

Transcriptome analysis and identification of regulators for long-term plasticity in *Aplysia kurodai*

Yong-Seok Lee^{a,1}, Sun-Lim Choi^{a,1}, Tae-Hyung Kim^b, Jin-A Lee^a, Hyong Kyu Kim^a, Hyoung Kim^a, Deok-Jin Jang^a, Jennifer J. Lee^a, Sunghoon Lee^b, Gwang Sik Sin^b, Chang-Bae Kim^c, Yutaka Suzuki^d, Sumio Sugano^d, Tai Kubo^e, Leonid L. Moroz^f, Eric R. Kandel^{g,2}, Jong Bhak^b, and Bong-Kiun Kaang^{a,2}

^aNational Creative Research Initiative, Center for Memory and Institute of Molecular Biology and Genetics, Department of Biological Sciences, College of Natural Sciences, Seoul National University, San 56-1 Silim-dong, Gwanak-gu, Seoul 151-747, Korea; ^bKorean Bio Information Center, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-333, Korea; ^cMajor in Life Science, College of Natural Sciences, Sangmyung University, Seoul 110-743, Korea; ^dHuman Genome Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan; ^eNational Institute of Advanced Industrial Science and Technology, Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology Tsukuba Central 2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8568, Japan; ^fThe Whitney Laboratory for Marine Bioscience, University of Florida, Augustine, FL 32080; and ^gCenter for Neurobiology and Behavior, College of Physicians and Surgeons, Columbia University, 1051 Riverside Drive, New York, NY 10032

Contributed by Eric R. Kandel, September 9, 2008 (sent for review January 4, 2008)

The marine mollusk *Aplysia* is a useful model organism for studying the cellular bases of behavior and plasticity. However, molecular studies of *Aplysia* have been limited by the lack of genomic information. Recently, a large scale characterization of neuronal transcripts was performed in *A. californica*. Here, we report the analysis of a parallel set of neuronal transcripts from a closely related species *A. kurodai* found in the northwestern Pacific. We collected 4,859 nonredundant sequences from the nervous system tissue of *A. kurodai*. By performing microarray and real-time PCR analyses, we found that ApC/EBP, matrilin, antistasin, and eIF3e clones were significantly up-regulated and a BAT1 homologous clone was significantly down-regulated by 5-HT treatment. Among these, we further demonstrated that the Ap-eIF3e plays a key role in 5-HT-induced long-term facilitation (LTF) as a positive regulator.

expressed sequence tag | long-term facilitation

The marine mollusk *Aplysia* is an important model organism for studying the cellular and molecular mechanisms underlying learning and memory (1–3). There are four major advantages of using *Aplysia* for the studies of synaptic plasticity, learning, and memory storage. First, *Aplysia* is capable of a variety of interesting behaviors that can be modified by various forms of learning to give rise to both short- and long-term memories. Second, *Aplysia* has a relatively simple nervous system, consisting of only $\approx 2 \times 10^4$ central nerve cells compared with the 10^{11} neurons of mammalian brains. Third, the giant neurons of *Aplysia* are among the largest somatic cell types in the animal kingdom, enabling one to conduct various genetic manipulations, such as the microinjection of plasmid, RNA, or protein on the level of a single neuron (4). Finally, many neurons are uniquely identifiable in every animal of the species and can be related to the animal's behaviors (5, 6). Because of these advantages, the cell and molecular biology of certain key neural circuits in the nervous system of *Aplysia* have been studied extensively.

However, molecular level analyses in *Aplysia* have been limited until recently by the lack of genomic information. As a first step, Moccia *et al.* characterized the gene expression profiles of the processes of *Aplysia* sensory neurons, thereby demonstrating the usefulness of high-throughput gene analysis in studying synaptic plasticity (7). Next, the whole mitochondrial genome of *A. californica* was sequenced (8) and a large scale expressed sequence tag (EST) analysis for this species was completed (9). Moroz, Kandel, and colleagues sequenced more than 200,000 ESTs, which represented over 65,000 nonredundant sequences from *A. californica* cDNA libraries derived from several sources

including the whole CNS, individual ganglia, identified neurons, and identified processes of identified neurons. Of those putative unique transcripts, they were able to annotate approximately 4,900 genes with clearly recognized orthologs in other organisms (9). Further characterization of neuronal transcripts in *Aplysia* requires both functional and comparative studies. As a first step in that direction, we have obtained complementary data from *A. kurodai*, a closely related species living in the northwestern Pacific. Both organisms represent a relatively recent speciation event and the topological organization of the central ganglia is nearly identical between two species in which the majority of individual neurons are clearly identifiable.

