

# Neuronal Transcriptome of *Aplysia*: Neuronal Compartments and Circuitry

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## SUMMARY

Molecular analyses of *Aplysia*, a well-established model organism for cellular and systems neural science, have been seriously handicapped by a lack of adequate genomic information. By sequencing cDNA libraries from the central nervous system (CNS), we have identified over 175,000 expressed sequence tags (ESTs), of which 19,814 are unique neuronal gene products and represent 50%–70% of the total *Aplysia* neuronal transcriptome. We have characterized the transcriptome at three levels: (1) the central nervous system, (2) the elementary components of a simple behavior: the gill-withdrawal reflex—by analyzing sensory, motor, and serotonergic modulatory neurons, and (3) processes of individual neurons. In addition to increasing the amount of available gene sequences of *Aplysia* by two orders of magnitude, this collection represents the largest database available for any member of the Lophotrochozoa and therefore provides additional insights into evolutionary strategies used by this highly successful diversified lineage, one of the three proposed superclades of bilateral animals.

## INTRODUCTION

*Aplysia* and related opisthobranchs are free-living representatives of the Mollusca (class Gastropoda), the second

largest phylum in the animal kingdom after the Arthropods. Molluscs have more than 100,000 extant species—marine, freshwater, and terrestrial—and trace their origin to the Cambrian period (Brusca and Brusca, 2003; Pojeta et al., 1987). Molluscs are of further phylogenetic interest because they are an exceedingly diverse and evolutionarily highly successful bilaterian lineage equivalent in their significance to chordates, arthropods, and nematodes. Their success is, however, based upon a different body plan and different adaptive strategies. To understand their strategies better, it is useful to have a better inventory of gene gain and loss in the major bilaterian lineages represented by *Aplysia*, *Drosophila*, or *C. elegans*. Despite their importance, however, the molluscs have not previously been studied on a genome-wide scale. Indeed, while there is now good genomic information on two of the three proposed superclades of bilaterian animals the Deuterostomia (echinoderms, hemichordates, and chordates) and Ecdysozoa (Arthropods, Nematodes, and four to five smaller phyla), evolutionary analysis is seriously limited by lack of genomic information of the third proposed superclade, the Lophotrochozoa (i.e., protostomes with ciliated larvae) to which Mollusca belongs (Aguinaldo et al., 1997; de Rosa et al., 1999; Halanych, 2004).

Although the Lophotrochozoa constitute more than 15 animal phyla (Halanych, 2004), none of the representatives of this group, the largest and systematically most diverse of these three presumed superclades of bilateral animals, has had its genome sequenced. In fact, expressed sequence tag (EST) collections from molluscs are very limited. As a result, the position of molluscs within the Lophotrochozoa and the relationships within the molluscan phylum among the phylogeny of bilateral animals, are all

poorly understood and controversial (Brusca and Brusca, 2003; Giribet et al., 2006; Grande et al., 2004a, 2004b; Halanych, 2004; Haszprunar, 2000; Lydeard et al., 2000; Nielsen, 2001; Passamaneck and Halanych, 2006; Valentine, 2004; Vonnemann et al., 2005).

Beyond phylogenetic relationships, our understanding of the basic molecular aspects of nervous system biology, such as genes controlling the maintenance of cellular diversity, the generation of specific neuronal circuitry, and the modifications of neural circuitry by learning and memory, suffers from limited knowledge about the repertoire of genes expressed by individual neurons. For example, we know little about the genes that distinguish the identities of one identifiable neuron from another, or even a motor neuron from a sensory neuron or from an interneuron. The anatomical complexity of most nervous systems poses obstacles for the detailed study of cellular identity in relation to behavioral functions.

These obstacles can be overcome in the *Aplysia* nervous system in part by the accessibility of individual ganglia and specific neurons within these ganglia and the ability to identify individual nerve cells that play roles in specific behaviors (Kandel, 1976, 1979). *Aplysia* has only about  $10^4$  central nerve cells (Cash and Carew, 1989; Kandel, 1976) compared to the  $2 \times 10^5$  neurons of *Drosophila* and about  $10^{11}$  neurons of mammalian brain. Moreover, in mouse, *C. elegans*, and *Drosophila*, most of the nerve cells are relatively small. By contrast, many of the neurons located in the *Aplysia* ganglia are large, and some are gigantic (100–1000  $\mu\text{m}$  in diameter). Because of this, *Aplysia* has been useful for cell and molecular biological studies of behavior, learning, and memory, complementing the genetic studies in *Drosophila* and mice. Many of these large identified cells, such as the metacerebral cells (MCC), have well-defined homologs even in distantly related species (Kandel and Tauc, 1966; Sakharov, 1974; Weiss and Kupfermann, 1976), a feature that lends itself to the comparative study of behavior at the cellular level.

As with *C. elegans*, the identified nerve cells in *Aplysia* form precise connections with one another. Thus, the connections between identified cells of a neural circuit can be mapped on a cell-to-cell basis for a variety of behaviors ranging in complexity from simple withdrawal reflexes to complex fixed action patterns such as locomotion, feeding, and defense reactions (Hening et al., 1979; Jahan-Parwar and Fredman, 1979; Kupfermann, 1974; Kupfermann and Kandel, 1969). Moreover, these behaviors are modulated by various forms of nonassociative and associative forms of learning, as well as by arousal and motivational states (Cleary and Byrne, 1993; Kupfermann and Weiss, 1982; Rosen et al., 1989; Fitzgerald et al., 1997; Kandel, 2001). Components of these defined neuronal circuits can be reconstructed in cell culture to study both specific cells and cellular compartments at high resolution (Kandel, 2001).

Despite these distinct advantages, research in *Aplysia* has been seriously handicapped by the deficit in genomic

information. As part of an attempt to correct this deficit systematically, we generated more than 175,000 ESTs and characterized the global transcriptome at three levels of neuronal organization: (1) the entire central nervous system (CNS) consisting of nine morphologically distinct ganglia (Figure 1C), (2) individual identified neurons, such as the sensory cells and motor cells of the gill withdrawal reflex and MCC serotonergic modulatory interneurons (Figure 1D), and (3) neuronal processes from the same MCC and sensory neurons (see also Moccia et al., 2003). In this way, we were able to identify general neuronal markers as well as markers for subcellular populations of transcripts, which provide a beginning for exploring genomic bases of neuronal identity and the roles of generalized and localized mRNA translation in synaptic specificity and growth. Gene clusters represented by these ESTs appear to comprise more than half of the protein coding genes of *Aplysia*, including several hundred novel genes potentially involved in cellular signaling, development, and synaptogenesis.

