Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference

Supattra Treerattrakool¹, Sakol Panyim¹,², Siu-Ming Chan³, Boonsirm Withyachumnarnkul⁴,⁵ and Apinunt Udomkit¹

¹ Institute of Molecular Biology and Genetics, Mahidol University, Nakhon Pathom, Thailand
² Department of Biochemistry, Mahidol University, Bangkok, Thailand
³ School of Biological Sciences, The University of Hong Kong, China
⁴ Department of Anatomy, Mahidol University, Bangkok, Thailand
⁵ Centex Shrimp, Mahidol University, Bangkok, Thailand

One of the important peptide hormones that control reproduction in crustaceans is gonad-inhibiting hormone (GIH). GIH is known to modulate gonad maturation by inhibiting synthesis of vitellogenin (Vg), the precursor of yolk proteins. In this study, a cDNA encoding a GIH (Pem-GIH) from the eyestalk of *Penaeus monodon* was cloned using RT-PCR and RACE techniques. Pem-GIH cDNA is 861 bp in size with a single ORF of 288 bp. The deduced Pem-GIH consists of a 17-residue signal peptide and a mature peptide region of 79 amino acids with features typical of type II peptide hormones from the CHH family. Pem-GIH transcript was detected in eyestalk, brain, thoracic and abdominal nerve cords of adult *P. monodon*. The gonad-inhibiting activity of Pem-GIH was investigated using the RNA interference technique. Double-stranded RNA, corresponding to the mature Pem-GIH sequence, can trigger a decrease in Pem-GIH transcript levels both in eyestalk ganglia and abdominal nerve cord explant culture and in female *P. monodon* broodstock. The conspicuous increase in Vg transcript level in the ovary of GIH-knockdown shrimp suggests a negative influence for Pem-GIH on Vg gene expression, and thus implies its role as a gonad-inhibiting hormone. This is the first report to demonstrate the use of double-stranded RNA to elucidate the function of GIH in *P. monodon*.

Female reproduction in crustaceans is controlled by an elaborate endocrine system. The prominent cellular activity that occurs during ovarian development is known as vitellogenesis, which is the process whereby vitellogenin (Vg), a yolk protein precursor, is accumulated in the developing oocyte [1]. Vitellogenesis is an essential step in ovarian maturation. Vg can be synthesized in the ovary and/or other nonovarian sites such as the hepatopancreas [2–5]. Vg synthesis and ovarian maturation are regulated by an eyestalk endocrine factor referred to as vitellogenesis-inhibiting hormone (VIH) or gonad-inhibiting hormone (GIH) [6,7].

Gonad-inhibiting hormone is a member of the neuropeptide family that is synthesized in neuroendocrine cells located in the eyestalk medulla terminalis ganglionic X-organ. Once produced, these neuropeptides are transported to the axon terminals that form a neurohaemal organ called the sinus gland, from where they are secreted [8]. This hormone family is known as the CHH family. Mature peptides of CHH family

**Abbreviations**

CHH, crustacean hyperglycemic hormone; GIH, gonad-inhibiting hormone; MIH, molt-inhibiting hormone; RT, reverse transcription; Vg, vitellogenin; VIH, vitellogenesis-inhibiting hormone.
members generally have 78–83 amino acid residues with a molecular mass of ~8–9 kDa. These hormones contain six cysteine residues that are aligned in conserved positions [9,10]. The CHH family can be divided into two types, type I and type II, as reflected by their primary structure [11–13]. The most abundant hormone in this family, crustacean hyperglycemic hormone (CHH), belongs to type I, whereas the other two hormones, molt-inhibiting hormone (MIH) and GIH, are categorized in type II. CHH or type I contains in its precursor sequence a short peptide called CHH-precursor-related peptide followed by a dibasic residue-processing site. By contrast, type II hormones are preceded directly by the signal peptides. In addition, alignment of the amino acid sequence reveals deletion of the amino acid glycine at the fifth position after the first cysteine residue in type I peptides.

