Brief Communication

Identification of genes related to learning and memory in the brain transcriptome of the mollusc, *Hermisenda crassicornis*

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The sea slug *Hermisenda crassicornis* (Mollusca, Gastropoda, Nudibranchia) has been studied extensively in associative learning paradigms. However, lack of genetic information previously hindered molecular-level investigations. Here, the *Hermisenda* brain transcriptome was sequenced and assembled de novo, producing 165,743 total transcripts. Orthologs of 95 genes implicated in learning were identified. These included genes for a serotonin receptor and a GABA-B receptor subunit that had not been previously described in molluscs, as well as an adenylyl cyclase gene not previously described in gastropods. This study illustrates the *Hermisenda* transcriptome's potential as an important genetic tool in future learning and memory research.

[Supplemental material is available for this article.]

Nervous systems of molluscs have been a focus of neuroscience research for many decades, yet their study has been impeded by lack of genetic information. The nudibranch *Hermisenda crassicornis* exhibits a simple form of associative learning, which has been studied extensively: it can learn to associate light changes with vestibular stimulation (Crow and Alkon 1978). The neural correlates of *Hermisenda*'s associative memories formed during light-movement pairings have been uncovered (Alkon 1980; Crow and Alkon 1980; Britton and Farley 1999; Tamse et al. 2003; Cavallo et al. 2014). *Hermisenda* has also been the subject of investigations related to many other aspects of neuroscience, including sensory and motor neuron physiology (Crow and Tian 2004; Nesse and Clark 2010; Jin and Crow 2011; Crow et al. 2013) and the evolution of behaviors and neurotransmitter systems (Lillvis et al. 2012; Newcomb et al. 2012; Lillvis and Katz 2013). A limited number of molecular-level studies have been performed on *Hermisenda* in these areas (Nelson and Alkon 1988; Crow et al. 1997). Broader-scale genetic experiments, such as identification of genes or specific genetic isoforms that produce proteins involved in *Hermisenda* associative learning, have been impossible without more extensive genetic information specifically from *Hermisenda*.

Transcriptomes have been sequenced in a small number of other gastropod species, including neuronal transcriptomes from *Aplysia californica* (Moroz et al. 2006; Fiedler et al. 2010; Heyland et al. 2011), *A. kurodai* (Lee et al. 2008; Choi et al. 2010), *Lymnaea stagnalis* (Bouetard et al. 2012; Sadamoto et al. 2012), and *Tritonia diomedea* (Senatore et al. 2015), which have been used in studies of learning and other aspects of neuroscience. The field of molluscan neurogenomics is growing thanks in large part to recent advances in sequencing technologies, which allow larger amounts of transcriptomic information to be sequenced at lower costs compared with technologies available a few years ago.

In this study, we describe the brain transcriptome of the sea slug *Hermisenda crassicornis*. The transcriptome was sequenced from central nervous system tissue mRNA, which included the cerebropleural ganglia, pedal ganglia, optic ganglia, eyes, and sta-

tocyst hair cells. Shotgun de novo assembly generated 165,743 total transcripts. To illustrate its usefulness as a genetic tool, we have identified orthologs of genes related to learning and memory from the *Hermisenda* transcriptome, including genes that were not previously identified in molluscs.

Paired-end sequencing of *Hermisenda* mRNA generated 109M 100-bp reads. Low-quality reads were trimmed using Sickle (Joshi and Fass 2011), and reads were assembled by de novo assembly using Trinity (Haas et al. 2013). The 165,743 total transcripts, or contigs, were grouped in the assembly into 99,944 Trinity Chrysalis components (e.g., comp1, comp2, etc.) and 115,126 Trinity Butterfly components (e.g., comp1_c0, comp1_c1 etc.). We consider the Chrysalis components as the completely nonredundant transcript groupings, since Butterfly components originated from Chrysalis-derived De Bruijn graphs, some of which subsequently partitioned during Butterfly due to low read support (Haas et al. 2013). The average contig length of the *Hermisenda* transcriptome was 778.81 bp (Table 1). The largest contig was 35,109 bp in length. Proteins were predicted in silico by TransDecoder. More than half of the predicted proteins with complete open reading frames (ORFs) were 300 amino acids (aa) or longer, indicating that the transcriptome assembly contains a large number of genes coding for full-length proteins (Supplemental Fig. S1A). Of the total number of TransDecoder-predicted proteins, 4912 proteins were complete, 3515 were missing the 5' end, 1851 were missing the 3' end, and 5404 were missing both the 5' and 3' ends (Supplemental Fig. S1A, inset).

