**In Silico** Prediction of Neuropeptides/Peptide Hormone Transcripts in the Cheilostome Bryozoan *Bugula neritina*

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**Abstract**

The bryozoan *Bugula neritina* has a biphasic life cycle that consists of a planktonic larval stage and a sessile juvenile/adult stage. The transition between these two stages is crucial for the development and recruitment of *B. neritina*. Metamorphosis in *B. neritina* is mediated by both the nervous system and the release of developmental signals. However, no research has been conducted to investigate the expression of neuropeptides (NP)/peptide hormones in *B. neritina* larvae. Here, we report a comprehensive study of the NP/peptide hormones in the marine bryozoan *B. neritina* based on *in silico* identification methods. We recovered 22 transcripts encompassing 11 NP/peptide hormone precursor transcript sequences. The transcript sequences of the 11 isolated NP precursors were validated by cDNA cloning using gene-specific primers. We also examined the expression of three peptide hormone precursor transcripts (BnFDSIG, BnILP1, BnGPB) in the coronate larvae of *B. neritina*, demonstrating their distinct expression patterns in the larvae. Overall, our findings serve as an important foundation for subsequent investigations of the peptidergic control of bryozoan larval behavior and settlement.

**Introduction**

The bryozoan *Bugula neritina* is a cosmopolitan marine fouling organism. *Bugula neritina* are colonial and have a complex life cycle that consists of a rather short free swimming larval phase followed by a sessile adult stage [1]. *B. neritina* produces coronate larvae, an oval-shaped type of larva with a ciliated epithelium, a pair of simple photosensory eyespots, an undifferentiated apical cell mass (the apical blastema), an internal sac, and a pyriform organ. All these major larval tissues are innervated and connected by the larval nervous system [2]. Prior to the initiation of permanent attachment, the coronate larvae have a relatively short pelagic phase for substratum exploration. Metamorphosis can be triggered by both endogenous and environmental...
signals [3]. It is generally believed that the induction of larval settlement in *B. neritina* is a neurally regulated process. For example, previous studies have demonstrated that different neurotransmitters can be either inductive or inhibitory to larval settlement of *B. neritina* [4, 5]. The pyriform organ, apical disc and ciliated epithelial layer have been suggested to play a sensory role during the exploration process [6]. During metamorphosis, *B. neritina* larvae undergo dramatic morphological and structural transformations [3]. Following a series of morphological changes during the initial phase of metamorphosis, the larva permanently attaches onto the substratum, and the larval body is re-organized. Reed and Woollacott have demonstrated that substantial muscular contractions initiate the initial morphogenetic process [7]. It is clear that a well-designed neural circuit precisely regulates the musculature-mediated structural rearrangement. Therefore, a comprehensive understanding of the larval neural element is necessary.

Neuropeptides (NP) are small peptides that are produced by neurons and are generally less than 50 amino acids in length. After being synthesized in the cell body of neurosecretory cells, they are transported to a specific location along the neurite, such as the synaptic junction, and are released from the neurite in response to neural stimulation. Their action depends on the expression of the corresponding receptor in target cells [8]. NP are the largest and most diverse class of signaling molecules and are involved in the regulation of many physiological functions [9]. The diversity of NPs varies in different taxa. In past decades, major efforts have focused on the discovery and functional characterization of NPs in different animal phyla [10, 11]. The conventional NP identification process depends on bioassay-guided discovery. Although this approach enables the separation and identification of several classes of classical NPs, a substantial amount of starting material, namely the protein sample from the neuron, is needed. Consequently, this technique is limited to animals such as decapods or insects, from which sufficient protein can be extracted for peptide fractionation and mass spectrometry identification [12]. With the advance of new technologies, direct imaging mass spectrometry (IMS) has become a powerful and specific technique for the discovery of NPs during the last decade [13–15]. The limitation of the MS technique, however, is that the accuracy of mass spectrometry is critical. Recently, the development of high-throughput next-generation sequencing technology [16–18], as well as increasing public availability of expressed sequence tag (EST) libraries, has enabled an alternative approach to the discovery of NPs. Briefly, homology searches using known NP peptide sequences were performed with relatively unrestrictive matching criteria to avoid the exclusion of candidate NP transcripts from the EST library. Candidate genes were then filtered based on a number of NP hallmark characteristics, such as the presence of the N-terminal signal peptide, dibasic cleavage sites, and prediction of the mature NP [19]. In conjunction with online software programs, it is possible to predict the post-translational processing of the deduced peptides. In short, *in silico* genome/transcriptome mining has been shown to be a successful and effective method for the discovery of NPs [19–21].