Compared with CHH and MIH, only a limited number of GIH have been characterized to date. The first peptide with in vivo GIH activity was isolated from the American lobster Homarus americanus [14]. Another peptide that has been shown to depress Vg mRNA expression in the ovary fragment is the Pej-SGP-III of Marsupenaeus japonicus [15]. Likewise, a similar approach was used to assay VIH activity in the crayfish Procambarus bouvieri [16]. MIH-B from the shrimp Metapenaeus ensis, although capable of extending the molting cycle, may be considered as another candidate for GIH because the mRNA levels of this peptide decrease sharply during the early phase of gonad maturation and increase continuously as the vitellogenic stages proceed [17]. The cDNA encoding GIH-like peptide is also found in a few other species such as the Norway lobster Nephrops norvegicus [18] and the prawn Macrobrachium rosenbergii [19]. However, whether the peptides encoded by these cDNAs function as GIH needs further verification.

In this study, a cDNA encoding GIH from Penaeus monodon and its potential role in vitellogenesis were studied. Functional knockdown of Pem-GIH by double-stranded (ds)RNA was applied to demonstrate the negative effect on Vg expression in the ovary of previtellogenic adult female and thus provides evidence for its role as a GIH.

Results

Cloning and characterization of Pem-GIH cDNA

A partial 3′ cDNA sequence encoding GIH from P. monodon (Pem-GIH) was amplified by several sets of degenerate primers (Fig. 1) designed from the conserved amino acid sequences of type II hormones in the CHH family. Nucleotide sequence analysis revealed that 7 of 213 recombinant clones harbored GIH-like nucleotide sequences, as judged by a unique feature of the amino acid sequences at the C-terminus, which are longer than and different from that of MIHs. To obtain the 5′ region of this cDNA, a set of specific primers was designed from the 3′ sequence of the cDNA as described in the Experimental procedures. In addition, full-length cDNA was amplified with specific primers, as shown in Fig. 1. The nucleotide sequences of the full-length Pem-GIH cDNA of eight individual recombinant clones were sequenced, and confirmed as representing identical clones. Fig. 2 shows the nucleotide sequence of Pem-GIH cDNA (GenBank accession no. DQ643389) and its deduced amino acid sequence.

Fig. 1. Schematic diagram showing the structure of Pem-GIH cDNA and locations of the primers used in this study. The 5′- and 3′-UTRs are shown as a thin line. The ORF is depicted by boxes: the unfilled box represents the signal peptide and the filled box represents the mature peptide.
The full-length cDNA encoding the putative GIH of *P. monodon* was composed of 861 nucleotides containing a 5′-UTR (93 bp), an ORF (288 bp), a stop codon (TGA) and a 3′-UTR (477 bp) with a potential polyadenylation signal AATAAA located 7 bp upstream of the poly(A) tail. The ORF of *Pem-GIH* codes for a protein of 96 amino acid residues. The signal peptide, predicted using the SignalP 3.0 server, consisted of 17 amino acids, whereas the remaining 79 amino acids comprised the mature Pem-GIH peptide. The deduced amino acid sequence of putative Pem-GIH showed the conservation of six cysteine residues in the mature peptide with a glycine residue at the fifth position after the first cysteine. Mature Pem-GIH showed 68% amino acid identity with the GIH of *M. ensis*, but 45 and 48% amino acid identity with that of *H. americanus* (Hoa-GIH) and *N. norvegicus* (Nen-GIH), respectively (Fig. 3).

**Tissue-specific expression of Pem-GIH**

*Pem-GIH* expression in several *P. monodon* tissues was examined by RT-PCR using a pair of primers specific for *Pem-GIH* cDNA. *GIH* transcripts at the expected size of 385 bp were detected in the eyestalk ganglia, brain, thoracic nerve cord and abdominal nerve cord of individual shrimp. No *GIH* transcript was found in other tissues examined. This expression profile is similar to that of *Mee-GIH* expression in mature female *M. ensis* [17]. Interestingly, expression of *Pem-GIH* in these tissues was found in both male and female of adult and adolescent *P. monodon* (Fig. 4).

