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# Characterization of the shrimp neuroparsin (MeNPLP): RNAi silencing resulted in inhibition of vitellogenesis



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## ABSTRACT

The full-length *Metapenaeus ensis* neuroparsin (MeNPLP) cDNA was cloned which encodes a shrimp protein homologous to the insect neuroparsin and vertebrate insulin-like growth factor binding protein (IGFBP). MeNPLP cDNA is 1389 bp in length and the longest open reading frame is 303 bp in length. The first 27 aa are predicted to be the signal peptide and aa 28–101 is the mature peptide with an estimated molecular weight of 7.83 kDa and pI of 5. It shows high amino acid sequence similarity (42–68%) to the neuroparsin of insects and N-terminal end of the IGFBP of vertebrates. The cysteine residues in MeNPLP responsible for disulfide bond formation are conserved as in other neuroparsin-like proteins. The expression level of MeNPLP is the highest in the hepatopancreas, followed by the nerve cord, brain, heart, ovary, and muscle. However, it was not expressed in the testis. Using an insect neuroparsin antibody, MeNPLP could only be detected in the hepatopancreatic tubules, suggesting that MeNPLP may be a secretory product. Although MeNPLP expression was stimulated in the ovary, it was inhibited in the hepatopancreas after treatment with neurotransmitter serotonin (5-HT). *In vivo* gene silencing of MeNPLP could cause a significant decrease of vitellogenin transcript level in the hepatopancreas and ovary. As a result, a corresponding decrease in vitellogenin protein level was observed in the hemolymph and ovary. In conclusion, this study has provided the first evidence that MeNPLP is involved in the initial stage of ovary maturation in shrimp.

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## 1. Introduction

Both insects and crustaceans belong to the Arthropod phylum and they share many similar taxonomic and physiological features. For example, their body is covered by a cutinized exoskeleton that restricts their growth and they produce ecdysteroids as molting hormone that control periodic molting [1,2]. However, insects and crustaceans also produce similar hormones that may have different functions. For example, insects and crustaceans produce a crustacean hyperglycemic hormone-like (CHH-like) neuropeptide from the neurosecretory neuron. In crustaceans, CHH or CHH-like are responsible for the metabolism of glucose whereas in insects the ion transport peptide (i.e. ITP or CHH equivalent) controls ionic and fluid homeostasis as well as body volume through fluid

absorption [3,4]. The insect neuroparsins are a group of neuropeptide initially identified in locust and recent studies have confirmed its widespread existence in other insects [5,6]. However, the discovery of similar proteins was only reported recently in crustacean [6,7]. Neuroparsins are known to be involved in reproduction and gregarious phasing of the locust [8,9]. During early maturation of the female locusts, the transcript level of the neuroparsin was low but the level increased to 5 times during the onset of vitellogenesis. In crustacean, the presence of neuroparsin was only reported initially from differential expression study and more recently from expressed sequence tag (EST) projects [5]. To date, research regarding the characterization of these crustacean neuroparsins is unavailable. Consequently, the functions of these neuroparsin-like peptides in crustacean are elusive.

In a differential display experiment of *Metapenaeus ensis*, we had cloned a partial cDNA encoding the putative neuroparsin-like (MeNPLP) from the ovary of an early mature shrimp injected with serotonin [10]. The partial cDNA shared high sequence homology to the insect neuroparsin and the vertebrate insulin-like growth factor binding protein. Since the partial cDNA was cloned from the ovary,

Abbreviations: 5-HT, serotonin; CHH, crustacean hyperglycemic hormone; EST, expressed sequence tag; GSI, gonadosomatic index; IGFBP, insulin-like growth factor binding protein; ITP, ion transport peptide; MeNPLP, *Metapenaeus ensis* neuroparsin; NP, neuroparsin

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it is possible that the protein encoded by the gene may be involved in female reproduction. Moreover, in the sand shrimp *M. ensis*, two vitellogenin genes (i.e. *MeVg1* and *MeVg2*) have been cloned [11]. These two genes showed a low level of homology to each other (i.e. <60% similarity). Since *MeVg1* is the predominant gene that expressed in both the hepatopancreas and ovary and the partial *MeNPLP* was originally isolated from the ovary of a 5HT-stimulated shrimp, we will focus on the role of *MeNPLP* on the expression of *MeVg1*. Furthermore, because of the established technique using RNA interference to study gene functions, the other objectives of this study are (i) to clone and characterize the cDNA for the shrimp neuroparsin and (ii) to use RNA interference technique to study the function of *NPLP* in female shrimp vitellogenesis.

## 2. Results

### 2.1. Characterization of the shrimp *MeNPLP*

A partial cDNA sequence for *MeNPLP* was obtained by PCR using primers CB21 and CB22 (Fig. 1). Gene specific primers *MeNPLP*-R1 and *MeNPLP*-F1 were designed for first round 5' and 3' RACE cloning; gene specific primers *MeNPLP*-R2 and *MeNPLP*-F2 were designed for second round 5' and 3' RACE cloning. The full-length *MeNPLP* cDNA sequence was obtained from several overlapping cDNA clones and it was also verified by re-amplifying the complete cDNA using gene-specific primers *MeNPLP*-F3 and *MeNPLP*-R3 (Fig. 1). The full-length *MeNPLP* cDNA is 1389 bp and the deduced protein consists of 101 amino acid residues (GenBank: KJ415256.1). It has a short 5' un-translated region (UTR) and a much longer 3' UTR (Fig. 1). Using SignalP Program ([www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)), AA1–27 is the predicted signal peptide that cleaves at residues VAG-AP to produce the mature peptide with an estimated molecular weight and pI values of the mature peptide of 7.83 kDa and 5.0, respectively ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)). BLASTX search analysis shows that *MeNPLP* is most similar to the neuroparsin of the locust (*Locusta migratoria*; GenBank # PRF: 226301, total 107 aa, 70% similarity, 61 aa overlap), and (*Schistocerca gregaria*; GenBank # CAC38869.1, total 107 aa, 68% similarity, 61 aa overlap), the neuroparsin-like molecules of the copepod (*Caligus rogercresseyi*; Genbank # ACO10490.1, total 97 aa, similarity 55.4%, 92 aa overlap), the spider (*Cupiennius salei*; single insulin-like growth factor-binding domain protein-2 [12] CusSIBD; Genbank # CCD22033.1 total 99 aa, 54% similarity, 71 aa overlap), the fish [*Oreochromis niloticus* HTRA1-like (XP\_003459047.1, 453 aa, 45% similarity, 88 aa overlap), the bee [13] queen brain selective protein ApmQBSP (*Apis mellifera*; GenBank # NP\_001035359.1, total 128 aa, 63% similarity, 61 aa overlap), ecdysteroidogenic hormone of the mosquito *Culex quinquefasciatus* CqQOEH (Genbank XP\_001870999.1, 144 aa, 42% similarity, 100 aa overlap), insulin-like growth factor binding protein-related protein 1 (OnmIGFBP) of the fish [14] (*Oncorhynchus mykiss*; Genbank # NP\_001118120.1, total 263 aa, 46% similarity, 79 aa overlap), human [15] (*HsHTRA3*; #NpH44272, total 452 aa, 45%, 88 aa overlap), *Litopenaeus vannamei* [16] single insulin binding domain protein (LivSIBD; Genbank # ACF93414.1, 94 aa, 60% similarity, 43 aa overlap), and the crayfish *Pacifastacus leniusculus* [17] crustacean hematopoietic factor (PleCHF; Genbank #: ADN06258.1).

