane domains are indicated by horizontal bars. *Potential sites for N-glycosylation. Black and gray shading correspond to identical and similar amino acid residues, respectively.

(Zhang et al., 2012), a BLAST search identified OYG_10025197 as the putative gene encoding the *Cg*-sNPFR-like receptor; however, this gene predicts a shorter protein with a slightly distinct C-terminal part, which might be a result of alternative splicing or incorrect gene deduction. A phylogenetic analysis showed that the *Cg*-sNPFR-like receptor clusters with insect sNPFRs and displays a phylogenetic proximity with human type 2 NPYR and zebrafish NPYR Y7. In contrast, the other chordate NPY, mollusk and insect NPY/F receptors cluster as separate branches (Fig. 2).

Differential expression of the *Cg*-sNPFR-like receptor encoding gene

We investigated the spatial distribution of *Cg*-sNPFR-like receptor transcripts by reverse transcription quantitative PCR (RT-qPCR) using RNA samples from adult tissues. The *Cg*-sNPFR-like receptor was mostly expressed in the visceral ganglia. Lower but significant expression was also measured in all other tested tissues (mantle, labial palps, digestive gland, gills and gonadic area) (Fig. 3A).

Cg-sNPFR-like receptor transcripts showed a stable expression in the visceral ganglia along the reproductive cycle, but with higher expression levels in females than in males (Fig. 3B). In the gonadic area, expression of *Cg*-sNPFR-like receptor transcripts was maximal in both males and females at the first stages of gametogenesis, when energy storage is maximal (stages 0 and 1), and declined as gametogenesis proceeds and storage tissue regresses (stages 2 to 3) (Fig. 3C). Because the feeding status of oysters is known to impact the efficiency of their gametogenesis (Chávez-Villalba et al., 2002), the expression of *Cg*-sNPFR-like receptor transcripts was compared in the visceral ganglia of fed and starved animals. Interestingly, transcript levels were significantly higher in three weeks starved animals compared to fed animals (Fig. 3D).

Activation of a *Cg*-sNPFR-like receptor by LFRFamide peptides

We used a calcium mobilization assay to identify the peptide ligand(s) of the *Cg*-sNPFR-like receptor (Beets et al., 2011).

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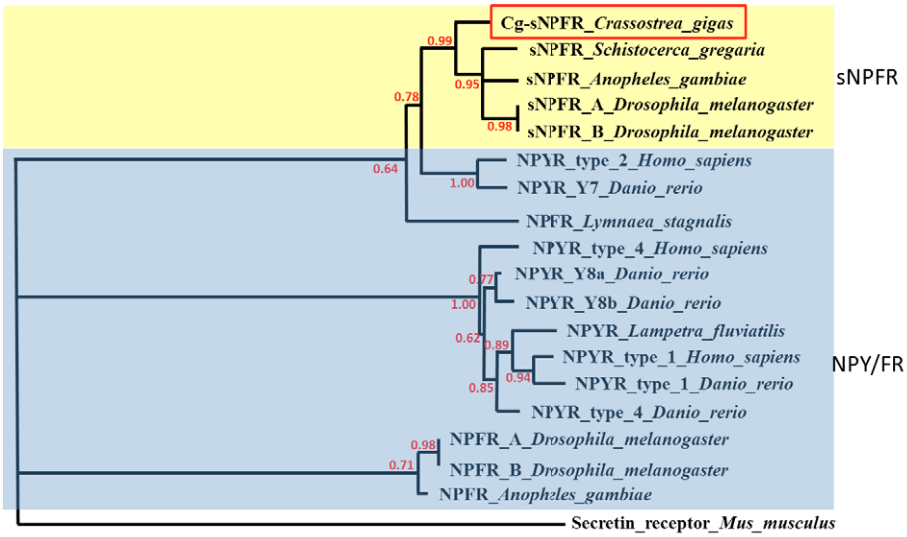

Fig. 2. Phylogenetic representation of the relationship between the *Cg*-sNPFR-like receptor and genuine sNPF and NPY/F receptors, as generated by a maximum likelihood method using the phylogeny pipeline (www.phylogeny.fr). The mouse secretin receptor was chosen as outgroup. Branch node labels correspond to likelihood ratio test values. Accession numbers: short neuropeptide F receptors (sNPFRs): *Anopheles gambiae* [ABD96049.1], *Drosophila melanogaster* isoform A [AAF49074.2] and isoform B [NP_001262086.1], and *Schistocerca gregaria* [AGC54822.1]; neuropeptide F receptors (NPFRs): *Anopheles gambiae* [AAT81602.1], *Drosophila melanogaster* isoform A [AAF51909.3] and isoform B [NP_001246945.1], and *Lymnaea stagnalis* [CAA57620.1]; neuropeptide Y receptors (NPYRs): *Homo sapiens* type 1 [NP_000900.1], type 2 [NP_000901.1], type 4 [NP_005963.3], *Danio rerio* type 1 [NP_001095861.1], type 4 [NP_571515.1], type Y7 [AAI63330.1], type Y8a [NP_571512.1], type Y8b [NP_571511.1], and *Lampetra fluviatilis* [AAL66410.1]; *Mus musculus* secretin receptor [NP_001012322.2].