**dsRNA-induced Pem-GIH knockdown in shrimp explant culture**

The role of *Pem-GIH* was investigated by dsRNA-mediated gene silencing via RNAi, using dsRNA specific to the *Pem-GIH*. The coding sequence for mature Pem-GIH was used as template in the synthesis of GIH-specific dsRNA. The efficacy of this GIH-dsRNA to knockdown GIH expression was first determined in GIH-expressing tissues. Briefly, eyestalk XOSG neurons and abdominal nerve cord explant were cultured in a medium that contained GIH-dsRNA. RT-PCR results showed barely detectable levels of GIH transcript in the GIH-dsRNA-treated eyestalk XOSG culture from adult female shrimp after 3 h (Fig. 5A) indicating that GIH expression could be efficiently inhibited by GIH-dsRNA. Similar results were also seen when abdominal nerve cord explant culture was incubated with GIH-dsRNA for 3 and 6 h (Fig. 5B,C). By contrast, the irrelevant dsRNA, GFP-dsRNA, failed to knockdown *Pem-GIH* mRNA expression as the abdominal nerve cord explant was incubated with GFP-dsRNA for 3 h (Fig. 5A) indicating that GIH expression could be efficiently inhibited by GIH-dsRNA. Similar results were also seen when abdominal nerve cord from either adult or adolescent female shrimp was incubated with GIH-dsRNA for 3 and 6 h (Fig. 5B,C). By contrast, the irrelevant dsRNA, GFP-dsRNA, failed to knockdown *Pem-GIH* mRNA expression as the abdominal nerve cord explant incubated with GFP-dsRNA. The numbers on the left and right of the sequences show the coordinate of nucleotides and amino acids in corresponding lines.
was a potent tool for functional study of Pem-GIH in the shrimp.

**Biological assay for vitellogenesis-inhibiting activity of Pem-GIH by dsRNA-mediated functional knockdown**

To test whether the knockdown of Pem-GIH expression by dsRNA would interfere with Vg gene expression, previtellogenic adult female P. monodon were injected with GIH-dsRNA and the level of Pem-GIH expression as well as the expression of Vg transcript in the shrimp was determined by RT-PCR.

In order to determine the silencing effect of GIH-dsRNA in the shrimp, eyestalk ganglia were collected from previtellogenic adult P. monodon on day 3, 5 and 7 subsequent to GIH-dsRNA injection. The results (Fig. 6A) show that 3 days after dsRNA injection,
shrimp administered with GIH-dsRNA showed drastically reduced *Pem-GIH* transcript levels in the eyestalk ganglia when compared with control shrimp injected with Tris/NaCl only. This comprehensive silencing lasted until day 5 before the expression of *Pem-GIH* began to recover to some extent on day 7. Moreover, expression of two other related genes, *Pem-CHH1* and *Pem-MIH1* of *P. monodon*, did not change in GIH-knockdown shrimp when compared with control shrimp (Fig. 6B), suggesting the specificity of *Pem-GIH* silencing by GIH-dsRNA. Subsequently, the consequence of the depletion in GIH transcript on *Vg* synthesis was investigated on day 5 after GIH-dsRNA injection. Fig. 7 shows that the *Vg* transcription level was increased more than ninefold in the ovary of GIH-knockdown previtellogenic adult shrimp when...
compared with that in control shrimp. The increase in the ratio of Vg to actin transcripts in the GIH-depleted background suggested that functional knockdown of Pem-GIH led to the induced expression of Vg in the ovary.

Discussion

Because of the lack of information on the GIH in penaeid shrimp, the attempt to clone GIH cDNA from P. monodon in this study was carried out using a RACE approach with degenerate primers designed from the conserved amino acid sequences among MIH/GIH from other species of crustaceans. To increase the possibility of obtaining the GIH cDNA of P. monodon, codons preferably used for CHH (GenBank accession nos AF233295, AY346379 and AY346380) and MIH (GenBank accession nos AY496454 and AY496455) genes of this species were also taken into consideration for primer design. In addition, mRNA from eyestalk neurons of adult female P. monodon at different vitellogenic stages as determined by gonadal somatic index were used as the template for cDNA cloning in this study. This is based on a previous study which showed high levels of GIH mRNA in the sinus gland during previtellogenesis and vitellogenesis [20]. A putative GIH cDNA of P. monodon (Pem-GIH) was successfully cloned using the aforementioned strategy. The deduced amino acid sequence of putative GIH from P. monodon possesses all the characteristics in agreement with a type II hormone from the CHH family [11–13]. Moreover, the C-terminus of Pem-GIH had an extension of two amino acid residues when compared with that of MIH. This is consistent with previously identified GIHs from other crustacean species (Fig. 3). Pem-GIH cDNA was thus subsequently examined for its gonad-inhibiting function by using a RNA interference (RNAi) technique.