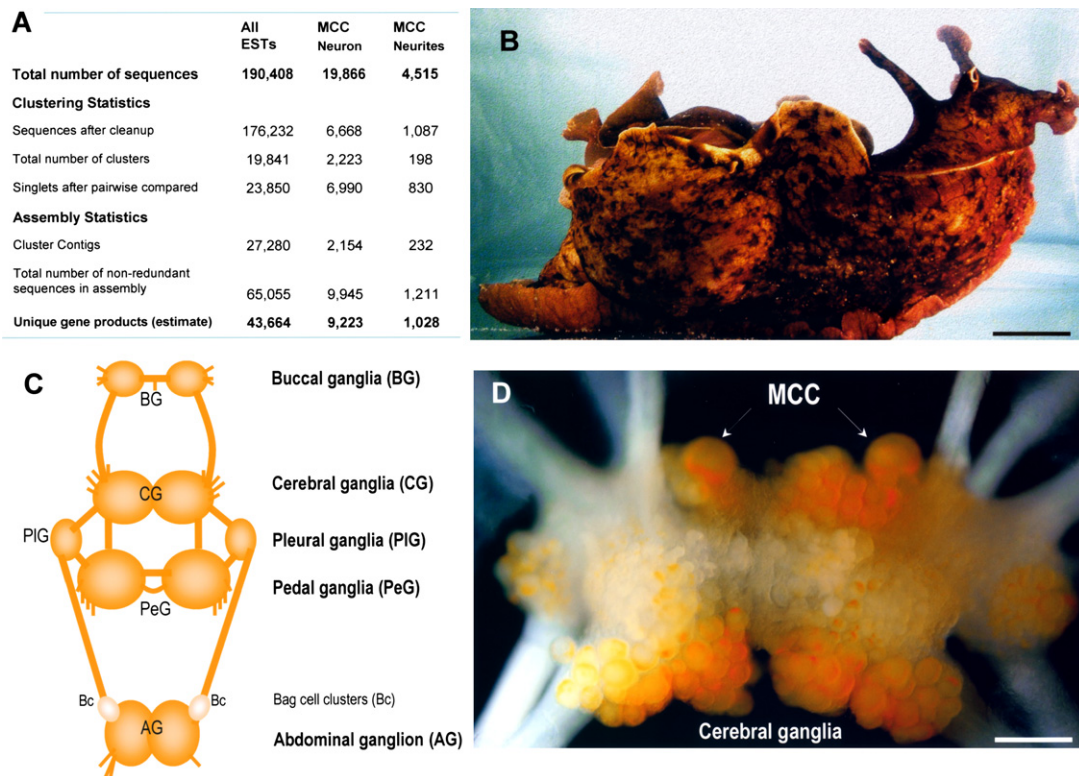
## RESULTS AND DISCUSSION

### Neuronal Transcriptome of *Aplysia californica*: Assembly Statistics and Development of a Neurotranscriptomics Database

We have generated 190,408 ESTs from normalized and nonnormalized cDNA libraries prepared from the *Aplysia* CNS. Figure 1A summarizes the assembly statistics for the ESTs obtained from the entire collection as well as from individual neurons and their processes. A list of the sources for each cDNA library is listed in Table S1. After removal of ribosomal, mitochondrial (Knudsen et al., 2006), and short sequences (less than 100 bases), the clustering and assembly of 176,232 neuronal ESTs yielded 19,814 total clusters and 65,055 total nonredundant sequences (Figure 1A). Of these, we detected a total of 24,422 open reading frames (ORFs  $\geq 100$  aa). More than 11,000 predicted *Aplysia* gene products had recognizable similarity to 3,047 unique protein domains (Pfam, Table S2).

As the next step, we have characterized transcriptome of individual cells. First, we used microarrays to identify transcripts in the sensory and motor neurons of the gill-withdrawal reflex. Second, we generated libraries from individual identified serotonergic neurons (MCC) and obtained 9,223 unique gene products expressed in these cells. Finally, we generated libraries from pure neuronal processes of the MCCs and obtained about 1,000 nonredundant sequences.

Figure 2 and Table S3 provide a breakdown of the annotated *Aplysia* sequences using characteristics based on mappings to gene ontology (GO): predicted “molecular function” and “biological process.” The largest functional categories for CNS transcripts were those related to binding proteins (36.1%) and enzymes (28.5%), followed by transporters (10.7%), signal transducers (7.7%), and developmental proteins (6.1%). About 30% of the annotated



**Figure 1. Construction of an *Aplysia* Neurotranscriptomics Database**

(A) Table summarizing statistics for the neuronal transcriptome project including collections of ESTs from identified MCC neuron and neuronal processes (neurites) of the same cell.

(B) *Aplysia californica*, a free moving animal. Scale: 4 cm.

(C) Schematic representation of the *Aplysia* central nervous system and its nine major ganglia, including paired buccal (BG), pedal (PeG), pleural (PiG), and cerebral (CG) as well as a single abdominal (AG) ganglion; a pair of neurosecretory clusters (Bag cells: Bc) is located in the abdominal ganglion and involved in the control of egg-laying behavior.

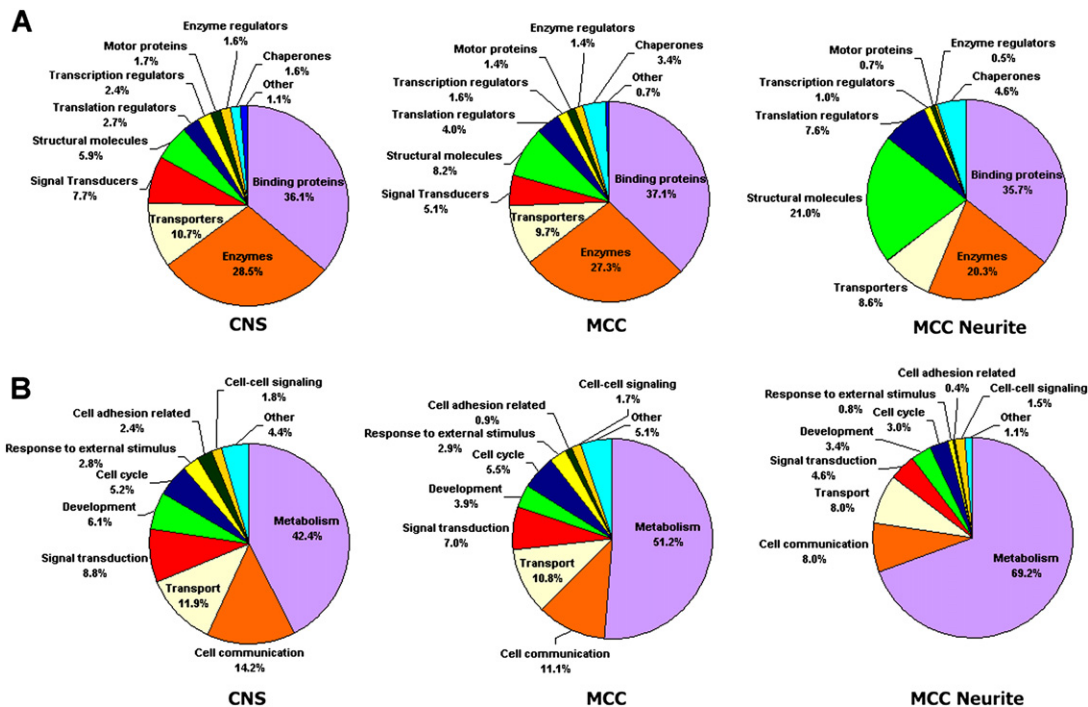
(D) Dorsal view of the left and right cerebral ganglia of *Aplysia* and position of serotonergic modulatory neurons (MCC). All connective tissues were removed to reveal individual neurons located at the surface of the ganglia. Scale: 400  $\mu$ m.

CNS transcripts in *Aplysia* were predicted to be membrane-associated (including 6% or 444 transcripts integral to the plasma membrane), and about 5% were putative extracellular secretory proteins and neuropeptides.

Sequencing of nearly 10,000 ESTs from nonnormalized libraries from the pedal-pleural ganglia allowed us to estimate the relative abundance of selected transcripts and screen for the presence of potential neuron specific markers that were previously unknown for molluscs and many other members of this clade (see Table S4). The most abundant class of ESTs contains neuropeptides (e.g., pedal peptide, pleurin, achatin, and FMRF-amide), followed by the cytoskeletal proteins (e.g., neuronal isoform of  $\beta$ -tubulin and  $\alpha$ -actin), glial secretory proteins (e.g., *Aplysia* glial protein [Ag] and Acetylcholine Binding Protein [AChBP]), and components related to protein synthesis. With the help of this EST collection, we obtained full-length cDNA sequences for several *Aplysia* genes relevant for future analysis of interneuronal signaling (Table S5). Web-accessible resources and annotated searchable

databases were created and are available from links provided at the end of this paper.

