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Transcriptomic Analysis of Neuropeptides and Peptide Hormones in the Barnacle *Balanus amphitrite*: Evidence of Roles in Larval Settlement

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Abstract

The barnacle *Balanus amphitrite* is a globally distributed marine crustacean and has been used as a model species for intertidal ecology and biofouling studies. Its life cycle consists of seven planktonic larval stages followed by a sessile juvenile/adult stage. The transitional processes between larval stages and juveniles are crucial for barnacle development and recruitment. Although some studies have been conducted on the neuroanatomy and neuroactive substances of the barnacle, a comprehensive understanding of neuropeptides and peptide hormones remains lacking. To better characterize barnacle neuropeptideome and its potential roles in larval settlement, an *in silico* identification of putative transcripts encoding neuropeptides/peptide hormones was performed, based on transcriptome of the barnacle *B. amphitrite* that has been recently sequenced. Potential cleavage sites and structure of mature peptides were predicted through homology search of known arthropod peptides. In total, 16 neuropeptide families/subfamilies were predicted from the barnacle transcriptome, and 14 of them were confirmed as genuine neuropeptides by Rapid Amplification of cDNA Ends. Analysis of peptide precursor structures and mature sequences showed that some neuropeptides of *B. amphitrite* are novel isoforms and shared similar characteristics with their homologs from insects. The expression profiling of predicted neuropeptide genes revealed that pigment dispersing hormone, SIFamide, calcitonin, and B-type allatostatin had the highest expression level in cypris stage, while tachykinin-related peptide was down regulated in both cyprids and juveniles. Furthermore, an inhibitor of proprotein convertase related to peptide maturation effectively delayed larval metamorphosis. Combination of real-time PCR results and bioassay indicated that certain neuropeptides may play an important role in cypris settlement. Overall, new insight into neuropeptides/peptide hormones characterized in this study shall provide a platform for unraveling peptidergic control of barnacle larval behavior and settlement process.


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Introduction

Neuropeptides constitute the largest class of intercellular messenger molecules and play key roles in many physiological processes, e.g. reproduction, homeostasis and locomotion [1]. In general, they are produced from endocrine cells or neurons as precursors (preprohormones) and become biologically active after post-translational modifications. Secreted neuropeptides can have autocrine, paracrine and hormonal effects, by binding to membrane receptors of organ systems [2]. The earliest traceable ancestral neuropeptides date back to primitive metazoans, i.e. cnidarians [3,4]. In arthropods, neuropeptide studies have so far been limited to insects and decapods. For instance, eclosion hormone and ecdysis triggering hormone are the most well-known neuropeptides extensively studied in the moth *Manduca sexta* and the fruit fly *Drosophila melanogaster* [5]. Prothoracotrophic hormone has been characterized in various insects and proposed to initiate larval metamorphosis through stimulating prothoracic glands via G protein-coupled receptor/cAMP [6]. The crustacean hyperglycemic hormone family, originally isolated from X-organ-sinus gland (XO-SG) complex of decapods, was involved in regulating energy and ionic metabolism, or inhibiting molting and reproduction [7]. Besides insects and decapods, only very limited information is available on arthropod neuropeptides.

Barnacles are common in intertidal communities worldwide, and often cause biofouling problems. The life-history of barnacles consists of six naupliar stages and one cypris stage when larvae become competent to attach to substratum and then metamorphose into sessile juveniles (collectively referred to as “settlement”). Cyprid is non-feeding stage and has evolved highly specialized features and behavior for settlement [8]. Besides the various exogenous inducers such as conspecific biogenic cues [9,10], various biogenic amines and hormones such as serotonin [11,12], dopamine, methyl farnesoate and 20-Hydroxyecdysone were also reported to regulate larval attachment and metamorphosis [13–16]. While extensive studies have been carried out on neurotransmitters/hormones in barnacle, only limited information is available on barnacle settlement.
Table 1. Neuropeptide/peptide hormones predicted based on transcriptome mining of Balanus amphitrite.

<table>
<thead>
<tr>
<th>Peptide family</th>
<th>Barnacle accession No.</th>
<th>Reference accession No.</th>
<th>RACE confirmed</th>
<th>E-value</th>
<th>Score</th>
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<tr>
<td>A-type allatostatin</td>
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<td>BAF64528.1</td>
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<td>1122</td>
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<td>NP_001036890.1</td>
<td>+</td>
<td>7E-22</td>
<td>249</td>
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<tr>
<td>C-type allatostatin</td>
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<td>P85798.1</td>
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<td>3E-15</td>
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</table>

*Reference accession No. is the accession No. of the known neuropeptide that has the highest hit against barnacle transcript.