RNAi, a post-transcription gene-silencing process in which dsRNA triggers sequence-specific suppression of its cognate mRNA [21], is a powerful tool for studying gene function [22–24]. In P. monodon, a dsRNA-induced gene-silencing phenomenon has been recently demonstrated [25], therefore it was selected as a tool for studying the functional knockdown of Pem-GIH cDNA in this study. GIH-specific dsRNA was synthesized from a 240 bp coding region of the mature Pem-GIH. The use of long dsRNA provides the possibility of generating more varieties of effective siRNA (21–23 nucleotides) molecules. Nevertheless, the nonspecific silencing, known as off-target phenomenon, may also occur from these diverse siRNA products of the long dsRNA [26,27]. To minimize this off-target silencing, the GIH-dsRNA sequence was used to search for a possible region of 21–23 consecutively identical nucleotides in the sequences of all P. monodon CHH and MIH. A nucleotide sequence comparison revealed no such region (data not shown) in either CHHs or MIHs, suggesting that the GIH-dsRNA should direct sequence-specific silencing of Pem-GIH with a minimal off-target effect on other related genes. Indeed, this was clearly showed by the result in Fig. 6B in which the shrimp administered with GIH-dsRNA still expressed CHH and MIH at the level comparable with that of the control shrimp. The efficacy of GIH-dsRNA to silence Pem-GIH expression was manifested by the dramatic depletion in Pem-GIH mRNA level in shrimp eyestalk ganglia and abdominal nerve cords as early as 3 h after incubating with GIH-dsRNA. This silencing was not affected by irrelevant dsRNA, thus indicating that Pem-GIH knockdown occurred in a sequence-specific fashion. Similar specific silencing of Pem-GIH by GIH-dsRNA was also demonstrated in adolescent female P. monodon (Fig. S1). Accordingly, any biological changes observed following GIH-dsRNA injection may be considered the consequence of Pem-GIH knockdown.

To date, no conclusive evidence about the mode of action of GIH on vitellogenesis has been established. Recently, the recombinant vitellogenesis-inhibiting hormone (VIH or GIH) of H. americanus has been
reported for its biological activity to inhibit Vg mRNA synthesis in the ovary of heterologous species, M. japonicus [28]. In addition to the ovary, hepatopancreas has been revealed as another site for Vg synthesis in shrimp [29,30]. Although the function of Vg originating from the hepatopancreas has not been clearly evidenced, it has been shown that Vg expression in the hepatopancreas is correlated with ovarian maturation [31]. After synthesis in the hepatopancreas, Vg undergoes post-translational processing into smaller subunits by a subtilisin-like endopeptidase, these subunits are then released into the hemolymph. These hemolymph Vg subunits are further processed by an unidentified enzyme before being sequestered by the ovary, and form yolk protein (vitellin) subunits [32]. The induced ovarian Vg expression of GIH-knockdown previtellogenic adult P. monodon evidently indicates the inhibitory function of Pem-GIH on Vg gene expression in the ovary. Our results are in concurrence with an increase in Vg expression after eyestalk ablation in M. japonicus [33,34] and L. vannamei [35]. In addition, the level of Vg expression in hepatopancreas was not appreciably affected following dsRNA-mediated knockdown of Pem-GIH (data not shown). This is not unanticipated because Vg synthesis was not induced in hepatopancreas of eyestalk-ablated shrimp either, especially within the first 7 days following eyestalk ablation [33]. In addition, Okumura et al. showed that the Vg mRNA level increased slowly in hepatopancreas at the start of vitellogenesis in naturally mature female of M. japonicus, compared with that in the ovary [36]. Empirically, our results support the postulation that ovarian Vg is required for early maturation of the ovary in crustaceans [5] and conform well to the precocious ovarian development after the main source of GIH synthesis was removed by eyestalk extirpation [37].

Although a similar strategy was used to analyze the function of CHH cDNA from L. schmitti [38], the effect of CHH silencing on glucose level was determined at 24 h after dsRNA injection. Our study clearly demonstrates that the effect of dsRNA silencing is effective for at least 5 days in shrimp, which provides further benefit to the use of dsRNA for analysis of genes whose function has long-term physiological influence.

Although the function of GIH has been studied mainly in female crustaceans, expression of Pem-GIH in male P. monodon, which is similar to previous reports [17,39], implies that GIH may play a more versatile role in the male as well. Eyestalk ablation in the crayfish Cherax quadricarinatus resulted in an overexpression of androgenic gland polypeptides, which had a direct effect on male reproductive system [40]. Whether Pem-GIH is involved in reproduction in male P. monodon needs further investigation.

