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Molecular characterization of a vitellogenesis-inhibiting hormone (VIH) in the mud crab (*Scylla olivacea*) and temporal changes in abundances of VIH mRNA transcripts during ovarian maturation and following neurotransmitter administration

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ABSTRACT

The vitellogenesis-inhibiting hormone (VIH), also known as gonad-inhibiting hormone, is a neuropeptide hormone in crustaceans that belongs to the crustacean hyperglycemic hormone (CHH)-family peptide. There is regulation vitellogenesis by VIH during gonad maturation in crustaceans. A full-length *Scylla olivacea VIH* (*Scyol-VIH*) was identified through reverse transcription polymerase chain reaction and rapid amplification of cDNA ends. The open reading frame consists of 378 nucleotides, which encodes a 126-amino acid precursor protein, including a 22-residue signal peptide and a 103-amino acid mature peptide in which 6 highly conserved cysteine residues are present. There was expression of the *Scyol-VIH* gene in immature female *Scylla olivacea* in the eyestalk, brain and ventral nerve cord. The *Scyol-VIH* gene expression was localized to the eyestalk X-organ, brain neuronal clusters 6 and 11, and in multiple neuronal clusters of the ventral nerve cord. The relative abundance of *Scyol-VIH* mRNA transcript in the eyestalk was relatively greater in immature stage females, then decreased as ovarian maturation progressed. Furthermore, eyestalk *Scyol-VIH* increased after dopamine (5 μg/g BW) injection. The present research provides fundamental information about *Scyol-VIH* and its potential effect in controlling reproduction.

1. Introduction

The mud crab, *Scylla olivacea*, is one of the most important commercial crab species in Thailand and the Indo-pacific area, primarily due to the high quality meats and eggs. As a result of decreasing wild populations, it has become increasingly important to study the mud crab central nervous system (CNS) and its molecular factors that are released that regulate numerous physiological activities, including reproduction (Tinikul et al., 2009).

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Neurotransmitters and neurohormones [e.g., serotonin (5-HT), dopamine (DA), red pigment-concentrating hormone (RPCH), crustacean hyperglycemic hormone (CHH) and vitellogenesis-inhibiting hormone (VIH; also called gonad-inhibiting hormone, GIH)] are synthesized and released from the eyestalks (Keller, 1992; Tinikul et al., 2011; Kornthong et al., 2013; Liu et al., 2018). For reproduction, two antagonistic hormones, VIH and gonad-stimulating hormone [GSH; also called vitellogenesis-stimulating hormone (VSH)] (Nagaraju, 2011) appear to be very important. There remains inconsistent thought about whether GSH is essential in controlling crustacean reproduction (Rotllant et al., 2018). In regard to neurotransmitters, evidence for 5-HT involvement in the regulation of crustacean reproduction is more conclusive, whereby it is known to stimulate or inhibit the synthesis and release of downstream reproductive hormones (Tinikul et al., 2009; Nagaraju, 2011). In *S. olivacea*, 5-HT stimulates neurohormone and reproductive hormone secretions such as RPCH, CHH and farnesoic acid O-methyltransferase (FAMeT) (Kornthong et al., 2013, 2014; Duangprom et al., 2017). The functions of DA in controlling neurohormones, however, is still unknown.

The VIH molecule is mainly produced in the XO/SG complex of the Norway lobster (*Nephrops norvegicus*), although the presence of VIH in the brain and subesophageal ganglion has been also reported in the Pacific white shrimp (*Litopenaeus vannamei*) (Edomi et al., 2002; Chen et al., 2014). Upon release, VIH regulates crustacean ovarian maturation by inhibiting the synthesis and uptake of vitellogenin (Vg) (Nagaraju, 2011; Chen et al., 2014; Liu et al., 2018). The process of vitellogenesis (also known as yolk deposition) is a prerequisite for oogenesis and embryonic development (Nagaraju, 2011; Jia et al., 2013). At the molecular level, VIH belongs to CHH-family peptide. Many putative VIH cDNAs and corresponding protein sequences have been identified and characterized from several crustacean species such as *Homarus americanus* (de Kleijn et al., 1994), *Macrobrachium rosenbergii* (Yang and Rao, 2001), *N. norvegicus* (Edomi et al., 2002), *Homarus gammarus* (Ollivaux et al., 2006), *Penaeus monodon* (Treerattrakool et al., 2008), *L. vannamei* (Tsutsui et al., 2013; Chen et al., 2014), *Macrobrachium nipponense* (Li et al., 2015), and *Scylla paramamosain* (Liu et al., 2018). To confirm functionality, knockdown of VIH has been performed in *P. monodon* and *L. vannamei*, providing evidence for its negative effect on Vg synthesis and uptake into the ovary (Treerattrakool et al., 2011; Feijo et al., 2016). The VIH protein has only been detected in a few crab species, primarily due to the difficulty of extraction and isolation (Liu et al., 2018).

The molecular identification and distribution of VIH in *S. olivacea* has not been reported, nor whether expression of the gene that encodes for this protein is altered following 5-HT or DA administration. In the current study, a cDNA encoding *VIH* from *S. olivacea* was cloned and sequenced. *S. olivacea VIH* mRNA relative abundance was then investigated, and there was evaluation of variations during ovarian maturation and after administration 5-HT and DA.

2. Materials and methods

2.1. Ethic statement

Female *S. olivacea* were caught by local fishermen in Ranong, Thailand and used in this study, therefore, there was no specific permission required for sample collections of mud crabs from the area and species for scientific research purpose. The experiments were performed according to the guidelines on the care and use of animals for scientific purpose provided by the Institutional Care and Use Committee of Thammasat University. This study was specifically approved by Animal Care and Use Committee of Thammasat University, National Research Council of Thailand (NRCT), Protocol Number 017/2558. All efforts were made to minimize the suffering of animals.

2.2. Experimental animals

Healthy immature and mature female *S. olivacea* were obtained from local farms in Ranong, Thailand. Immature and maturities of female mud crabs were determined using the previously described methods (Ikhwanuddin et al., 2011). Based on size, immature female mud crabs were 7 to 8 cm in carapace width (CW) and 70 to 120 g in body weight (BW), while mature female mud crabs were 9 to 12 cm in CW and 150 to 300 g in BW. The ovarian maturation of female mud crabs was examined and classifications of the stage of the ovary were based on the morphological appearance of the ovary (Islam et al., 2010; Ghazali et al., 2017; Hidir et al., 2018). *S. olivacea* were maintained in concrete tanks using procedures that have been previously described (Duangprom et al., 2017, 2018).