Comparison of our database against National Center for Biotechnology Information's KOG (eukaryotic clusters of orthologous groups) database (Tatusov et al., 2003) revealed that our ESTs have homologs to about 55% of KOGs shared among all seven represented organisms (*Caenorhabditis elegans*, *Drosophila melanogaster*, *Homo sapiens*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Encephalitozoon cuniculi*) and 40% of the KOGs when we restrict the analysis to human, *Drosophila*, and *C. elegans*. These comparisons imply that the annotated sections of our database cover roughly 50% of commonly expressed genes, including housekeeping genes and other genes expressed in nearly all organisms and about 40% of commonly expressed genes found only in organisms with a nervous system. Table 1 presents a comparison of signal transduction components currently identified in *Aplysia* with those from the genomes of *Drosophila*, *C. elegans*, and humans. Based



**Figure 2. Comparison of ESTs Identified from CNS, MCC Somata, and MCC Neurites**

(A and B) Annotated ESTs broken out according to major GO categories by both molecular function (A) and biological process (B) for ESTs obtained from the whole CNS, MCC somata, and MCC neurites. While the majority of categories stay relatively constant in each mRNA source, there are significantly fewer signal transducers and more structural molecules found in MCC neurites.

on the comparison of ion channels and homologs to KEGG metabolic and signaling pathways (Table S6), we estimate that the entire *Aplysia* EST database represents ~50%–70% of the expressed genes in the nervous system.

### *Aplysia* in an Evolutionary Context

The availability of the *Aplysia* transcriptome allows us to address questions about the phylogenetic position of molluscs among other animal phyla. All bilaterians are thought to be grouped into three major superclades (Aguinaldo et al., 1997; de Rosa et al., 1999; Peterson and Eernisse, 2001; Philippe et al., 2005). The Deuterostomes are proposed to occupy the basal branch of the tree, along with the two more derived sister superclades, the Ecdysozoa and the Lophotrochozoa (containing more than 15 animal phyla including molluscs). This tripartite classification, initially based on analysis of rRNAs and selected HOX genes (de Rosa et al., 1999; Halanych, 2004; Passamanek et al., 2004), has now been challenged by recent molecular studies (Philip et al., 2005; Rokas et al., 2005; Wolf et al., 2004) and by data from developmental biology (Nielsen, 2001, 2005). As a result of the limited genomic information available from the representatives of many invertebrate phyla, even the existence of two monophyletic protostome superclades is controversial (Passamanek and Halanych, 2006).

We used the sequence data obtained from *Aplysia* to re-examine whether molluscs are a sister taxon of Ecdysozoa or whether they are best grouped together with Deuterostomes, as suggested by Davidson and coworkers (Chen et al., 2004) based on their interpretation of new Precambrian fossils. For this phylogenetic analysis, we selected a cnidarian (*Hydra*) for outgrouping the bilaterian animals, two insect species, two worms, the zebrafish (*Danio*), the ascidian (*Ciona*) and an echinoderm, the sea urchin (*Strongylocentrotus*). The latter two were chosen as members of basal chordates and deuterostomes, respectively. These species represent all major clades, for which genome or large-scale EST projects have been performed. We analyzed 45 protein families (Table S15) based on their known conservative evolution and wide distribution among species (Wolf et al., 2004).

The results of this phylogenetic analysis (Figure 3A) are consistent with a model of three major superclades of bilaterians and positions *Aplysia* as a sister to the arthropod/nematode clade. This finding also supports the monophyly of bilaterians (i.e., the same common ancestor for humans, *Aplysia*, *Drosophila*, and *C. elegans*). Furthermore, our analysis supports the idea that the Lophotrochozoans (to which Mollusca belongs) and the Ecdysozoa are sister groups. Thus, the proximity of *Hydra* to the bilaterians makes it a significantly better outgroup for the bilateral animals than the yeast/plant outgroup.



Figure 3A also illustrates that the evolutionary distance from *Aplysia* to human is shorter than the distance from *Drosophila* and *C. elegans* to human. This indicates that the amino acid replacement rate has been lower in the lineage leading to *Aplysia* than in the lineages leading to *Drosophila* and *C. elegans*. To test this idea further, we measured the corresponding distances among the individual protein families (Table S7) according to the JTT model of evolution. This analysis also showed a significantly shorter distance from humans to *Aplysia* than to both *C. elegans* and *Drosophila*. Figure 3B illustrates the overall comparison between *Aplysia* ESTs and protein coding genes in the three other organisms. Greater similarity was found between human and *Aplysia* proteins, further confirming that the amino acid replacement rate has been lower in the molluscan lineage than in the lineages leading to *Drosophila* and *C. elegans*. Similarly, annelid and human proteins were also found to be more closely related to each other than to their ecdysozoan orthologs (Raible et al., 2005).

#### Predicted Gene Loss in Bilaterians

The topology of the bilaterian phylogenetic tree (Figure 3A) and novel *Aplysia* sequences make possible an analysis of gene loss within each of the four bilaterian lineages leading to humans, *Aplysia*, flies, and *C. elegans*. Indeed, the presence of a shared gene ortholog between *Aplysia* and humans as well as its absence in the sequenced genomes of *Drosophila* or *C. elegans* suggest the loss of these orthologs in the lineages of Ecdysozoa rather than its improbable independent origin in molluscs and deuterostomes. As an illustrative example of such an analysis, ORFs from each of the *Aplysia* nonredundant sequences were compared against protein databases from the other model organisms using blastx. Selected genes identified in this analysis can also be found in Tables S8 and S9.

We focused in particular on proteins from about 40 annotated transcripts relevant to brain development and functions that are only present in humans and *Aplysia* and are not present in *C. elegans* or *Drosophila*. For example, we found an *Aplysia* homolog of Churchill, a zinc finger transcription activator that in vertebrates acts as a switch between different roles of the fibroblast growth factors and regulates the transition between gastrulation and neurulation (Sheng et al., 2003). Interestingly, the Churchill homolog, though not found in nematodes, arthropods, or *Ciona*, was recently found in two cnidarians, the coral *Acropora* (Kortschak et al., 2003) and the freshwater polyp *Hydra*. The origin of this gene can be traced to the most basal metazoan predecessors, and it has apparently been lost in more derived ecdysozoans, such as nematodes and arthropods.

A similar fate can be seen for the P2X receptor genes that encode ATP-gated cationic channels known to be key elements in purinergic transmission involved in pain and certain forms of long-term plasticity. We found a homolog of this gene in *Aplysia* and *Hydra*, yet homologs are apparently absent from sequenced ecdysozoan ge-

nomes. Other genes found in *Aplysia* but lost in insects and nematodes include Cystatin B encoding a specific cysteine protease inhibitor in humans (Brannvall et al., 2003), which is associated with a progressive myoclonic type of epilepsy that leads to mental deterioration and dementia (Lehesjoki, 2003). Neuronal transcripts shared by *Aplysia* and humans also include a unique group of Selenoproteins (e.g., the predicted selenoprotein N homolog of *Aplysia* has no known homologs in other invertebrates), Ependymin-like neurotrophic factor, Major Vault proteins with RNA binding capacities but unknown neurological functions, and proteins related to innate immunity (e.g., complement components).

Some newly identified *Aplysia* transcripts have only been reported in basal deuterostomes and were apparently lost in the entire vertebrate lineage of deuterostomes as well as ecdysozoans with sequenced genomes. One such example is the apexrin-like secretory protein found in the *Aplysia* CNS. In the sea urchin *Heliocidaris erythrogramma*, apexrin is involved in establishing cell polarity, ectoderm development, and metamorphosis (Haag et al., 1999). The fact that apexrin is found in such divergent lineages as echinoderms, cephalochordates, and molluscs implies it had a role in the predecessor of all bilaterians.