Here, we describe and annotate the collection of 11,493 ESTs generated from the central nervous system of *A. kurodai*. Using this collection, we constructed custom microarrays composed of approximately 7,000 neuronal cDNA clones. These arrays were used to identify genes differentially expressed in response to 5-HT known as a key neurotransmitter for LTF in *Aplysia* (10). Finally, we characterized a gene, *Aplysia* eukaryotic translation initiation factor 3 (Ap-eIF3e), as a molecular switch that converts short-term facilitation (STF) to LTF.

Results

Generation and Assembly of *Aplysia kurodai* ESTs. By sequencing cDNA libraries from the CNS of *A. kurodai*, we constructed a representative EST database that facilitates the mining of genes related to basal neuronal functions and plasticity. Sequence analyses of the previously cloned *Aplysia* genes showed that many transcripts contained relatively long UTRs in their 3' ends (11, 12) and might cause problems in the initial annotation of protein-coding genes. Therefore, we generated bidirectional CNS cDNA libraries using an oligo-capping method that enabled us to obtain full-length and 5'-end-enriched cDNAs (13–15). To increase the complexity of cDNA clones, we made three additional libraries: a nondirectional random-primed CNS library, a

Author contributions: Y.-S.L., S.-L.C., J.-A.L., and B.-K.K. designed research; Y.-S.L., S.-L.C., T.-H.K., J.-A.L., H.K.K., H.K., J.J.L., S.L., G.S.S., C.-B.K., Y.S., S.S., and T.K. performed research; Y.-S.L. and T.-H.K. contributed new reagents/analytic tools; Y.-S.L., S.-L.C., T.-H.K., J.-A.L., H.K.K., H.K., and D.-J.J. analyzed data; and Y.-S.L., S.-L.C., H.K., D.-J.J., J.J.L., L.L.M., E.R.K., J.B., and B.-K.K. wrote the paper.

The authors declare no conflict of interest.

¹Y.-S.L. and S.-L.C. contributed equally to this work.

²To whom correspondence may be addressed. E-mail: kaang@snu.ac.kr or erk5@columbia.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0808893105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

Table 1. *Aplysia kurodai* contig statistics

Total sequences analyzed	11493
Number of ESTs in contigs	7728
Number of contigs	1094
Number of singletons	3765
Number of putative transcripts (assembled sequences)	4859
Number of contigs in size of	
< 1 kb	780
1 kb ≤ size < 2 kb	286
2 kb ≤ size < 3 kb	27
3 kb ≤ size < 4 kb	2
Average contig size	949 bp

5-HT treated CNS library that is 3' end-enriched, and a buccal muscle library. A total of 11,755 sequences was obtained, trimmed of vector and low-quality sequences, yielding 11,493 ESTs that measured an average 595 bp (see [supporting information \(SI\) Table S1](#)).

These 11,493 high-quality ESTs were assembled and clustered by using the nonsupervised clustering method, assuming that the clustered ESTs originated from the same gene. Part (32.8%) of the original ESTs (3,765) was composed of singletons (or occurred only once in the collection). The remaining 7,728 clones fell into 996 clusters with sizes ranging from two (515 clusters) to 2,274 (one cluster), while 57.3% of the total ESTs belonged to cluster sets of 10 or fewer. The clustered ESTs were assembled by using the CAP3 assembly program (16), resulting in 4,859 nonredundant transcripts (1,094 contigs and 3,765 singletons), ranging up to 3,200 bp in length (Table 1).