2.3. RNA isolation

The eyestalk, brain, VNC, muscle, gill, gut, hepatopancreas, heart and ovaries were collected from female *S. olivacea*, and immediately frozen in the liquid nitrogen before being stored at −80 °C until use. Total RNA was extracted from each tissue using TriPure Isolation reagent (Roche, Germany), following the manufacturer's protocol. Total RNA obtained from the eyestalk was further purified using GeneJET RNA purification kit (Thermo Scientific, USA) to remove the eye pigment. Total RNA concentration and purity were determined by using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA).

2.4. Molecular cloning of vitellogenesis-inhibiting hormone (VIH)

Total RNA (2 μg) extracted from the eyestalks of immature female *S. olivacea* was used for first-strand cDNA synthesis using QuantiNova Reverse Transcription Kit (Qiagen, Germany), following the manufacturer's protocol. For polymerase chain reaction (PCR), there was amplification of *VIH* transcript, and primers (Table 1) were designed from a VIH transcript derived from *S. paramamosain* (Liu et al., 2018). Complementary DNA was subsequently used as a template for PCR using forward (VIH-F) and reverse

Table 1
Primers used for RT-PCR, 5' and 3'RACE, and quantitative RT-PCR.

Primer	Direction	Nucleotide Sequence
VIH-F	Forward	5'GATGATCGACGATGAGTGCCCGA 3'
VIH-R	Reverse	5'CGTTTGATGACTGTAGAACATCTC 3'
Actin-SO-F	Forward	5'-GAGCGAGAAATCGTTCGTGACAT- 3'
Actin-SO-R	Reverse	5'-CCCATGGTGATGACCTGGCCGT-3'
5VIH-R1	Reverse	5'- TACCAATAAGGTTCGGGCACTC -3'
3VIH-F1	Forward	5'-CACTCACCAATAGGAGACATTTC-3'
Scyol-VIHsp-F	Forward	5'-AGGAGGAACTGCTTCTACAACGAGG
Scyol-VIHsp-R	Reverse	5'-GAGTGAATAATGTGAGATGTGGCTA-3'
Scyol-VIHq-F	Forward	5'- CACGTGGTGCATCAGCGCGA -3'
Scyol-VIHq-R	Reverse	5'-GTACCGTCGTCAGCATGAGGGCG -3'

(VIH-R) primers with details being included in Table 1. Thermocycling condition used for PCR amplification was set as follows: 1 cycle at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C, with a final extension of 10 min at 72 °C. To identify the full-length *VIH* gene sequence, 5'RACE and 3'RACE were performed using SMART^{TM} cDNA library construction kit (Clontech, CA, USA), following the manufacturer's protocol. Universal primer mix was provided in the kit and gene specific primers to amplify 5' and 3' ends (5VIH-R1 and 3VIH-F1) were used to obtain the complete cDNA of VIH (Table 1). Thermocycling conditions used for PCR amplification were set as follows: 1 cycle at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR product was further analyzed by gel electrophoresis using 2% agarose gel. The amplicon with predicted full-length size was purified using a GeneJET gel extraction kit (Thermo Scientific, USA), and inserted into a pDrive vector (Qiagen, Germany). Plasmids with insert sequences were purified using GeneJET Plasmid Mini-prep Kit (Thermo Scientific, USA) before these were submitted for sequencing (Macrogen, Korea).

2.5. Sequence analysis of Scyol-VIH cDNA

The full-length of *Scyol-VIH* nucleotide sequence was used for Basic Local Alignment Search Tool (BLAST) search against National Center for Biotechnology Information (NCBI) Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for defining similar sequences. *Scyol-VIH* nucleotide sequence was translated to an amino acid sequence using the Expasy bioinformatics resource portal-translate tool (http://web.expasy.org/translate/). Protein sequences of VIH from related species were retrieved from Entrez (NCBI). For protein sequence comparison, multiple alignment of amino acid sequences was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Presence of a signal peptide sequence was determined using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen, 2017). A phylogenetic analysis of VIH was conducted using MEGA7 software (Kumar et al., 2016) based on the Neighbor-joining method with an interior branch test.

2.6. Transcript distribution by reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization

The cDNA from various *S. olivacea* tissues, were used as templates for PCR. Each PCR was performed according to the previous described protocol (Duangprom et al., 2017, 2018). To investigate *Scyol-VIH* expression, gene-specific primers (Scyol-VIHsp-F and Scyol-VIHsp-R) were used (Table 1). The RT-PCR exponential phase was determined within 30 cycles to allow the comparison of cDNAs developed from an internal control (*S. olivacea-β-actin)*.

In situ hybridization (ISH) was performed following the previously described protocol (Duangprom et al., 2017, 2018). The *Scyol-VIH* riboprobe was synthesized using a DIG-oligonucleotide labeling kit (Roche, Germany). Sections were observed and photographed using a Leica compound microscope equipped with a digital camera (Leica, Germany).

2.7. Relative abundance of Scyol-VIH mRNA transcript during ovarian development

The eyestalks were collected from intermolt stage *S. olivacea* at different ovarian maturation stages (10 animals per stage) based on classification methods described by Islam et al. (2010) and Hidir et al. (2018). The ovarian maturation stage of this species was classified according to gross morphology of the ovary. The ovary could not be observed in the immature stage female. Ovaries were transparent to white in color at Stage I, pale or light yellow color at Stage II, orange in Stage III, and orange to reddish orange in Stage IV, respectively. The cDNAs were then prepared as described in the previous section of this manuscript. Real-time PCR was performed in duplicate for every sample. Gene-specific primers (Scyol-VIHq-F and Scyol-VIHq-R) were used for amplification of mud crab *S. olivacea-VIH* (Table 1). The *β-actin* gene was used as a normalization control (Kornthong et al., 2013, 2014; Duangprom et al., 2017, 2018). Amplifications ($n = 10$ for each stage) were conducted on a CFX-96 (Bio-Rad, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad, USA). Relative abundance of *Scyol-VIH* mRNA transcripts was calculated and analyzed following the previously described protocol (Duangprom et al., 2018). A statistical significance analysis was performed using the SPSS program (Statistical Product and Service Solutions; version 19) with a one-way analysis of variance (ANOVA) being conducted. A probability value less than 0.05 ($P < 0.05$) indicated a significant difference.