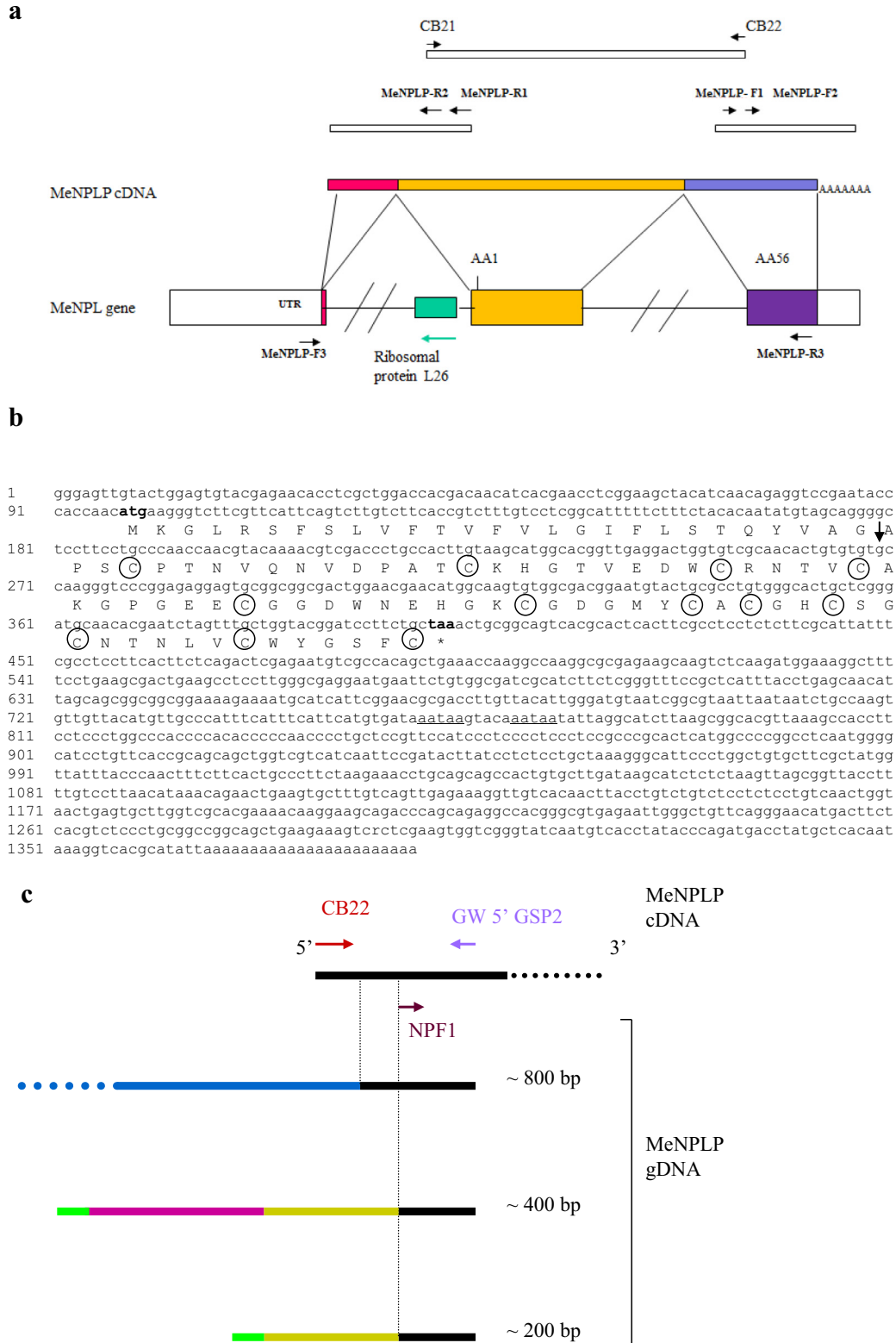
To study the alignment and phylogenetic relationships of the *MeNPLPs* with neuroparsin-like of other crustacean, insect and vertebrate insulin binding protein, selected *NPLP* including the sequences obtained from other shrimp EST projects were obtained from the Genbank database. They included *NPLP* sequences from the shrimp *Litopenaeus setiferus* (LisNPLP; GenBank #: BE846730.1), *Penaemon monodon* (PemNPLP; EST-derived, GenBank #: BI784456.1), *L. vannamei* (LivSIBD; EST-derived, GenBank #: BE188518.1), the lobster *Homarus americanus* (HoaNPLP;

EST-derived, GenBank #: CN854288.1), the mosquito *Anopheles gambiae* (AngOEH; GenBank #: EAA06579.3), the crayfish *Cherax quadricarinatus* insulin-like androgen gland hormone binding protein (CqIAGBP; GenBank #: 78412) and others (see above). In this study, only the insulin binding domain of the larger proteins was used for analysis. They were first aligned with the multiple alignment program CLUSTALW (<http://www.genome.jp/tools/clustalw/>). The result indicated that despite the low overall similarity among these neuroparsin-like molecules, there were specific regions that showed high degree of conservation (Fig. 2). Inspection of *MeNPLP* amino acid sequence revealed that it consists of 12 cysteine residues and 6 of them aligned perfectly well with the cysteine residues of neuroparsin-like molecules from other crustacean, insect and vertebrates. The phylogenetic tree revealed that these crustacean neuroparsin-like molecules are highly diversified as the sequence diversified among all the crustacean *NPLPs* included in the test (Fig. 3). Moreover, these “crustacean neuroparsin-like molecules” may represent a larger group of different related peptides that were evolved from a yet unknown ancestor molecule. Based on different crustacean neuroparsin-like sequences, the crustacean *NPLP* can be divided into two clusters and that the whole group of crustacean *NPLP* diverged very early during evolution. Although there were several shrimp *NPLP* proteins in the EST databases, *MeNPLP* is most closely related to the neuroparsins of the insect.

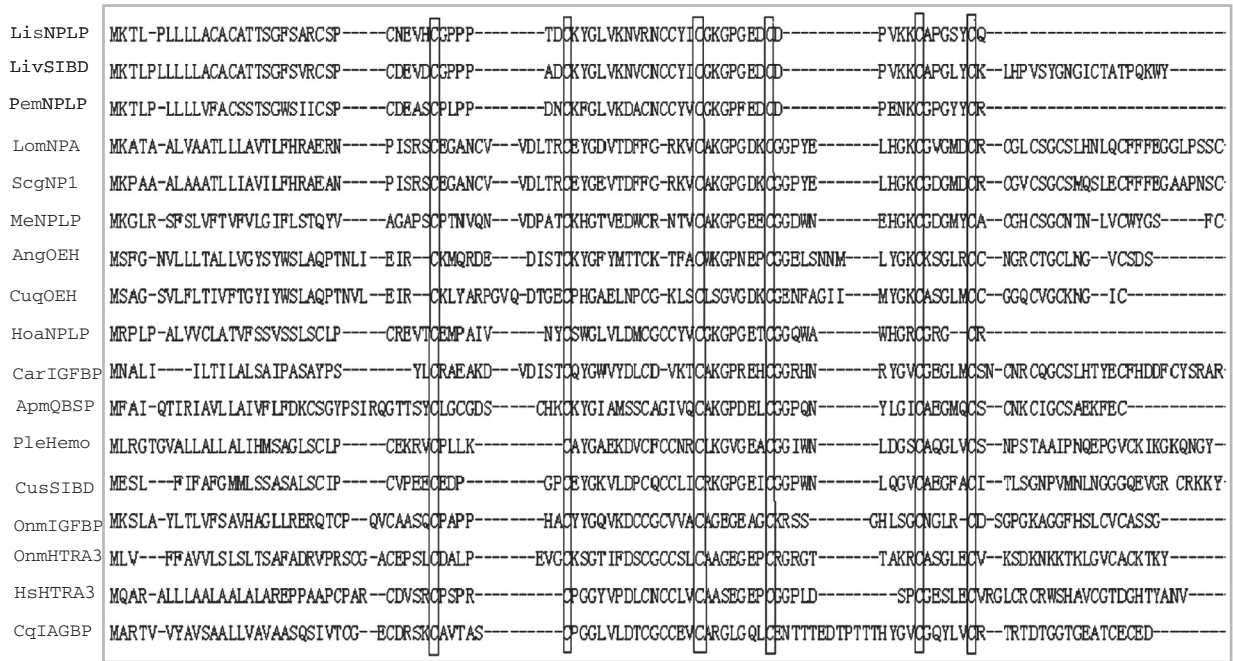
### 2.2. Expression of *MeNPLP*

The ovary development stages of *M. ensis* can be divided into five stages (stage I to stage V) based on the gonadosomatic index (i.e. 100% × ovary weight/total body weight) [11]. Initially female shrimp of the more advanced gonad maturation stages (GSI >9%) were used to study the tissue distribution of *MeNPLP*, but the results showed that *MeNPLP* transcripts could not be detected in the ovary, epidermis, gut, heart, hemocytes and muscles (data not shown). Since the expression level of *MeNPLP* is low in other tissues, a larger quantity of total RNA was loaded (30 µg). The slow migration of the ribosomal RNA in the nerve cord and brain is probably due to the larger quantity of glycogen in the RNA extract of the two tissues as compared to other tissues (Fig. 4a). Total RNA of the samples are not degraded as the signal of the *MeNPLP* looked intact without smeary appearance. When shrimp at early stage of gonad maturation (GSI = 1.1%) were used, *MeNPLP* transcripts could be detected in many tissues including the nerve cord, brain, eyestalk, heart, hepatopancreas, and ovary. The hepatopancreas, however, consisted of the highest level of *MeNPLP* transcripts in both the males and females (Fig. 4a). The expression of *MeNPLP* in the hepatopancreas was 20–30 folds higher than those in the ovary and nerve cord (Fig. 4a). As a comparison, we also analyzed the expression of *MeNPLP* in the male and the result indicated that the level of *MeNPLP* in the hepatopancreas of male was relatively constant. The patterns of *MeNPLP* expression profile at different gonad maturation stages were also investigated. In the hepatopancreas of early ovary maturation stage (i.e. stage I), *MeNPLP* expression was the highest and the level decreased gradually to less than half of the amount when the shrimp advanced from stage III to stage V. However, in stage V hepatopancreas, no expression of *MeNPLP* was detected by Northern blot. In the ovary of the female, *MeNPLP* transcript and expression level increased sharply when shrimp reached ovary stages II to IV and the level dropped again to undetectable level at stage V. As for the nerve cord, although the level was much lower than those of the ovary and hepatopancreas, it could be seen that the highest level of *MeNPLP* was recorded during early stage of gonad maturation and the level decreased to minimum from stages IV to V (Fig. 4b).