In summary, this study identified and characterized Pem-GIH cDNA of P. monodon in both molecular and biological aspects. The system of a functional–knockdown study was exploited using GIH-specific dsRNA, and revealed, for the first time, the influence of Pem-GIH on vitellogenin transcript levels in the ovary, which directly linked GIH to expression of Vg mRNA. Finally, our results demonstrated that dsRNA-mediated gene silencing has a potential as a powerful tool for functional study of other genes in crustaceans.

**Experimental procedures**

**Animals**

Wild adult female P. monodon, at different vitellogenic stages, were caught from the Gulf of Thailand, Chonburi Province, Thailand. They were used in cDNA cloning experiments. Previtellogenic adult female P. monodon used for the GIH functional assay were domesticated shrimp provided by Bangkok Aquaculture Farm Company (BAFCO, Huasai, Nakhon Si Thammarat Province, Thailand).

Experiments involving animals were carried out in accordance with animal care and use protocol of the Mahidol University Animal Care and Use Committee (MUACUC).

**Total RNA preparation and first-strand cDNA synthesis**

Eyestalk neurons were dissected from individual eyestalks of adult female shrimp at various stages of reproductive cycle and homogenized in TRI-REAGENT® (Molecular Research Center, Cincinnati, OH, USA). Total RNA of eyestalks was extracted by using TRI-REAGENT® according to the manufacturer’s protocol.

The reverse transcription (RT) step was performed with ImProm-II™ reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s protocol using 500 nM of oligo(dT)16 or PRT primer (5'-CCGGATATCT AACCTCTAGAGCATCCTTTTTTTTTTTTTT-3') to prime cDNA synthesis at 42 °C for 60 min.

**RACE**

The degenerate primers used in 3'-RACE of Pem-GIH cDNA were designed from the conserved amino acid sequences of MIH/GIH from several species of crustacean.

In the first round of PCR, a 1 µl aliquot of cDNA was amplified with 3'-RACE-GIH1A [5'-TG(TC)(AC)C1G(GG) T1ATGGG(TC)AA(C)GG1GA-3'] and PM1 (5'-CCGG AATTCAAGC1TCTAGAGGATCC-3') primers in 25 µL
of a reaction mixture containing 10 mM Tris/HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 200 nM of each primer, 200 μM each of dATP, dCTP, dGTP, dTTP and 1.25 units of Taq DNA Polymerase in storage buffer B (Promega). Amplification was performed in a DNA thermal cycler (GeneAmp System 2400; PE Applied Biosystems, Foster City, CA, USA) with 35 cycles of 94°C for 30 s, 50°C for 30 s and 74°C for 1 min followed by 7 min incubation at 74°C as a final extension. Subsequently, nested amplification was performed with 200 nM of 3′-RACE-GIH1B [5′-ATGGGG(TC)AC(AC)GGGACG(AC)GTGGA(AC)TICA(TC)TITA(GTGCAG)TTTAACGGAAAATTAAT-3′] and PM1 primers to obtain the specific product.

For 5′-RACE, first-strand cDNA synthesis was performed in a reaction as described above, except that 1 μM of 5′-RACE-GIH1 primer (5′-CCACCGGCCCCGGCCGTCGATTGAG-3′) was substituted for PRT primer. The reaction was carried out using two-step RT. The first RT step was incubated at 50°C for 5 min. For the second RT step, 1 μM of dATP in 30 μL of 100 mM cacodylate buffer (pH 6.8), 1 mM MgCl₂, 0.1 mM dithiothreitol, 200 μM dATP and 20 units of terminal deoxynucleotidyl transferase (TdT) (Promega). The reaction was incubated at 37°C for 20 min and TdT was heat-inactivated at 65°C for 10 min. The first round PCR with 3 μL of the dA-tailed cDNA template was carried out as described for 3′-RACE using 200 nM of 5′-RACE-GIH2 (5′-GGCCCTCGCGCTTGGCCGAGTG-3′) and PRT primers, except that annealing was performed at 55°C for 30 s. Second-round PCR was performed with 200 nM of 5′-RACE-GIH3 (5′-TGATTTCTGTGACACAAACGATCCAGGCTG-3′) and PM1 primers to obtain specific amplified product.