Recently, a transcriptome database of various stages of metamorphosis of *B. neritina* was constructed [22]. This database provided the foundation for *in silico* transcriptome mining of NP expressed in *B. neritina* larvae. Here, we aimed to provide the first comprehensive report of peptide hormones in *B. neritina* larvae using *in silico* transcriptome mining. We identified 22 transcripts encompassing 11 NP precursor sequences. The expression of 11 NP precursors was validated by cDNA cloning. We successfully identified the spatial expression patterns of three NP transcripts in the coronate larva of *B. neritina*. This is the first neuropeptidome study of the bryozoan *B. neritina*.
Materials and Methods

Larval sample preparation

Adults from *B. neritina* colonies were collected from the floating rafts and other submerged objects at a fish farm in Trio Bench, Hong Kong (22°21'09 N, 114°16'15 E) from winter 2013 to spring 2015 and then maintained in an aquarium at 21°C with a flow-through seawater system at the Coastal Marine Laboratory of Hong Kong University of Science and Technology for less than seven days before use. Permission to collect *B. neritina* colonies from the fish farm was provided by the fish farm owner. Prior to larval release, adult colonies were transferred to a transparent fish tank containing 10 L of 0.22-μm filtered seawater. Spawning of free swimming larvae were triggered and concentrated by a point source illumination. For in situ hybridization, larval specimens were fixed in 4% paraformaldehyde in 0.22-μm autoclaved filtered seawater (AFSW). For RNA extraction, approximately 1000 larvae were transferred to a 1.5-mL Eppendorf tube. After removal of residual sea water, larval specimens in the tube were immediately transferred to a liquid nitrogen storage tank. RNA samples from larval specimens were extracted two months after cryogenic storage.

Transcriptome mining of NPs/peptide hormone precursors

The transcriptome database generated from our previous study was used to screen for NP hormone candidate transcripts. Database searches to discover NPs/peptide hormones were conducted using methods that were modified according to recent publications [19, 23–27]. Protein sequences of the known NPs and peptide hormones in invertebrates were downloaded from Uniprot Knowledgebase (http://www.uniprot.org/) using "NP", "hormone" or "neurohormone" as search keywords without "receptor", "receptor associated", "hypothetical", "uncharacterized", "signal", "cytochrome", "esterase", "transferase", "binding protein", "hydrolase", "lipase", "inducible protein", "methyltransferase" or "transmembrane". The screened sequences were transformed into FASTA format to generate a local peptide database. The transcriptome of *B. neritina* was blasted against the known peptide database mentioned above in tblastn mode to mine for putative cDNA sequences encoding NPs/peptide hormones. For each peptide query, the first three with the lowest E-value (less than 0.01) were chosen as candidates and manually checked for homology to known peptides.

Prediction of mature peptide structures

The structures of the selected candidate sequences were predicted based on previously established procedures [19]. These sequences were translated using the ExPASy translate tool (http://www.expasy.org/). Each deduced peptide was assessed for typical NP precursor features, including start and stop codons, the presence of an N-terminal signal peptide sequence, and pro-hormone convertase processing sites [28]. Signal peptide prediction was conducted using the online program SignalP 4.1 Server [29]. Pro-hormone cleavage sites were predicted based on the information supplied by Veenstra [30] and the Neuropred online program (http://neuroproteomics.scs.illinois.edu/cgi-bin/neuropred.py), as well as homology to known NP precursors. The web-based software DiANNA 1.1 was used to predict disulfide bridges in putative mature peptide sequences [31]. The online program Sulfinator was used to predict the sulfation state of Tyr residues [32]. In some cases, disulfide bridge formation and other post-translational modifications, e.g., cyclization of N-terminal Glu/Cln residues and C-terminal amidation at Gly residues, were predicted mainly based on homology to known peptide isoforms [24].
RNA extraction and cDNA synthesis

Total RNA was extracted from the free-swimming stage, the early pre-ancestrulae stage, the mid pre-ancestrulae stage and the juvenile stage of *B. neritina* using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the standard protocol provided by the manufacturer. To remove DNA contaminants, total RNA was treated with a TURBO DNA-freeTM kit (Ambion Inc., Austin, TX, USA). First-strand cDNA was prepared from the total RNA from each stage using M-MLV reverse transcriptase (USB® Product, Cleveland, OH, USA) with oligo(dT) primers [33].