To compare the *Hermisenda* brain transcriptome with previously published genetic information, BLAST searches were run against published data sets. Matching components were then filtered to select the best hit per component (BHPC). The *Hermisenda* transcriptome yielded 20,152 nonredundant BHPC matches with *E*-values below 1×10^{-6} by tBLASTx (i.e., protein-

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Table 1. *Hermisenda crassicornis* brain transcriptome assembly statistics. The de novo assembly of trimmed reads. (A) Transcriptome size and content. (B) Contig size and quality based on entire assembled transcriptome, or (C) based on longest isoform per component.

(A) Description of transcripts	
Total transcripts	165,743
Total Chrysalis components	99,944
Total Butterfly components	115,126
Percent GC	39.51
(B) Based on all transcripts (bp)	
Median contig length	389
Average contig	778.1
Total assembled bases	128,965,468
Contig N ₁₀	4851
Contig N ₂₀	3341
Contig N ₃₀	2483
Contig N ₄₀	1880
Contig N ₅₀	1400
(C) Based on longest isoform per subcomponent (bp)	
Median contig length	329
Average contig	604.5
Total assembled bases	69,593,541
Contig N ₁₀	3831
Contig N ₂₀	2566
Contig N ₃₀	1842
Contig N ₄₀	1300
Contig N ₅₀	903

level alignment) against a database created from *T. diomedea* brain transcriptome mRNA and 16,995 matches against an *A. californica* mRNA data set (Supplemental Fig. S1B). The invertebrate RefSeq protein data set matched the highest number of *Hermisenda* translated nucleotide transcripts, compared with mammalian and nonmammalian vertebrate RefSeq protein data sets, as well as the SwissProt protein data set (Supplemental Fig. S1C). These BLAST results indicate that the *Hermisenda* transcriptome is complete in its genetic coverage relative to other published transcriptome assemblies.

Using translated protein BLAST results from the comparison with the SwissProt protein data set, components were filtered to remove hits with *E*-values of 1×10^{-3} or greater, in order to remove matches made by chance alone. The 12,081 resulting transcripts were subsequently uploaded to BLAST2GO (Conesa et al. 2005), which used a cutoff value of 1×10^{-6} to infer homology with SwissProt database sequences, followed by InterProScan, GO-SLIM, and Enzyme Code Mapping. The resulting 8916 genes were assigned gene ontology (GO) terms (Ashburner et al. 2000). GO assignments were generated for each of the three major GO branches at BLAST2GO Level 2: molecular function, biological process, and cellular component (Supplemental Fig. S2A). GO terms were similar at level 2 compared with the previously published *L. stagnalis* (Sadamoto et al. 2012) and *T. diomedea* (Senatore et al. 2015) assemblies.

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000), enzymatic pathways were mapped via BLAST2GO-KEGG: 103 biological pathways were identified. These pathways incorporated 1143 transcriptome sequences, including 579 enzyme sequences. Seventeen pathways were identified as containing one or more enzymes related to learning (Supplemental Fig. S2C). Together, these analyses indicate that the *Hermisenda* transcriptome contains a large amount of genetic information, meaning that it can be used as a new tool in the study of molluscan learning.

Several intracellular mechanisms have been identified as mediating light-turbulence associative learning in *Hermisenda*. Hair cells activated by turbulence release γ -aminobutyric-acid (GABA) through a mechanism mediated by adenylyl cyclase (AC), protein

kinase A (PKA), and calcium (Alkon et al. 1993; Tamse et al. 2003). GABA release activates type B photoreceptor metabotropic GABA receptors, and when paired with light stimulation enhances photoreceptor response. Serotonin (5-HT) stimulation from interneurons paired with light also enhances photoreceptor responses. Within the photoreceptors, the light-turbulence pairing and subsequent GABA and 5-HT release activates pathways involving IP3/DAG, protein kinase C (PKC), and mitogen activated protein kinase (MAPK), and elevation of arachidonic acid (AA) (Supplemental Fig. S3). This leads to an increase in calcium within photoreceptors, which amplifies their excitability and is a correlate of memory storage (Blackwell 2006; Blackwell and Farley 2008). The specific genes underlying these protein interactions have not, however, been identified previously in *Hermisenda*. We have used the proposed model for *Hermisenda* associative learning as a basis to identify genes that code for proteins related to these changes (Supplemental Table S1), and have highlighted three categories of genes using phylogenetic analyses here.