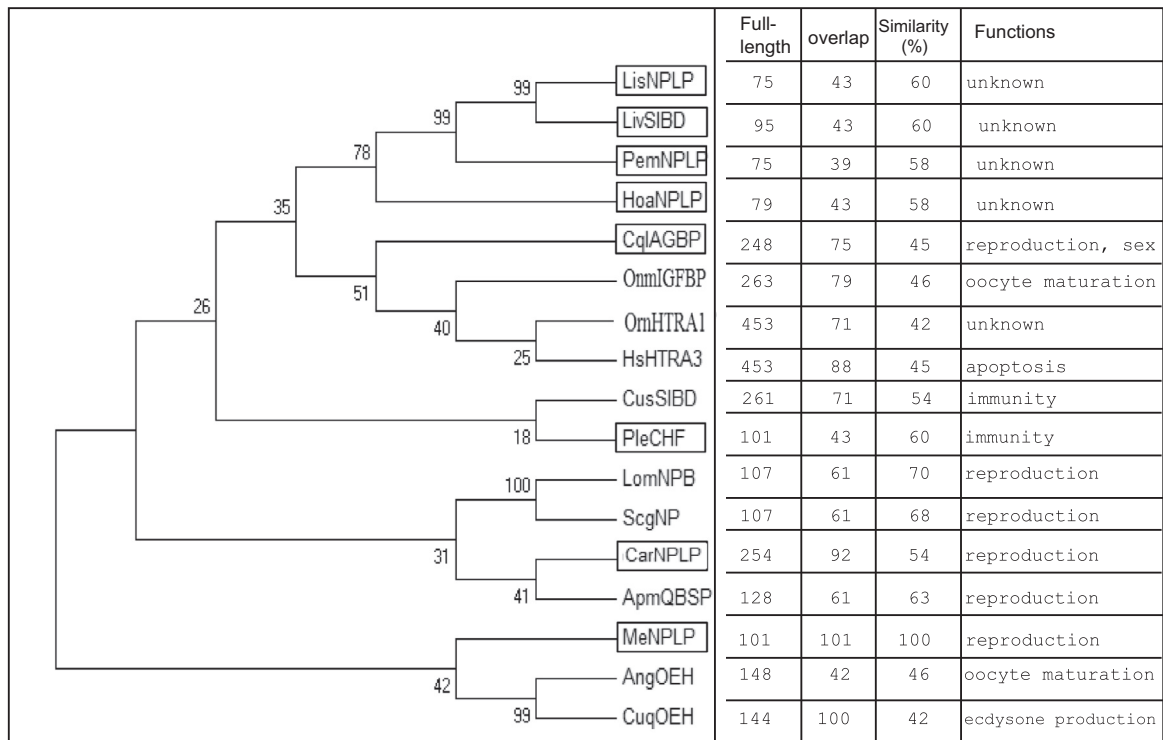
By immunohistochemical detection approach, intense immunopositive signals could be detected in the hepatopancreas. Most of



**Fig. 1.** (a) Cloning strategy for the MeNPLP cDNA. Partial MeNPLP cDNA is obtained by RT-PCR cloning of the ovary cDNA using primers CB21, CB22. Gene specific primers MeNPLP-F1/R1 and MeNPLP-F2/R2 were used for generation of overlapping cDNA clones. The final cDNA consist of three regions derived from three exons. Exon 1 encode for the 5' untranslated region (dark gray) and the exon 2 encoded some 5' untranslated region immediately followed by the coding sequence (gray) of the prepro-peptide and AA56; exon3 encode for the rest of the coding sequence plus the 3'-untranslated region. (b) The Full-length cDNA (1389 bp) and deduced amino acid sequence of MeNPLP. The predicted ORF is from nt 98–400. The start (ATG) and stop (TGA) codons are in bold letters. Conserved cysteine (C) residues are in small circles. The putative cleavage site for the signal peptide is indicated by a downward arrow. The putative polyadenylation signal is underlined. (c) Cloning of 3 different MeNPLP genes using a genome PCR walking kit (Invitrogen, USA). PCR using primer GW 5' GSP2 and genomic DNA (gDNA) of single shrimp resulted in three gene fragment of 200, 400, and 800 bp, with slightly different DNA sequence. Same color lines indicated genomic DNA fragments with identical nucleotide sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

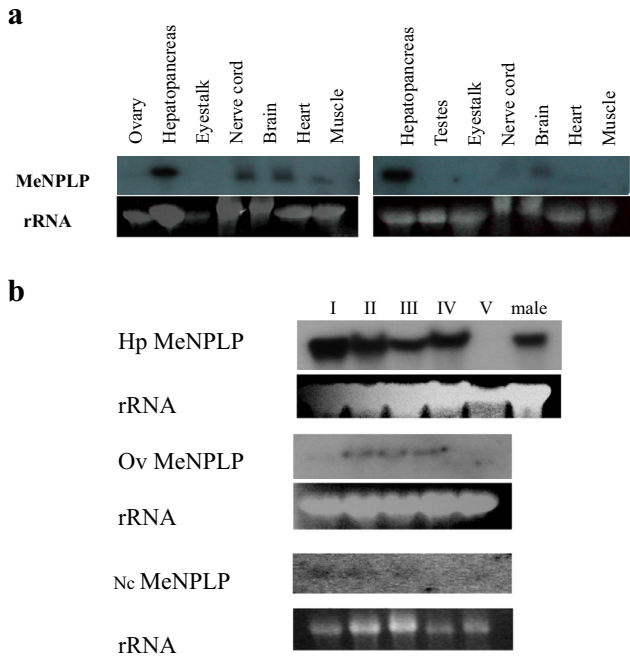


**Fig. 2.** Multiple amino acid sequence alignment of shrimp neuroparsin like molecules, insect neuroparsin, and N-terminal region of insulin-like growth factor binding protein of the vertebrates using Clustalw. The conserved cysteine is boxed. The sequences (<http://www.ncbi.nlm.nih.gov/>) used in the analysis included NPLP sequences from the shrimp *L. setiferus* neuroparsin-like LisNPLP (GenBank: BE846730.1), *P. monodon* PmNPLP (EST-derived, GenBank: BI784456.1), the lobster *H. americanus* HoaNPLP (EST-derived, GenBank: CN854288.1), the copepod *C. rogerscresseyi* CarNPLP (GenBank # AC010490.1), *L. vannamei* single insulin LivSIBD (EST-derived, GenBank: BE188518.1), the spider *C. salei* single insulin domain binding protein CusSIBD (Genbank # CCD22033.1), tilapia *O. niloticus* OrnHTRA3 (Genbank # XP\_003459047.1), the locust neuroparsin ScgNP1 (GenBank # CAC38869.1) and LomNPA (GenBank #CAA76829.1), the mosquito ovarian ecdysteroidogenic hormone I of *A. gambiae* AngOEH (Genbank # EAA06579.3) and *C. quinquefasciatus* CuqOEH (Genbank # XP\_001870999.1); the fish *O. mykiss* insulin growth factor binding protein OnmIGFBP (Genbank # NP\_001118120.1) and human *H. sapiens* HsHTRA1, GenBank # ABA33953.2), the crayfish crustacean hematopoietic factor PleCHF (Genbank # ADN06258.1).