RACE sequencing and peptide confirmation

Because NP precursor sequences generated from the transcriptome are often fragmented or incomplete, the rapid amplification of cDNA ends (RACE) technique was performed to obtain full-length open reading frames (ORFs) of the predicted genes for further confirmation. Two sets of gene-specific primers were designed based on the partial cDNA sequences from the *B. neritina* transcriptome database. The sequences of the reads and the corresponding primers are listed in **S1 Table**. For 5' RACE, an oligo(dC) tail was added to the 3' cDNA ends of the cDNA template by terminal deoxynucleotidyl transferase (TdT) (USB, Cleveland, Ohio, United States). Gene-specific reverse primers and an oligo(dG) (10) adaptor primer were used to perform nested PCR. The 3' RACE was performed in the same manner as described for 5' RACE, except an oligo(dT) adaptor primer and gene-specific forward primers were used. The primer sequences are listed in **S1 Table**. PCR products were gel-purified and then ligated into the pMD18-simple T vector (TaKaRa Bio Inc., Dalian, China), transformed and sequenced (BGI Company, China). NCBI Blastx was performed to check the homology between the full-length ORFs and the predicted NP/neurohormone amino acid sequences. If a cloned sequence did not match the predicted NP/neurohormone, it was excluded. All NP precursor transcript sequences were submitted to NCBI GenBank, and the accession numbers are shown in **Table 1**.

Sequence analysis

Corresponding NP sequences were downloaded from the NCBI protein sequence database, and the known NP genes and proteins were compiled. Predicted mature peptide sequences together with reference sequences were used for the alignment using the bioinformatics software Mega 6.0 [34].

In situ hybridization (ISH) (whole-mount) and section ISH (SISH)

Each target NP gene transcript was amplified using the transfected vector carrying the target gene fragment acquired by 3' RACE, and the corresponding gene-specific forward and reverse primers are listed in **S1 Table**. The T7 promoter sequence was conjugated to the 5’ end of the gene-specific reverse primer (**S1 Table**). The anti-sense digoxin-labeled probes were synthesized using the DIG RNA labeling kit (Roche Diagnostics, Nutley, NJ, USA). Swimming larvae were collected and fixed in freshly prepared 4% paraformaldehyde overnight at 4°C. The fixed samples were then washed three times with PBS to remove the fixative, dehydrated with 100% methanol and stored at -20°C until further processing. The experiment was conducted based on a previously described method [35] with minor modifications. The samples were rehydrated by sequential dilution series of methanol in PBS. The samples were washed three times with PBST (PBS-0.1% TritonX100) and then permeabilized with 10 μg/mL of proteinase K (Invitrogen) in PBST at room temperature for 7 minutes. The specimens were post-fixed in 4% PFA and then washed five times with PBST. After pre-hybridization at 56°C for 1 hour in a
hybridization mix, RNA probe hybridization was performed at 56°C overnight. Washing was conducted with pre-warmed hybridization mix (without heparin and salmon sperm DNA) using sequential concentrations followed by three washes with PBST. Prior to immunostaining, the hybridized samples were incubated with blocking solution (4% BSA in PBST) for 1 hour at room temperature. The samples were then incubated with anti-DIG-AP antibody (Roche Applied Science, Indianapolis, IN) at a concentration of 1:5000 in blocking solution at 4°C overnight with gentle rotation. Next, the samples were washed six times with PBST and then incubated in alkaline Tris buffer (0.1 M Tris-HCl pH 9.5, 50 mM MgCl2, 0.1 M NaCl, 1% Tween-20) containing NBT and BCIP (Promega) at room temperature in the dark. The samples were washed with stop solution until a deep blue or purple color developed. After mounting with 80% glycerol, the specimens were observed using a light microscope (Olympus, Tokyo, Japan). Because the whole-mount samples were not completely transparent, paraffin sectioning of the hybridized samples was performed to visualize the internal expression pattern.