Amplification of full-length Pem-GIH cDNA

Total RNA extracted from one pair of eyestalks from an adult female shrimp in stage IV of vitellogenesis, as described above, was used to synthesize a cDNA template for the cloning of full-length cDNA of Pem-GIH. A 1 μL aliquot of cDNA was amplified with GIHF (5′-GAACGTCGTCGATTTTATATTTAAACGGTACCG-3′) and GIHR (5′-GGTCGACTTTATTTAAACGGAATATAAT-3′) primers in a 25 μL reaction containing 1× Phusion HF buffer including 1.5 mM MgCl₂, 500 nM of each primer, 200 μM each of dATP, dCTP, dGTP, dTTP and 0.25 units of Phusion DNA Polymerase (Finnzymes, Espoo, Finland). Amplification was performed in a DNA thermal cycler (GeneAmp System 2400; PE Applied Biosystems) with 35 cycles of 98°C for 10 s, 50°C for 30 s and 72°C for 1 min followed by 7 min incubation at 72°C as a final extension.

RT-PCR

To detect tissue-specific expression of Pem-GIH, total RNA extracted from several P. monodon tissues, including eyestalks, brain, thoracic nerve cord, abdominal nerve cord, heart, hepatopancreas, ovary, and muscle, was used as a template for RT with PRT primer as described above. The specific transcript of Pem-GIH was amplified with GIHF and 5′-RACE-GIH1 primers to detect Pem-GIH transcript level in all experiments. The reaction was amplified with 35 cycles of 94°C for 30 s, 50°C for 30 s and 74°C for 1 min followed by 7 min incubation at 74°C as a final extension. To detect Vg transcript in the ovary, Vg-F (5′-CTAAGGCCATATATCTGTGCTG-3′) and Vg-R (5′-AACGTTGGCAATGATCTTCCTTT-3′) primers designed from the EST clone containing Vg sequence from P. monodon ovary (GenBank accession no. EE332453) were used in a reaction with 32 cycles of 94°C for 30 s, 50°C for 30 s and 74°C for 1 min followed by 7 min incubation at 74°C. The actin transcript was amplified with PmActin-F (5′-GACTCTGACGTCCGCGCAGG-3′) and PmActin-R (5′-AGCACGCGTGTCATACCTCGCT-3′) primers in a reaction with 21 cycles of 94°C for 30 s, 55°C for 30 s and 74°C for 1 min followed by further incubation at 74°C for 7 min. The expected sizes of Pem-GIH, Vg and actin transcripts are 385, 354 and 539 bp, respectively.

Preparation of GIH-dsRNA

Production of GIH-dsRNA by in vitro transcription

Two DNA templates for dsRNA of GIH that span the coding sequence of the mature Pem-GIH, each containing T7 promoter sequence at the 5′-end on different strands were synthesized by PCR from full-length Pem-GIH cDNA. Two separate PCR reactions were set up, one with T7-containing forward primer (5′-TAAATACGACTCACTATAGGGAGAAACATCCTGGACATCGAAATGCAGGG-3′) and reverse primer (5′-CCGGCATTGGAGATGCTGAT-3′) for the sense-strand template, the other with forward primer matGIHF (5′-AACATCTCTGGACAGCAAATGCAGGG-3′) and T7-containing reverse primer (5′-TAAATACGACTCACTATAGGGAGAACGGATCATGGATGCTGAT-3′) for the antisense-strand template. The reaction consisted of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 74°C for 1 min for 9 cycles with a 1°C decrease in annealing temperature per cycle; the annealing temperature then remained at 48°C for another 30 cycles and followed by a final extension at 74°C for 7 min. The expected PCR product were excised and purified with a gel extraction kit.
Production of GIH-dsRNA by in vivo expression in Escherichia coli

In order to obtain large quantity of dsRNA for the in vivo functional assay, GIH-dsRNA was produced as hairpin-RNA precursor in *E. coli* following a previously described method [25] with some modifications. A 340 bp DNA template for the sense strand of dsRNA connecting with a loop region was amplified with primers sense-GIHFF1-Xbal (5′-GCTCTAGAACATCCCTGGCAGCA-3′) and sense-GIHR1-BamHI (5′-CGGGATCTAGCCAGGCGGA GA-3′). Another DNA template for the antisense strand of dsRNA, 240 bp, was amplified with primers as-GIHF-SalI (5′-CGGTCAACAGATCTCCCTGGCAGCA-3′) and as-GIHR-BamHI (5′-CGGGATCTCGACACGCGGGCG CGGCC-3′). The antisense template was first cloned into pET17b vector at BamHI and XhoI sites, followed by the sense template at Xbal and BamHI. The resulting recombinant plasmid was constructed and propagated in *E. coli* DH5α and its nucleotide sequence was verified by automated DNA sequencing.