Transiently transfected HEK293T cells expressing the oyster receptor and the promiscuous G protein $G\alpha_{16}$ were challenged with a series of *C. gigas* synthetic peptides, whose sequences were retrieved from a personal *C. gigas* peptide database (Table 1). Unfortunately, no sNPF-like peptides with a characteristic C-terminal RLRFamide motif were found. A similar RPRFamide pattern was detected in oyster mature NPY/F, originating from a full-length NPY/F precursor (CU983945) that was identified in GigasDatabase (Fleury et al., 2009), and in some of its truncated forms characterized by mass spectrometry (MS) (data not shown). These peptides were tested in addition to a set of FMRFamide-like neuropeptides, carrying the RFamide motif that is generally thought to be essential for receptor activation in this peptide family (Kulathila et al., 1999). Only LFRFamide-related peptides were able

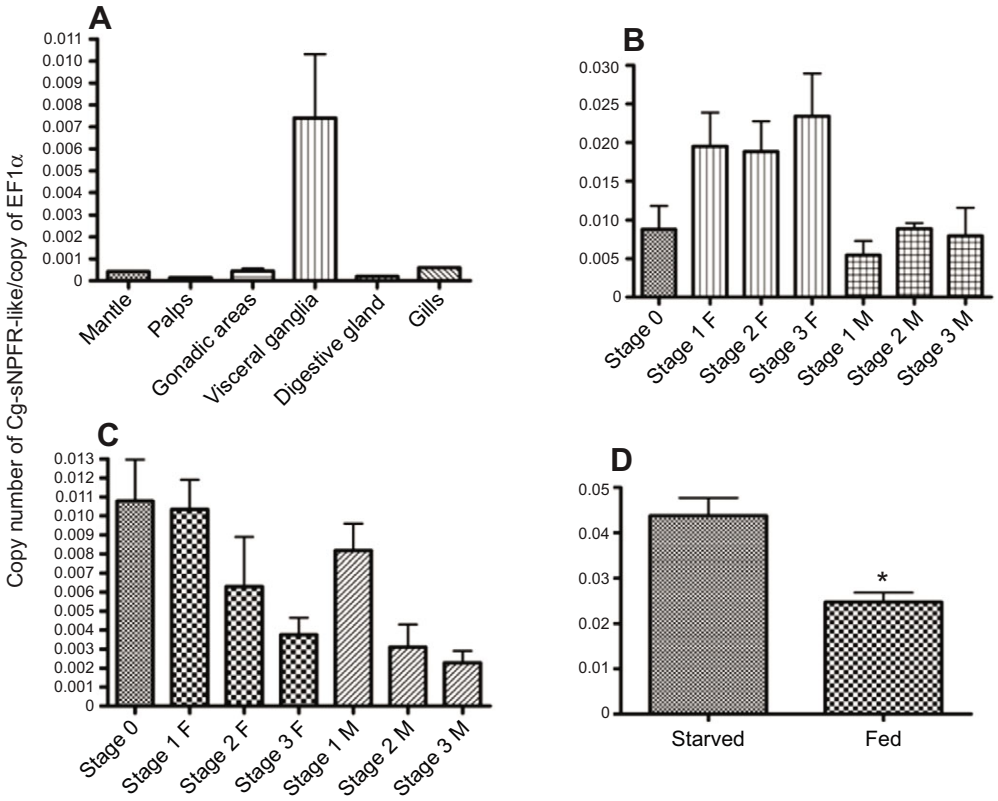

Fig. 3. Expression of the *Cg*-sNPFR-like receptor monitored by RT-qPCR. Expression levels were determined as the number of copies of *Cg*-sNPFR-like receptor transcripts per copy of elongation factor 1α (EF1α) mRNA. (A) Expression in adult tissue samples. (B) Expression in visceral ganglia and (C) in the gonadic area of *C. gigas* adults collected at different stages of gametogenesis. Each value is the mean of five pools of tissues, each from six individuals. Stage 0: sexual resting stage; Stage 1: gonial multiplication stage; Stage 2: tubule development and maturation stage; Stage 3: sexual maturity stage. (D) Expression in the visceral ganglia of starved and fed animals (n=15) (Student's t-test, *P<0.05). Vertical bars represent s.e.m.