Results and Discussion

NPs and peptide hormones predicted in *Bugula neritina*

In this study, putative NP-encoding transcripts were predicted *in silico* from the *B. neritina* transcriptome reported in a previous study, and then verified by molecular cloning. This is the first study to attempt to identify NP and peptide hormones in bryozoan species. In summary, 11 NP precursor sequences were predicted, and 10 of them were confirmed by transcript cloning (Table 1).

| Name         | Peptide family                  | Contig No. (From *B. neritina* transcriptome) | Transcript length (bp) | Reference accession No. | 5' RACE confirmed | 3' RACE confirmed | E-value | Score | GenBank accession No. |
|--------------|---------------------------------|-----------------------------------------------|------------------------|-------------------------|-------------------|-------------------|---------|-------|----------------------|
| BnVP-like    | Arginine-vasotocine             | BN_contig_97286, BN_contig_87480              | 599                    | AAX18227.1              | +                 | +                 | 4.00E-09 | 61.2  | KX095876             |
| BnPEP-like   | Pedal peptide/orcokinin neuropeptide | BN_contig_141741, BN_contig_166562, BN_contig_98929 | 792                  | AHB62384.1              | +                 | -                 | 1.00E-05 | 55.1  | KX095877             |
| BnFDSIG-like | Pedal peptide/orcokinin neuropeptide | BN_contig_220841, BN_contig_55496             | 697                    | AEE25644.1              | +                 | +                 | 1.00E-10 | 72     | KX095878             |
| BnNPF-like   | Neuropeptide Y                  | BN_contig_222301, BN_contig_184693            | 476                    | AGM46557.1              | +                 | +                 | 8.00E-10 | 61.6  | KX095879             |
| BnGHB        | Glycoprotein hormone-beta5-I    | BN_contig_4814                                | 949                    | CAR95348.2              | +                 | +                 | 6.00E-08 | 57.4  | KX095880             |
| BnILP-A      | Insulin-like peptide            | BN_contig_62648, BN_contig_127297             | 1220                   | XP_008642491.1          | +                 | +                 | 3.00E-27 | 112    | KX095882             |
| BnINS_B      | Insulin-like peptide            | BN_contig_36942                               | 371                    | Q9W7R2.1                | -                 | -                 | 4.00E-25 | 44.3  | KX095885             |
| BnINS_C      | Insulin-like peptide            | BN_contig_203950                              | 675                    | CAC20109.1              | +                 | +                 | 3.00E-04 | 59.7  | KX095886             |
| BnILP-D      | Insulin-like peptide            | BN_contig_236733                              | 1029                   | XP_011415724.1          | +                 | +                 | 5.00E-08 | 59.3  | KX095881             |
| BnILP-E      | Insulin-like peptide            | BN_contig_208700, BN_contig_236692            | 696                    | XP_012388351.1          | +                 | -                 | 1.00E-11 | 69.3  | KX095883             |
| BnILP-F      | Insulin-like peptide            | BN_contig_8730                                | 547                    | ABB23272.1              | -                 | +                 | 5.00E-17 | 83.6  | KX095884             |

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Because many NPs are relatively fragmented in the transcriptome dataset, the BLAST scores tended to be low (with a high E-value). This issue has been reported in previous studies and was not critical for the application of in silico methods [19]. Full-length cloning of NP transcripts was used to confirm the in silico predictions. The NP precursor sequences and predicted structures are listed in Table 1 and Fig 1, respectively.

The predicted NP transcript precursors identified in this study included six insulin-like peptides (BnILP-A, BnILP-B, BnILP-C, BnILP-D, BnILP-E, BnILP-F), two peptide-like sequences, BnPedal/orcokinin-like (BnPEP-like) and BnFDSIG-like, one vasopressin-like peptide sequence (BnVP-like), and one NP F-like sequence (BnNPF) and glycoprotein hormone-beta 5-I (BnGPB) (Table 1). Multiple sequence alignment analysis clearly demonstrated that the predicted protein sequences of BnVP-like, BnNPF, BnGPB and all six BnILP precursor transcripts were homologous to known NPs (Table 2, details discussed below). Their presence in the B. neritina transcriptome requires further confirmation. Although the spatial localization of the RYamide and FMRFamide NPs has been examined by immunohistochemistry in previous studies [36, 37], the transcripts of these two NPs were not detected in our study. NP production occurs mainly in the cell body of neurosecretory cells. In B. neritina swimming larvae, however, the nervous system is rather simple, comprising a small number of neurons and, potentially, only a few neurosecretory cells [2]. Thus, NP transcripts may be present at relatively low abundances in the transcriptome library prepared from whole swimming larvae. Rather low coverage of the NP transcripts was expected. The NP transcripts were rather fragmented and could not be recovered using our in silico prediction method. We predicted that the RYamide and FMRFamide NP transcripts were expressed but at very low abundances; therefore, they could not be recovered from the transcriptome. Relatively low transcript abundance may also explain our high failure rate (8 out of 11) in detecting the expression of NP...
### Table 2. Predicted neuropeptide/peptide hormones from *Bugula neritina*.