**Fig. 3.** Phylogenetic tree analysis of selected neuroparsin-like molecules in shrimp, insects and some vertebrates. The analysis is based on the Insulin-like binding (IB) domain of the selected proteins. For large proteins with more amino acids, only the IB domain is used for the analysis (about 100 amino acid residues). All crustacean NPLP proteins are boxed. The neighbor-joining tree was constructed from multiple sequence alignments with other crustacean NPLPs, insect NPs, crustacean protein sequences derived from the GenBank database (see legend for Fig. 2) using the molecular evolutionary genetics analysis (MEGA 4.2) software. Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions. The bootstrap value (%) is indicated at the branch of the tree. The table on the left of the tree shows the full-length sequence, the overlapping region and potential function(s) of the proteins.



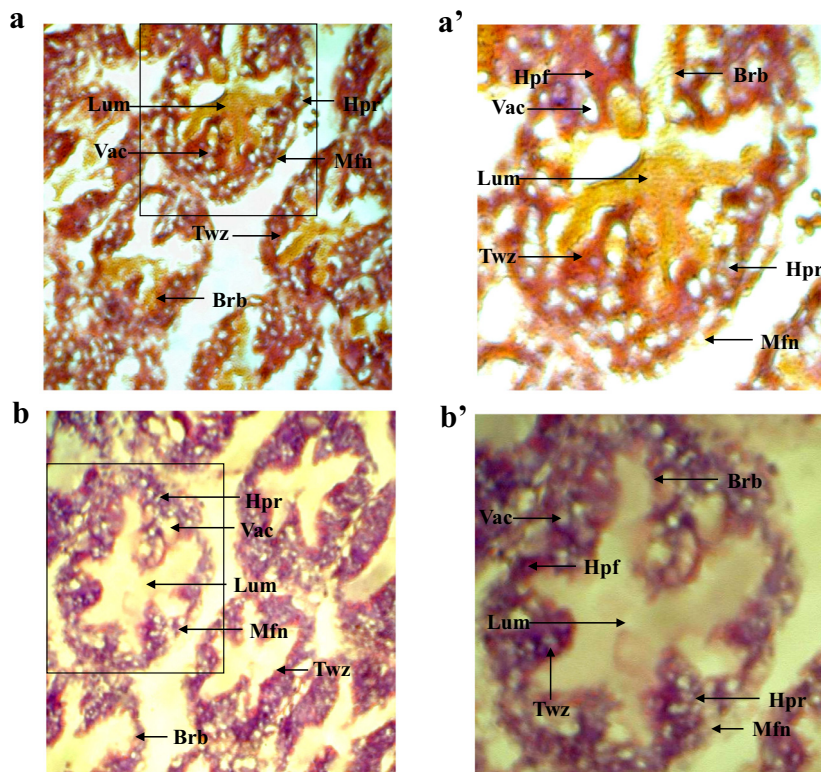


**Fig. 4.** Expression studies of MeNPLP. (a) Northern blot detection of MeNPLP expression in female (left) and male (right) shrimp in different tissues including the ovary, eyestalk, nerve cord, brain, hepatopancreas, heart, muscle and testes (b) Expression profile of MeNPLP in the hepatopancreas (Hp), ovary and nerve cord during the ovary maturation cycle of the female. The ovary maturation stages are: stage I (GSI <1%), stage II (GSI = 1–2%), stage III (GSI = 2–4%), stage IV (GSI = 4–7%), and stage V (gonadosomatic index 7–12%). The rRNA are of the corresponding samples are used to indicate RNA integrity and loading concentrations. A male Hp sample is also included.

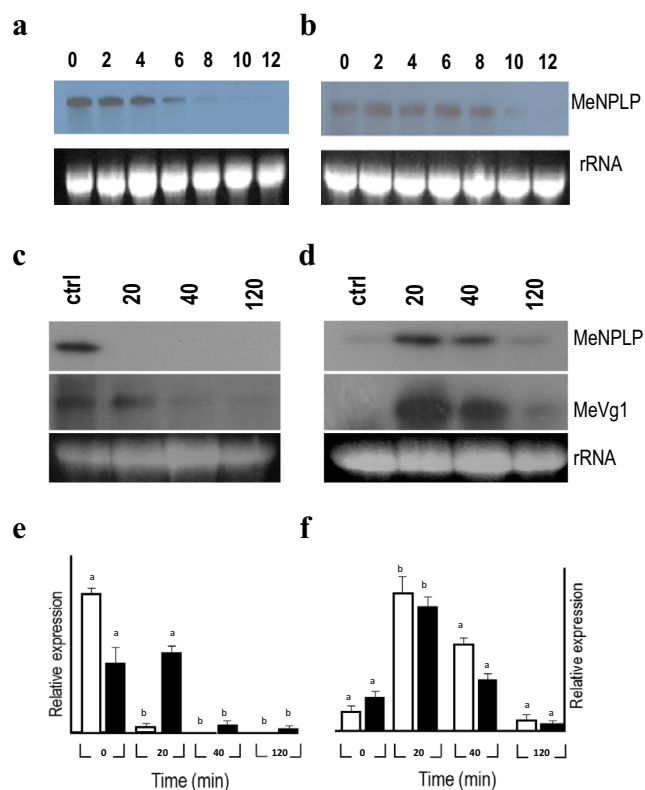
the hepatopancreatic tubules reacted positively with insect antibodies and all of those signals were concentrated in the lumen of the hepatopancreas.

### 2.3. *In vitro* effect of 5HT on MeNPLP and MeVg1 gene expression

Serotonin (5-HT) is one of the neurotransmitters reported to stimulate reproduction in shrimp [19]. Since the initial isolation of partial MeNPLP cDNA was from the ovary of 5-HT injected shrimp, and the hepatopancreas showed the highest expression level of MeNPLP during early stage of vitellogenesis; therefore, we performed an *in vitro* hepatopancreas and ovary explant assays to study the effect of 5-HT on MeNPLP and MeVg1 gene expression. Before the 5-HT test, a preliminary experiment was performed to determine the optimal culture time for this assay. Female shrimp at early vitellogenic stage (stage I, GSI <1.5) were dissected for hepatopancreas and ovary explants. The tissues were cultured at 0, 2, 4, 6, 8, 10, 12 h. The results indicated that both the hepatopancreas and ovary explants were viable as the rRNA remained intact for both tissues for at least 12 h. The hepatopancreas explants began to express less MeNPLP transcript after 4 h. For the ovary explants, MeNPLP transcript level began to decrease which might be due to the diminishing nutrient content in the medium. In summary, a 2 h culture time was optimal for the explants and subsequently all the assays were performed for 120 min with intermediate sampling at 20 and 40 min (Fig. 4a). The results indicated that when hepatopancreas fragments were treated with 5HT, the expression of MeNPLP was inhibited and the transcript level of the MeVg1 also decreased (Fig. 6c and e). Unlike the hepatopancreas, when the ovary fragments were treated with 5HT, a significant increase of MeNPLP transcript was detected. MeNPLP expression increased from 20–40 min and then decreased to a much lower level at 120 min. Furthermore, the increase was



**Fig. 5.** Cellular localization of MeNPLP protein in medial region of shrimp hepatopancreatic tubules in transverse sections. Enlarged view of the medial region of hepatopancreatic tubules sectioned transversely. (a). Immunostained with anti-Lom-NPA serum (1:1000). (a') Higher magnifications (see rectangle in a). (b). Negative control of adjacent section by a pre-immuned rabbit serum. (b') Higher magnifications (see rectangle in b). [Brb brush border or microvilli; Hpf hindgut folds; Hpr hepatopancreatic R-cell; Lum lumen; Mfn myo-epithelial fiber nucleus; Twz terminal web region; Vac vacuole].