2.8. Effect of serotonin (5-HT), dopamine (DA) and spiperone (SP) on relative abundance of Scyol-VIH mRNA transcript

The effect of neurotransmitters 5-HT, DA and the DA antagonist, spiperone (SP), were conducted to investigate changes in *Scyol-VIH* expression. Immature *S. olivacea* females at the intermolt stage were divided into four groups: (1) sham control group (n = 15) in which the animals were administered with vehicle control (100 µl of 0.9% normal saline); (2) 5-HT treatment group (n = 15) in which the animals were administered with 100 µl of 5-HT at 5 µg/g BW dissolved in 0.9% normal saline; (3) DA treatment group (n = 15) of 100 µl of DA at 5 µg/g BW dissolved in 0.9% normal saline; (4) SP treatment group (n = 15) in which the animals were administered with 100 µl of SP at 5 µg/g BW dissolved in DMSO. Administration was performed by injection of solution into the muscle at the base of the fifth walking leg. Animals were sacrificed at 3 h post-injection, then the eyestalk, brains and VNCs were collected. Tissues were briefly frozen in the liquid nitrogen before being storage at −80 °C until use. Real-time PCR for amplification of *S. olivacea VIH* transcript was performed in duplicate for each sample (n = 15 per group). Abundance of *β-actin* mRNA was used as a normalization control (Kornthong et al., 2013, 2014; Duangprom et al., 2017, 2018). A list of gene-specific primers is provided in Table 1. Relative abundances of the *VIH* gene transcript relative to the abundance of *β-actin* mRNA transcript and a variance between individual treatments were performed, calculated and statistically analyzed using the methods described in Kornthong et al. (2013, 2014) and Duangprom et al. (2017, 2018).

3. Results

3.1. Cloning and sequence analysis of S. olivacea VIH cDNA

A partial sequence of *Scyol-VIH* was amplified using primers corresponding to the conserved region of crustacean VIH. The PCR product was 457 nucleotides in length which encoded 102-amino acid protein. The BLAST searches of a deduced Scyol-VIH against the NCBI GenBank database indicated Scyol-VIH shares more than 75% sequence identity to known crustacean VIHs (data not shown). This partial sequence was subjected to 3'-RACE and 5'-RACE procedures to obtain a full-length sequence of *Scyol-VIH*, which contained 689 nucleotides and encoded a 126-amino acid Scyol-VIH precursor protein (Fig. 1). This nucleotide sequence is available on the Genbank database (accession number: MH882453). A signal peptide for the Scyol-VIH precursor was predicted, with cleavage occurring at position A22 and A23. A 126-amino acid Scyol-VIH prohormone was predicted to be cleaved into a putative VIH mature peptide (Fig. 1). The Scyol-VIH molecule contains 6 cysteine residues (Cys29, Cys46, Cys49, Cys62, Cys66, and Cys75) (Fig. 1), which are highly conserved in crustacean VIH prohormones (Fig. 2). A comparison of the deduced full-length VIH prohormone of *S. olivacea* with other crustacean VIHs indicated *S. olivacea* VIH share the greatest sequence similarity to VIH of the mud crab, *S. paramamosain* (99.20%), but less with the VIH of the Norway lobster (*N. norvegicus*; 46.46%), European lobster (*H. gammarus*; 46.46%), caridean shrimp (*P. carinicauda*; 46.39%), sand shrimp (*Metapenaeus ensis*; 37%), giant tiger prawn (*P. monodon;* 35.11%), and whiteleg shrimp (*L. vannamei*; 34.04%) (Fig. 2). The VIH/GIH prohormone alignment there was a large amount of amino acid sequence identity, especially within the VIH mature peptide region, and conservation of 6 cysteine residues (Fig. 2). With a phylogenetic tree assessment, there was Scyol-VIH of *S. olivacea* that clustered with other VIHs/GIHs from related crustacean species and that these might