| Precursor transcript Name | Predicted mature peptide (variant) name | Predicted mature peptides |
|---------------------------|----------------------------------------|--------------------------|
| **BnFDSIG-like**          | P1                                     | SFDSIGNGPIGLKR            |
|                           | P2                                     | SFDGIGNGPIGLKR            |
|                           | P3                                     | SRDSIGFGPIGLKR            |
|                           | P4                                     | SFGGIGGRPASYKR            |
| **BnPep-like**            | P1                                     | YLERIGSGNALISKR           |
|                           | P2                                     | YLERIGSGNSVKK             |
|                           | P3                                     | QHLKGSGENSLPKKR           |
|                           | P4                                     | YLERIGSGNELVGKRR          |
|                           | P5-partial                             | YLERIGSGND                |
| **BnVP-like**             | **Vasopressin-like**                   |                          |
|                           | **Neurophysin**                        | TVEGKPALCRACGPGLAGQCYNGSACYCCGYFGCYNYRKTDDECQISSPTQLCKGFSCPFTIPGGSCSANGFCCCNSDTCRPSLTCVRLTNLYGEPEKHVR  |
|                           | **BnPep-like**                         | TPVFFPASGCLNUTRLTNVNGANHCVGFDGGCYCESYDILPEPYRDHRHQCFCGFAYETHVIELANCHQGYSPEVFKFPKTVSCTSCCRECDPMREVCMB  |
|                           | **BnNPFL**                             | GPPEPQSKFKSEELQYKLSLEEY(SOH)LAY(SOH)NDPVRFGKR  |
|                           | **BnILP-A**                            | DFEHMCHEDFSRSAGYSSCGRLTSVLKCDGNYLASYRKRR  |
|                           | Chain B                                | DLEKISLTETSEANSYLPRKR  |
|                           | Chain A                                | NYEYVEDGMGVIGICECCVTNTIRELDQYCESKR  |
|                           | **BnILP-B**                            | GTSAEEYKVTICDKSNPRMGCSQVFQYIVICNGNIGSSGFLTSDDR  |
|                           | Chain B                                | AIRPHVDSSNNRINTANSFLSQYKR  |
|                           | Chain A                                | QEFLEDERDRARMMGHICCECCHTCTELEKQYECGSKR  |
|                           | **BnILP-C**                            | TFSAAEHTYERTLSTYGRMRSRCGELDYLSCGYQYQYYRDRR  |
|                           | Chain B                                | AVSSLNNTPDOKVIQQEQETTANAHSVSHKASENFLFRQKR  |
|                           | Chain A                                | QHGFGFPSNSQGYCECCVYCGNDEIMQYCEEFKRR  |
|                           | **BnILP-D**                            | LERICHMDQSNKDPQCLRRQLYLSRIGNDICTPK  |
|                           | Chain B                                | HNHTEOLSEXSEGNNPTTANPIGSISSRETANLFHNKRR  |
|                           | Chain A                                | QOAEDSVNVTFSGIGICECCFHCPCSEYLEYGSKGKR  |
|                           | **BnILP-E**                            | DFEHTQTDDVDQSPYTFCGQLTNMVDFCKGDER  |
|                           | Chain C                                | LSQLAVLDVGTASITHDFISAKK  |
|                           | Chain A                                | ANSLIMFSAGLANAESGYCECCVNRCVTVELEQYCNPLAAIQPRLRR  |
|                           | **BnILP-F**                            | PYINKRR  |
|                           | Chain B (partial)                      | AFDDDSKROIQRVSNIFVSKNHSNFNLKPR  |
|                           | Chain A                                | NGHERSYGVVCECCINPCTLREM/DAVCAYKRR  |