5-HT modulates *Hermisenda* photoreceptors and interneurons during paired light and turbulence associative learning (Jin et al. 2009). Therefore, we investigated 5-HT receptor genes in *Hermisenda*. Four families of 5-HT receptor genes (families 1, 2, 4, and 7) had been previously identified in molluscs (Nagakura et al. 2010), and a fifth family (family 6) had been predicted from the *Aplysia* genome (XP_005105784.1). The 5-HT1 gene has undergone a duplication event in molluscs, resulting in 5-HT1a and 5-HT1b receptors (Nagakura et al. 2010). No 5-HT receptor genes had been previously identified in *Hermisenda* or other Nudibranchs.

The *Hermisenda* brain transcriptome revealed putative orthologs of each of the previously identified receptor genes, 5-HT1a, -1b, -2, -4, and -7. It also uncovered a 5-HT6 ortholog, which had previously not been identified as expressed from mRNA in any invertebrate, to our knowledge. A maximum likelihood phylogenetic tree shows that all of the families cluster with other known receptor genes (Fig. 1A). As previously shown, 5-HT2 receptors clustered with dopamine D2 receptors, whereas 5-HT6 genes were more closely related to dopamine D1 receptors (Mustard et al. 2005; Nagakura et al. 2010; Spielman et al. 2015).

We confirmed the existence of the 5-HT6 gene in the *Aplysia* (aplysiaenatools.org) and *Tritonia* brain transcriptomes. DNA plasmids were generated by three or more independent PCR reactions, followed by cloning to pGEM-T Easy vectors. Each plasmid was sequenced, and sequences were aligned using ClustalW to verify that sequence identity matched between the transcriptomes and the cDNA. *Hermisenda* 5-HT 1a, 1b, 4, 6, and 7 receptor genes were sequenced. *Tritonia* 5-HT 1a, 1b, 4, 6, and 7 receptor genes were also sequenced. The resulting PCR-generated DNA sequences were ~98% identical to the transcriptome sequences by ClustalW alignment (data not shown).

The transcriptome also contained a novel isoform of the 5-HT2 family, referred to here as 5-HT2b. The previously identified molluscan 5-HT2 type gene will be referred to as 5-HT2a here. The 5-HT2b_{Herm} gene fragment predicted a 335 amino acid-long protein containing transmembrane domains 1–5, only, and aligned by BLAST to arthropod 5-HT2b receptors. The 5-HT2a_{Herm} gene was fragmented between two contigs: comp74520_c0_seq1 predicted a 314 amino acid protein containing transmembrane domains 1–5, while comp77190_c0_seq1 predicted a 383 amino acid protein, which coded for intracellular loop 5 and transmembrane domains 6–7. To our knowledge, 5-HT2b has not been previously identified in any other mollusc. To be assured that the gene is molluscan, we identified 5-HT2b homologs using BLAST searches in the *Aplysia* (aplysiaenatools.org) and *Tritonia* brain transcriptomes. Phylogenetic analysis and BLAST comparisons revealed that the molluscan 2b receptor clustered more closely with

Figure 1. 5-HT receptor phylogenetic relationships. (A) Maximum likelihood phylogenetic tree showing relationships between receptor subtypes. Bootstrap values are shown for each node. Subunits were aligned using transmembrane regions 1–5 and 6–7, or 1–5 only for fragmented receptor subtypes. Receptors that were identified from *Hermisenda* or *Tritonia* are shown in bold and marked with an asterisk. Receptors that were identified as novel in molluscs (5-HT2b) or invertebrates (5-HT6) are shown in light and underlined text. The dopamine receptors D1 and D2 are also shown, as they are part of the superfamily of aminergic receptors. (B) 5-HT receptor model showing seven transmembrane domain regions with location of predicted ligand binding site and G-protein activation site shown. (C) 5-HT2b ClustalW alignment shows location of predicted ligand binding conserved cysteine residue, shaded in light gray, and predicted G-protein activation site shaded in light gray for DRY motif, dark gray for DRF motif. Species abbreviations: *Hermisenda crassicornis* (Herm), *Tritonia diomedea* (Trit), *Aplysia californica* (Aply), *Drosophila melanogaster* (Dros), *Rattus norvegicus* (Rat), *Procambarus clarkii* (Proc).

the arthropod 5-HT2b gene than with the molluscan 5-HT2a gene. Note that although the 5-HT2 receptors in invertebrates and vertebrates form a cluster, the nomenclature for 5-HT2 subtypes in vertebrates and invertebrates does not correspond because the genes diverged in each group after the protostome/deuterostome split (Peroutka and Howell 1994).