Bioinformatics-based mining of neuropeptides from Expressed Sequence Tag (EST) library, transcriptome and genome has been recently conducted and numerous novel peptides have been uncovered in several species [17, 18]. The barnacle has been subjected to deep sequencing of EST recently [19]. In our study, we obtained the transcriptome of the barnacle *Balanus amphitrite* using 454 pyrosequencing technology, which contained more than 90,000 predicted open reading frames [20]. This rich source of transcriptomic information made large-scale in silico discovery of peptides feasible since we can overcome the difficulties of collecting and sectioning enough amounts of nervous tissues from *B. amphitrite* larvae for mass spectrometry analysis. In this study, we conducted in silico transcriptome mining of neuropeptides/peptide hormones in *B. amphitrite*, and quantified their expression levels at different developmental stages. We then examined the effect of proprotein convertase inhibitor on larval settlement to explore the possible function of the neuropeptides characterized. Our results provide a comprehensive catalog of neuropeptideome of *B. amphitrite*, and insights on the possible functional roles of some neuropeptides in barnacle larval settlement.

Materials and Methods

1. Sample Preparation

Adult barnacles were collected from the Pak Sha Wan public piers, Hong Kong (22°21'45" N, 114°15'35" E). No specific permits were required for the field collection. The field studies did not involve any endangered or protected species. Broods were isolated from adult barnacle in the laboratory and nauplii were hatched and cultured according to Thiyagarajan & Qian 2008 [21], and larvae were collected once they reached cypris stages after 4 days’ culture. For expression analysis of peptide precursor genes, nauplii II, nauplii VI, cyprids, newly metamorphosed juveniles, and adults were collected. Total RNA extraction and cDNA synthesis were conducted according to Chen et al. 2011 [20]. Briefly, total RNA of barnacles of different developmental stages was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Trace DNA contaminants were removed by TURBO DNA-free™ Kit (Ambion Inc, Austin, TX, USA). cDNA was synthesized using M-MLV reverse transcriptase (USB, Cleveland, OH, USA) with oligo dT primer for Rapid Amplification of cDNA Ends (RACE) reactions and real-time PCR assays.

2. Database Mining of Neuropeptide Precursors

Several methods from recent publications [17,18,22] were combined and modified to search for neuropeptide/peptide hormone encoding genes in barnacle transcriptome, which contains 23,451 contiguous sequences including 182 contigs, 23,269 isotigs and 77,785 singletons [20]. Protein sequences of the known neuropeptides and peptide hormones in arthropod were obtained from UniProt Knowledgebase (http://www.uniprot.org/) using “neuropeptide”, “hormone” and “peptide” as search keywords without "receptor", "signal anchor", or "transmembrane". NCBI non-redundant protein sequences (http://www.ncbi.nlm.nih.gov/) were also used for known arthropod peptides extraction, since different databases tend to use different key-word searching criteria which may lead to different results. After removing the unrelated sequences, such as enzymes and transcription factors, the remaining sequences were transformed into FASTA format to generate a local arthropod neuropeptide database. The program “BLASTn” was used to mine for putative cDNA sequences that encode for active peptides in the barnacle transcriptome via queries using
arthropod neuropeptide sequences mentioned above. For each query, the top three blast hits with an E-value lower than 0.01 were screened out and chosen as candidates and manually checked for homology to known peptides.

3. Peptide Prediction

Neuropeptide candidate sequences generated by database mining were translated using ExPASy translate tool (http://web.expasy.org/translate/). Three typical neuropeptide precursor criteria, which are signal sequence, pro-hormone processing sites and length less than 300 amino acids, were applied to evaluate candidate sequences. Signal peptide identification was deduced by online program SignalP 3.0, using both the neural networks and Hidden Markov Model algorithms [23]. Pro-hormone cleavage sites were predicted based on work by Veenstra 2000 [24], and Neuropred online program (http://neuroproteomics.scs.illinois.edu/neuropred.html) and/or by homology to the known arthropod precursors. Sulfation state of Tyr residues was predicted using the online program Sulfinator [25] and/or by homology to known arthropod neuropeptides. In some cases, other post-translational modifications, e.g. cyclization of N-terminal Glu/Gln residues and C-terminal amidation were predicted mainly by homology to known peptide isoforms.

4. RACE Sequencing and Peptide Confirmation

Since neuropeptide precursor sequences generated from transcriptome are usually fragmented or incomplete, further confirmation on the predicted neuropeptide candidates by full length open reading frame (ORF) is required. Two sets of specific primers were designed from the partial cDNA sequences obtained from the transcriptome database. For RACE, a first run of amplification...
was performed using gene specific primer 1st and adaptor with oligo (dT)/oligo (dG) primer. Then gene specific primer 2nd (up or down to primer 1st) and adapter primers were used for second run PCR amplification. RACE products were cloned and sequenced. Complete amino acid sequences of candidate genes were submitted to NCBI BLAST again and checked manually for the precursor structure. If BLAST result of the new sequence didn’t match the corresponding neuropeptide genes, it was excluded.