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cgaaagctctttactaagtgtaataatgcgtggtgatcttttcatttatcatcctggaacg      61
gatttttgttcctgttctgcatccatgtcctcttttaaactatttcataaatggttcaga     121
gagacgagaaacattatccctctcttcatctccgtctgacagaggatgtggctgctggcc     181
                                          M  W  L  L  A            5
gtggtggtcatggccgccctctggagcctcagtgtccagcgaacagcggcgaggatgatc     241
 V  V  V  M  A  A  L  W  S  L  S  V  Q  R  T  A  A  R  M  I      25
gacgatgagtgcccgaaccttattggtaacagggacctttatgagaaagtagactggatc     301
 D  D  E  C  P  N  L  I  G  N  R  D  L  Y  E  K  V  D  W  I      45
tgcgaagactgtgctaacatcttccgcaaaatccgcatgggttatcgatgcaggaggaac     361
 C  E  D  C  A  N  I  F  R  K  I  R  M  G  Y  R  C  R  R  N      65
tgcttctacaacgaggaattcacgtggtgcatcagcgcgacggagaacacgggacacaag     421
 C  F  Y  N  E  E  F  T  W  C  I  S  A  T  E  N  T  G  H  K      85
aaggaactgatgcaatgggtcgccatcctgagggccacgccagagaggcaccgctctttc     481
 K  E  L  M  Q  W  V  A  I  L  R  A  T  P  E  R  H  R  S  F     105
ttgacgtccatccagggaggcgctgcaattcgccctcatgctgacgacggtactgcttac     541
 L  T  S  I  Q  G  G  A  A  I  R  P  H  A  D  D  G  T  A  Y     125
tgaggcactgaacatcattaaacctttcactgttacttgccacatctcacattattcact     601
 *                                                               126
caccaataggagacatttctacatgaaatgttctacagtcatcaaacgatgaattaatta     661
gaaattggaaaaaaaaaaaaaaaaaaaaaa                                   691
```

Fig. 1. Nucleotides corresponding with deduced amino acid sequence of the full-length *S. olivacea* vitellogenesis-inhibiting hormone (Scyol-VIH) are shown; Within 689 nucleotides of *Scyol*-VIH cDNA sequence, 378 were predicted to be an open reading frame, and 313 untranslated regions; The open reading frame was translated into 126 deduced amino acids; Numbers indicate nucleotide (black) and amino acid (black bold) positions; Small and capital letters indicate nucleotide and amino acid sequences, respectively; A black bold italic M letter represents a start codon, while an asterisk a stop codon; Signal peptide sequence of Scyol-VIH is indicated by underlined italic letters; Blue bold C letter in the circle indicates cysteine residues.

Fig. 2. Amino acid alignment of vitellogenesis-inhibiting hormone (VIH) of *S. olivacea* with other known VIH/GIH Gaps (-) are included to allow alignment '*', identical amino acid; ':'conserved substitution (similar shapes); Species abbreviation and GenBank accession numbers: *Scypa*, *Scylla paramamosain* (AHE40786); *Penmo, Penaeus monodon* (ACX47134); *Litva*, *Litopenaeus vannamei* (AGX26044); *Met*en, *Metapenaeus ensis* (AAL33882); *Palca*, *Palaemon carinicauda*, (AIJ49750); *Nepno*, *Nephrops norvegicus* (AAK58133); *Homga*, *Homarus gammarus* (ABA42181), and *Scyol*, *Scylla olivacea* (AZF98733.1); Signal peptide and mature peptide regions are indicated with arrows; Conserved cysteine sites are indicated with bold letters.

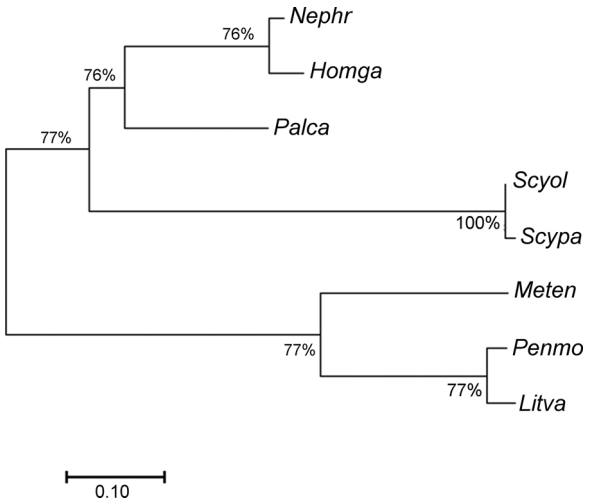

Fig. 3. Phylogenetic tree analysis of VIHs/GIHs in *S. olivacea* and other crustaceans; Phylogenetic tree was constructed based on neighbour-joining method with interior branch test; The number at the nodes represent bootstrap values at an approximate level of confidence (0–100%) from 1000 replicates in the monophyly of the respective branch Abbreviation: *Scypa*, *Scylla paramamosain*; *Penmo, Penaeus monodon*; *Litva*, *Litopenaeus vannamei*; *Met*en, *Metapenaeus ensis*; *Palca*, *Palaemon carinicauda*; *Nepno*, *Nephrops norvegicus*; *Homga*, *Homarus gammarus*, and *Scyol*, *Scylla olivacea.*

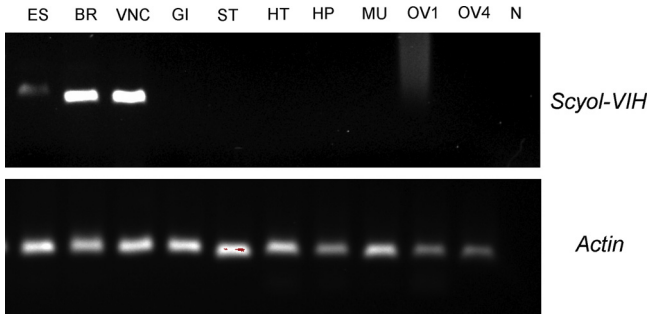

Fig. 4. Tissue-specific relative abundances of *Scyol-VIH* mRNA transcript in immature female using RT-PCR; *Scyol-VIH* mRNA transcript is detected in the eyestalk (ES), brain (BR) and ventral nerve cord (VNC); *β-actin* mRNA abundance was used as internal controls; Abbreviations: ES, eyestalk; BR, brain; VNC, ventral nerve cord; GI, gill; ST, stomach; HT, heart; HP, hepatopancreas; MU, muscle; OV1, ovary stage 1; OV5, ovary stage 5; and N, negative control.