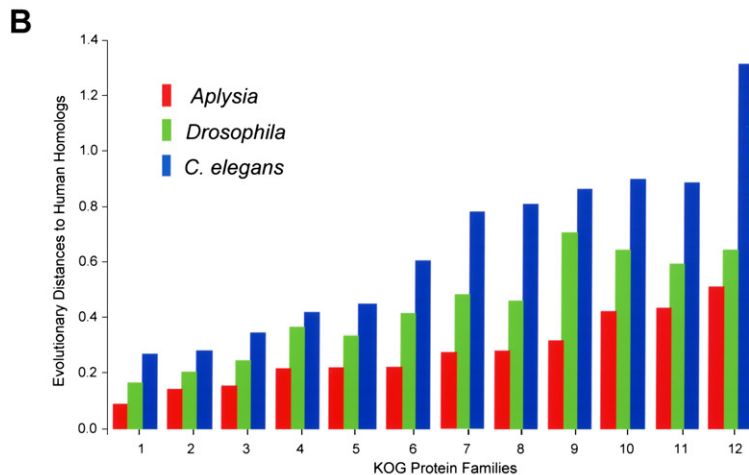
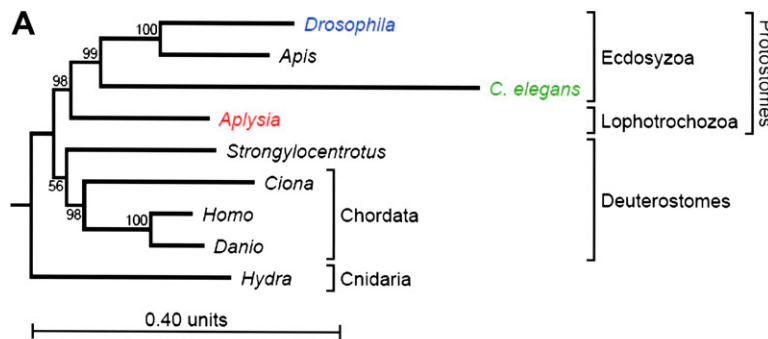
We conservatively estimate that there are at least 20 genes expressed in the CNS of *Aplysia* that are shared by *Aplysia* and *Hydra* (prebilaterian organism, phylum Cnidaria) that have no obvious homologs in vertebrates, arthropods, or nematodes (Table S9), suggesting that these 20 genes were a part of the most basal gene pool present in early metazoans even before the origin of bilaterian animals. Some of these 20 transcripts from this list have homologs only within the basal or unicellular eukaryotes (e.g., the apicomplexans (*Cryptosporidium parvum*) or the slime molds (*Dictyostelium discoideum*)).

In *Aplysia* we identified seven nonredundant sequences (UspA) of one ancient superfamily of “Universal Stress Proteins” (Usp, Table S9), a well-known and conserved group of proteins that are found in Archaea, Eubacteria, and plants. However, the functions of these stress proteins in eukaryotes are currently not known. In bacteria, these proteins act as global regulators of gene expression triggered by a large variety of environmental insults. In *Escherichia coli*, for example, an integrated adaptive response has been described resulting in changes of cell motility, adhesion, and oxidative stress resistance (Nachin et al., 2005). Recently, Usp-like ESTs were also found in the cnidarians *Nematostella* and *Acropora* (Technau et al., 2005) as well as in the parasitic flat worm *Schistosoma japonicum*. This implies greater conservation of UspA proteins in basal metazoans and lophotrochozoans but a noticeable loss of this class of proteins during the evolution of echinoderms, chordates, nematodes, and some arthropods.

In summary, information from the *Aplysia* transcriptome together with recent comparative data (Kusserow et al., 2005; Miller et al., 2005; Technau et al., 2005) support the idea that there was a common bilaterian ancestor (Urbilateria) that had a complex genome. As a result, more

**Table 1. Comparison of Gene Orthologs Relevant to Neuronal Functions among Major Model Organisms and Humans**

Gene Orthologs	Aplysia neuronal transcripts	Drosophila genome	C. elegans genome	Human genome
Protein Kinases	452	240	454	518
Protein Phosphatases	67	66	106	100
Ionotropic Glu Receptors	15	11	10	16
Metabotropic GluR	3	2	3	8
Ionotropic ACh receptors	16	12	56	17
Voltage gated Ca channels	8	9	12	10
Voltage gated Na channels	2	4	4	10
K channels	14-16	22	64	78
Amiloride-sensitive Na channels	2	24	27	11
Cyclic nucleotide gated channels*	4	7	4	10
Cadherins/Protocadherins	12	17	16	113
Synaptotagmins	8-10	3	5	30
Semaphorins	4-5	6	2	2
Fragile X MRP (Mental Retardation Protein)	1	1	1	2
Plexins	3	2	2	16
Innexins/Pannexins (gap junctions)	9	9	29	3
Nitric oxide synthase#	2	1	0	3
Nuclear hormone receptors	6	21	270	48
ITP/ryanodine receptors	2-3	2	4	8
Serotonin transporter	1	1	1	1
5-HT receptors	6	4	5	20
DA receptors	3	5	3	6
GABA A & B receptors	5/1	4/3	4/2	19/4
Gly Receptors	2	4	3	6
Histamine receptors	2	0	0	4
Receptors guanylyl cyclases	7	0	31	5
Ephrin & EphR	2	1/1	3/1	16
Importins	7	3	10	20
Notch/Delta	3-4/2	1/1	2/1	4/3
Integrins/disintegrin	29	7	4	67
Laminins	10	3	3	17
Prominins (stem cell marker)	2	1	1	11
Microcephalin (MCPH)	1	1	1	1
P2X purinoreceptors#	2	0	0	10
Major Vault Complex#	3	0	0	3
Ependymin#	1	0	0	1
RAG-1 like (Recombination-Activating Gene)#	1-5	0	0	1
DNA methyltransferase associating protein 1#	1	0	0	1
Theromacin (cys-rich antimicrobial peptides)#	1	0	0	0
Universal Stress Proteins (UspA)#	7	0	0	0



	KOG ID	Protein Name
1	KOG3181	40S Ribosomal Protein S3
2	KOG0877	40S Ribosomal Protein S2/30S
3	KOG0479	DNA Replication Licensing factor, MCM3 component
4	KOG1628	40S Ribosomal Protein S3A
5	KOG0830	40S Ribosomal Protein SA (P40)/Laminin receptor 1
6	KOG0182	20S Proteasome, regulatory subunit $\alpha$ type PSMA6/SCL1
7	KOG0180	20S Proteasome, regulatory subunit $\beta$ type PSMB3/PUP3
8	KOG0175	20S Proteasome, regulatory subunit $\beta$ type PSMB5/PSMB8/PRE2
9	KOG0863	20S Proteasome, regulatory subunit $\alpha$ type PSMA1/PRE5
10	KOG0179	20S Proteasome, regulatory subunit $\beta$ type PSMB1/PRE7
11	KOG0174	20S Proteasome, regulatory subunit $\beta$ type PSMB6/PSMB9/PRE3
12	KOG2314	Translation Initiation Factor 3, subunit $\beta$ (eIF-3 $\beta$ )