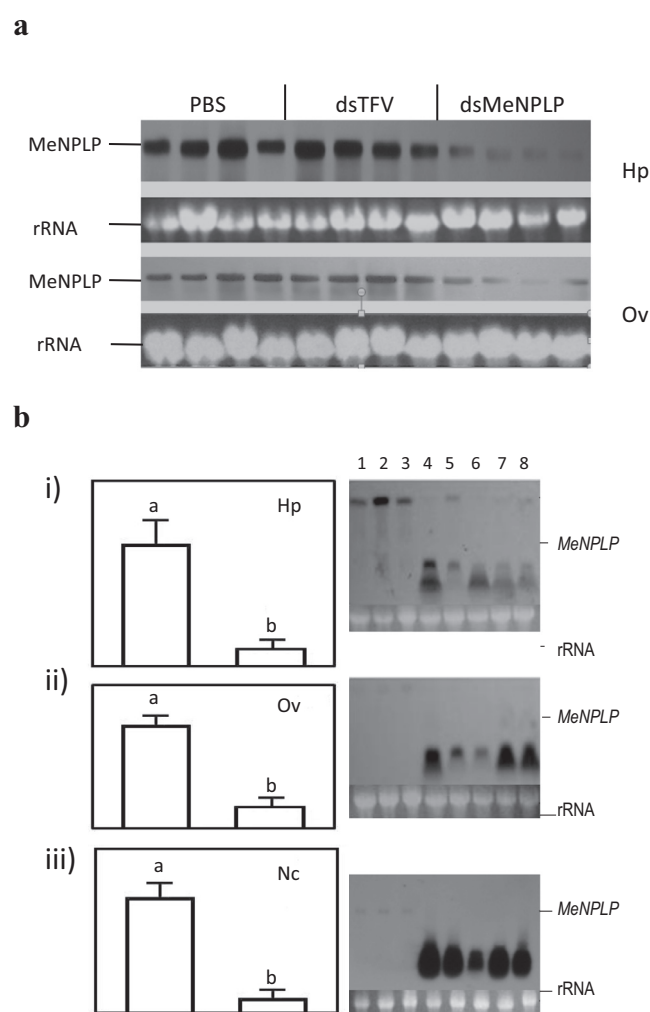


**Fig. 6.** *In vitro* effect of 5-HT on the hepatopancreas (a, c, e) and ovary (b, d, f) expression of MeNPLP and MeVg1. Initial time course experiment of neuroparsin MeNPLP expression in hepatopancreas (a) and ovary (b) at different time intervals (0–12 h); effect of 5-HT incubation on expression of MeNPLP and MeVg gene by the hepatopancreas (c) and ovary explants (d). Tissue fragments were incubated in a nutrient medium (M199) and the tissues in the well of the 24-well plate were harvested at 20, 40 and 120 min. The expression of the vitellogenin (MeVg1) and MeNPLP of the shrimp were analyzed by Northern blot with the corresponding MeNPLP and MeVg1 probes. The rRNA of the corresponding samples are used to indicate RNA integrity and loading concentrations. The bar diagram show the relative expression level (with standard deviation) of the MeNPLP (open bar) and the MeVg1 (dark bar) from ovary (e) and hepatopancreas of 8 shrimp.

accompanied by an increase of MeVg1 transcript (Fig. 6d, f). The result of this study indicated that the hepatopancreas and ovary responded very differently to 5HT treatment in terms of MeNPLP and MeVg1 expression.

#### 2.4. RNA interference study of MeNPLP function

From several previous RNAi studies, we have shown that 3  $\mu$ g double-stranded RNA (dsRNA) per gram of shrimp is effective to knock down several genes and all the non-specific dsRNA used as control would not affect the expression of the gene examined [20]. In this study, shrimp at early vitellogenic stage (i.e. GSI <2) were selected for RNAi experiment as MeNPLP was expressed mainly in these stages. Using a concentration of 3  $\mu$ g dsRNA/gm shrimp, the Northern blot result revealed that the amount of dsRNA taken by different tissues was different. Injection of PBS (or control dsRNA) and MeNPLP dsRNA had no apparent harmful effect on the shrimp as the survival rate of the shrimp was 100%. When shrimp were injected with dsRNA for MeNPLP, the MeNPLP transcript level in hepatopancreas decreased 4–5 folds. Also, the hepatopancreas and ovary of shrimp injected with MeNPLP dsRNA had moderate amounts of residual dsRNA. However, the nerve cords showed a much stronger hybridization signal resulted from residual dsRNA remained in the tissue which suggested that the uptake of dsRNA by nervous tissue was much higher than the hepatopancreas and the ovary (Fig. 7). In revealing the gene silencing effect of the dsRNA, the hepatopan-

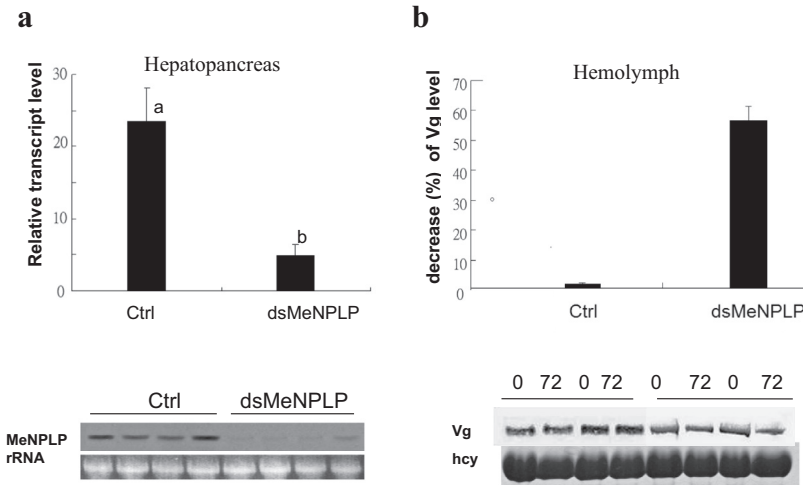


**Fig. 7.** RNAi gene silencing of MeNPLP expression. (a) Preliminary experiment in which shrimp are injected with PBS, dsRNA from tiger frog virus (dsTFV) (a non specific dsRNA control) and dsMeNPLP. Individual lane indicated RNA sample obtained from a single individual. The rRNA of the corresponding shrimp is shown. (b) MeNPLP gene silencing by dsMeNPLP injection. The bar diagram on the left indicated the estimation of MeNPLP transcript level (+ standard deviation) from animal injected with PBS or with dsMeNPLP. Different letters indicated significant difference in relative intensity ( $N = 8$ ,  $P < 0.05$ ). On the right is the corresponding Northern blot detection of MeNPLP expression in (i) hepatopancreas (Hp), (ii) ovary (Ov), and (iii) nerve cord (Nc) of shrimp after MeNPLP dsRNA injection. The lower panel of the Northern blot shows the ethidium bromide staining of rRNA. The smear in lanes 4–8 indicated the residual dsMeNPLP RNA labeled by the specific MeNPLP probe. Lanes 1–3 indicated PBS injected control shrimp and the lanes 4–8 indicated sample from dsRNA injected shrimp.

creas, ovary and nerve cord of the females injected with dsRNA showed a much lower level of MeNPLP transcript which indicated that the expression of MeNPLP was suppressed in the dsRNA injected animals. In the control, however, there was no change in the MeNPLP transcript level as compared to the dsRNA injected shrimp (Fig. 7b). In other words, MeNPLP expression was clearly unaffected in those animals. Furthermore, the probe did not produce any hybridization signal or smearing signal which indicated the absence of residual dsRNA in the control (Fig. 7b).

#### 2.5. Effect of MeNPLP gene silencing on MeVg1 expression by the hepatopancreas and ovary

To study the potential function of neuroparsin in shrimp reproduction, the expression of vitellogenin (MeVg1) in dsRNA injected females was monitored. The result indicated that the expression



**Fig. 8.** Effect of MeNPLP gene silencing on MeVg1 gene expression in the hepatopancreas (a) and the hemolymph level of MeVg1 (b). Shrimp were injected with MeNPLP dsRNA and MeNPLP transcript level in hepatopancreas (a) was estimated; The histogram (top) show the relative level of MeVg1 transcript analyzed (total animal N = 8 for each group). Different letters indicated significant difference in relative intensity (*t*-test, N = 8, *P* < 0.05). A typical Northern blot of 4 control and 4 treated animals was shown (below) I. Individual lanes show the hepatopancreas RNA sample from each animal. (b) The top histogram show the estimated changes of relative MeVg1 protein level (%) by in the hemolymph of dsRNA injected shrimp by Western blot analysis. Different letters indicated significant difference in relative intensity (*t*-test, N = 8, *P* < 0.05). A typical Western blots contained 4 control (ctrl) and 4 treatment animals was shown with the Vg detected by anti-MeVg antibodies and the lower panel with hemocyanin as a reference control. Each lane represent sample obtained from one animal.