have resulted from a common ancestor from the same molecule from an evolutionary perspective (Fig. 3).

3.2. Tissue abundances of VIH mRNA transcript using RT-PCR and in situ hybridization

The relative abundance of *Scyol-VIH* mRNA transcript in different *S. olivacea* tissues was examined using RT-PCR using primers specific for *Scyol-VIH*. The *Scyol-VIH* transcripts were detected in the eyestalk, brain, and ventral nerve cord of individual shrimp at the expected size of 250 base pairs (Fig. 4). There were no *Scyol-VIH* transcripts detected in other tissues examined (gill, stomach, heart, hepatopancreas, muscle, ovary stage 1, ovary stage 5) as well as in the negative control samples.

To localize *Scyol-VIH* transcript in the eyestalk, brain and ventral nerve cord, *in situ* hybridization were performed. A hematoxylin and eosin (H&E) stained median sagittal section of the left eyestalk provided for assessment of the anatomy of the eyestalk and its neurons (Fig. 5A). Using consecutive sections of the eyestalk, *Scyol-VIH* mRNA transcript was detected in regions of the X-organ (XO) (Fig. 5B, 5C and D). There were no positive signals detected in eyestalk neuronal clusters 1, 2, and 3 (Fig. 5C and D). In the sense-strand riboprobe, used as a negative control, there were no positive signals within the eyestalk section (Fig. 5E). A H&E stained section of the brain when assessed at low magnification, allowed for evaluation of the anatomy of the brain (Fig. 6A). There were positive signals for *Scyol-VIH* mRNA transcript detected primarily in the brain neuronal clusters 6 and 11 (Fig. 6B). There were positive signals in neurons of various sizes, including small-, medium- and large-sized neurons of clusters 6 (Fig. 6C-F) and 11 (Fig. 6G-J). There was no positive signal for *Scyol-VIH* in the other part of the brain such as protocerebral tract, protocerebral bridge, and median antenna neuropil. In the sense-strand riboprobe, there was no positive signals within the brain section (Fig. 6K). A H&E stained section of the VNC allowed for assessment of the anatomy of the VNC (Fig. 7A), which includes the subesophageal ganglion (SEG), five fused thoracic ganglia, a thoracic artery (TA) and abdominal ganglia (AG). There was *Scyol-VIH* transcript detected in various parts of the VNC (Fig. 7B). There was *Scyol-VIH* mRNA transcript detected in the small- and medium-sized neurons within the SEG (Fig. 7C and D). In the thoracic ganglia (TG), *Scyol-VIH* transcript was also detected in small- and medium-sized neurons (Fig. 7E). In the AG, *Scyol-VIH* transcript was detected in small-, medium-, and large-sized neurons (Fig. 7F). In negative control sections using a sense-strand riboprobe, there was no positive signals in the VNC section (Fig. 7G).

3.3. Differential relative abundances of VIH mRNA transcript during ovarian maturation

To further elucidate the expression profile of *Scyol-VIH* in female *S. olivacea*, there were relative abundances of *Scyol-VIH* mRNA transcript assessed in the eyestalks of immature and mature (different ovarian maturation stages included) female mud crabs using real-time PCR. The abundance of *β-actin* mRNA transcript was used for normalization of relative abundances of *Scyol-VIH* mRNA transcript. There were large relative abundances of *Scyol-VIH* mRNA transcript in the eyestalks of immature females, while there was a lesser relative abundance in the eyestalks of mature females. The relative abundance of *Scyol-VIH*, however, was markedly different only in the eyestalks of immature and stage II mature females ($P < 0.05$) (Fig. 8). There was no difference in relative abundance of *Scyol-VIH* mRNA in the eyestalks of mature females at different ovarian developmental stages.

3.4. Effect of serotonin (5-HT), dopamine (DA) and spiperone (SP) on relative abundance of Scyol-VIH mRNA