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precursor transcripts by in-situ hybridization. To improve the recovery rate of NP transcripts, the most direct strategy is to increase the coverage depth of the *B. neritina* larval transcriptome. Deep coverage of the transcriptome has the advantage of facilitating the recovery of full-length transcripts and of increasing the possibility of resolving transcripts with a low abundance. However, this approach requires much higher sequencing costs and computational power. An alternative technique would be to isolate the neurons from swimming larvae and perform a transcriptomic analysis of the isolated neurons. Okano et al. has developed an enzymatic cocktail to dissociate neurons from *B. neritina* swimming larvae [38]. The development of a technique to isolate *B. neritina* larval neurons may enable high-resolution transcriptome profiling and facilitate the discovery of novel NP transcripts.

NP sequences and spatial expression pattern analysis

**FD$\text{SIG}$ NP and Pedal/orcokinin-like NP transcripts.** Two pedal peptide/orcokinin NP precursor transcripts, BnFD$\text{SIG}$-like and BnPep-like, were predicted. BnFD$\text{SIG}$-like consisted of two partial transcripts in the transcriptome reference base (Table 1). These two transcripts were aligned with the LYamide/FDS$\text{SIG}$ NP and pedal peptide precursor 2 from the marine polychaete *Platynereis dumerilii* [39], respectively. The full-length BnFD$\text{SIG}$-like transcript was acquired by 5′ and 3′ RACE. The deduced protein precursor of the BnFD$\text{SIG}$ transcript encodes a 293-amino-acid protein, in which the first 24 amino acids are the signal peptide. *In silico* cleavage of the predicted precursor protein yields eight mature peptides with 12 amino acids comprising four peptide variants (P1/2/3/4) (Fig 2A, peptide sequences listed in Table 2). Similar to the *P. dumerilii* LYamide/FDS$\text{SIG}$ NP, the predicted mature peptide variant P1 contains the C-terminal SFDS$\text{IG}$ motif, whereas the other three peptide variants possess one or two amino acid substitutions in the FDS$\text{IG}$ motif, such as SFD$\text{G}$IG in P2, SRDS$\text{IG}$ in P3 and SFG$\text{G}$IG in P4 (Fig 2B, Table 2). While these putative mature peptides of BnFD$\text{SIG}$-like greatly resembled the FDS$\text{IG}$ motif of the *P. dumerilii* LYamide/FDS$\text{IG}$ NP, they lacked the LYamide motif (Fig 2B). The predicted BnFD$\text{SIG}$ peptide variants P1, P2 and P3 exhibited a basic SxDxIGxGPIGL structure, whereas variant P4 shared little similarity with the other three variants (Fig 2B).

For another pedal peptide precursor transcript, BnPedal/orcokinin-like, although we were unable to reach the 3′ end of the transcript, *in silico* prediction of the deduced precursor partial protein sequence yielded at least six mature peptides (one partial sequence) with five peptide variants constituting 12–14 amino acid residues (peptide sequences listed in Table 2). The predicted mature peptides of BnPedal/orcokinin-like exhibited a basic C-terminal YLER-IGSGNxxxx(x)-NH2 motif structure. The predicted mature peptides of BnFD$\text{SIG}$-like and BnPedal/orcokinin-like shared a C-terminal xxxGxxxx structure (IGxG motif highlighted in the purple square in Fig 2B). Instead of a FDS$\text{G}$xG motif, the predicted mature peptides of BnPedal/orcokinin-like exhibited a LERIGSGN motif in the counterpart region (Fig 2B). Such motif structures exhibited remarkable similarity to orcokinin peptides from the Pacific Oyster *Crassostrea gigas*, which exhibited a (F/L)DRLGSG motif (Fig 2B). Interestingly, *C. gigas* pedal peptides shared much less similarity to the pedal peptides from the molluscan *Aplysia californica* and *Lymnaea stagnalis* compared with the putative BnPedal/orcokinin-like peptides (Fig 2B).