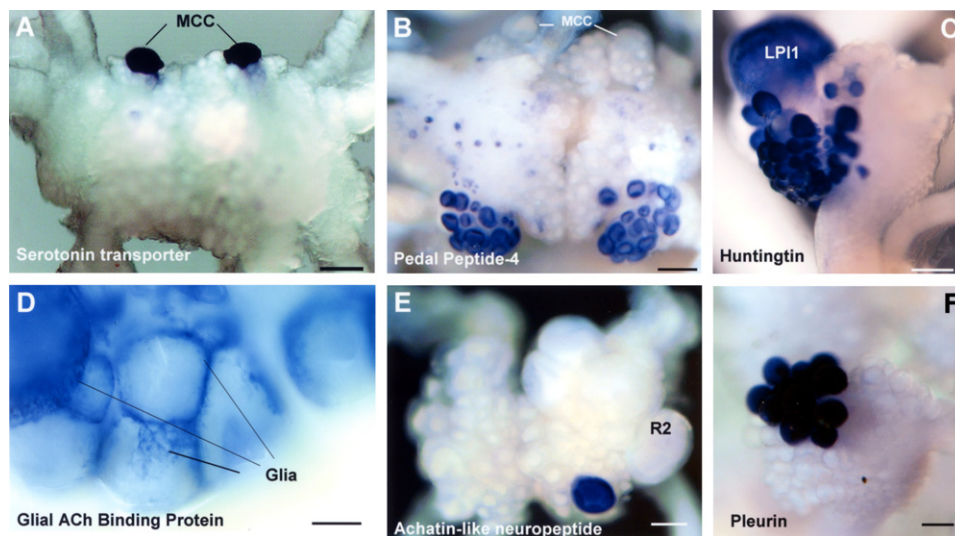
**Figure 3. *Aplysia* in Evolutionary Context**

(A) Phylogenetic relationship between *Aplysia* and other genomic model animals reconstructed based on the KOG (Tatusov et al., 2003) sequence analysis. The mollusc *Aplysia californica* (a representative of the Lophotrochozoa clade) is placed as a sister to the Arthropod (the fruit fly, *Drosophila melanogaster*, and the honey bee, *Apis mellifera*), and nematode (*Caenorhabditis elegans*) phyla representing the clade Ecdosyzoa. The clade of Deuterostomes is represented here by chordates (*Homo sapiens*, the zebrafish *Danio rerio*, the ascidian *Ciona intestinalis*) and the sea urchin (*Strongylocentrotus purpuratus*). Therefore, this evolutionary analysis shows that *Aplysia* is more closely related to nematodes and insects than to vertebrates. The evolutionary distance (measured as branch length) from human to *Aplysia* is shorter, however, than the distance from human to *Drosophila* and *C. elegans*, suggesting that the amino acid replacement rate has been lower in the lineage leading to *Aplysia* than in the lineages leading to *Drosophila* and *C. elegans*.

(B) Comparison of relative evolutionary distances for selected protein families between model organisms (*C. elegans*, *Drosophila*, *Aplysia*) and human homologs. The relative distances (the y axis) are measured for each KOG protein family (Tatusov et al., 2003) according to the JTT model of evolution (Jones et al., 1992). The distance units are expected amino acid replacements per site (for further details see Supplemental Data). Note that rates of evolutionary protein changes in the *Aplysia* lineage (red bars) were slower when compared to *C. elegans* or *Drosophila* lineages (blue and green bars).

derived genomes of insects and nematodes represent a substantial level of gene loss from an ancestral state as compared to slower evolved genomes within selected lophotrochozoan (e.g., molluscs, annelids) and deuterostome (vertebrates) lineages. Furthermore, the fact that many ancient regulatory systems are expressed in neurons is important in the re-evaluation of novel hypotheses about the evolution of neural systems (Holland, 2003).

Counts for *Drosophila*, *C. elegans*, and human gene orthologs were determined by manually screening Ensembl, Flybase, Wormbase, and NCBI. Genes for which no orthologs were found are counted as 0. P2X-type ATP receptors, Vault proteins, ependymin, and RAG-like transcripts, etc. that were identified in *Aplysia* and human genomes but were not found in *C. elegans* or in *Drosophila* are marked # (see details in the Supplemental Data). One HCN channel, one CNG channel, and two CNG-like ESTs were found in *Aplysia* (marked \*). There are four HCNs in humans and one in *Drosophila*, but no HCN channels were found in *C. elegans*.



**Figure 4. Expression Patterns of Selected Neuronal Genes in the CNS of *Aplysia californica* (In Situ Hybridization)**

RNA probes were designed based on cloned sequences to examine their expression in individual *Aplysia* ganglia.

(A) Expression of serotonin transporter (AAK94482) in paired MCC neurons, in a dorsal view of the cerebral ganglion.

(B) Novel isoform of pedal-type neuropeptide (DQ479396) is predominantly expressed in two symmetrical clusters of motoneurons (A/B groups) in the cerebral ganglion.

(C) Expression of Huntingtin in the left pleural ganglion (LPI1 is the giant cholinergic neuron involved in control of mucus release from the body wall).

(D) Acetylcholine Binding Protein (AAL37251) is exclusively expressed in glial type cell of *Aplysia* (the caudal part of the abdominal ganglion).

(E) Expression of Achatin-like neuropeptide precursor (AY842441) in the abdominal ganglion.

(F) Highly localized expression of Pleurin neuropeptide precursor (AY833131) in the right pleural ganglion. Scales: 300  $\mu$ m (A and B); 200  $\mu$ m (C); 100  $\mu$ m (D); 350  $\mu$ m (E); 120  $\mu$ m (F).

### The Transcriptome of the *Aplysia* Nervous System: Overview of Predicted Neuronal Genes

As expected, the *Aplysia* neuronal transcriptome is enriched for many developmentally related genes. The transcriptome reflects nearly every aspect of neuronal signaling and includes genes such as Microcephalin and Abnormal Spindle-like Microcephaly associated (ASPM) that determine brain size and glial markers such as AChBP (AAL37251, Figure 4D), *Aplysia* glial protein (Ag), and glia maturation factor  $\beta$ . Perhaps most surprising is the finding of markers for various human disorders (Table S10). We illustrate the diversity of new information contained within this database with several selected examples.

#### Neurotransmitter Systems

The *Aplysia* neuronal transcriptome contains transcripts encoding genes for the synthesis of most major transmitters, their receptors, and essential components of their signal transduction pathways (Table 1) and provides the first insight into the existence of purinergic transmission in molluscs. The availability of many of these markers allowed us to map cholinergic and octopaminergic neurons in *Aplysia* (Figure S2).

#### Disease-Relevant Gene Products

A comparative analysis of 146 human genes implicated in 168 neurological diseases (Cravchik et al., 2001) allowed us to identify 104 orthologs (71%) of these genes in *Aplysia* (Table S10), including genes relevant to Parkinsons and Alzheimer's disease, thus affording a unique opportu-

nity to define their functions in simpler networks. Eleven *Aplysia* homologs of human genes, relevant to different neurodegenerative diseases, are differentially lost either in *Drosophila* (e.g., Cochilin, Fukutin, CLN8, GM2 activator) or *C. elegans* (e.g., Huntingtin [Figure 4C], Fragile X Mental Retardation Protein, Hyperpolarization-activated cyclic nucleotide-modulated ionic channel, and Wolframin) lineages. These comparisons suggest that the distinctive features of the CNS of *Aplysia* present opportunities to study molecular and cellular functions of these genes, as well as to develop relevant models for these diseases in *Aplysia*. Interestingly, *Aplysia* has an ortholog of the mammalian antiaging hormone, *Klotho*. In mice, a defect in *Klotho* gene expression accelerates the aging process, while its overexpression extends the life span (Kurosu et al., 2005).