To study the effect of 5-HT, DA and SP on the relative abundance of *Scyol-VIH* mRNA transcript in the CNS (including the eyestalk, brain and VNC), immature female *S. olivacea* were injected and served as a sham control (100 µl of 0.9% normal saline), 5-HT (5 µg/g BW), DA (5 µg/g BW) and SP (5 µg/g BW) group and there were samples collected at 3 h post-injection. The relative abundance of *Scyol-VIH* mRNA was normalized using abundance *β-actin* and differential relative abundance of *Scyol-VIH* among the treatment and control group was subsequently evaluated. After DA administration, relative abundance of *Scyol-VIH* in the eyestalk was greater at 3 h post-injection when compared with relative abundance of *Scyol-VIH* in the eyestalk of sham controls. There was an increase in relative abundance of *Scyol-VIH* mRNA transcript in the brain and VNC after DA injection, although it was not significantly different

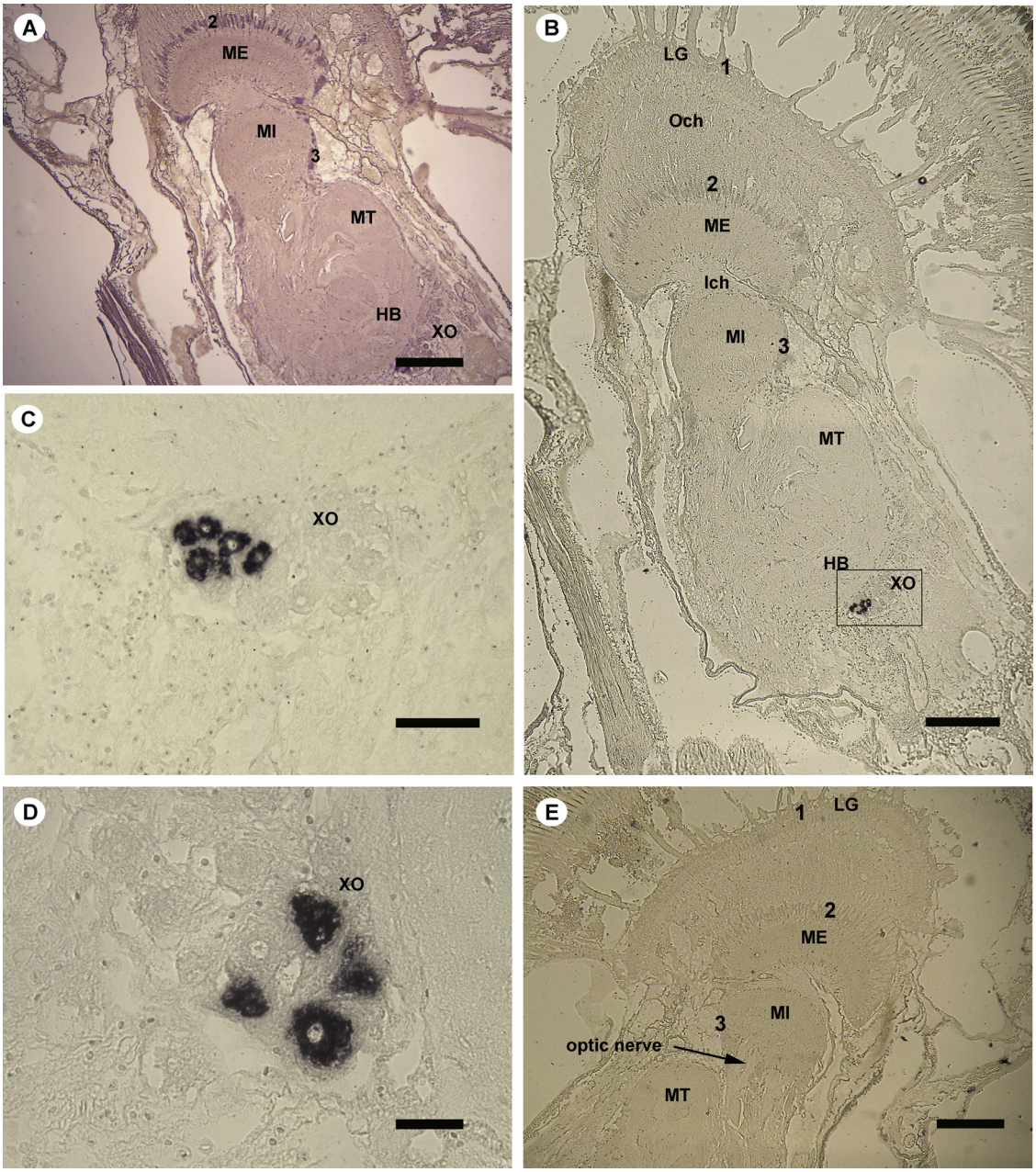

Fig. 5. Localization of *Scyol-VIH* mRNA transcript in the eyestalk of *S. olivacea* using *in situ* hybridization (A) A sagittal section of the eyestalk stained with H&E showing the location of neuronal clusters (numbered) and neuropils; (B) An overview of *Scyol-VIH* transcript abundance in eyestalk (dark purple); (C–D) High magnification micrographs showing positive signals in the X-organ (XO); (E) Negative control micrograph using a DIG-labeled sense-strand *Scyol-VIH* riboprobe with no positive signal in the cytoplasm of neurons; Scale bars: 200 µm in A, B and E; 50 µm in C; 20 µm in D; Abbreviations: LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; XO, X-organ; HB, hemielipsoid body; Och, outer optic chiasm; Ich, inner optic chiasm, 1, Cluster1; 2, Cluster 2; and 3, Cluster 3.

to that of the sham controls. Injection of 5-HT and SP did not have any effect on the relative abundance of *Scyol-VIH* mRNA transcript as compared with the sham controls (Fig. 9).

4. Discussion

Reproduction in crustaceans is regulated by neuropeptides and neurohormones produced by the central nervous system (eyestalk, brain and VNC) (Tinikul et al., 2009; Kornthong et al., 2014). In the present study, VIH of *S. olivacea* was initially identified through the molecular cloning techniques. Then, tissue-specific abundances (by RT-PCR) and spatial localization (by *in situ* hybridization)

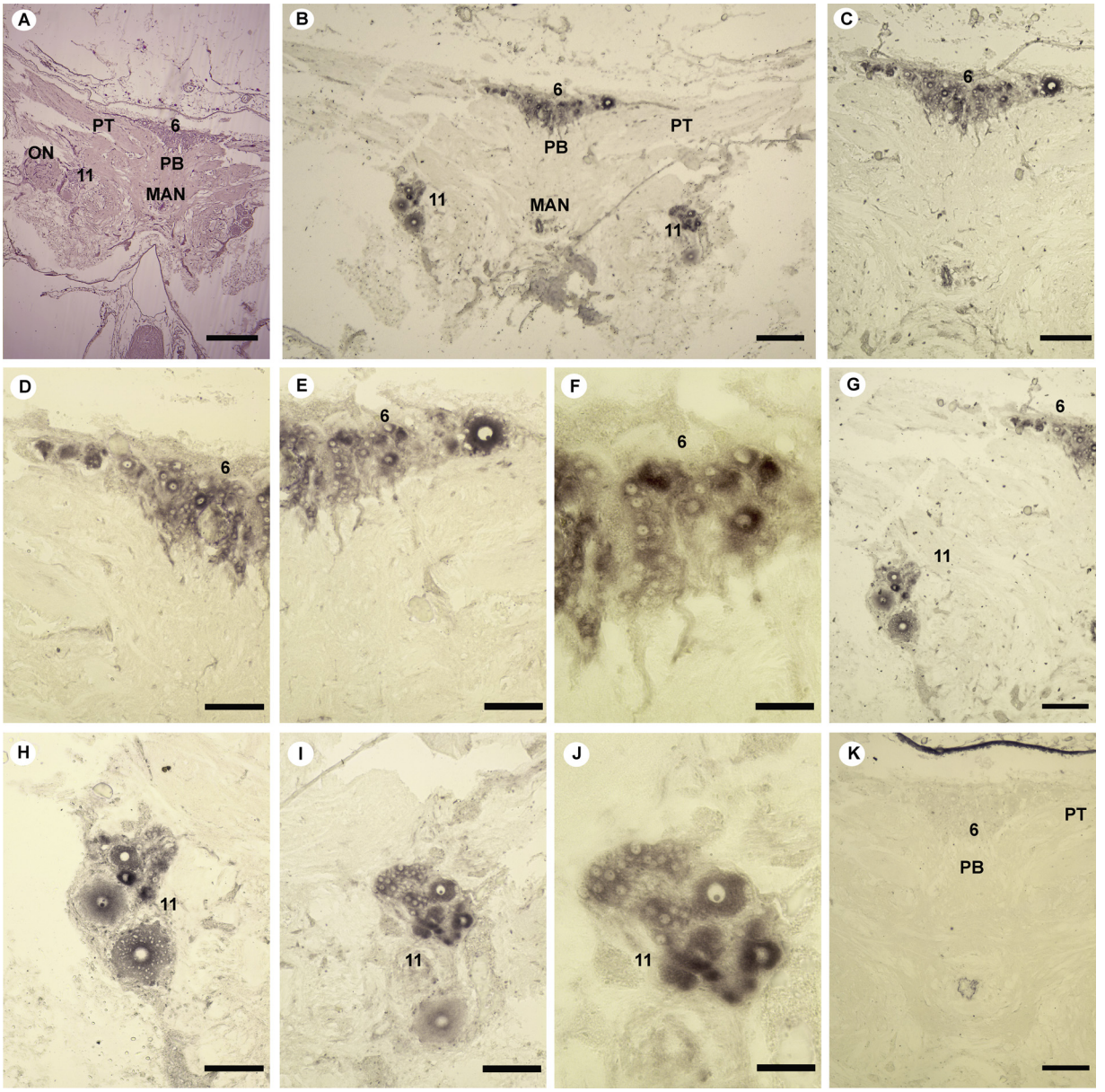

Fig. 6. *In situ* hybridization of *Scyol-VIH* in the brain of *S. olivacea*; (A) A mid horizontal section of the brain stained with H&E showing the locations of neuronal clusters (numbered) and neuropils; (B) Low magnification micrograph showing the overall positive signals in various neuronal clusters within the brain, including Clusters 6 and 11; (C–F) High magnification micrographs showing positive staining in small-, medium- and large-sized neurons of Cluster 6; (G–J) High magnification micrographs showing positive staining in small-, medium- and large-sized neurons of Cluster 11; (K) Negative control micrograph using a DIG-labeled sense-strand *Scyol-VIH* riboprobe detecting no positive signal in the cytoplasm of neurons; Scale bars: 200 μm in A, B and K; 100 μm in C and G; 50 μm in D, E, H and I; and 20 μm in F and J; Abbreviations: PT, protocerebral tract; PB, protocerebral bridge; MAN, median antenna neuropil; ON, olfactory neuropil.

provided for a more precise assessment into the functions of VIH in *S. olivacea*, including following administration of neurotransmitters 5-HT and DA.

The characterization and function of VIH in the brachyurans, including the true crabs such as the mud crab and blue swimming crab, is quite limited. Only a single VIH transcript has been identified in the *S. paramamosian* (Liu et al., 2018), which shares 91% similarity in nucleotide sequence to *Scyol-VIH*. A number of VIH/GIH mRNA sequences have been previously identified for the other malacostracans such as the lobster *H. americanus*, norway lobster *N. norvegicus*, giant fresh water prawn *M. rosenbergii*, white leg shrimp *L. vannamei*, and black tiger shrimp *P. monodon* (Soyez et al., 1987; Yang and Rao, 2001; Edomi et al., 2002; Treerattrakool et al., 2008; Chen et al., 2014). The VIH molecule belongs to the CHH/MIH/GIH neuropeptide family based on primary and precursor molecule structures (Lacombe et al., 1999; Webster et al., 2012). The results for phylogenetic analysis of VIH, inclusive of Scyol-VIH,