5. Sequence Analysis

Corresponding neuropeptide sequences from crustaceans and insects were searched and collected. All the crustacean neuropeptide genes and proteins discovered through in silico data mining, cloning or mass spectrometric approach were compiled. Predicted mature peptide sequences were used for alignment using ClustalW (version 2.0) with default parameters and manually checked.

6. Quantitative Real-time PCR

Gene specific primers were designed manually based on nucleotide sequences from the barnacle transcriptome database. Details of the primers are listed in Table S3 as additional information. The cytochrome b gene was used as the inner reference for normalizing the expression levels of target genes [26]. All real-time PCR assays for each peptide-encoding gene were performed on Stratagene Mx3005P QPCR System (Agilent, Santa Clara, CA, USA), using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KAPA Biosystems, Woburn, MA, USA). For each neuropeptide gene, three replicates were conducted using each batch of larvae, and three batches of larvae per treatment and control. This compound is a highly specific and potent inhibitor against proprotein convertases responsible for maturation of bioactive peptides [28]. Specifically, stock solution (20 mM) was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) and stored at −20°C. Experiments were conducted in triplicates and repeated three times with different batches of cyprids. Around 20 cyprids were added to each well of a 24-well plate (#3047, BD Falcon™, Franklin Lakes, NJ, USA) consisting of three concentrations (1, 10, and 100 μM). Two negative controls, i.e. autoclaved filtered seawater (AFSW) only and 0.5% DMSO in AFSW. The plates were incubated at 28°C in darkness for 48 hours, and the number of metamorphosed barnacle cypris larvae was counted. Metamorphosis was defined as settlement by the barnacle cypris larvae with the formation of a aboral peduncle and haptic rhinophore, which was visually evident by the end of the experiment. Data were analyzed by 2−ΔΔCT method [27] and further tested by using one-way ANOVA, followed by Tukey test post-hoc analysis. Gene expression level in juvenile stage was standardized in order to better characterize genes that are up-regulated in cypris stage and down-regulated after metamorphosis, which might be involved in settlement regulation.

7. Proprotein Convertase Inhibitor Assay

To test the hypothesis that neuropeptides/peptide hormones are involved in larval settlement, we incubated cyprids in solution of peptidyl chloromethylketone (Enzo life sciences, Farmingdale, NY, USA) and compared the percentage of metamorphose between the treatment and control. This compound is a highly specific and potent inhibitor against proprotein convertases responsible for maturation of bioactive peptides [28]. Specifically, stock solution (20 mM) was prepared by dissolving the compound in dimethyl sulfoxide (DMSO) and stored at −20°C. Experiments were conducted in triplicates and repeated three times with different batches of cyprids. Around 20 cyprids were added to each well of a 24-well plate (#3047, BD Falcon™, Franklin Lakes, NJ, USA) each containing 1 mL of test solution. The treatment group consisted of three concentrations (1, 10, and 100 μM), with two negative controls, i.e. autoclaved filtered seawater (AFSW) only and 0.5% DMSO in AFSW. The plates were incubated at 28°C in darkness for 48 hours, and the number of metamorphosed barnacle cypris larvae was counted. Metamorphosis was defined as settlement by the barnacle cypris larvae with the formation of a aboral peduncle and haptic rhinophore, which was visually evident by the end of the experiment. Data were analyzed by 2−ΔΔCT method [27] and further tested by using one-way ANOVA, followed by Tukey test post-hoc analysis. Gene expression level in juvenile stage was standardized in order to better characterize genes that are up-regulated in cypris stage and down-regulated after metamorphosis, which might be involved in settlement regulation.

**Figure 3. Comparison of barnacle tachykinin-related peptide with that of insects and crustaceans.** (A) TRP sequence alignment. Decapods TRP are from: Cancer borealis [72] and Homarus americanus ACB41786; isopod TRP is from Eurydice pulchra CO869025; branchiopod TRP is from Daphnia pulex [38]. Insects TRP are from: Tribolium castaneum EFA90176, Drosophila melanogaster AAF89172, Bombyx mori NP_001124364 and Rhypharobia maderae Daphnia pulex EFA09176, castaneum. Figure 4. Comparison of barnacle orcokynin with other arthropods.** (A) Sequence alignment mature orco. (B) Precursor structure of barnacle orco compared with other crustaceans and insects. Sequences of insects Orco are from: Nasonia vitripennis XP_0034266062, Bombyx mori NP_001124366, Danaus plexippus EH177769, Harpegnathos saltator EFN80782 and Apis mellifera XP_001120650; copepods Orco are from: Callus rogercresseyi and Lernaearca branchialis [22]; decapods Orco are from: Litopenaeus vannamei [1], Marsupenaeus japonicas [73], Homarus americanus ACD13197, Procambarus clarkii [73], Carcinus maenas [74] and Orconectes limosus [75]. Branchiopod Orco is from Daphnia pulex EFX70781. Asterisks “**” represent typical motif residues of each peptide. doi:10.1371/journal.pone.0046513.g003
and swimming cyprids was counted under a dissecting microscope every 24 hours. Three replicates were conducted for each batch of larvae, and in total three batches of larvae were collected for bioassay. Percentage of larval metamorphosis was calculated and arcsine transformed prior to one-way ANOVA analysis followed by Tukey post hoc analysis.