#### Immunity and Posttranslational Gene Silencing in CNS

Orthologs of 25 predicted antimicrobial, antiviral, and immune-related proteins have been found in the *Aplysia* EST database, including lysozymes, transcripts similar to blood coagulation factors, Ig heavy chain precursors, and complement precursors. Until now, complement genes were only found in vertebrates (as an illustrative example of vertebrate lineage-specific innovations related to adaptive immunity), and their discovery in *Aplysia* suggests that orthologs of these genes were lost in Ecdysozoa (nematode and arthropod) lineages.



Although RNAi is broadly found in different organisms, there previously has been no evidence in molluscs for the presence of proteins involved in RNAi. We have identified the main components of RNAi in *Aplysia*, including homologs to RNase III, the Dicer & Argonaute gene family.

#### **Identification of New Signaling Pathways in Invertebrate Nervous System**

Our analysis has identified two previously undescribed signaling pathways in the invertebrate CNS: thyroid hormone (TH) related signaling (Heyland et al., 2006 and Table S11) and CpG methylation. TH hormones are involved in the development of the nervous system in a variety of vertebrate taxa. We find a peroxidase ortholog, potentially involved in TH metabolism, expressed in the cerebral ganglion. The presence of this gene in the CNS of *Aplysia californica*, along with several other putative transcripts from the TH-like signaling pathway, suggests the presence of TH-like signaling in molluscs as well (Heyland et al., 2006).

DNMT1 and DNA methyltransferases in general have been found to be lacking in *C. elegans*, *Schistosoma* (Rosado Fantappie et al. 2001), and dipteran insects (Goll et al. 2006), which all are known to have only trace levels of 5-methyl cytosine at CpG sites if any (Simpson et al. 1986, Rae and Steele 1979). We find in the *Aplysia* EST database homologs for the DNA methyltransferase DNMT1, DMAP1 (DNA methyltransferase associating protein), and the transcriptional repressor MBD2 (Methyl-CpG binding Domain Protein 2). These findings demonstrate that *Aplysia* has many of the fundamental components for transcriptional regulation by CpG methylation.

#### **Portrait of the Transcriptome from Individual Neurons and Processes**

How does the mRNA expression profile of one neuron differ from that of different neuronal types? How does the mRNA population in the cell body of a neuron differ from the mRNAs present in the neuronal processes? As a first step to address these questions, we have examined the composition of the components of the gill-withdrawal reflex: the sensory neuron and the motor neuron. In addition, we examined a model serotonergic cell capable of modulating the strength of the synaptic connections.

#### **Identification of Circuit-Specific Transcripts: Transcriptomic Profiling of Sensory and Motor Neurons Using Representative *Aplysia* Microarrays**

Using custom-made highly representative *Aplysia*-specific oligonucleotide (60-mer) microarrays, we examined a critical monosynaptic component of the neural circuit of the gill-withdrawal reflex, the motor neuron L7, and the mechanosensory neurons. After performing significance analysis with a 5% false discovery rate and 2-fold change cut-off, we identified 853 differentially expressed transcripts: 362 were enriched in L7 and 491 were enriched in sensory neurons (see illustrative examples in Figure 5 and Tables S12 and S13). This approach has provided new ideas about the specificity of neurotransmitter action and identification of candidate cell-specific signaling molecules.

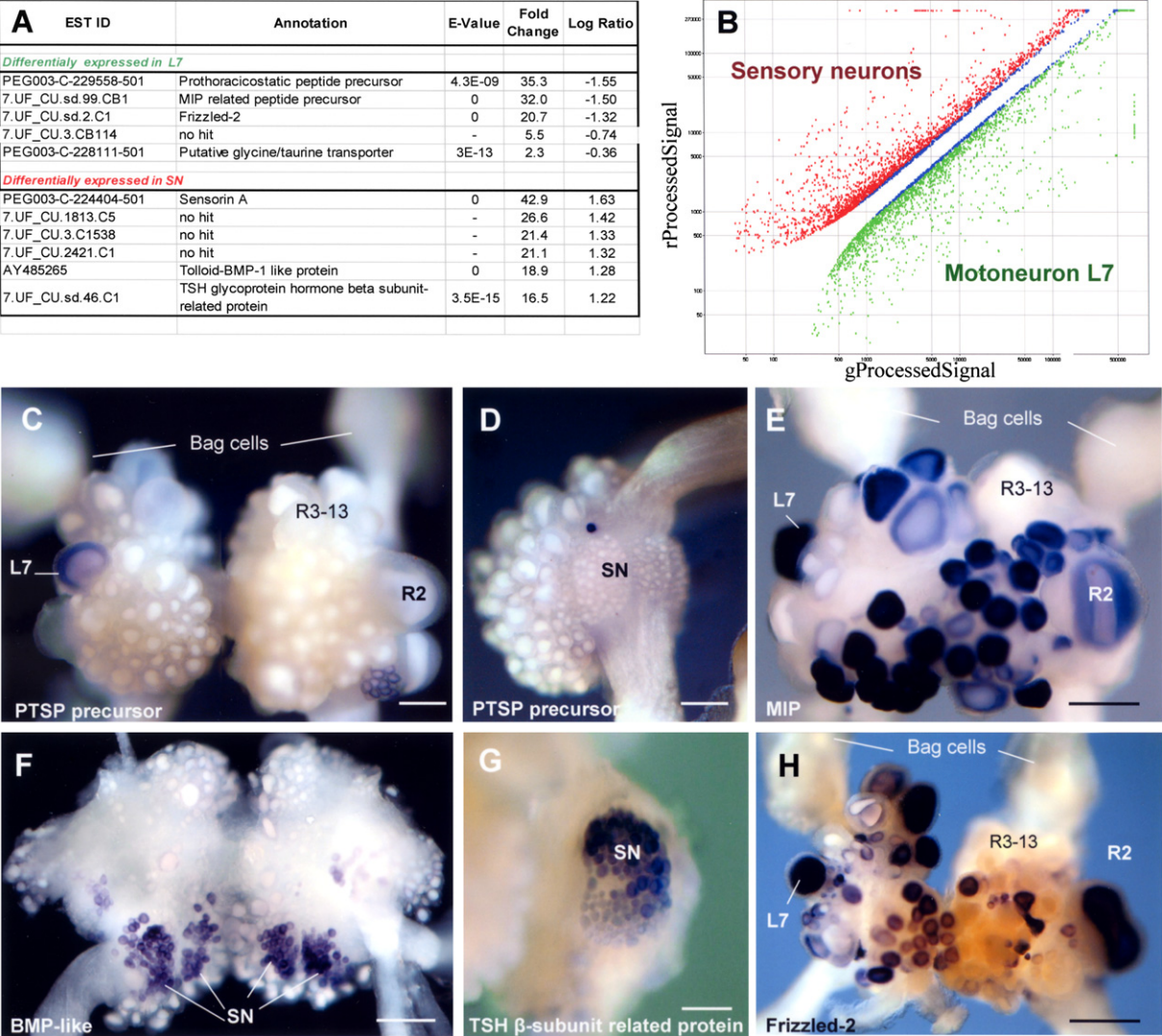
Our initial analysis pointed to three L7 specific proteins as candidate transmitter molecules. The first is the Mytilus inhibitory peptide (MIP) related precursor (Figure 5E), initially cloned from bivalves and later from *Aplysia* (Fujisawa et al., 1999). This precursor can produce opioid-like peptides known to be specific modulators in molluscan neurons. The second candidate is a novel *Aplysia* neuropeptide precursor (PTSP, Figures 5C and 5D), distantly related to a prothoracicostatic hormone in the silkworm *Bombyx mori* that controls insect development via cAMP/Ca<sup>2+</sup> signaling cascades. Using whole-mount in situ hybridization (Figure 5), coupled with Lucifer yellow injection of L7 (not shown), we demonstrated that the MIP related peptide precursor, the PTSP, and a third putative neurotransmitter transporter are differentially expressed in L7 but not sensory neurons, confirming the microarray data.