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Table 1. Potency of *Crassostrea gigas* peptides to activate the *Cg*-sNPFR-like receptor

Peptide sequence	Receptor activation
NDSLLPPNRPSRFSSPGQLRQYLKALNDYYAIVGRPRFamide (NPY/F)	–
ALNDYYAIVGRPRFamide (sNPY/F2)	–
YAIVGRPRFamide (sNPY/F1)	–
FLRFamide*	–
FMRFamide*	–
ALSGDHYIRFamide*	–
GALFRFamide	+
GSLFRFamide	+
SSLFRFamide	+
SVDNEKPHTPFRFamide	–

+, activation; –, no activation, peptide concentration: 10^{-5} mol l^{-1}.
*Deduced from *C. gigas* FMRFamide precursor [Hs246379].

to activate the receptor and induced intracellular calcium mobilization independently of the expression of $G\alpha_{16}$, which suggests coupling of the oyster GPCR to the G_q pathway. Calcium responses were not evoked in cells transfected with an empty vector, or by any other peptide tested on *Cg*-sNPFR-like receptor expressing cells (data not shown). LFRFamide family members activated the receptor in a dose-dependent manner, though with different potencies (Fig. 4). Calculated EC_{50} values were 1.1, 2.1 and 4.1 µmol l^{-1} for GSLFRFamide, SSLFRFamide and GALFRFamide, respectively.

Characterization and expression of LFRFamide peptides and their encoding transcript

The sequence of the open reading frame encoding an LFRFamide precursor was obtained from GigasDatabase (CU994925) (Fleury et al., 2009), and by a sequence similarity search in the *C. gigas* genome database (Zhang et al., 2012). A perfect match was found for the OYG_10020740 predicted gene, which overlaps with EST CU994925 from nucleotide 156 and was considered to encode the oyster LFRFamide precursor. The deduced precursor protein displays an organization similar to that of the *L. stagnalis* LFRFamide precursor (Hoek et al., 2005) and consists of 159 amino acids, including a 23 amino acid signal peptide and nine predicted endoproteolytic cleavage sites. Complete processing of the peptide precursor would generate four distinct and likely amidated peptides due to the presence of a C-terminal glycine residue: three LFRFamide hexapeptides (GSLFRFamide, GALFRFamide and SSLFRFamide) and a single FRFamide tridecapeptide (SVDNEKPHTPFRFamide) (Fig. 5A). With the exception of the C-terminal end, these peptides show only minor sequence similarity with arthropod sNPFs. Comparison of oyster peptides with their presumed counterparts in annelids (Veenstra, 2011) or with putative sNPF neuropeptides from nematodes (Nässel and Wegener, 2011) displays a substantial degree of sequence variability even between peptides from the same animal clade (Fig. 5B). Using direct mass spectrometric analyses, all four peptides were found to be present in visceral ganglia peptidic extracts (Fig. 6), and their sequences were confirmed by tandem mass spectrometry (data not shown).

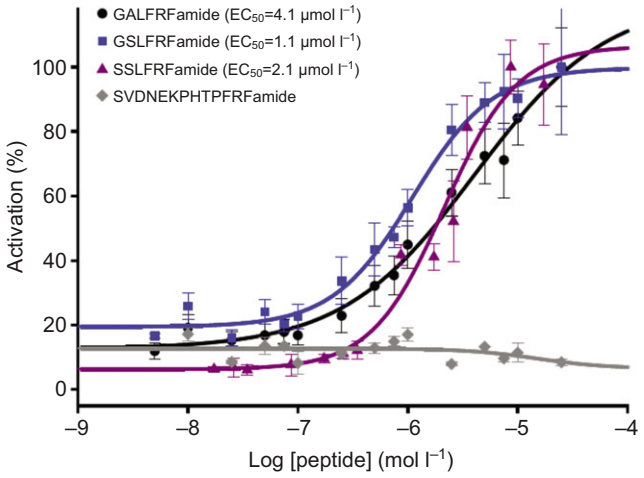

Fig. 4. Concentration-dependent calcium responses evoked by GSLFRFamide, SSLFRFamide, GALFRFamide and SVDNEKPHTPFRFamide in *Cg*-sNPFR-like receptor expressing HEK293T cells. Concentration–response data are shown as relative (%) to the highest value (100% activation) for a given peptide, and were performed at least in triplicate. Vertical bars represent s.e.m.

To gather information on the potential role of LFRFamide peptides in the oyster *C. gigas*, the distribution of transcripts encoding their precursor was studied in various tissues by RT-qPCR. LFRFamide transcripts were expressed at relatively high levels in the visceral ganglia, and at basal levels in all other tissues (Fig. 7A). No significant variation was measured along the reproductive cycle in male or female visceral ganglia (Fig. 7B). In contrast, as for the gene encoding its cognate receptor, the LFRFamide precursor gene showed higher expression levels in starved oysters compared with fed ones, though this increase in precursor expression was not statistically significant (Fig. 7C). The processing of a neuropeptide precursor protein usually results in end products being transported to the nerve terminals. Therefore, we compared the tissue distribution of LFRFamide precursor transcripts with that of LFRFamide peptides, directly measured on different tissue extracts by MALDI-MS. All LFRFamide peptides were unambiguously detected in most adult tissues, with exception of the labial palps, whereas the FRFamide tridecapeptide from the same precursor was found only in the visceral ganglia fractions (Table 2).