Pedal peptide/orcokinin (PP/OK)-type peptides are a family of structurally related NPs. Pedal peptides were first isolated from the pedal ganglion of *Aplysia californica* [40]. However, orcokinin was initially isolated from the abdominal nerve cord of the crayfish *Orconectes limosus* [41]. PP/OK-type peptides have been attributed a range of functions in different taxa, including myoactivity, seasonal physiology and endocrine regulation in arthropods,
locomotion in both annelids and mollusks [40, 41, 42, 43], and circadian regulation in the cockroach *Leucophaea maderae* [44–46]. Currently, nothing is known about the physiological functions of PP/OK-type peptides in bryozoans. Whole-mount *in situ* hybridization (WISH) and sections of WISH (Fig 3) of the BnFDSIG-like precursor transcript revealed expression at the junction between the epidermal and mesodermal blastemal layer of the apical organ as well as at the neuromuscular junction in the root region of the internal sac (Fig 3A & 3B). The BnFDSIG-like precursor transcript was also strongly expressed in the apical organ, with much weaker signal detected at the equatorial nerve cord and in the internal sac of both the epidermal and mesodermal blastemas (Fig 3A & 3B). The equatorial nerve cord was suggested to play both a sensory and locomotor role in swimming larvae [47]. While neurites from the equatorial nerve cord direct innervate ciliated corona cells, innervation of intercoronal sensory cells has also been reported [47]. Expression of the BnFDSIG-like precursor transcript in the equatorial
nerve ring (Fig 3A) suggested the involvement of BnFDSIG-like peptides in locomotion in *B. neritina* larvae. The absence of BnFDSIG-like precursor transcript expression in the centric part of the apical organ as well as the centric part of the larvae suggested that these peptides were not produced by the anterior nerve cord, which connects the sensory apical ciliated cells to the central nerve ring [2, 47]. The role of BnFDSIG-like precursor transcript expression in the junction between the epidermal and mesodermal blastemal layer of the apical organ is currently unclear.

Glycoprotein hormone

One putative glycoprotein hormone-β5 (GPB5) transcript was predicted by *in silico* prediction (Table 1). The full-length transcript exhibited weak but significant homology to GPB5 in *Bombyx mori*. This transcript encoded a 130-amino-acid protein, in which the first 24 amino acids were the predicted signal peptide (S1 Table). No dibasic cleavage sites were detected in this hormone precursor. Alignment with glycoprotein hormone beta homologs from vertebrates and invertebrates revealed nine highly conserved Cys residues (Fig 4). The GPB5 found in *B. neritina* contained conserved Cys residues, which likely form a cysteine knot structure in common with other glycoprotein hormone subunits [48]. The results of the glycoprotein hormone WISH (Fig 3A) and a section of WISH (Fig 3B) are shown in Fig 5. The spatial gene expression patterns of BnGPB5 were similar to those of BnFDSIG-like. However, this hormone was also expressed in the wall region of the internal sac (Fig 3B).

The glycoprotein hormone is highly homologous to other cysteine knot growth factor (CKGF) families, including bursicon, which is well known for its effects on cuticle tanning/melanization during the ecdysis process in insects [49]. In *B. neritina*, the spatial gene expression patterns of GPB were detected in the apical organ and the wall region of the internal sac, which form the epidermis of the body wall and secrete a cuticular exoskeleton that undergoes
calcification [6]. These observations suggest that BnGPB5 might be involved in the development of the adult body wall.

**Insulin-like peptides**

The insulin family comprises several members, including insulin, insulin-like growth factor (IGF) and relaxin in vertebrates [50–52], insulin-like peptide in insects [53] and the molluscan insulin-related peptide (MIP) in mollusks. Insulin in invertebrates is produced by
neuroendocrine cells in the central nervous system (CNS) [54]. Classical insulin precursor contains contiguous B-C-A peptides, in which the A- and B-chain peptides are linked together by three disulfide bonds after maturation. The C-chain peptide assists the formation of the linkage and is subsequently removed from the cleavage site [55].

Six putative insulin-like peptide precursor transcripts (BnILP-A, BnILP-B, BnILP-C, BnILP-D, BnILP-E and BnILP-F) were found in B. neritina. Among these genes, the full-length sequences of BnILP-A, BnILP-C and BnILP-D were isolated and sequenced using RACE (Table 1). Although only two of the six putative insulin-like peptide precursor transcripts covered the full ORF, multiple sequence alignment with insulin and insulin-like peptides homologs revealed a clear B-C-A chain peptide structure in all six putative mature insulin-like peptides, with a putative dibasic cleavage site at the junction between each putative chain (Fig 5). All six putative insulin-like peptide were predicted to form four cysteine bridges (Fig 5). The WISH results localized BnILP1 to the blastema of the apical organ (Fig 3), similar to the results obtained for BnFDSIG and BnGPB5. During metamorphosis the apical organ undergoes substantial development. We speculate that BnILP-A may regulate the development of the polypide during metamorphosis.