Results and Discussion

1. Neuropeptides Predicted from *B. amphitrite*

In this study, a combination of *in silico* prediction of putative neuropeptide-encoding genes of *B. amphitrite* and molecular cloning verification were performed using known peptides in Arthropoda as queries. As a result, 16 neuropeptide families were predicted, and 14 of them were confirmed by peptide homology and gene cloning (Table S1). Since many neuropeptides are relatively fragmented (10–30 amino acids) in the transcriptome dataset, the BLAST score tended to be low with a high E-value. Their full length open reading frames were cloned by RACE, which not only served as a secondary proof of the *in silico* prediction, it was extremely conserved among arthropods and only differ at one N-terminal residues [32]. Gly-SIFamide peptide discovered in *B. amphitrite* in this study was highly similar to that of other insects and decapods, but with one amino acid Pro⁵ being changed into Thr⁶ (Figure 1B). Likewise, NPF of *B. amphitrite* possessed extra five-residues insertion between positions 19–23 from C-terminal, which was a novel isoform for NPF peptide family (Figure 1C). Furthermore, clear differences existed between sulfakinins from barnacle and their arthropod counterparts. The precursor of barnacle sulfakinin encoded for two more mature peptides than that of other arthropods (Figure 2B). In addition, compared with C-terminal typical signature YGHM/LRFamide with sulfated or nonsulfated Tyr in other species, sulfakinin-2/3/4 in *B. amphitrite* possessed Lys⁴ rather than the ubiquitous Arg⁴, and Met⁷/Phε⁸ instead of His⁶ in all known arthropod sulfakinin variants (Figure 2A). Further support was derived from barnacle ASTC with the mature sequence of SYWKQCSFNAVSCFamide (Table S1). The typical motif of ASTC is either X6CYFNPISCF with N-terminal residues [32]. Gly-SIFamide peptide discovered in *B. amphitrite* in this study was highly similar to that of other insects and decapods, but with one amino acid Pro⁵ being changed into Thr⁶ (Figure 1B). Likewise, NPF of *B. amphitrite* possessed extra five-residues insertion between positions 19–23 from C-terminal, which was a novel isoform for NPF peptide family (Figure 1C). Furthermore, clear differences existed between sulfakinins from barnacle and their arthropod counterparts. The precursor of barnacle sulfakinin encoded for two more mature peptides than that of other arthropods (Figure 2B). In addition, compared with C-terminal typical signature YGHM/LRFamide with sulfated or nonsulfated Tyr in other species, sulfakinin-2/3/4 in *B. amphitrite* possessed Lys⁴ rather than the ubiquitous Arg⁴, and Met⁷/Phε⁸ instead of His⁶ in all known arthropod sulfakinin variants (Figure 2A). Further support was derived from barnacle ASTC with the mature sequence of SYWKQCSFNAVSCFamide (Table S1). The typical motif of ASTC is either X6CYFNPISCF with N-terminal residues [32]. Gly-SIFamide peptide discovered in *B. amphitrite* in this study was highly similar to that of other insects and decapods, but with the broadly conserved Ala⁷ being substituted by Ser⁷.

During evolution, the non-synonymous mutations may either radical, or promote or impair the neuropeptide’s biological function [34]. Plenty of structural function studies on neuropeptides suggested even small variations of amino acid sequence can lead to substantial functional changes in the potency of the peptides, depending on the position of changes [35]. For instance, the allatostatin Pea-AST2 of the cockroach *Periplaneta americana* was reported to be more potent than Pea-AST1 over a 400-fold range, in terms of their ability of inhibiting juvenile hormone synthesis [36]. Thereby, unique neuropeptide structures found in barnacle may imply that their functional efficiency has been altered. Whether, and in what way modified peptide sequences would alter their bioactivity and subsequently physiological processes in barnacles remains to be investigated.