DISCUSSION

By sequence similarity and phylogenetic analyses, we identified in this study a mollusk GPCR in the Pacific oyster that is orthologous to the insect sNPFR family. Consistent with the theory of peptide-receptor co-evolution (Park et al., 2002), this finding suggests the presence of sNPF-related peptides in mollusks, whereas sNPF distribution up to now appears strictly restricted to arthropods (Nässel and Wegener, 2011). In the fruit fly, the consensus C-terminal sequence xPxLRLRxamide was found important for sNPFR activation, with a crucial role of the Arg residue at position 4 from the amidated C terminus (Mertens et al., 2002). No such perfect match was found in any known oyster peptides, although a

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A

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1     ATG GAG ACA AAA ACG TTA CTT ATC TGG GTT TTT GCA GCA TTC CTG   45
1      M   E   T   K   T   L   L   I   W   V   F   A   A   F   L   15

46    GCC TTC ATT GAA GTA TCC ACA GCT TCC AAA GAA TCA AAA CTA AAT   90
16     A   F   I   E   V   S   T   A   S   K   E   S   K   L   N   30

91    CAT GAA GAA TCG GAA GTG AAG TCA TTA AAA AGG CGG TCG AAT CCA   135
31     H   E   E   S   E   V   K   S   L   K   R   R   S   N   P   45

136   ATG GAT GAC GTC ACA GAC ACG AT GAT TTT TCG GAC GAA GAT TTA   180
46     M   D   D   V   T   D   T   Y   D   F   S   D   E   D   L   60

181   AAT GAA CTT ATG AAA AGA GGT TCA CTT TTC CGG TTT GGA AAA CGG   225
61     N   E   L   M   K   R   G   S   L   F   R   F   G   K   R   75

226   GGA GCA TTG TTC CGA TTC GGC AAA AGA AGC GAA GAA GAT AAG CGT   270
76     G   A   L   F   R   F   G   K   R   S   E   E   D   K   R   90

271   GGA TCT CTT TTC AGG TTT GGG AAA CGT ATG GAC GGT TAT TAT GTA   315
91     G   S   L   F   R   F   G   K   R   M   D   G   Y   Y   V   105

316   TCA CCA TAT GCA AGT AGT GAA AAT TTT CCG GAA AAA GAT GAT AAA   360
106    S   P   Y   A   S   S   E   N   F   P   E   K   D   D   K   120

361   AGA GGA AGC CTT TTC CGG TTT GGA AAG CGA TCC TCG TTG TTC CGA   405
121    R   G   S   L   F   R   F   G   K   R   S   S   L   F   R   135

406   TTT GGA AGA AGT GTC GAT AAT GAA AAA CCC CAT ACG CCA TTT CGT   450
136    F   G   R   S   V   D   N   E   K   P   H   T   P   F   R   150

451   TTT GGA CGT GAG GAG GAA GAT GAA ATA TAA   480
151    F   G   R   E   E   E   D   E   I   *
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B

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L  C. gigas LFRFa1                                  GSLFRF
   C. gigas LFRFa2                                  GALFRF
   C. gigas LFRFa3                                  SSLFRF
   C. teleta FRYa1                                    IFRY
   C. teleta FRYa2                                    LFRY
   C. teleta FRYa3                                  AAIFRY
   C. teleta FRYa4                                  VPIFRY
   C. teleta FRWa1                                  QAIFRW
   C. teleta FRWa2                                    LFRW
   C. teleta FRWa3                                DGAPVFRW
   C. teleta FRWa4                                 DSPVFRW
   C. teleta FRWa5                                  SNIFRW
   C. teleta FRWa6                                  SSPAFRW
E  C. elegans FLP-27      EASAFGDIIGELKGKGLGGRMRF
   H. glycies FLP-27        DSDYDELLTEFRSKGSRMRF
   H. americanus sNPF1                  SMPSLRLRF
   D. melanogaster sNPF1              AQQRSPSLRLRF
   D. melanogaster sNPF3                  KP-QRLRW
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Fig. 5. Diversity of LFRFamide peptides in *Crassostrea gigas* and sequence alignment with sNPF family members. (A) Nucleotide and deduced amino acid sequence of the open reading frame encoding a *Crassostrea gigas* LFRFamide precursor (OYG_10020740) retrieved from the oyster genome database (http://oysterdb.cn/home.html). The nucleotide sequence in bold and italics indicates the overlapping sequence with EST CU994925. The putative signal peptide is indicated in italics. Potential proteolytic processing sites are highlighted in gray and a C-terminal glycine (G) for amidation is in bold. The predicted mature peptides are underlined. (B) Sequence alignment of *C. gigas* LFRFamide precursor predicted peptides with their potential orthologs from annelids (Veenstra, 2011), arthropod sNPF family members, and their putative nematode counterparts (Nässel and Wegener, 2011). All peptides share an amidated C terminus. The arginine residue (R) important in insect receptor-specific activation is indicated in bold. Black and gray shading indicate amino acids identical or similar to *C. gigas* peptides, respectively. E: Ecdysozoa, L: Lophotrochozoa.