Interestingly the crustacean 5-HT2b protein contains a highly derived DRF motif, which causes constitutive activity of the associated G-protein activation site (Clark et al. 2004), instead of a DRY-motif G-protein activation site, which is more common in 5-HT receptors. The DRF-motif was also found in the molluscan 5-HT2b predicted proteins (Fig. 1C), which might be further indication that the 5-HT2b gene evolved before the lophotrocozoan-ecdysozoan split. Alternatively, it could indicate parallel evolution of G-protein activation site motifs. The identification of a 5-HT2b gene may provide useful information about the functions of 5-HT receptors in molluscan learning.

We PCR amplified, cloned, and sequenced 5-HT2a and 5-HT2b genes from cDNA generated from *Hermisenda* and *Tritonia* brains and from *Hermisenda* whole-body cDNA. In *Hermisenda*, the initial PCR amplification indicated that 5-HT2a

and 2b genes were more highly expressed in whole-body cDNA compared with brain cDNA. This indicates that while 5-HT2a and 2b are expressed at very low levels in the *Hermisenda* brain, they are expressed at higher levels elsewhere in the body. RSEM (Li and Dewey 2011), a program that estimates relative abundance of genes in the transcriptome by providing transcripts per million (TPM) values, indicated that these genes were in very low abundance in the brain (see RSEM Relative Transcriptome Abundance, Supplemental Table S1; *Tritonia* RSEM abundance not shown). To verify the RSEM data, we compared 5-HT2a^{Herm} (Supplemental Table S1, TPM 3.38) with the *Hermisenda* 5-HT transporter (Supplemental Table S1, TPM 166.17), and the *Hermisenda* Pedal Peptide 3 Precursor (contig ID comp64571_c0_seq1, TPM 1264.32). Using absolute real-time quantitative PCR (qPCR), we found that there was a strong correlation between the RSEM-predicted expression level in the transcriptome and the amount measured from whole-brain tissue by qPCR (Supplemental Fig. S4).

GABA is another neurotransmitter that modulates light-movement paired learning (Schultz and Clark 1997; Blackwell 2002). No GABA receptor genes had been previously described from *Hermisenda*. Metabotropic GABA type B receptor subunits 1 (GABA-BR1) and 2 (GABA-BR2) were predicted from the *Aplysia* genome (XM_005092746.1 and XM_005109637.1) and a GABA-BR1 subunit gene was described in the *Tritonia* brain transcriptome (Senatore et al. 2015). The BR1 and BR2 subunits most likely function as heterodimers, as they do in other species (Kammerer et al. 1999). A GABA-BR3 subunit was identified in *Drosophila* (Mezler et al. 2001), but had

not been previously identified in any molluscs. We identified GABA-BRs in the *Hermisenda* transcriptome, and verified sequences by tBLASTx or BLASTx against NCBI and UniProt/SwissProt databases. GABA-BR1 genes were identified in the *Hermisenda*, as well as the *Aplysia* brain transcriptomes. GABA-BR2 genes were identified in *Hermisenda*, *Tritonia*, and *Aplysia* transcriptomes. A GABA-BR3 subunit was also identified in the brain transcriptomes of each of the three species, which aligns more closely with the *Drosophila* GABA-BR3 subunit than it does with molluscan GABA-BR1 or GABA-BR2 subunits (Fig. 2). The function of the GABA-BR3 gene in *Drosophila* is unknown, however (Mezler et al. 2001), so we cannot predict whether this gene is involved in molluscan learning, although the newly identified GABA-BR1^{Herm} and GABA-BR2^{Herm} genes most likely play roles in mediating *Hermisenda* light-vestibular stimulation pairing.

Adenylyl cyclase (AC) is part of the second messenger system required for memory formation, and is involved in changes within the presynaptic hair cells that occur during *Hermisenda* associative learning (Tamse et al. 2003). In *Aplysia*, four AC genes had been previously identified from families known as AC1, AC5/6, AC2/4/7, and AC9 (Sossin and Abrams 2009; Lin et al. 2010). Of

Figure 2. Phylogenetic relationships between GABA-B receptor subunits. (A) Diagram of GABA-B receptor subunits, with predicted GABA-BR3^{Herm}. The seven transmembrane domains, heterodimeric carboxy terminals, and venus-flytrap motif amino terminals are shown. (B) Maximum likelihood phylogenetic tree of GABA-B receptor subunits. Subunits were aligned using transmembrane regions 1–7. Bootstrap values are shown for each node. Receptor subunits identified for *Hermisenda*, *Tritonia*, or *Aplysia* are shown in bold and marked with an asterisk. Receptor subunits that were identified as novel in molluscs are shown in bold and underlined text. Species abbreviations: *Hermisenda crassicornis* (Herm), *Tritonia diomedea* (Trit), *Aplysia californica* (Aply), *Drosophila melanogaster* (Dros), *Rattus norvegicus* (Rat).