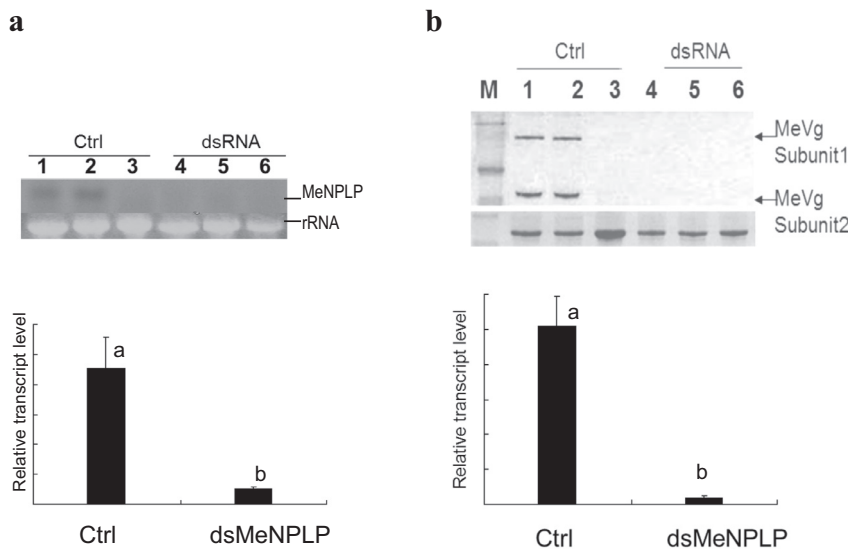
level of *MeVg1* in the hepatopancreas of the dsRNA injected female (Fig. 8a) had greatly reduced (i.e. 8×). Since MeVg1 is produced in the hepatopancreas, released in the hemolymph for ovary uptake, the vitellogenin level in the Hemolymph of the dsRNA treated shrimp were analyzed by Western blot analysis. Hemolymph of control shrimp receiving no dsRNA injection had a relative constant level of vitellogenin (only <3% changes) whereas vitellogenin level in hemolymph of dsRNA injected female dropped 60% (Fig. 8b). When the expression of MeNPLP in the ovary was analyzed by Northern blot, it was revealed that there was a significant reduction of the MeNPLP transcript level in the dsMeNPLP treated females (Fig. 9a). The result confirmed that silencing of *MeNPLP* gene in shrimp of early vitellogenesis could cause a decrease in vitellogenin production by the ovary. For example, in the two

control received no dsRNA, the anti-MeVg antibody recognize the MeVg (subunits 1 and 2). However, the ovary extracts from the dsMeNPLP injected shrimp showed the absence of signal (Fig. 9b). In summary, the results from RNA interference suggest that *MeNPLP* is important for initial vitellogenesis in the hepatopancreas.

**3. Discussion**

*3.1. cDNA characterization and comparison*

The current report describes the identification, characterization and functional role of MeNPLP. MeNPLP contains 12 cysteine residues and 11 of them are in the conserved positions identical to that



**Fig. 9.** Effect of MeNPLP dsRNA injection on ovary synthesis and expression of MeVg1. (a) Top: Northern blot show the ovary relative transcript level for the MeVg1 gene in the control (ctrl) and dsRNA injected shrimp (dsRNA). The diagram shows a typical Northern blot results. Each lane represent the sample of one animals (total N = 8 for each treatment). Below: estimated % change in MeVg1 transcript level in dsRNA injected shrimp and control (N = 8). (b) Coomassie staining of ovary protein (top) and Western blot detection of MeVg in ovary from the control (ctrl) and dsRNA injected shrimp (bottom). Individual lane shows the samples obtained from one animal. Top panel indicated the position of the MeVg subunits recognized by the anti-MeVg polyclonal antibody and lower panel with hemocyanin as a reference control.



of the locust NPLPs as revealed by amino acid sequence comparison (Fig. 2). In the locust (*L. migratoria*), neuroparsin is a neurohormone first isolated from the nervous tissues of the corpus cardiacum and recent studies suggest that neuroparsin-like protein is present in many insects [21–23]. The information accumulated from EST databases and molecular cloning results in this study have confirmed the presence of insect-like neuroparsins in decapod crustaceans. MeNPLP is most similar to the copepod neuroparsin-A like molecule. The copepod cDNA was isolated from the EST project and there is little information on this neuroparsin-like molecule. The spider single insulin-like growth factor-binding domain protein (SIBD) was isolated from the hemocyte and it may be involved in the innate immunity of the spider [12]. The results from phylogenetic tree analysis indicate the presence of two clusters of neuroparsin like proteins in one species. The NPLP identified in the decapods are from EST cloning of different shrimp. These sequences consist of LisNPLP and LivSIBD. They are closely related to the fish IGFBP, the vertebrate HTRA and the crayfish CqIAGBP. It is obvious that multiple neuroparsin-like molecules may exist in shrimp. For example, MeNPLP is more closely related to the insect neuroparsins such as ScgNP and LomNPB. Together they form a more distantly related group with the copepod, mosquito ecdysteroid substance, and *Apis* queen substance, etc. MeNPLP is more distantly related to the other crustacean “neuroparsin-like” molecules (i.e. PemNPLP, LivNPLP, HoaNPLP, CqIAGBP) which form a closer phylogenetic relationship with the vertebrate IGFBP and fish (OrnHTRA1) (Fig. 3). The results have suggested that more neuroparsins or neuroparsin-like molecules will be identified in a single shrimp species. The presence of multiple forms of neuroparsins in a single species has also been reported in insects. For example, in the locust two forms of NPs were identified in the insect, namely NPA and NPB. They are encoded by the same precursor called LomNP. Both NPA and NPB of the locust are multi-functional. They regulate reproduction by acting as the anti-juvenile hormone to prevent oocyte growth and inhibit ovary maturation. Similar function may occur in shrimp as the expression of MeNPLP in the shrimp decreases drastically towards the end of gonad maturation phase of the females. In fact, the existence of a NPLP-like substance in the hemocytes has been reported in the shrimp *L. vannamei* (LivSIBD) [3,4].

At present, there are at least five putative neuroparsin-like peptides as derived from EST sequences of the shrimp *L. vannamei*, *L. setiferus*, and *P. monodon*. The MeNPLP consists of 101 aa which is the longest of all NPLPs identified in shrimp so far. Moreover, the alignment result indicates that MeNPLP is more closely related to the locust NP and the vertebrate IGFBP than to other shrimp NPLPs sequence selected in this study. For example, the shrimp neuroparsins are not clustered together as a group in the phylogenetic tree (Fig. 3). Despite the difference in alignment and phylogenetic studies, most of these sequences display pronounced similarities to the locust neuroparsins and they possess a characteristic pattern of positional conserved cysteine residues. This is further supported by the phylogenetic tree analysis as the shrimp neuroparsin-like protein from different shrimp clustered with different related groups in the tree. Furthermore, preliminary genomic study was also performed by cloning the gene sequence of the shrimp neuroparsin. Using a PCR-based genome walking approach, at least three different copies of MeNPLP genes have been identified (Fig. 1c) which further confirms the presence of multiple MeNPLP-related genes in *M. ensis*. For example, in the GenBank Blastp search study, MeNPLP is most similar to the insect neuroparsins (i.e. ScgNP1 and LomNPA), followed by the NPLP-like of the copepod. MeNPLP is also related to the *Apis* queen brain selective protein which is important for reproduction and larval development [13] and the mosquito AgOEH for the production of steroid [18]. However, in the phylogenetic tree analysis, MeNPLP

is grouped together with the mosquito sequences (i.e. AngOEH and CuqOEH) (Fig. 3).

The result suggests that MeNPLP may be a secretory product of the Hp (Fig. 5). In contrast, no immunopositive signal could be detected in the nerve cord, ovary and muscles (data not shown). We reasoned that this may be due to the much lower level of MeNPLP expression in other shrimp tissues and/or the lower cross-activity of the insect antibody to the shrimp molecule.

Unlike the insect neuroparsin which is expressed mainly in neuronal tissues, these decapod neuroparsin-like transcripts are widely distributed. Although the hepatopancreas shows the highest expression level, other tissues such as the ovary, brain, heart and muscle also show low level of expression suggesting its widespread function in different tissues.

On the other hand, the N-terminal end of other crustacean NPLP consists of higher degrees of cysteine conservation when compared to that of the vertebrate OnmIGFBP (Fig. 3). In the vertebrate, the human HsHTRA3 gene encodes for a protein which is much larger than the rest of NPLPs and NPs. HTRA1 is composed of four distinct protein domains: a carboxyl-terminus, an insulin-like growth factor binding domain, a kazal domain, a trypsin-like peptidase domain and a PDZ domain. The amino acid sequence of MeNPLP is similar to the N-terminal end of the HTRA3 of the vertebrate. For example, the phylogenetic tree revealed the divergence in amino acid sequence of neuroparsin from different groups of animal. Such divergence may be due to the incomplete cloning of neuroparsin-like molecule from each species.