In situ hybridization with choline acetyltransferase (ChAT, a marker for cholinergic neurons) revealed that L7 is negative for ChAT and thus not likely a cholinergic motor neuron (Giller and Schwartz, 1971). No GABA immunoreactive neurons have been observed in the abdominal ganglion (Diaz-Rios et al., 1999), and this essentially rules out the possibility that L7 is GABAergic. This leaves both taurine-/glycine-type transporters as candidates for L7 specific uptake mechanisms. In addition, the microarray data suggested L7-specific expression of several molecules known to be key regulators in morphogenesis and cell-cell signaling. Of these, we cloned Frizzled-2 (AY535406) and confirmed its high and specific expression in L7 but not sensory neurons (Figure 5H).

Microarray data for the presynaptic (sensory) neuron confirmed the presence of bone morphogenetic (BMP) type/tolloid protein (confirmed by in situ hybridization, Figure 5F), a candidate involved in structural changes associated with cell-cell communication. A second major cell-specific transcript in the sensory neurons is the sensorin prohormone. Other predicted gene products differentially expressed in sensory neurons also include genes known to be involved in the pathways leading to short- and long-term plasticity (Tables S12 and S13).

#### **Serotonergic Modulatory Cells (MCC)**

Normalized and nonnormalized cDNA libraries from five to eight individual MCC neurons were analyzed to obtain good representation of both common and rare cell-specific transcripts. The assembly of ESTs obtained from MCC neurons resulted in 9,945 nonredundant sequences, suggesting that roughly 10,000 unique gene products are expressed in a single *Aplysia* neuron. Of these, 1,263 genes were assigned to GO categories based on their predicted molecular function or biological process (Figure 2A and Table S3). Among the most abundant transcripts in MCC neurons are those for serotonin transporter and predicted pheromone receptors. The transcriptome of MCC also contains putative photoreceptors, suggesting that these interneurons might also be involved in light sensitivity or regulation of circadian clocks (supported by the expression of Nocturnin homologs).



**Figure 5. Identification of L7 and Sensory Neuron-Specific Transcripts Using Microarrays**  
(A) Selected differentially expressed transcripts in L7 and sensory neurons.  
(B) Expression results from oligoarray experiments comparing L7 and sensory neurons. Spots representing transcripts common to the two neuronal types have been removed for clarity.  
(C–H) Neuron-specific expression (in situ hybridization) of selected transcripts identified in the microarray experiments (C, E, and H: abdominal ganglion; D and G: pleural ganglia; F: cerebral ganglia). Transcripts encoding *Aplysia* prothoracicostatic peptide (PTSP) homolog (DQ375548, C and D), MIP-like neuropeptide (AAF80382, E) and Frizzled-2 (AY535406, H) are expressed in L7 (but not in sensory neurons, e.g., D), while BMP-like (AY485265, F) and TSH glycoprotein hormone β subunit-related protein (AAX35673, G) genes are expressed in sensory neurons. Note that MIP-like peptide prohormone and Frizzled-2 are also expressed in a subset of abdominal neurons, including some gill motoneurons and R2 neuron. Scales: 300 μm (C); 250 μm (D); 400 μm (E and H); 200 μm (F); 150 μm (G).

**Subcellular Transcriptome of a Neuron: EST Collection from MCC Neurites and Comparison to the Neurites of Sensory Neurons**  
Since these peripheral processes sustain the requirement of local protein synthesis at the synaptic sites, a cohort of mRNAs must be transported to the peripheral processes. We addressed this issue by sequencing normalized and nonnormalized cDNA libraries from neuronal processes (neurites) of MCC. The assembly of 4515

ESTs resulted in 198 different clusters of transcripts and 830 singlets. We annotated 268 unique nonoverlapping sequences in MCC processes and assigned them to predicted molecular functions using GO categories (Figure 2A and Table S3).  
Table 2 shows several transcripts, including actin, ubiquitin, and several ribosomal proteins that are enriched several-fold in the MCC processes in comparison to the MCC somata. The enrichment of several ribosomal

**Table 2. Abundance of Transcripts in MCC Somata (MCC\_N) and MCC Neuronal Processes (MCC\_P)**

EST Annotation	Blast Hit Accession	Blast E-value	MCC_N%	MCC_P%
Tubulin B-alpha-1	Q71U36	0	1.3	1
<b>Soma Ferritin</b>	P42577	1E-76	1.1	1.5
<b>Similar to putative pheromone receptor</b>	XP_578826	6E-16	1.1	1.1
<b>Thymosin beta</b>	Q9W7M8	4E-12	0.5	0.9
Sodium-dependent serotonin transporter *	P51143	3E-51	0.5	0.07
Synaptotagmin-1 (p65)	P41823	1E-88	0.4	0.07
<b>7B2</b> [ <i>Lymnaea stagnalis</i> ]	AAB41699	1E-107	0.3	0.6
<b>Receptor of activated protein kinase C</b>	O42248	1E-160	0.3	0.4
Calmodulin	P62153	1E-79	0.3	0
Eukaryotic translation initiation factor 1A	P47813	1E-41	0.3	0
Major vault protein (MVP100)	Q90405	3E-48	0.3	0
60S ribosomal protein L6	Q02878	2E-66	0.2	0.07
40S ribosomal protein S15A	P48149	3E-62	0.2	0
Spectrin beta chain, brain 1	Q01082	1E-174	0.2	0
Aromatic-L-amino-acid decarboxylase (AADC) *	P05031	1E-51	0.2	0
<b>60S ribosomal protein L3</b>	O16797	1E-115	0.1	0.4
<b>40S ribosomal protein S2</b>	Q90YS3	8E-85	0.1	0.3
Translationally controlled tumor protein (TCTP)	O18477	4E-11	0.1	0.1
60S ribosomal protein L7	P18124	1E-75	0.1	0.07
Amelogenin	Q28462	2.00E-07	0.1	0
<b>60S ribosomal protein L5</b>	Q26481	3E-66	0.05	0.6
<b>Nucleoside diphosphate kinase NBR-A</b>	P52174	5E-58	0.05	0.4
<b>60S ribosomal protein L7a</b>	O57592	7E-99	0.05	0.3
<b>40S ribosomal protein S8</b>	Q90YR6	7E-86	0.02	0.3
<b>Unknown transcript</b>	-	-	0.02	0.4
<b>60S ribosomal protein L9</b>	P50882	3E-67	0.02	0.3
<b>Hypothetical protein Bd1086</b>	NP_968019	1.00E-08	0.02	0.3
<b>Cytochrome c oxidase polypeptide Vb</b>	P19536	9E-15	0.02	0.3
<b>Ubiquitin</b>	P68201	6E-30	0.02	0.3
<b>Actin, cytoplasmic</b>	Q964E0	1E-166	0.02	0.2
<b>60S ribosomal protein L10</b>	O96647	1E-103	0.02	0.1
<b>60S ribosomal protein L13a</b>	P40429	1E-68	0.02	0.1
<b>40S ribosomal protein S3B</b>	P47835	1E-116	0.02	0.1
<b>Tax_Id = 9606 47 kDa protein</b>	ENSP00000348114	2.00E-07	0.02	0.07
<b>60S ribosomal protein L13</b>	Q90Z10	3E-64	0.02	0.07
<b>40S ribosomal protein S27</b>	Q6ZWU9	2E-34	0.02	0.05
<b>40S ribosomal protein S5</b>	P46782	3E-96	0.02	0.05
<b>60S ribosomal protein L18B (L14B)</b>	P02412	1E-79	0.02	0.04
Non-neuronal cytoplasmic intermediate filament protein (IF)	P22488	2E-24	0.02	0.02

Transcripts enriched in MCC neuronal processes are marked in bold. Markers of serotonin containing cells are labeled with asterisk (\*).

proteins in the MCC processes agrees with recent information on the processes of the sensory neuron and suggests that in the processes the proteins present on the ribosome surface may be replenished directly and have the capability for local protein synthesis (Moccia et al., 2003). Interestingly, the subcellular transcriptome of MCC also contains several cell communication and signal transduction components, including mRNAs encoding four kinases (diglyceride kinase, delta; calcium independent protein kinase C; and two serine-threonine protein kinases similar to PAK-1 and CDC2L), inositol-1,4,5-triphosphate receptor, NMDA receptor, presenilin, hedgehog homologs, Slit homolog 2, RNA binding ELAV-like protein, cadherin, and FMRP.