The recombinant plasmid of hairpin-RNA of *Pem-GIH* was subsequently transformed into an RNaseIII-deficient *E. coli* HT115 strain. Expression of hairpin-RNA was induced with 0.4 mM isopropyl thio-β-D-galactoside for 2 h in 2x YT medium. Cells were harvested by centrifugation and resuspended in 100 μL NaCl/P; containing 0.1% SDS. The sample was boiled for 2 min and then snapped cool on ice. To eliminate endogenous RNA from bacterial cell and single-stranded RNA in the loop region of GIH hairpin-RNA, the cell lysate was treated with RNaseA at 37 °C for 30 min. dsRNA of *Pem-GIH* was extracted by using TRI-REAGENT® (Molecular Research Center) and resuspended in Tris/NaCl (10 mM Tris/HCl pH 7, 10 mM NaCl).

The quantity of dsRNA was determined by the UV spectrophotometry at an absorbancy of A260.

dsRNA-mediated *Pem-GIH* knockdown in shrimp explant culture

Eyestalk ganglia or abdominal nerve cords of *P. monodon* were dissected from individual shrimp. The eyestalk from a single shrimp was used in each experiment. The XOSG neuron from the left eyestalk was used as a negative control whereas that from the right eyestalk was treated with dsRNA as described below. Nerve cord from the same shrimp was cut into ~ 0.8–1 cm pieces and used in one set of the experiment. The explant samples were incubated in a well of 24-well plate filled with 1.5 mL of modified M199 culture medium consisting of M199 powder in crab saline (440 mM NaCl, 11 mM KCl, 13.3 mM CaCl2, 26 mM MgCl2, 26 mM Na2SO4 and 10 mM Hepes pH 7.2) supplemented with 100 μg/mL penicillin–streptomycin antifungus and 40 μg/mL gentamicin sulfate. The samples were added to 3 μg of GIH-dsRNA and cultured with shaking at 20–24 °C for the appropriate length of time. Samples were then washed with modified M199 medium plus antibiotic before collected for RNA extraction.

The level of *Pem-GIH* transcript was detected by RT-PCR with GIHF and 5′-RACE-GIH1 primers as described earlier.

Functional knockdown assay for *Pem-GIH* activity

Previtellogenic adult female *P. monodon* at the intermolt and early premolt stages (C–D2) (~ 85–120 g each) were cultured in tanks filled with artificial seawater (~ 30 p.p.t. salinity). Shrimp were divided into two groups, each containing five shrimp. The control group was injected through the arthrodial membrane of the second walking leg with Tris/NaCl (~ 3 μL·g⁻¹ body weight) and the experimental group was injected with GIH-dsRNA (~ 3 μg·μL⁻¹) at 3 μg·g⁻¹ body weight. The level of *GIH* and *Vg* transcripts in eyestalk ganglia and ovary, respectively were detected by RT-PCR 5 days after being administered with dsRNA.

Statistical analysis

Results are presented as mean ± SEM. Statistical significance between values was determined by Levene's test of independent sample t-test from SPSS for Windows 11.5.

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References


Supplementary material

The following supplementary material is available online:

Fig. S1. Silencing of Pem-GIH in adolescent female P. monodon. Adolescent shrimp (22–23 g) were divided into three groups. Control shrimp (buffer) were injected with Tris/NaCl, whereas shrimp in the experimental group (dsGIH) were injected with GIH-dsRNA at a concentration of 2 μg/10 g shrimp. The third group (dsGFP) was injected with GFP-dsRNA at the same concentration as GIH-dsRNA. Eyestalk ganglia were collected from individual shrimp in each group at 72 h after injection for RNA extraction and detection of Pem-GIH and actin transcripts by RT-PCR. –ve represents negative PCR.

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