related signature (RPRFamide) was found in *C. gigas* long NPY/F and its N-terminally truncated forms that have been chemically characterized. As neither long *Cg*-NPY/F nor truncated forms activate the *Cg*-sNPFR-like receptor, the potential activity of RFamide family members was investigated based on the statement that the RFamide motif is usually crucial for receptor activation (Kulathila et al., 1999). Although other peptide ligands might exist, LFRFamide peptides derived from a single *C. gigas* precursor protein specifically activate the *Cg*-sNPFR-like receptor (LFRFamide receptor). Compared with most arthropod sNPFs, *C. gigas* LFRFamide peptides share the C-terminal RFamide motif, but exhibit only subtle similarity and lack the crucial Arg residue found in all known sNPFs (Nässel et al., 2008). Considering the time of divergence between Lophotrochozoa and Ecdysozoa and the possible relaxation of receptor-ligand binding constrains, a rapid peptide-receptor co-evolution may have washed out clear structural relationships between sNPFs and LFRFamide peptides despite their evolutionary relatedness. A similar explanation was suggested for the evolution of the AKH/GnRH signaling system in Bilateria (Grimmelikhuijzen and Hauser, 2012).

The sNPF family together with a great diversity of RFamide peptides have evolutionarily conserved roles in the regulation of feeding behavior and energy balance (Bechtold and Luckman, 2007; Nässel and Wegener, 2011). Interestingly, in all mollusk species investigated so far, LFRFamide peptides appear to regulate similar physiological processes. The first peptide (GSLFRFamide) was discovered in the prosobranch mollusk *Fusinus ferrugineus* (Kuroki et al., 1993), but the function of this peptide family was initially investigated in the marine mollusk *Aplysia californica*. In this species, LFRFamide peptides that are expressed in buccal neurons decrease the size of the accessory radula closer muscle via a combination of presynaptic and postsynaptic commands (Cropper et

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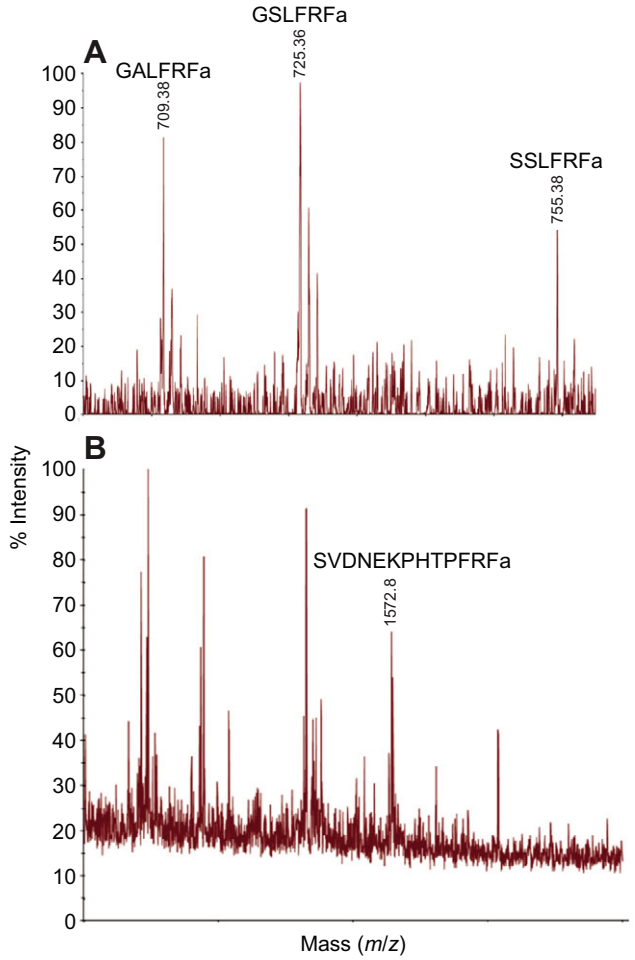

Fig. 6. Mass analyses of the four peptides predicted from the *C. gigas* LFRFamide precursor in a visceral ganglia peptidic extract. (A) MS spectrum of three LFRFamide peptides – GALFRFamide, GSLFRFamide and SSLFRFamide – and (B) MS spectrum of the fourth tridecapeptide, SVDNEKPHTPFRFamide. MS/MS spectra confirm the sequence of the four predicted *C. gigas* peptides (data not shown).

al., 1994). Similar inhibitory effects were also shown on central neurons in the snail *Helix aspersa* (Chen et al., 1995). In the snail *L. stagnalis*, an LFRFamide encoding gene was strongly upregulated upon schistosome parasitic infection, a biological event known to disturb host growth, energy metabolism and reproduction (Hoek et al., 1997). Alteration of these functions is thought to result from the inhibitory action of LFRFamide peptides on *L. stagnalis* caudo-dorsal and light green cells, two cell clusters that regulate female reproduction, and growth and metabolism, respectively (Hoek et al., 2005). More recently, a new LFRFamide peptide with potent activity on the contraction of the rectum was identified in the cephalopod *Sepia officinalis* (Zatylny-Gaudin et al., 2010). The identification of an LFRFamide peptide precursor gene in the limpet *Lottia gigantea* genome (Veenstra, 2010) confirms the widespread distribution of this peptide family in the mollusk phylum.