The vertebrate IGFbps are regulator of the IGF in vertebrates [24]. Its N-terminal insulin/IGF-binding domain is highly conserved within the IGFBP family and is very similar to the well-conserved sequence of arthropod neuroparsin related peptides. Furthermore, there is an indication that IGFBP-related peptides also occur in another invertebrate phylum. For example, Perlustrin, a nacre protein from the mollusc (*Haliotis laevis*), also displays homology to the N-terminal domain of mammalian IGFbps, and it binds IGF with a similar affinity as the N-terminal module of IGFBP-5 [25].

### 3.2. Expression studies of MeNPLP

The expression of MeNPLP, similar to the insect such as locust, is widely expressed in many tissues especially during reproductive phase. Since the 5 stages of ovary development cover a wide range of gonadosomatic index (GSI: percentage ovary weight/total body weight) of the animal, there may be variations in the expression patterns of the MeNPLP in these tissues for a particular GSI. Further research may require to confirm if there is any significant change in expression within a small change of GSI of a particular ovarian development.

In this report, we have provided *in vitro* evidence that MeNPLP transcript level decreases in the hepatopancreas but is up-regulated in the ovary after 5-HT treatment (Fig. 6). We have also demonstrated that 5-HT can cause a rapid decrease of MeNPLP and MeVg1 transcripts level in the hepatopancreas explants culture *in vitro*. In contrast, there are different responses of MeVg1 gene expression by the ovary. The *in vitro* result suggests that the regulation of vitellogenin synthesis in the hepatopancreas and ovary may be under the control of different mechanisms. Since MeNPLP gene expression level decreases as the gonad development continues and the expression level drops to undetectable level toward the middle to late maturation. It can be hypothesized that MeNPLP may be important for the initial stimulation of vitellogenin. As the process of vitellogenesis was in progress, neuroparsin transcript level decreased again and the level remained relatively low throughout the late vitellogenesis and oviposition [5,6]. An age-dependent increase in the neuroparsin transcript levels was



also observed in male fat body of the insect, whereby the highest level occurred just before the age of male sexual maturity with yellow coloration of the cuticle and copulation [8].

The expression of *MeNPLP* in the hepatopancreas and ovary is correlated with the maturation cycle which further supports our notion that it has a potential role in shrimp female reproduction. In adult females, the *MeNPLP* transcript level in hepatopancreas decreases from pre-vitellogenic to mid-late vitellogenic stage; in the ovary, *MeNPLP* transcript level increases from pre-vitellogenic to the maximum level at early-mid vitellogenic stage and drops to an undetectable level from middle ovarian stage to maturation spawning at stage V. Since the hepatopancreas and ovary are major organs involved in vitellogenesis of female shrimp, the high expression level of *MeNPLP* in these tissues suggests that *MeNPLP* is needed for the early stage of reproduction. In decapods crustaceans, gonad maturation in females are characterized by rapid synthesis of the major yolk protein (vitellogenin) in the process called vitellogenesis [26,27].

The expression patterns of *MeNPLP* in different tissues of shrimp during the reproductive cycle (Fig. 5), and the significant effects after 5-HT injection (Fig. 6) strongly suggests that it plays a role in female vitellogenesis. In many decapods, the neurotransmitter 5-HT has been implicated in the stimulation of reproduction in shrimp. The results from *in vitro* 5-HT study also indicate that the hepatopancreas and ovary respond differently to 5-HT. While the hepatopancreas expression of *MeNPLP* was inhibited by 5-HT, the ovary *MeNPLP* transcript level increased significantly after 5-HT treatment. The increase of *MeNPLP* transcript level was maintained until 120 min after treatment. Because the apparent difference in the response of ovary and hepatopancreas to 5-HT treatment *in vitro*, It is possible that both the ovary and hepatopancreas is under the control of different endocrine factor. Moreover, we only test the early stage since the initial cDNA clone was isolated from the 5-HT induce ovary. More work need to be done on the 5-HT effect on the ovary and hepatopancreas at different maturation stages.

With regards to the detection of the *MeNPLP* protein, using antibody of the locust neuroparsin, no signal was detected in the eyestalk, nerve cord, brain and ovary. Since the result from Northern blot analysis indicated the presence of transcript in stage II to IV, the absence of immunopositive signal in the ovary suggest that *MeNPLP* transcript may not be translate but are deposited in the ovary as maternal message for later usage. The lack of *MeNPLP* signal in these tissues in immunohistochemical study may be due to the low specificity of the insect hormone in the recognition of shrimp neuroparsin. Since *MeNPLP* expression level is the highest in the hepatopancreas, the locust antibody may be able to recognize as larger quantity of the *MeNPLP* is present in the hepatopancreas.

### 3.3. RNA interference and functional study of *MeNPLP*

In this study, RNAi is used to study the function of *MeNPLP*. Since the initial time course experiment indicated that a culture time of 2 h would have minimal effect on the expression of *MeNPLP*, all the subsequent culture were perform for a total time of 120 min. Moreover, similar to other dsRNA interference experiment, the tiger frog virus, as a non-specific dsRNA to demonstrate the absence of off-target effect. This study represents the first RNAi study on the function of this group of small peptide. In other animal model such as the desert locust, knock-down of the ScgNPs or ScgIRP affected vitellogenin transcript levels and oocyte growth in a positive and negative way, respectively. The findings are indicative for a role of ScgNPs and ScgIRP in the control of vitellogenin synthesis [7]. In *M. ensis*, injection of ds*MeNPLP* could knock-down the expression of *MeNPLP* in female shrimp. The dsRNA appeared

to be very stable as it can be detected in shrimp after 72 h. In fact, in another studies, we also demonstrated that RNA interference effect could last for 120 h [20]. In shrimp, the hepatopancreas is the extra-ovarian vitellogenin synthesis site. Vitellogenin, after its synthesis in the hepatopancreas, is secreted to the hemolymph and transported to the ovary for uptake by the vitellogenin receptor through receptor mediate endocytosis [28]. Injection of dsRNA for *MeNPLP* can cause a significant decrease in the *MeNPLP* transcript level in the hepatopancreas and the reduction in vitellogenin production lead to a decrease in vitellogenin level in the hemolymph. Similarly, Western blot analysis confirmed the absence or reduction in vitellogenin level in the ovary of the dsRNA injected shrimp. The result indicates that *MeNPLP* is required for shrimp gonad maturation. In the insect, knock-down of the ScgNPs or ScgIRP affected vitellogenin transcript levels and oocyte growth in a positive and negative way, respectively. The current findings are indicative for a similar role of ScgNPs and ScgIRP in the control of vitellogenin synthesis as in the shrimp.

In conclusion, with the cloning of a neuroparsin precursor-like cDNA in *M. ensis* and the expression study of *MeNPLP* in different tissues (including the hepatopancreas, ovary, nerve cord, brain and heart), further analysis of the gene structure, the gene family, the effect of 5HT on neuroparsin expression and its potential role in reproduction or vitellogenesis will be important to advance our knowledge in this group of neuropeptide. Moreover, as the sand shrimp express two different vitellogenin (*MeVg1* and *MeVg2*), it would be interesting to know if the *MeNPLP* would have similar effect on *MeVg2* expression.

## 4. Experimental procedures

### 4.1. Materials

#### 4.1.1. Animals

The sand shrimp *M. ensis* were purchased from a local seafood market and acclimated at 25 °C in a 12 h light and 12 h dark photoperiod.

### 4.2. Cloning of the shrimp neuroparsin cDNAs and genes

Total RNA was prepared by guanidine isothiocyanate extraction method [29]. RNA concentration was determined by spectrophotometry using a UV-160A machines (Shimadzu, Japan). RNA was analyzed by denatured formaldehyde agarose gel electrophoresis. For reverse transcription PCR (RT-PCR), the first strand cDNA was synthesized by reverse transcription in a mix containing 5 µg of total RNA, 2 pmol of oligo-dT<sub>17</sub> primer, 10 mM DTT, 2 mM dNTP mix, 2.5 mM MgCl<sub>2</sub> and 1 unit of Superscript II reverse transcriptase (Life Technologies, USA) at 42 °C for 3 h. The primer used in the initial cloning of *MeNPLP* was CB21 and CB22 [10] designed originally for the expression study of CHH gene in *M. ensis* (Fig. 1). For 5' cDNA cloning of *MeNPLP*, a rapid amplification of cDNA ends (RACE) kit (Roche, Germany) was used with gene specific primers *MeNPLP*R1 and *MeNPLP*R2 (Fig. 1; Table 1). For cloning of 3' end, gene specific primers *MeNPLP*-F1 and *MeNPLP*-F2 were used for the 3' end of *MeNPLP*. Both 5' and 3' RACE were performed according to the manufacturer's instruction manual. Similar to the RT-PCR cloning, RACE products were subcloned into a TA vector (Promega, USA) and DNA sequence determination was performed using the dideoxynucleotide chain termination method on a DNA sequencer (Model 373A, Applied Biosystem). Full-length cDNA of *MeNPLP* was obtained from sequences of the overlapping clones. RT-PCR products were subcloned into pGEM-T vector and sequenced. Sequences obtained from initial degenerative RT-PCR together with the 5' and 3' RACE were combined to generate partial

**Table 1**

The sequences of the primers used in the PCR cloning RT-PCR and 5' and 3' RACE.