Of the total assembled putative transcripts, 1,648 sequences matched proteins from the GenBank nonredundant database at a BLASTX e-value cutoff of 10^{-5} . These sequences were further classified by using gene ontology (GO) categories (17) as described in [Tables S2 and S3](#). Original ESTs were submitted to GenBank under accession numbers EY392795–EY396002 and EY416138–EY424374; all analyzed data and detailed information are available through a website (<http://seahare.org>).

Comparison of Neuronal Transcripts Between Two *Aplysia* Species.

First, we mapped *A. kurodai* contigs to 65,055 putative *A. californica* nonredundant contigs so as to identify orthologous sequences. Of the 4,859 *A. kurodai* contigs, 3,267 (67%) were matched to known *A. californica* sequences and 1,592 (33%) had no significant matches (BLASTN; $e \leq 10^{-10}$) (Fig. 1). It is likely that many of these nonmatching sequences are fragments of nonoverlapping transcripts. Fig. 1B summarizes the proportion of these *A. californica* nonmatching *A. kurodai* sequences with and without BLASTX (18) matches in the nonredundant protein (nr) database ($e \leq 10^{-3}$) from National Center for Biotechnology Information. The majority (1,278 of 1,592; 80%) of sequences had no matches in public databases. Of the 314 BLASTX-matching transcripts, only 3% were homologous to known protein sequences from Mollusca, including the genus *Aplysia* (Fig. 1C).

Differentially Expressed Genes in Response to 5-HT. To monitor the changes in gene expression in response to 5-HT stimulation, we constructed *A. kurodai* cDNA microarrays. A total of 6,912 clones was successfully amplified by PCR and printed on glass slides. To investigate the changes of gene expression by 5-HT treatment, *Aplysia* were treated with 5-HT *in vivo* (250 μ M, 2 h) (11), and pleural ganglia that contain sensory clusters were isolated. Amplified RNAs from the pleural ganglia of 5-HT treated or nontreated animals were used to generate a fluorescence-labeled probe and hybridized to the microarray (see details in the Methods section and/or SI). After filtering out low