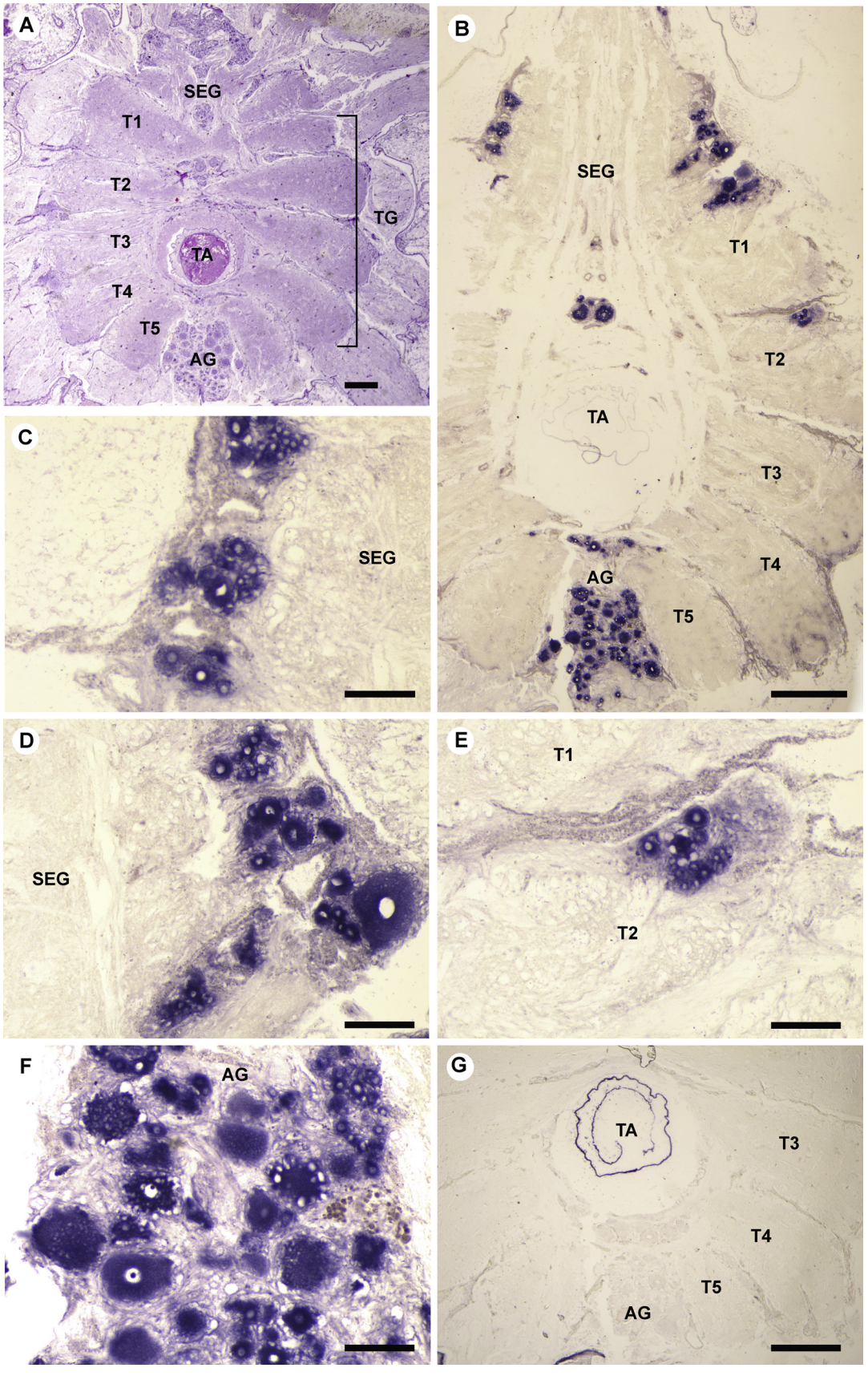

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Fig. 7. *In situ* hybridization of *Scyol-VIH* in the mud crab ventral nerve cord (VNC) of *S. olivacea*; (A) H&E staining of VNC showing the locations of neuronal clusters and thoracic neuropils; (B) Low power micrograph detecting positive signals in the various locations, including SEG, TG, and AG; (C) High magnification micrograph detecting stained neurons in the SEG, including small- and medium-sized neurons; (D) High magnification micrograph detecting positive signals in small-, medium- and large-sized neurons of the SEG; (E) High magnification micrographs detecting positive signals in small- and medium-sized neurons of the TG, between T1 and T2; (F) High magnification micrograph showing positive signals in small-, medium- and large-sized neurons of the AG. (G) Negative control micrograph using a DIG-labeled sense-strand *Scyol-VIH* riboprobe showing no positive signal in the cytoplasm of neurons; Scale bars: 500 μm in A; 200 μm in B and G; and 20 μm in C, D, E and F; Abbreviations: SEG, sub-esophageal ganglion; T1-T5, thoracic neuropils 1–5; TG, thoracic ganglion; AG, abdominal ganglion; and TA, thoracic artery.

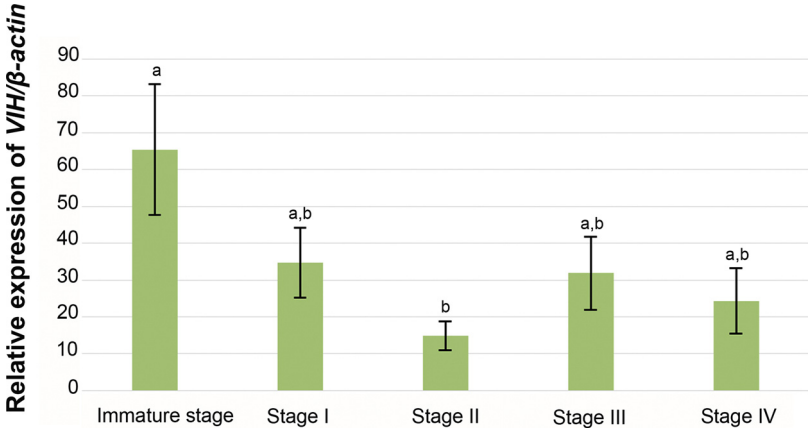

Fig. 8. Relative abundance of *Scyol-VIH* mRNA transcript in the eyestalk during ovarian development; *Scyol-VIH* mRNA relative abundance in the eyestalk of immature female mud crab depicted as being in the greatest amount ($n = 10$ per each stage) comparing to those of Ovarian Stage 1–4; The groups with different letter codes (a code and b code) indicate differences ($P < 0.05$).

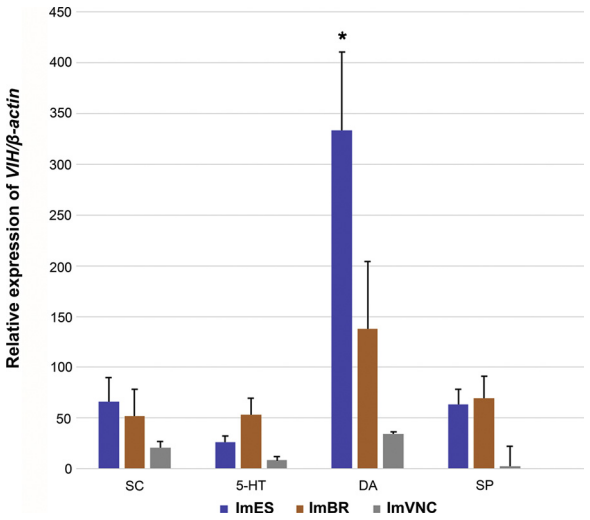

Fig. 9. Effect of 5-HT, DA and SP priming on VIH mRNA transcript abundance using quantitative RT-PCR; *Scyol-VIH* relative abundances were determined at 3 h post injection ($n = 15$); Asterisk indicates significant differences (at $P < 0.05$) when compared with the sham control group: Abbreviations: SC, sham control; 5-HT, serotonin; DA, dopamine; and SP, spiperone.

indicate *S. olivacea* may have a common ancestor molecule to the other VIH/GIH, although there was not a phylogenetic analysis of Scyol-VIH comparing to other members of CHH superfamily in the present study.

It has been proposed that the X-organ is the major site of VIH synthesis and release (Chen et al., 2014) because there is VIH immunoreactivity in the XO/SG complex of various crustaceans (Edomi et al., 2002; Webster et al., 2012; Urtgam et al., 2015). In the present study, *Scyol-VIH* mRNA transcripts were present in the X-organ of the eyestalk, various neuronal clusters of the brain and VNC, but not in other organs examined. This finding is supported by VIH studies in other crustaceans such as *L. vanamei, S. paramamosian, M. rosenbergii, P. monodon* and *N. norvegicus* (Yang and Rao, 2001; Edomi et al., 2002; Treerattrakool et al., 2008; Chen et al., 2014; Liu et al., 2018). In the present study, *Scyol-VIH* mRNA transcripts were present within the small-, medium-sized and some of large-sized neurons of brain clusters 6 and 11. In addition, these mRNA transcripts were detected in the various sizes of

neurons of subesophageal ganglion (SEG), thoracic ganglion (TG), and abdominal ganglion (AG) of VNC.