2. Neuropeptide Sequences Analysis

Although most of the neuropeptides identified in the present study are widely distributed among arthropod species, we found some isoform variants that appeared to be unique, including those that had previously been thought to be highly conserved in arthropods. For instance, SIFamide family consists of two major isoforms, namely Gly-SIFamide and Val-SIFamide, which are extremely conserved among arthropods and only differ at one N-terminal residues [32]. Gly-SIFamide peptide discovered in *B. amphitrite* in this study was highly similar to that of other insects and decapods, but with one amino acid Pro⁵ being changed into Thr⁶ (Figure 1B). Likewise, NPF of *B. amphitrite* possessed extra five-residues insertion between positions 19–23 from C-terminal, which was a novel isoform for NPF peptide family (Figure 1C).

3. Some Neuropeptides of *B. amphitrite* Indicate Closer Relationship with Insects Rather than Decapods

Insects and decapods are two major groups of arthropods widely studied in comparative endocrinology and neuropeptide physiology. Thorough comparison of neuropeptide structures and sequences revealed that some neuropeptides of barnacle were structurally similar to their insect homologs. The first instance was...
based on precursor structure of both the TRP and Orco genes of *B. amphitrite*. Decapods’ TRP precursor generally gave rise to several copies of a single TRP isoform or only one additional TRP isoform [37], while the TRP precursor of insects tended to encode multiple diverse isoforms [38]. The TRP precursor of *B. amphitrite* contained four copies of different TRP mature peptides (Figure 3, Table S2), which is structurally more similar to insects. In the case of Orco, mature Orco of both insects and *B. amphitrite* encoded for 14 amino acids, while the Orco of decapods was strictly 13 amino acids long (Figure 4A). Besides, comparison of their precursor structures also indicated that barnacle Orco resembled that of insects, with less mature peptides than crustaceans (Figure 4B).

The barnacle ASTB revealed in this study (Figure 5) lends further support to our postulation above. Among the 10 peptide isoforms encoded by ASTB precursor of *B. amphitrite*, 9 of them showed high similarity with the ASTB of insects. In addition, sequence alignment of IRP also indicated a similar result (Figure 6). In general, the IRP precursor contains contiguous B-C-A peptides. After maturation, A- and B-chain peptides are linked together by two interchain disulfide bonds and one intrachain disulfide bond. C-chain peptide assists the formation of linkage and will be clipped off at the cleavage sites afterwards [39]. IRP of *B. amphitrite* structurally resembled that of insects, with one more amino acid between the two Cys on B-chain and four amino acids gap between the second and third Cys of the A-chain (Figure 6). One contradiction came from the ASTA of *B. amphitrite*, which resemble that from decapods. Among predicted ASTA peptides of *B. amphitrite*, ASTA-1/4/5/6/8/9 were similar to ASTA peptides from the giant fresh water prawn *Macrobrachium rosenbergii*, while the rests shared similarities with the lobster *Panulirus interruptus* (Figure 7). Sequence comparison of other neuropeptides including BurA/B, Calci and EH were shown in Figure 8 and 9, revealing their conserved structures among arthropod species.

Based on molecular studies in the past decades, it is now widely accepted that hexapods are associated with crustaceans, forming a group called Pancrustacea within arthropods. However, the inner relationships among Pancrustacean constituent lineages are far from being resolved [40]. A recent phylogenetic analysis of protein-coding nuclear genes demonstrates that Hexapoda is most closely related to the crustacean Branchiopoda, Cephalocarida and Remipedia, while Malacostraca including decapods are grouped with Cirripedia (barnacles) making the traditional

![Figure 6. Peptide alignment of insulin-related peptide of *Balanus amphitrite* with arthropods. Isopods IRP are from: Armadillidium vulgare AB029615 and Porcellio dilatatus AB089811; decapods IRP are from: Marsupenaeus japonicus AB029615, Penaeus monodon GU208677.1 and Cherax quadricarinatus DQ851163; branchiopod IRP is from Daphnia pulex [38]; insects IRP are from: Bombyx mori NP_001233285, Locusta migratoria P15131, Drosophila melanogaster CG14173, Anopheles gambiae AAQ89693 and Camponotus floridanus EFN61735. Asterisks “*” mark the conserved Cysteine residues, black frame indicates specific amino acids insertion and deletion in IRP of isopod and decapod species, compared to insects, branchiopod and barnacle. doi:10.1371/journal.pone.0046513.g006](image-url)
crustacean class Maxillopoda with copepod [41]. However, in
B. amphitrite, neuropeptides such as TRP, Orco, IRP and ASTB,
were much similar to their homologs from insects rather than
decapods, as supported by structure comparison and sequence
alignment. High sequence similarity among different species may
represent independent evolution under shared evolutionary
constraints, which maintains structural and functional conserva-
tion of protein products [38]. The closer relationship of these
neuropeptides between B. amphitrite and insects inferred much
about their similar function in these two groups. Since molecular
neuroendocrine information of arthropods other than decapods
and insects is limited, data on neuropeptides from other taxa
would be helpful.