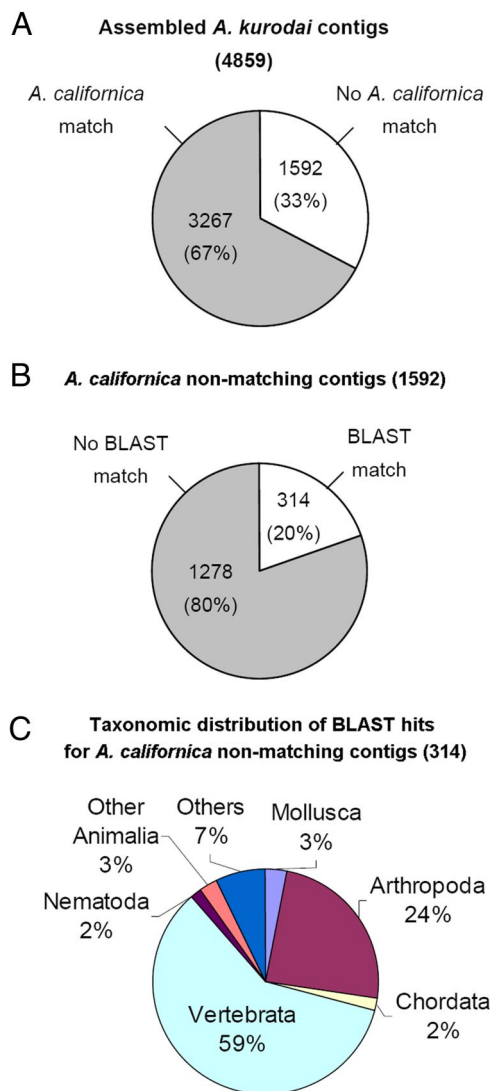


Fig. 1. Comparative analysis of *A. kurodai* with *A. californica*. (A) The proportion of *A. kurodai* contigs with and without matches in *A. californica* EST set (BLASTN; $e \leq 10^{-10}$). (B) *A. kurodai* contigs without matches in *A. californica* EST were separately analyzed for BLASTX matches in nr protein database (BLASTX; $e \leq 10^{-3}$). (C) *A. californica* nonmatching-*A. kurodai* contigs with matches in nr database were classified by the homologous proteins and their species. Twenty-four percent of the sequences were homologous to the sequences from Arthropoda, including *Drosophila* and *Apis*. A large portion, 59% of BLASTX-hit genes, showed homology to sequences from a variety of species in Vertebrata, from *Danio rerio* (zebrafish) to *Homo sapiens*. Others were mostly related to proteins from Nematoda or other Animalia. It is interesting that some (7%) matched to bacterial (*Mycobacterium*, *Bacillus*, and *Silicibacter*) or viral (*entomopoxvirus* and *bacteriophage*) protein sequences. These sequences might result from the infection or contamination of *Aplysia* nervous system.

quality spots, 27 clones were found to be differentially expressed in 5-HT-treated pleural ganglia. (Table S4). Table 2 shows the list of selected annotated clones. The previously characterized immediate-early gene, ApC/EBP, was also found to be up-regulated after 5-HT treatment.

We also performed the qRT-PCR analysis to further validate predicted 5-HT regulated genes (Fig. 2). Based on BLASTX search results, 13 clones that have the matching proteins in the database were selected for qRT-PCR analysis. qRT-PCR confirmed that four clones (ApC/EBP, matrilin, antistasin, and eIF3e) were significantly up-regulated by 5-HT treatment, and

Table 2. Real-time PCR confirmation about differentially expressed clones by >2-fold 2 h after 5-HT treatment in microarray

EST name	BLASTX result*	GenBank accession number	Ratio of medians	Short description of real-time PCR results†
Up-regulated clones				
5CAP092402_H04_760	Matrilin (AAN61407, 1.00E-33)	EY418286	5.377	Significant
5CAP090501-pMES_D10_142	Antistatin (P38977, 2.00E-25)	EY417467	4.830	Significant
5CAP031402_pMES_B12_1600	Cathepsin L-like cysteine proteinase precursor (AAQ22984, 5.00E-05)	EY417083	4.419	No change
5CAP090504-pMES_E03_435	alpha tubulin 2 (AAM09674, 6.00E-48)	EY417749	2.984	No change
5CAP092402_B10_694	LOC443610 protein (AAI28922, 7.00E-55)	EY418226	2.838	No change
ApC/EBP (positive control)	ApC/EBP (AAA18286, 0)		2.594	Significant
5HTCNS122105-T3-C10-628	Eukaryotic translation initiation factor 3, subunit 6 (NP_001559, 5.00E-73)	EY416763	2.436	Significant
5CAP090501-pMES_F08_164	Hypothetical protein (XP_001176996, 6.00E-33)	EY417489	2.230	No change
Down-regulated clones				
5CAP101001_F09_1029	Kazal proteinase inhibitor (ABL74453, 2.00E-22)	EY418526	0.346	No change
5CAP120302-pMES_C06_1166	unnamed protein product (CAG08644, 2.00E-14)	EY419713	0.356	Reverse trend
5CAP031405_pMES_G09_1891	hCG1999844 (EAX04582, 0.013)	EY417337	0.460	No change
5CAP101001_H07_1051	BAT1 homolog (AAQ13472, 2.00E-26)	EY418548	0.486	Significant

*BLASTX results are described as: gene description (accession number of BLASTX matched gene, E-value).

†Significant means $P < 0.05$ by student's t test; trend or reverse trend means $0.05 < P \leq 0.10$ by student's t test; no change means $P > 0.10$.

one clone (BAT1 homolog) was significantly down-regulated by 5-HT treatment (Fig. 2). The expression of the remaining clones will be examined in a future study.

Role of *Aplysia* eIF3e in 5-HT-Induced, Long-Term Facilitation at the Sensorimotor Synapse. As the next step, we tested whether a 5-HT-regulated gene, eIF3e/subunit 6 (Int6) (Table 2), contributes to the long-term plasticity. First, we obtained a full-length cDNA from *A. kurodai* nervous system (for the cloning information, see Materials and Methods). A cloned Ap-eIF3e/Int6 (GenBank accession number EU791459) showed 56–73% amino

acid sequence identity in comparison with eIF3e/Int6 of other species (Fig. S1). The C-terminal PCI domain, which is involved in translation initiation and protein degradation, is also well conserved in Ap-eIF3e (19, 20).