High expression of both oyster LFRFamide peptides and their receptor in the visceral ganglia supports a role of this signaling system in neuromodulation or neurotransmission. Furthermore, we found differential expression of peptide and receptor genes along the reproductive cycle and according to the feeding status. This suggests that LFRFamide pathways control neural networks in the oyster central nervous system (CNS) involved in the regulation of reproduction and feeding, reminiscent of related peptide functions in other species (Chen et al., 1995; Cropper et al., 1994; Hoek et al., 2005). Except in *S. officinalis* (Zatylny-Gaudin et al., 2010), peripheral activity of the LFRFamide family of peptides has not been investigated. In this study, we detected all three oyster LFRFamide peptides in a majority of peripheral tissues, which argues for local peptide release from nerve terminals. As such, in contrast to when the peptide is released into the body cavity, a rather high concentration of peptide can be expected *in vivo* at the vicinity of the receptors. This hypothesis is consistent with the relatively high half-maximal effective concentrations (EC_{50}) characterizing oyster LFRFamide receptor/ligand activity. Oyster LFRFamide receptor physicochemical values are congruent with the EC_{50} of 2.2 μmol l^{-1} determined by whole-cell voltage-clamp recordings of *L. stagnalis* neurons for the sequence ortholog peptide (GGSLFRFamide), and with the micromolar range concentrations of LFRFamide peptides required to elicit half-maximal current responses in *A. californica* muscle fibers (Cropper et al., 1994, Hoek et al., 2005). The wide distribution of *C. gigas* LFRFamide peptides and receptors in peripheral tissues suggests that this peptide family is a functional regulator of a diversity of organs. Of particular interest is the sexual differential expression of the *Cg*-sNPFR-like receptor in the CNS, and increased receptor expression in the gonadic area when storage is maximal. The profile of receptor expression decline singularly paralleled the regression of storage tissues in the gonad (Berthelin et al., 2000). This observation suggests that LFRFamide receptors are likely expressed in the glycogen storage cells of the gonad and control their activity. Differences between males and females might reflect sex-related differential energy requirements for completing gametogenesis in this sequential hermaphroditic species. However, it remains to be investigated whether LFRFamide signaling favors storage or regulates other metabolic pathways in these cells. Increased expression of LFRFamide peptides and their receptor in the CNS of starved animals stresses a possible role as nutrient sensors to allow a balanced nutrient content in the animal. Interestingly, in the cockroach *Periplaneta americana*, sNPF contents were increased as the result of starvation. In this species, sNPF signaling is thought to act as a nutrient sensor by negatively controlling the activity of digestive enzymes (Mikani et al., 2012). Although opposite effects on feeding behavior have been shown in the desert locust, *Schistocerca gregaria* (Dillen et al., 2013), overexpression of sNPFs promotes food intake and regulates body size in *D. melanogaster* (Lee et al., 2004). Here, sNPF signaling affects growth and carbohydrate levels by controlling insulin signaling (Lee et al., 2008). Given the recent findings that insulin signaling responds to nutrient levels in the *C. gigas* gonad (Jouaux et al., 2012), and the control of insulin producing cells by LFRFamide peptides in *L. stagnalis* (Hoek et al., 2005), it is conceivable to consider that mollusk LFRFamide peptides represent functional analogues of arthropod sNPFs.

A fine control of energy allocation is crucial in sessile species subjected to large and fluctuating environmental parameters. This is especially true during gametogenesis, a period of negative energy budget (Soletchnik et al., 1997) that appears to be genetically correlated with susceptibility to summer mortality (Huvet et al., 2010), which is one of the current major burdens of oyster aquaculture. A variety of neuroendocrine signaling pathways operate to cope with these constraints, of which some have already been identified in the oyster (Bigot et al., 2012) (Jouaux et al., 2012). Our results on the characterization of an oyster *Cg*-sNPF-like receptor provide insights into novel neuroendocrine pathways regulating key