For initial cloning	
CB21:	CTATCTAGATGGTCGCGTCCGA
CB22:	GGGAGTGTACTGGAGTGTAC
For 5' RACE	
MeNPLP-R1:	GGTATTCGGACCTCTGTGATG
MeNPLP-R2:	GCCCCTGCTACATATTGTGTAG
For 3' RACE	
MeNPLP-F1:	ACTGGAACGAACATGGCAAG
MeNPLP-F2:	GGATGCAACCAATCTAGTT
For sequence confirmation	
MeNPLP-F3:	TTGTACTGGAGTGTACAGAA
MeNPLP-R3:	TGTTCCCTGAACGCCAAT
For RNA interference	
Forward:	5'-TAATACGACTCACTATAGGTACTACCAACATGAAGGGTCTTCGTTTC-3'
Reverse:	5'-TAATACGACTCACTATAGGTACTCGCAGTTTACGAGAAGGATCCGTAC-3'
For gene walking	
GW5'GSP:	CAGGAAGGAGCCCTGCTACATATTGTGTAG

cDNA and full-length sequences of MeNPLP. DNA and amino acid sequences derived from these clones were compared and analyzed by NCBI BLAST search. The NPLP, selected insect neuroparsin (NP), insulin-like androgen binding protein, insulin binding protein of vertebrates (IGFBP, HTRA) were used for the alignment analysis using CLUSTALW. For phylogenetic tree construction, neighbor-joining tree was constructed from multiple sequence alignments with other protein sequences derived from the GenBank database using the molecular evolutionary genetics analysis (MEGA) software, version 4.2. Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions.

#### 4.3. Expression study of MeNPLP

RNA samples were analyzed on a 1.2% formaldehyde denatured gel. After electrophoresis, the RNAs were transferred onto a Nylon membrane. For Northern blot, the membrane was hybridized to a Dig-labeled non-radioactive probe obtained from the synthesis of RT-PCR produced using MeNPLP-F1 and MeNPLP-R1 primers. The denatured probe was added to pre-warmed High-SDS hybridization buffer and hybridized for 16–20 h at 50 °C. The membrane was washed in 2× SSC with 0.1% SDS twice for 15 min and then in 0.1× SSC with 0.1% SDS twice for 15 min at 58 °C. The membrane was then incubated in 1:20,000 anti-DIG-AP conjugate in 1× blocking buffer. For signal detection, 200 µl CDP-Star (Roche) was added onto the membrane and luminescent signal was detected by exposing the membrane onto a film which was then developed by a Kodak film processor.

#### 4.4. Effects of serotonin (5HT) on MeNPLP expression

A shrimp *in vitro* explant culture system was used to study the effect of 5HT on the expression of MeNPLP. Briefly, hepatopancreas and ovary were dissected, cut into small fragments (i.e. 50 µl in volume) and placed in the wells of 24-well plate containing 1.5 ml Medium 199 (Sigma, USA). Serotonin (Sigma, USA) (1 µg/ml) was added to the culture medium with the tissue fragments and the plates were incubated at 25 °C for 4 h. At the end of the culture period, the tissues were collected for total RNA extraction followed by Northern blot analysis as described above. For quantitative analysis of MeNPLP expression, the signals ( $N=8$ ) corresponding to the MeNPLP cDNA on the film were quantified by the Image J analysis program (<http://rsb.info.nih.gov/ij/>). The relative expression level of MeNPLP was calculated after normalization with the amount of rRNA stained of ethidium bromide from the same sample with the rest of the RNA samples. Student's *t*-statistic

(*t*-test) was used to assess the level of significance with the SPSS (Version 13.0), and *P* values less than 0.05 was considered to be statistically significant.

#### 4.5. Immunohistochemical detection of MeNPLP

Different tissues of the shrimp (hepatopancreas, muscle, eye-stalk, etc.) were dissected and embedded into paraffin. The blocks are cut to 7 mm sections, mounted onto the slides, and dewaxed twice in xylene solution. Dehydration of slides was performed in 0%, 20%, 40%, 60%, 80%, 100% ethanol. After incubating in a PBS blocking buffer containing 0.5% gelatin, 0.5% BSA and 0.1% NaN<sub>3</sub> for 30 min at room temperature, the sections were blocked in the same solution containing 1:400 normal goat serum (1 h). The slides were incubated in fresh blocking buffer containing the first antibody against insect neuroparsin (1:5000) overnight. After washing the slides in PBS twice for 20 min, they were incubated (1 h room temperature) in the PBS containing an alkaline phosphate conjugated goat anti-rabbit antibody (1:5000). To remove the excess antibody, the slides were first washed in PBS twice for 10 min and in an alkaline phosphatase buffer (0.1 M tris, pH9.6, 0.1 M NaCl and 50 mM MgCl<sub>2</sub>) twice for 10 min. Color signals were developed by incubating the slides in 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in fresh alkaline phosphatase buffer. To stop color development, the slides were gently rinsed in large amount of running tap water. Similar procedures were used for the control except that it was incubated in a incubated in pre-immunized rabbit serum. Before microscopic observation, the slides were counter-stained in eosin, dehydrated and cleared with xylene.

#### 4.6. Functional study of MeNPLP by RNA interference

To prepare DNA template for dsRNA synthesis, DNA corresponding to the mature peptide of MeNPLP was amplified by PCR using T7 promoter linked primers (Forward: T7-linked AACATCA-GAACCTCGGAAGCTA; reverse: T7-linked CTTCCATCTTGA-GACTTGCTT). For PCR, the final reaction mix consisted buffer 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 0.5 µM MeNPLP-T7 FP, 0.5 µM MeNPLP-T7RP and 0.25 µl Taq DNA polymerase (Life Technologies, USA). PCR conditions included denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1.5 min for 30 cycles. PCR products were analyzed by 2% agarose gel electrophoresis and the targeted PCR product band (approximately 320 bp) was purified by the GENECLEAN® II Kit (Biogene, CA). For transcription of MeNPLP dsRNA, 1–2 µg of purified T7 promoter linked MeNPLP cDNA was used as template in the *in vitro* transcription reaction with T7 Megascript RNAi Kit (Ambion, USA) according to manufacturer's recommendations. In 20 µl reaction, MeNPLP DNA template was mixed with appropriate amount of nuclease-free water, 2 µl 10× T7 Reaction Buffer, 2 µl ATP solution, 2 µl CTP solution, 2 µl GTP solution, 2 µl UTP solution and 2 µl T7 enzyme mix. The mixture was incubated at 37 °C for 18 h. During the transcription, the two RNA strands were hybridized to form dsRNA.

For the RNA interference experiment, female shrimp (25–35 g) in early stage of maturation with similar GSI (2–3%) were visually examined and acclimated in culture tank overnight prior to injection. For injection experiment, MeNPLP dsRNA (3 µg in 1× phosphate buffered saline) was injected into shrimp through the arthroal membrane of the periopods by a Hamilton syringe. The controls received equal volume of PBS injection (or injection of a non-specific dsRNA). Shrimp were returned to the tanks for culture before sacrifice and for total RNA preparation from different tissues. The relative expression level of MeNPLP in the nerve cord was used as an indication of the RNAi effect between the

treatment and control groups. SDS–PAGE and Western blot analyses of the ovary and hemolymph protein from shrimp treated by dsRNA for MeNPLP. The ovary of female with GSI = 2–3.2% was dissected and extracted for total soluble protein by a 1 × PBS buffer. To demonstrate the change in the production of vitellogenin from the hepatopancreas, hemolymph samples were withdrawn from dsRNA treated shrimp and the total protein was analyzed by SDS–PAGE and Western blot using *M. ensis* vitellogenin polyclonal antibody [11]. Blots were incubated in a PBST solution containing the Anti-MeVg (titer 1:15,000) overnight. The signals representing the MeVg were quantified by Image J program and statistical analysis was performed as described in 4.5 from the above.

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