4. Neuropeptides that are Specifically Up/down
Regulated in Cyprid Stage
To further explore the potential involvement of neuropeptide
genes in larval settlement of B. amphitrite, late nauplius VI, cyprid,
early juvenile and adult were chosen to assess developmental
variation of neuropeptide genes. Fourteen predicted neuropeptide
genes were subjected to quantitative real-time PCR (Figure 10).
Since the expression level of bursicon z subunit has already been
measured by Chen et al. [20], this gene was omitted in the current
developmental profiling. Using gene expression level in the
juvenile stage as a standard, calcia (A isoform of calcitonin-like
diuresis hormone), sf (SIFamide), pdh (pigment dispersing
hormone) and astb (B-type allatostatin) were found to be
specifically up-regulated in either late naupliar stage or cypris
stage but down-regulated in young juvenile and adult stages (Tukey
test, p<0.05). tp (Tachykinin-related peptide) was down-regulated
in cypris and early juvenile stage compared to naupliar and adult
stages (Tukey test, p<0.05); irp (Insulin-related peptide) was down
regulated in early juvenile compared to other stages (p<0.05, One-
way ANOVA); expression level of npf (neuropeptide F), burb
(bursicon subunit B), calcb (B isoform of calcitonin-like diuresis
hormone), orco (orcokinin), sulf (sulfakinin), eh (eclosion hormone),
asta and astc (A- and C-type allatostatin) remained unchanged
among naupliar VI, cypris, juvenile stages, but were down-
regulated in adults. The down regulation of neuropeptide genes in
adults could be due to the degenerated neural system since the
central nervous system of adult barnacle is highly reduced in the
sessile mode [29,42]. There was, however, an exception, which is
the high expression of tachykinin-related peptide (tp) in both
naupliar VI and adult stages. Since the nervous system of cyprid
larva is specifically cater for sensing settlement cues [43], we thus
expect that peptide B-type allatostatin, A isoform of calcitonin-like
diuresis hormone (Calc-A), pigment dispersing hormone and
SIFamide are involved in cypris attachment and metamorphosis,
or at least performs a specific function during these two stages,
since they were higher expressed in larval stage but down-
regulated after metamorphosis.

4.1 B-type allatostatin. All three families of ASTs were
found in the transcriptome of B. amphitrite, but only ASTB was up-
regulated in both naupliar VI and cypris stages, being almost 3-
fold higher than the juvenile stage (Figure 10B). Different
developmental expression patterns of these three types of ASTs in
B. amphitrite indicates that each type may be respectively
involved in different physiological processes, such as juvenile
hormone synthesis, stomatogastric or cardiac neuromuscular
functions reported in insects [44,45]. One major function of ASTs
in the insects studied is inhibiting juvenile hormone (JH) synthesis
[46]. JH is responsible for the maintenance of juvenile character-
istics during development, and prevents metamorphosis during
larval stage. Methyl farnesoate (MF), the unepoxidised form of JH
III discovered in crustacean, was recently considered as function-
ally equivalent to insect JH [47]. In barnacle, high concentration
of exogenous MF induced precocious metamorphosis without
attachment [14], while physiologically-relevant concentration of
natural isomer of MF inhibited larval settlement [16]. Furthermore,
Yamamoto et al. [13] suggested that the balance of JH and
20-Hydroxyecdysone could regulate cypris metamorphosis, espe-
cially molting. Since ASTB was reported to have JH-inhibiting

Figure 7. Sequence comparison of barnacle A-type allatostatin with homologs in arthropods. Decapod ASTAs are from Macrobrachium
rosenbergii Q1AHE3, Panulirus interruptus A6BL33 and Procambarus clarkii Q3L153; insect ASTAs are from Bombyx mori NP_001037036, Drosophila
melanogaster AAF97792. Asterisk ‘*’ represents typical motif residues of ASTA peptide.
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In our study, two isoforms of calcitonin-like diuretic hormone (CalciA and CalciB) located at different transcripts were identified and distinct expression patterns were detected. CalciA was highly expressed in both Nauplii VI and cyprid compared with juvenile stage, while CalciB's expression didn't significantly change during settlement (Figure 10E, F). Calci belongs to the diuretic hormone (DH) family and could promote fluid secretion of Malpighian tubule in insects [48], acting as mosquito natriuretic peptide that can stimulate Na+ rich urine [49], or modulated diuresis-related hindgut activity [50]. In crustaceans, Calci was characterized in the American lobster *Homarus americanus* and functioned as an intrinsic modulator of cardiac output [51]. Thus barnacle CalciA may be involved in