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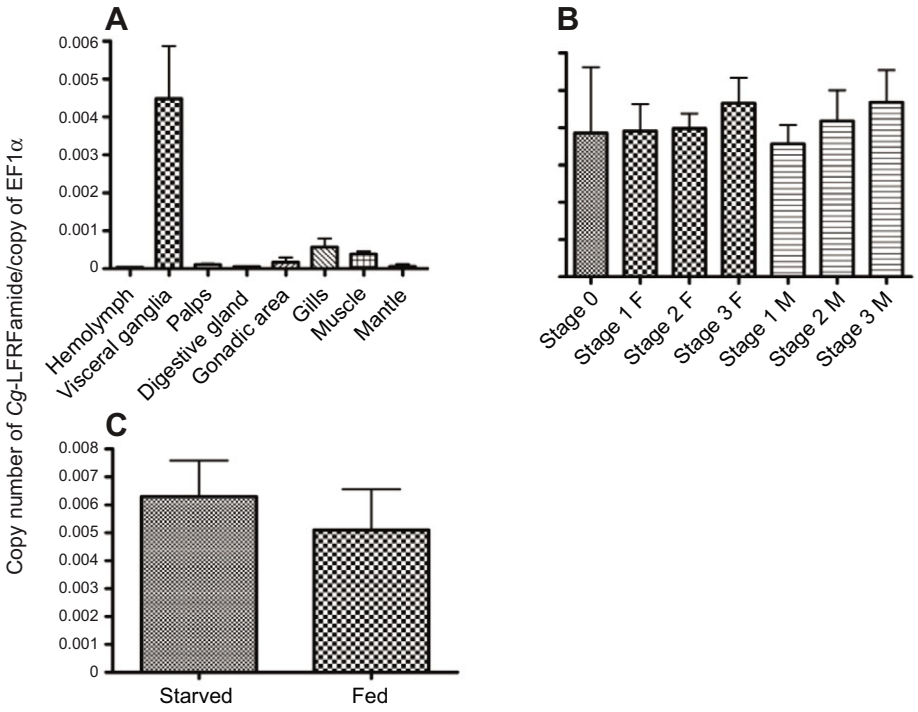

Fig. 7. Expression of the LFRFamide precursor measured by RT-qPCR. Expression levels were calculated as the number of copies of LFRFamide transcripts per copy of elongation factor 1α (EF1α) mRNA. (A) Expression in adult tissue samples. (B) Expression in visceral ganglia of adults of *C. gigas* collected at different stages of gametogenesis. Each value is the mean + s.e.m. of five pools of tissues, each from six individuals. Stage 0: sexual resting stage; Stage 1: gonial multiplication stage; Stage 2: tubule development and maturation stage; Stage 3: sexual maturity stage. (C) Expression in the visceral ganglia of starved and fed animals (n=15). Results were analysed using a Student's t-test; no statistical difference was observed (P>0.05).

physiological functions in one of the most important aquaculture species worldwide. These issues will greatly benefit from an ongoing neuropeptidomic survey and the recent publication of the oyster genome sequence (Zhang et al., 2012).

MATERIALS AND METHODS

Animals and tissue sampling

Two-year-old adult oysters *C. gigas*, purchased from a local farm (Normandie, France), were used for peptide characterization, transcription analyses and immunohistochemistry. Stages of reproduction (Stage 0: resting undifferentiated stage; Stage 1: gonial multiplication stage; Stage 2: maturation stage; Stage 3: sexual maturity) were determined by histological analysis of gonad sections as described previously (Rodet et al., 2005). To study the influence of trophic conditions, 1-year-old adult oysters were reared in water tanks either in the absence of food or with a 12% diet {12% of oyster dry mass in algal [mixture containing *Isochysis galbana* (clone T-ISO), *Chaetoceros calcitrans* and *Skeletonema costatum*] dry mass per day} in controlled experimental conditions during 3 weeks at Ifremer experimental facilities located in Argenton (Brittany, France). Tissues were sampled (visceral ganglia were carefully dissected out under a stereomicroscope, thus limiting any contamination from adjacent adductor muscles) and immediately frozen in liquid nitrogen and stored at −80°C until use.

Peptide synthesis

All peptides were custom-synthesized by GeneCust (Luxemburg). The sequences of *C. gigas* peptides were obtained from an in-house peptide database yielded by MS analyses of tissue extracts and data mining (P.F., unpublished results).

In silico analyses

Multiple sequence alignment was performed using ClustalW (Thompson et al., 1994). To determine the relationship between receptor sequences, a phylogenetic tree was generated by the maximum likelihood method using the phylogeny pipeline (www.phylogeny.fr) (Dereeper et al., 2008) connecting the following programs: MUSCLE for multiple alignment (full processing mode), Gblocks for alignment curation (minimum length of a block after gap cleaning: 10, no gap positions allowed in the final alignment, all segments with contiguous non-conserved positions higher than eight rejected, minimum number of sequences for a flank position: 85%), and PhyML for phylogeny (the default substitution model was chosen assuming an estimated proportion of invariant sites and four gamma-distributed rate categories to account for rate heterogeneity across sites; the gamma shape parameter was estimated directly from the data model). The reliability of internal branches was evaluated using an approximate likelihood-ratio test. TreeDyn was used for tree drawing.