Regarding VIH function, it inhibits the expression and function of Vg (Feijo et al., 2016). This hypothesis has been confirmed by the results of previous research, where Vg mRNA transcripts were detected in the cultured ovary but was not detected when the cultured ovary was incubated with a recombinant *L. vannamei* VIH (Tsutsui et al., 2013; Bae et al., 2017). Based results when using real-time PCR in the present study, relative abundance of *Scyol-VIH* transcript in the eyestalk was greatest in the females before there was ovarian maturation, suggesting that Scyol-VIH is in greatest abundance at the immature developmental stage and may be have its most important functions during this developmental stage. Because VIH has an inhibitory function on vitellogenesis (Nagaraju, 2011; Chen et al., 2014), findings in the present study indicate that in immature female crabs, VIH is inhibiting ovarian development and maturation. This finding is consistent with results from previous studies in *P. monodon* and *S. paramamosian,* where there was a greater abundance VIH in the eyestalks and hemolymph of immature female animals (Urtgam et al., 2015; Liu et al., 2018).

The control of VIH secretion is regulated by biogenic amines, which function as neurotransmitters and neurohormones in crustaceans (Tinikul et al., 2009). The 5-HT and DA molecules are detected in the CNS and functions (e.g., controlling of larval development and reproduction) have been studied in crustaceans (Tinikul et al., 2009, 2011; Khornchatri et al., 2015). In the present study, there was a stimulatory effect of DA on relative abundance of *Scyol-VIH* mRNA transcript indicating the expression of the gene encoding for this protein in CNS may be greater extent as a result of this treatment. There is inhibition by DA of gonad maturation by inhibiting the release of reproductive hormones and stimulating the release of VIH (Sarojini et al., 1995a, 1995b; Fingerman, 1997). There is an antagonist function of DA on the ovary-stimulating action of 5-HT in the red swamp crayfish, *Procambarus clarkii* (Sarojini et al., 1996). Furthermore, DA could selectively function in stimulation of a population of neurons in the XO/SG complex in *P. clarkii*, indicating this neuromodulator may have important functions in the XO/SG complex (Alvarez Alvarado et al., 2005). Based on those previous studies and results from the present study, it is suggested that DA might inhibit ovarian maturation through stimulation and induction of VIH neuronal activity in the X-organ of *S. olivacea*. In addition, the study of SP, which is a DA antagonist, was also conducted to investigate functions of DA. Injection of SP increased the gonadsomatic index (GSI) and oocyte diameter in *P. clarkii* and *M. rosenbergii,* and could shorten the duration of ovarian maturation in *M. rosenbergii* (Rodriguez et al., 2002; Tinikul et al., 2009). The 5-HT molecule has a stimulatory effect on ovarian maturation in *S. olivacea* through the release of reproductive hormones, including CHH, RPCH, and FAMeT (Kornthong et al., 2013, 2014; Duangprom et al., 2017). The 5-HT and SP might not, however, affect VIH relative abundance of mRNA transcript in *S. olivacea*. A direct reproductive function of VIH in *S. olivacea*, for example, may occur on ovarian maturation or vitellogenesis and there needs to be further research to elucidate whether there are these direct effects in this species.

5. Conclusion

In the present study, there was initially identification of a VIH gene in S. *olivacea*. The *Scyol-VIH* molecule contains 378 nucleotides and encodes a 126-amino acid Scyol-VIH precursor protein. The Scyol-VIH molecule of S. *olivacea* has a large amount of sequence similarity to VIH in other crustaceans. The *Scyol-VIH* mRNA transcript was exclusively present in the eyestalk, brain and VNC. Furthermore, with administration of DA there was a greater relative abundance of *Scyol-VIH* mRNA transcript which indicates there is greater expression of the gene that encode for this protein in the eyestalk and brain of immature females at 3 h post-injection of DA, when compared with the sham control. A large abundance of *Scyol-VIH* mRNA transcript in the eyestalk of *S. olivacea* immature females during ovarian maturation indicates that may be an involvement of DA in inhibiting Vg production.

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