**Figure 8. Sequence alignment of barnacle bursicon peptides.** (A) Alignment of bursicon α subunit. Sequences are from: euphausiacean *Euphausia superba* [22]; decapods *Homarus gammarus* ADI86242.1 and *Carcinus maenas* EU139428; branchiopod *Daphnia pulex* [38]; insects *Tribolium castaneum* DQ138190, *Drosophila melanogaster* NM_142726, *Apis mellifera* NM_001098234, *Musca domestica* EF424614 and *Bombyx mori* NM_001098375; chelicerate *Ixodes scapularis* XM_002407468. (B) Alignment of bursicon β subunit. Sequences are from: decapods *Homarus americanus* ADI86243.1, *Carcinus maenas* EU139429; branchiopod *Daphnia pulex* [38]; insects *Tribolium castaneum* DQ156997, *Drosophila melanogaster* NM_135868, *Apis mellifera* NM_001040262, *Musca domestica* EF424613 and *Bombyx mori* NM_001043824; chelicerate *Ixodes scapularis* XM_002407469. Asterisk ‘*’ mark the conserved cysteine that will form dimers. doi:10.1371/journal.pone.0046513.g008
maintaining ionic homeostasis of hemolymph during barnacle development.

4.3 Pigment dispersing hormone. The expression level of PDH was 4-fold higher in cyprid than that in juvenile (Figure 10K). Crustacean PDH is homolog to pigment dispersing factor (PDF) discovered in insects. In insects, PDF served as a major output signal in the biological clock for fruit fly and cockroach [52–54], which in turn regulated physiological processes and behavior related to daily rhythms. PDF was also located at visual interneurons in the synaptic neuropil (lamina) underlying the compound eye of the housefly Musca domestica [55]. In crustaceans, PDH has been reported to induce pigment movements in chromatophores and retinal pigment cells [56], or affect electrical response to photic stimulation of the compound eyes [57]. According to Webster 1998 [30], strong PDH immunoreactivity was found in perikarya on the surface of the neuropil of the ventral ganglion and supra-esophageal ganglia in adult barnacle species, and thus PDH was suggested to have neuromodulatory roles in somatic extensions in adult barnacles. At this moment no information is available for localization of the PDH in barnacle larvae. The nervous system of cyprid is more complicated than adult, and only cypris larva has a pair of morphologically well-differentiated compound eyes. Higher expression of PDH in the cypris stage and its general function in vision suggest that it may be related to the photoreception of compound eyes in cyprids during larval settlement.

4.4 SIFamide. The expression level of SIFamide was nearly 6-fold higher in the cypris stage than in juvenile (Figure 10L). SIFamide peptide family is broadly distributed among arthropod and highly conserved. It has diverse functions and acts as a local autocrine/paracrine modulator. In Drosophila, SIFamide could mediate sexual behavior [58], while in crustaceans, it was related to dominance hierarchy of the prawn Palaemonetes monodon [22], or modulating pyloric neural circuit in the lobster Homarus americanus [60]. Immunohistochemistry work showed that SIFamide was densely accumulated in the olfactory lobe in the crayfish Procambarus clarkii, indicating its function in olfactory systems [59]. Another study confirmed the presence of SIFamide in the eyestalk neuropils of a crayfish and suggested its role in visual signal processing [61]. The choice of substratum for permanent attachment of competent cyprid relies on sensitive response to both physical and chemical characteristics of environment as well as conspecific biogenic cues [62]. Since SIFamide is related to processing high-order, multimodal input and transmitting tactile, olfactory and visual stimuli [32], a higher expression level in cypris stage is required for transmitting neural signals and detecting exogenic cues in the settlement processes.

4.5 Tachykinin-related peptide. Expression of TRP in barnacle was down-regulated in the cypris and juvenile stages, compared to its relatively high expression in naupliar VI and adult stages (Figure 10N). The TRP family represents one of the largest neuropeptide families in the animal kingdom and is widely distributed across invertebrate, protochordate, and vertebrate species [63]. Previous researches suggested that TRP might function as both central neuromodulators and circulating hormones [64]. The TRPs display multiple functions in the nervous system and different kinds of muscle, and most importantly in gut tissue among insects [65]. TRP is related to
Neuropeptides in the Barnacle *Balanus amphitrite*

Developmental stages of *Balanus amphitrite*
feeding status in locust evident by a decrease in immunoreactiviy after 48 hours of starvation [66]. In crustaceans, TRP was first discovered in the crab Cancer borealis and exactly the same sequence was then found in other seven crab species [37]. In general, TRP is related to food intake and digestion related functions. Since the cyprid larvae do not feed, the subsequent habitat selection and settlement behavior are dependent on energy reservation [67], i.e. lipids and vitellin-like protein [68]. During settlement, barnacle larvae undergo tremendous morphological changes and begin to feed from 2 to 5 days afterward [69]. TRP expression attained a relatively low level in the two non-feeding stages, cyprid and early juvenile, indicating its paracrine/hormonal control of feeding-related behavior of barnacles.