To examine the role of Ap-eIF3e in long-term facilitation, we overexpressed Ap-eIF3e in a sensory neuron of sensory to motor cocultured synapse. Overexpression of Ap-eIF3e itself did not affect the synaptic strength one day after microinjection (% change in EPSP amplitude, 92.7 ± 4.6 , $n = 17$ and 100.0 ± 6.4 , $n = 14$ for Ap-eIF3e overexpressing and control synapses, respectively; $P = 0.351$, Fig. 3A). One pulse of 5-HT treatment,

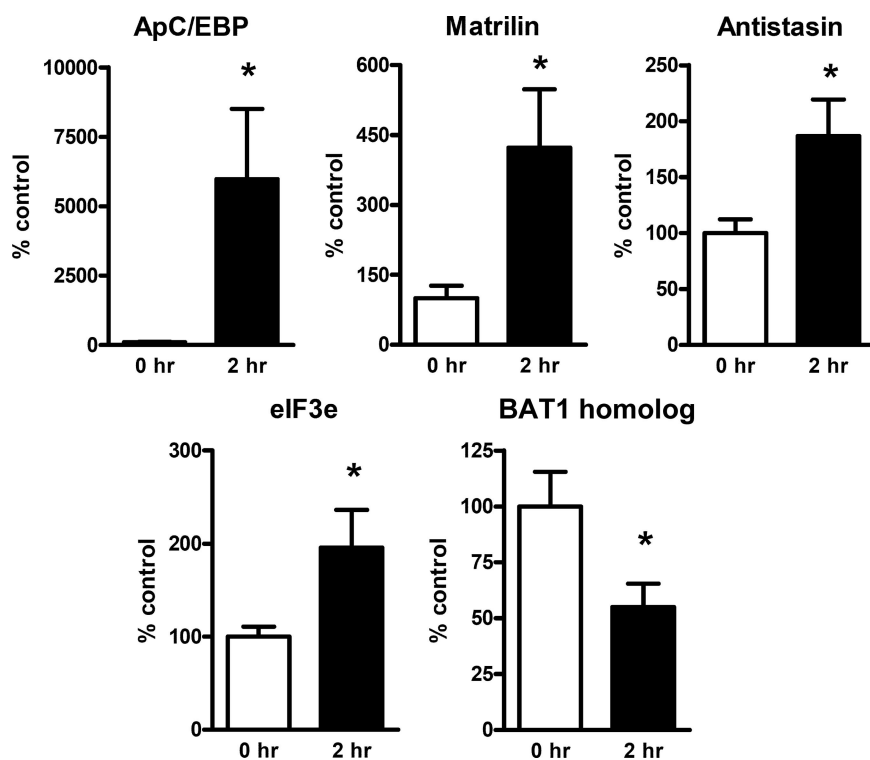


Fig. 2. Real-time RT-PCR analyses on the selected 5-HT regulated genes. Four genes (ApC/EBP, matrilin, antistatin, and eIF3e) were found to be significantly increased, and one gene (BAT1 homolog) was significantly decreased in response to 5-HT treatment ($P < 0.05$, Student t test).