Neuropeptide F

Neuropeptide F (NPF) was first identified in the tapeworm Moniezia expansa [56], and it was considered to be homologous to neuropeptide Y in vertebrates. Mature NPFs are typically 36 amino acids long and possess the C-terminal motif –RXRFamide (commonly RPRFa) and two tyrosine residues at positions 10 and 17 from their C-termini [57]. Two neuropeptide F-like transcripts were recovered from the B. neritina transcriptome (Table 1). The full-length cDNA sequence of these two contigs exhibited homology to NPF from Nasonia vitripennis. The precursor of the neuropeptide F-like transcript in B. neritina (BnNPFL) contains 116 amino acids, among which the first 25 amino acids are the predicted signal peptide. The mature BnNPY contains 35 amino acid residues (Fig 6, Table 2). Alignment of the mature BnNPFL with homologs from mollusks, insects and vertebrates revealed that the two tyrosine residues that are known to undergo sulfation are conserved in the putative BnNPFL peptide. However, the putative BnNPFL peptide was unique; the highly conserved C-terminal Arg residues within the RXRF motif were replaced with Pro (Fig 6). Neuropeptide Y/F or NPY-related peptides were collectively isolated from the central nervous system of both vertebrates and invertebrates [58–60]. The nervous system of B. neritina larvae is much simpler than that of vertebrates and insects. Although there is no well-defined CNS in the larvae, the neural plexus, which appears to be the only integration center for all sensory inputs, is generally considered to be the CNS equivalent [2, 5]. It will be interesting to assess whether BnNPY peptide expression is restricted to the neural plexus or occurs throughout the whole neural network.

Vasopressin-type peptide

Vasopressin is one of the earliest characterized NPs. It was first discovered in the human pituitary gland [61]. Recent advancements in molecular biology have led to the discovery of vasopressin-type peptides in insects and crustaceans [62]. The vasopressin-oxytocin peptide family has a very ancient lineage and is structurally highly conserved from hydra to humans [63]. NPs within this family generally consist of 12 amino acids, with a disulfide bridge between the cysteine residues at positions 1 and 6 [61,64]. We recovered two transcripts that matched arginine-vasotocine from the B. neritina transcriptome database. Subsequently, we isolated the full-length transcript sequence of the precursor of this VP-type NP, which we labelled as BnVP-like. Multiple sequence alignment of the putative BnVP-like with oxytocin and vasopressin from both the protostome and deuterostome clades revealed a good correlation of cysteine motifs in both the carrier peptide neurophysin and the mature
vasopressin-like peptide (Fig 7). The putative BnVP-like peptide is unique to other VP homologs in which the mature peptide consists of 14 rather than 12 amino acids, with two hydrophobic residues (Leu and Ile) in between the C-terminal Gly₄ and Gly₃. In addition, unlike vertebrate neurophysin homologs, the putative neurophysin peptide skipped the hydrophilic region between the first C-terminal cysteine and the conserved hydrophobic region (Fig 7). To our knowledge, this is the first report of a VP-type peptide in Bryozoa.
Conclusion

We recovered the transcripts of 15 putative B. neritina NPs and peptide hormones by in silico transcriptome mining. Analysis of the mature structures and sequences of the predicted NPs provides the first description of bryozoan NPs. Most of the NPs identified in this work have obvious homologs in mollusks, insects and polychaetes. The spatial expression of three NP precursor transcripts (BnFDSIG-like, BnILP-B, BnGPB5) in B. neritina larvae was successfully characterized by in situ hybridization. Overall, the results of the present study extend our knowledge of peptidergic expression in bryozoans. Future comparative studies could focus on the shelled planktotrophic larvae that predated the non-feeding larvae in the evolution of gymnolaemate Bryozoa [65].

Supporting Information

S1 Table. RACE gene specific primers for candidate B. neritina NP precursor transcripts isolation. (DOCX)
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Author Contributions
Conceived and designed the experiments: LY YHW PYQ.
Performed the experiments: LY YHW.
Analyzed the data: LY YHW GZ LSH PYQ.
Contributed reagents/materials/analysis tools: LY YHW GZ LSH PYQ.
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