We took advantage of our *Aplysia* transcriptome database to further annotate a set of 668 ESTs, independently collected from pure neuronal processes of pleural sensory neurons (Moccia et al., 2003) and to compare them to the subset of extrasomatic transcripts identified in MCC. 564 ESTs derived from sensory neuronal processes matched *Aplysia* transcripts in the present EST database (495 of them were identical or nearly identical and 169 had significant similarity). As a result, we annotated 66 novel transcripts, including ribosomal proteins, RNA binding proteins, and signaling proteins.

Direct comparison of the MCC neurite transcriptome to the transcriptome of the neurites of the sensory neuron suggests that a subset of extrasomatic transcripts is neuron-specific, including transcripts encoding signaling molecules and neuropeptides characteristic of a single cell type. Several identified transcripts that are associated with synaptic plasticity were found in sensory neuron processes, but not in MCC. These are the frizzled related protein, TGF  $\beta$ -inducible protein, ephrin type receptor, receptor for activated protein kinase C, the apoptosis inhibitor protein, CREB, and fasciclin. In addition, sensory neuron neurites are enriched for mRNAs that encode caveolin and the neuropeptide sensorin. By contrast, MCC neurites contain mRNAs encoding the serotonin transporter and serotonin synthetic enzyme tryptophan 5-monooxygenase that were not present in sensory neuron processes.

## Conclusions

As a result of large-scale sequencing of the *Aplysia* neuronal transcriptome, we have established a database for future gene discoveries, expression profiling, and characterization of signaling pathways. Our evolutionary analysis has suggested the Lophotrochozoa are closer to nematodes and arthropods than to chordates and other deuterostomes. Indeed, we have been able to clarify some of the evolutionary relationships and have identified genes that have been lost in the three major lineages of bilaterian animals. Thus, our neural transcriptome data have helped delineate a set of genes derived from a common bilaterian ancestor. Genes found in this database include many formerly unknown in molluscs, including many associated with human neurological diseases that are not found in

other classic model organisms. Further, the initial gene identification and expression analyses conducted on individual neurons and their processes in *Aplysia* provide a bridge between genomics at the level of identified neurons and physiological analysis within a defined cellular network and its appropriate linked behaviors. As the emphasis in neural science shifts from cellular to circuit studies, *Aplysia* can provide a number of interesting behaviors accessible for detailed investigation. The *Aplysia* transcriptome thus reveals many potential targets for investigation, particularly in terms of the more complex neural circuitry of behaviour, such as the fixed action potential patterns of feeding and locomotion.

## EXPERIMENTAL PROCEDURES

### cDNA Library Construction

Specimens (5–80 g) of *Aplysia californica* (Opisthobranchia: Anaspi-dea) were obtained from the NIH/University of Miami National Resource for *Aplysia*. Prior to dissection, animals were anesthetized by injecting a volume of isotonic  $\text{MgCl}_2$  (337 mM) equivalent to 50%–60% of their weight. Normalized and nonnormalized cDNA libraries were constructed from four types of tissues: (1) the whole CNS, (2) pedal-pleural ganglia, (3) individual MCC neurons, and (4) neuronal processes from MCC neurons. All details of cell isolation and culture protocols have been described elsewhere (Moccia et al., 2003; Lovell and Moroz, 2006). Normalization of the CNS, MCC, and MCC process libraries was performed using both a novel cDNA normalization method (duplex-specific nuclease or DSN normalization [Zhilidov et al., 2004]) and standard techniques (Fu et al., 2002; Soares et al., 1994). See additional details, including a complete list of libraries (Table S1), in the Supplemental Data.

### DNA Sequencing and Sequence Analysis

After cloning cDNA libraries into pBluescript, individual clones were amplified using either PCR or TempliPhi rolling circle amplification (GE Healthcare) and analyzed on either an ABI3730 (Applied Biosystems) or a MegaBACE 1000 (GE Healthcare) DNA sequencer.

All sequences were checked for quality prior to downstream analysis using custom PERL scripts. Using Paracel TranscriptAssembler (PTA) version 2.7.0 (Paracel Inc, Pasadena, CA), ribosomal RNA sequences, *E. coli* contamination, and mitochondrial genes were removed, and all vector and adaptor sequences were masked. Resulting sequences were then clustered and sequence clusters were assembled to yield a unique set of sequences (800 bases average length). Unique sequences were annotated using Paracel Blast through blastx searches against the SwissProt/Trembl and NCBI nr databases using an E-value cutoff of  $10^{-10}$ . Further details concerning EST annotation and analysis can be found in the Supplemental Data.

All annotation information, clustering analysis, and sequence information are stored in a MySQL database. Public access is available through two project websites at <http://aplysia.uf-genome.org> and <http://aplysia.cu-genome.org>. Data can be accessed through annotations that are broken down into GO categories for easy browsing or through blast searches with user-input sequences. Databases are also linked to graphical representations of metabolic pathways and relevant references in PubMed. Further detailed descriptions of the databases are available at the indicated websites.

### Phylogenetic Analysis

Reference sequences from the latest release of the KOG database (Tatusov et al., 2003) were obtained from NCBI. Orthologs for each KOG were extracted from publicly available protein and EST databases as well as the *Aplysia* EST database for each of the species



analyzed. EST sequences were translated into all reading frames, and the protein translation with the highest homology was extracted. This yielded a total of 45 protein families (see Table S15) with at least 11 orthologous proteins in each. Alignments of orthologous sequences were performed with T-Coffee and manually verified to ensure accuracy. Phylogenetic trees were determined from the alignments (excluding regions with more than two gaps) with the PHYML program using the JTT model and  $\gamma$  distributed rates (Guindon and Gascuel, 2003; Jones et al., 1992; Yang, 1994). Bootstrap analyses were performed to evaluate the reliability of the phylogenies. Further details can be found in the Supplemental Data.

### In Situ Hybridization

Sense and antisense probes were generated with the DIG RNA Labeling Kit (Roche Diagnostics); all details for in situ hybridization protocols have been described elsewhere (Jezzini et al., 2005; Jezzini and Moroz, 2004).

### Microarray Experiments

Two custom 44,000 oligonucleotide arrays were constructed in collaboration with Agilent Technologies using 60-mer oligonucleotide sequences designed from each nonredundant sequence in the *Aplysia* EST database. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE4628. Additional details about the protocols used including a list of features on each array can be found at the GEO web site and are summarized in the Supplemental Experimental Procedures.

### Supplemental Data

Supplemental Data include fifteen tables, two figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://www.cell.com/cgi/content/full/127/7/1453/DC1/>.

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#### Accession Numbers

The *Aplysia* EST sequences have been deposited in GenBank with accession numbers EB187504-EB359573, BF707524-BF708380, BF713631-BF713632, BI273615-BI273627, CK327631-CK329175, and CK320902-CK325388 (see Table S5 for full-length genes).