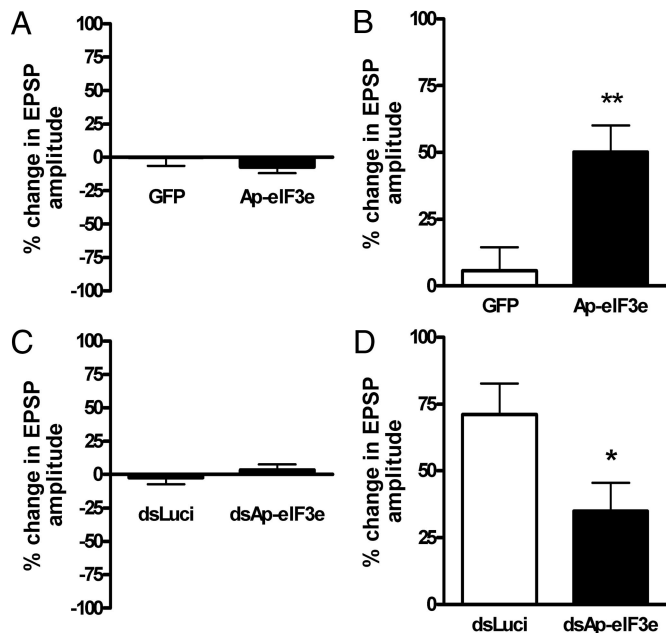


Fig. 3. Effect of Ap-eIF3e overexpression and knock-down on 5-HT induced LTF. (A) Bar graph representing the effect of overexpression of Ap-eIF3e on basal synaptic transmission. Overexpression of Ap-eIF3e by injection of pNEX δ -Ap-eIF3e into the sensory cell did not produce any significant changes in synaptic strength. The height of each bar corresponds to the mean percentage of change \pm SEM in EPSP amplitude tested 24 h after microinjection. (B) Bar graph representing the effect of overexpression of Ap-eIF3e on LTF. Overexpression of Ap-eIF3e in the sensory cell produced LTF when it was combined with the application of 10 μ M 5-HT for 5 min (one pulse, 1 \times) that can induce only STF. The height of each bar corresponds to the mean percentage of change \pm SEM in EPSP amplitude tested 24 h after 5-HT (10 μ M) treatment. **, $P < 0.01$ (C) Effect of Ap-eIF3e dsRNA on basal synaptic transmission. EPSP amplitudes were measured 24 h after Ap-eIF3e or luciferase dsRNA injections. (D) Effect of Ap-eIF3e dsRNA on 5 \times 5-HT-induced LTF. EPSP amplitudes were measured 24 h after 5 \times 5-HT treatment *, $P < 0.05$.

however, significantly increased the synaptic efficacy as measured by EPSP amplitude 24 h after 5-HT treatment only in Ap-eIF3e overexpressing synapses (% change in EPSP amplitude, 150.2 ± 9.9 , $n = 17$ and 105.7 ± 8.9 , $n = 14$ for Ap-eIF3e overexpressing and control synapses, respectively; **, $P < 0.01$) (Fig. 3B). These data suggest that Ap-eIF3e is a positive regulator, which consolidates STF into LTF.

To investigate whether Ap-eIF3e is a necessary factor for LTF, expression of Ap-eIF3e was blocked by microinjecting dsRNA against Ap-eIF3e in a sensory neuron and then, five pulses of 5-HT were applied. Knock-down of Ap-eIF3e did not produce any significant change in the basal synaptic transmission one day after microinjection (% change in EPSP amplitude, 103.6 ± 3.9 , $n = 15$ and 97.7 ± 5.0 , $n = 13$ for Ap-eIF3e dsRNA and control luciferase dsRNA, respectively; $P = 0.351$, Fig. 3C). However, synapses injected with Ap-eIF3e dsRNA showed decreased facilitation 24 h after five pulses of 5-HT (% change in EPSP amplitude, 134.8 ± 10.7 , $n = 15$; Fig. 3D). This facilitation was significantly different from the increase in EPSP amplitude measured in control dsRNA-injected synapses treated with five pulses of 5-HT (% change in EPSP amplitude, 171.2 ± 11.6 , $n = 13$; *, $P < 0.05$, student's t test; Fig. 3D). These data suggest that Ap-eIF3e plays a critical role in the consolidation of LTF.

Discussion

We have generated and analyzed 11,493 *A. kurodai* ESTs. Although over thirty-five species of the genus *Aplysia* have been reported throughout the world, only one species *A. californica*

has been studied extensively because it is commonly available along the US coasts and has been successfully bred in the laboratory (21, 22). *A. kurodai* is another commonly found species in the Asian Pacific area, along the Korean and Japanese coasts. Interestingly, although *A. kurodai* and *A. californica* are classified into two different subgenera *Varrta* and *Neoaplysia*, respectively, the nervous system of *A. kurodai* appears almost identical to *A. californica* both anatomically and physiologically (6, 22). Furthermore, the molecular mechanisms underlying synaptic plasticity are well conserved in both species. For example, the neurotransmitter 5-HT can induce both short- and long-term synaptic facilitation in both species, depending on the number of treatments. In addition, common sets of signaling molecules and pathways play essentially identical roles in synaptic facilitation in both species (23–26).