5. Prohormone Convertase Inhibitor Effectively Delayed Larval Settlement of *B. amphitrite*

Neuropeptides are derived from larger proprotein precursors which carry one or more mature peptides. Highly regulated posttranslational transformation is required for generating mature peptides with biological functions. After cleavage of N-terminal signal peptide, proprotein convertase (PC) cleaves at the mono- or diabasic cleavage sites of the remaining part of precursor, giving rise to peptide products that will undergo subsequent peptidase modification [70]. Two members of proprotein convertase family, PC2 and PC1/PC3, appear to play a preeminent role in neuroendocrine precursor maturation process in both mammalian and invertebrates [71]. In Caenorhabditis elegans, HPLC-MALDI-TOF analysis indicated a drastic reduction of types and abundance of neuropeptides in KPC-2/KPC-3 (PC homologs) mutant strains compared to wild type strains [70]. The kpc-2/egl-3 mutant was still viable, but its responsiveness to mechanical stimuli and egg-laying behavior were impaired [70].

To further explore peptidergic control of larval settlement of *B. amphitrite*, we performed settlement This inhibitor is the most potent commercial compound that specifically inhibits peptide production and maturation [28]. Bioassay result showed that this inhibitor effectively delayed larval attachment and metamorphosis of *B. amphitrite*, on a dose dependent manner. After 24 hours, larval metamorphosis was significantly inhibited when the inhibitor concentration ≥10 μmolL$^{-1}$ (Tukey test, **p<0.01, ***p<0.001), compared with cyprids incubated in AFSW or 0.5% DMSO as the control, while no significant effect was observed at 1 μmolL$^{-1}$ (Tukey test, p=0.81). The inhibition was unlikely to be caused by toxicity of PC inhibitor since the unsettled cyprids in treatment group were swimming normally. After incubation for 48 hours, most of the swimming cyprids in the treatment group settled and metamorphosed normally into early juveniles, and larval metamorphosis percentage among the controls and treatments was not different (Tukey test, p>0.05) (Figure 11). No mortality was observed for all the tested concentrations within the experimental duration. We may deduce that PC inhibitor restrained peptide maturation and thus cyprids delayed metamorphosis into juvenile. The real concentration of the compound in cyprids might be lower than the nominal concentration [11], and the peptide may be degraded by enzymes in hemolymph 48 hours after treatment. The complimentary peptide maturation pathways in addition of
PC could be another reason why settlement was not completely blocked by inhibitor.

Conclusion
In conclusion, we discovered fourteen neuropeptide and peptide hormone families/subfamilies through in silico transcriptome mining of *Balanus amphitrite*. The analysis of mature structure and sequence of the predicted neuropeptides provided a new evidence on evolution of barnacle neuropeptides. B-type allatostatin, calcitonin, pigment dispersing hormone and SFamide were up-regulated in cypriate stage and down-regulated after metamorphosis. Together with our bioactivity result of propionate converting inhibitor, we demonstrated the involvement of neuropeptides in larval metamorphosis. Our neuropeptide data also provide a platform for further elucidating the physiological functions of individual peptide. Specifically, synthetic peptide could be raised based on the predicted peptide structure, for exploring their spatial expression pattern through specific antibodies, or for in vivo test of their functions in barnacle through peptide treatment. Given that *B. amphitrite* is an important biofouling species worldwide, neuropeptide genes and their postulated functional role in larval settlement revealed in this study may shed light on the future development of novel antifouling compounds.

References

Supporting Information

Table S1 Mature neuropeptides/peptide hormones predicted from *Balanus amphitrite*. (PDF)

Table S2 Precursor sequences of neuropeptides and peptide hormones from *Balanus amphitrite*. Signal peptides are marked as green letter, precursor related peptides are marked as blue letter, and mature neuropeptides are marked as red letter. The putative mono-, di- or tribasic cleavage sites are underlined, and amino acid residues that predicted to be sulfated are shaded as pink. (PDF)

Table S3 Primers used for real-time PCR amplification. (PDF)

Author Contributions
Conceived and designed the experiments: X-CY P-YQ. Performed the experiments: X-CY Z-FC. Analyzed the data: X-CY JS ZC-F KM. Wrote the paper: X-CY Z-FC JS KM RW P-YQ.
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