Reverse endocrinology

Molecular cloning of the *Cg*-sNPFR-like receptor and transfection of mammalian cells

In silico screening of GigasDatabase (Fleury et al., 2009) resulted in the identification of a partial cDNA encoding a *Cg*-sNPFR-like receptor (CU993362). The full-length cDNA sequence was obtained by 5′- and 3′-RACE using the GeneRacer kit (Invitrogen) on cDNA (MMLV Reverse

Table 2. Distribution of peptides predicted by the LFRFamide precursor in *C. gigas* tissues

Tissue	GALFRFamide	GSLFRFamide	SSLFRFamide	SVDNEKPHTPFRFa
Visceral ganglia	✓	✓	✓	✓
Gonadic area	✓	✓	n.d.	n.d.
Digestive gland	✓	✓	✓	n.d.
Labial palps	n.d.	n.d.	n.d.	n.d.
Mantle	✓	✓	✓	n.d.
Mantle edge	n.d.	✓	✓	n.d.
Muscles	✓	✓	✓	n.d.
Gills	n.d.	✓	✓	n.d.

✓, peptide present; n.d., not detected.

Transcriptase, Promega) synthesized from *C. gigas* visceral ganglia mRNA extracted using the RNA Nucleospin II kit (Macherey Nagel). The open reading frame of the *Cg*-sNPFR-like receptor gene was amplified by PCR (Pfu DNA polymerase, Promega) from visceral ganglia cDNA using gene-specific sense (5′-CACCATGGCTCAGAACGT-3′) and antisense (5′-TCACACGGAATCGTCGGTGGTATCAG-3′) primers. The resulting PCR product was directionally cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) and verified by sequencing. Human embryonic kidney (HEK293T) cells were transiently transfected with the *Cg*-sNPFR-like receptor/pcDNA3.1 construct using Lipofectamine Ltx with Plus reagent (Invitrogen) according to the manufacturer's instructions. Co-transfection was carried out with an expression construct for the human $G\alpha_{16}$ subunit, a promiscuous G protein that can direct intracellular signaling of GPCRs to the release of calcium via the phospholipase C_β pathway, regardless of the endogenous G protein coupling of the receptor (Mertens et al., 2004). To measure receptor activity independent of $G\alpha_{16}$, calcium responses were measured in cells expressing only the *Cg*-sNPFR-like receptor. Cells for negative control experiments were transfected with empty pcDNA3.1 and $G\alpha_{16}$/pcDNA3.1 constructs.

Fluorescence assay

Activation of the *Cg*-sNPFR-like receptor by candidate peptide ligands was monitored using a fluorescence-based calcium mobilization assay as previously described (Beets et al., 2011). Briefly, transfected HEK293T cells were loaded with Fluo-4-AM (Molecular Probes) for 1 h. Excitation of the fluorophore was performed at 488 nm. The calcium response was measured for 2 min at 525 nm using a FLEXstation (Molecular Devices) at 37°C. Data were analyzed using SoftMax Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of 10^{-5} mol l^{-1}. Concentration–response measurements of activating ligands were conducted in triplicate and for at least two independent experiments. EC_{50} values were calculated from concentration–response curves that were constructed using a computerized nonlinear regression analysis with a sigmoidal dose–response equation (SigmaPlot 11.0).

Reverse transcription and quantitative PCR

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 20 min at 37°C with 1 U of DNase I (Sigma-Aldrich) to prevent genomic DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random hexanucleotidic primers (Promega), 0.5 mmol l^{-1} dNTPs and 200 U MMuLV Reverse Transcriptase (Promega) at 37°C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70°C for 10 min. The iQ™ SYBR Green Supermix PCR kit (Bio-Rad) was used for real-time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95°C/15 s, 60°C/15 s) with the following primers: Qs-*Cg*-sNPFR-like receptor 5′-GCTTACTCGTCATTGTGTCAA-3′ and Qa-*Cg*-sNPFR-like receptor 5′-CATTCTTCAGCGCACCTGT-3′ as sense and antisense primers for *Cg*-sNPFR-like receptor cDNA, and Qs-*Cg*-LFRFa 5′-CAGGTTTGGGAAACGTATGG-3′ and Qa-*Cg*-LFRFa 5′AAACCGGA-AAAGGCTTCCT-3′ as sense and antisense primers for *Cg*-LFRFamide cDNA. Accurate amplification of the target amplicon was checked by performing a melting curve. Using Qs-*Cg*-EF1α (5′-ACCACCCTGGTG-AGATCAAG-3′) and Qa-*Cg*- EF1α (5′-ACGACGATCGCATTTCTCTT-3′) primers, a parallel amplification of oyster Elongation Factor 1α (EF1α) transcript [BAD15289] was carried out to normalize the expression data of each transcript. The relative level of each gene expression was calculated for one copy of the reference elongation factor 1α gene according to the following formula: $N=2^{(Ct_{EF1\alpha}-Ct_{oystercDNA})}$.

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 and gills) were carefully dissected out and 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 direct MS analysis.

Mass spectrometry analysis

MS analyses 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) and 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 the latest version of *C. gigas* GigasDatabase (Fleury et al., 2009) (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.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.B., I.B. and M.-P.D. performed the experiments, analyzed the data and wrote the paper; P.B., L.S. and P.F. planned the experiments, analyzed the data and wrote the paper.

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