those genes, the AC1 family is calcium-sensitive, and is involved in learning (Abrams 1985). Orthologs of each of the four AC genes were identified in the *Hermisenda* and *Tritonia* transcriptomes (Fig. 3). The *Hermisenda* and *Tritonia* AC genes were fragmented into several contigs, which spanned portions of the C1 region or transmembrane spanning domains. It may be that the transcriptome assembly did not detect the entire genes, since it would be unlikely that all known gene orthologs are truncated in these species. The fragmentation of the molluscan genes may have caused the resulting phylogenetic tree in Figure 3 to indicate that *Drosophila* and rat AC genes are more closely related to one another than they actually are. *Hermisenda* and *Tritonia* AC genes were named here using the naming convention previously published for *Aplysia* (Sossin and Abrams 2009).

In addition to the four previously identified AC genes, a fifth family, known as AC3, has been predicted from *Aplysia* genomic DNA (NCBI reference XM_005108194.1) and was also identified in the *Lottia gigantea* genome (Sossin and Abrams 2009), but had not been identified as being expressed as mRNA in molluscs. The *Hermisenda* transcriptome contained an ortholog of the AC3 gene. A similar gene was also found in the *Aplysia* transcriptome, although no AC3 gene was found in the *Tritonia* transcriptome (Fig. 3). *Tritonia*'s missing AC3 gene may be explained by an incomplete transcriptome, but could also be because the ortholog was lost in that species.

In conclusion, we have sequenced and de novo assembled the *Hermisenda crassicornis* brain transcriptome, generating 165,743 total transcripts. Using BLAST annotations of the assembled transcriptome and its TransDecoder-predicted proteins, the *Hermisenda* transcriptome was found to be highly similar in

gene content to other published transcriptomes. BLAST searches against SwissProt and RefSeq, in combination with BLAST2GO annotation, revealed a large number of transcripts that are likely orthologs of previously published genes. Although a portion of the transcripts revealed fragmented genes, TransDecoder analysis showed that at least 50% of predicted proteins greater than 300 amino acids in length were full-length proteins. As assembly methods continue to improve, greater assembly coverage may be attained using this transcriptome, therefore we have published the raw transcriptome data to NCBI BioProject PRJNA270545, and have published a BLAST-searchable assembly at <http://neuroscience.gsu.edu/blast/>.

To illustrate its power as a tool for learning and memory research, we selected genes that produce proteins involved in learning and mined their orthologs from the *Hermisenda* transcriptome. We identified 95 genes by BLAST analysis as orthologs of learning-related genes. Of those, we highlighted three families of genes by phylogenetic analysis: 5-HT receptors, GABA-B receptors, and ACs. Within each gene family, one or more genes were identified that are novel for molluscs. Their identification indicates that the *Hermisenda* transcriptome is not only able to identify orthologs of *Aplysia* learning and memory genes, but can advance the field of molluscan learning and memory studies by identifying genes previously not described in molluscs. Gastropod molluscs are useful research subjects because their brains contain relatively simple neural circuits composed of identifiable neurons, but their value as a genetic research model has

Figure 3. Phylogenetic relationships between adenylyl cyclase (AC) genes. Maximum likelihood phylogenetic tree showing relationship between AC families. Bootstrap values are shown for each node. Genes identified as novel for *Hermisenda* and *Tritonia* are shown in bold and marked with an asterisk. Genes that were identified as novel in gastropods are shown in bold underlined text. Fragments of the *Hermisenda* and *Tritonia* genes, and the *Aplysia* AC3 gene, were identified from their transcriptomes and aligned against full-length orthologs from *Aplysia*, *Drosophila*, and rat to determine their orientation. Most fragments aligned to the C1 region of the gene or to one of the transmembrane domain spanning regions. For previously published species, full-length proteins were used in the alignment. Species abbreviations: *Hermisenda crassicornis* (Herm), *Tritonia diomedea* (Trit), *Aplysia californica* (Aply), *Lottia gigantea* (Lott), *Drosophila melanogaster* (Dros), *Rattus norvegicus* (Rat).

been limited to a few species. The *Hermisenda* transcriptome will increase knowledge of the neural correlates of learning by providing new genetic information and a basis for gene manipulation that was not previously possible, therefore making it a beneficial tool for learning and other molluscan research in the future.

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