Despite those similarities between two species and of the relatively larger size of EST database of *A. californica*, the current analysis shows that >30% of *A. kurodai* ESTs do not have homologous sequences in *A. californica* (Fig. 1). However, this might be an overestimate because of the limited genomic information from mollusks. *A. kurodai* transcripts, which had no *A. californica* matches or no BLAST matches, can be divided into three groups: (i) the genuine *A. kurodai* specific genes, (ii) noncoding RNAs, and (iii) intron related sequences. The data showing that 80% of *A. californica* nonmatching transcripts did not find matches in protein databases across all phyla (Fig. 1B) strongly suggests that most of the nonmatching sequences are from noncoding regions, which are less conserved or not sequenced in *A. californica* yet. In addition, of the remaining 20% BLAST-matching transcripts, only 3% had matches to known protein sequences from Mollusca, while 59% were homologous to vertebrates (Fig. 1C), which might be because of the incompleteness of molluscan databases. Taken together, it is less likely that the 33% of *A. californica* nonmatching transcripts are genuine *A. kurodai*-specific genes. We expect that an ongoing *A. californica* whole genome project will improve the quality of the ortholog-mapping procedure in the near future. Indeed, addition of the *A. kurodai* EST resources described here should facilitate the assembly and annotation of any forthcoming genomic sequences.

Because 5-HT is critically involved in both memory and synaptic facilitation in *Aplysia*, there has been a substantial amount of effort to identify differentially transcribed genes in response to this key neurotransmitter (11, 12, 27–29). In the present study, we used a microarray technique as a primary screening to identify 5-HT-regulated genes in *Aplysia* pleural ganglion. We monitored the gene expression profile 2 h after the onset of 5-HT treatment, which is a critical time period for the synthesis of macromolecules required for LTF (10). To validate the results of the primary screening and exclude false positives for the further studies, we performed qRT-PCR analysis. Because we used the low stringent experimental conditions in the primary screening (e.g., small number of arrays, no dye-swapping) to allow more chances to detect as many novel 5-HT responsive genes as possible, relatively large number of genes from the primary screening turned out to be false positives (Table 2). Nevertheless, we could identify a set of interesting 5-HT-regulated genes, which might be involved in synaptic facilitation.

The expression level of matrilin was increased in 5-HT treated pleural ganglia to more than fivefold than that of the control (Table 2 and Fig. 2). Matrilin belongs to a family of fibril-forming extracellular matrix proteins with four paralogues in the human genome (30). It participates in the formation of fibrillar or filamentous structures and is often associated with collagens. Moreover, it mediates interactions between collagen-containing fibrils and other matrix constituents (31). Although the role of matrilin in the nervous system has never been demonstrated, recent evidence suggests that extracellular matrix molecules,

such as laminin, reelin and tenascins, are involved in synaptic plasticity by interacting with ion channels or neurotransmitter receptors (32). Another gene antistasin that also indirectly regulates cytoskeleton structure was found to be up-regulated (33), suggesting that up-regulation of matrilin and antistasin may play a role in synaptic plasticity, possibly by regulating cytoskeleton structures or other membrane proteins. In addition, BAT1 homolog was down-regulated by 5-HT treatment and confirmed by qRT-PCR (Fig. 2). BAT1 is known as an essential RNA splicing factor and also plays an important role in exporting mRNA from nucleus (34, 35). *Aplysia* BAT1 homolog may regulate mRNA transport and/or splicing of some synaptic plasticity-related genes. The role of this down-regulated gene in LTF remains to be investigated.

To examine the possible role of differentially expressed genes in synaptic facilitation, we performed a functional analysis on one of the up-regulated genes, Ap-eIF3e. Previously, we have shown that the overexpression of an immediate early gene ApC/EBP in presynaptic sensory neuron enables a single pulse of 5-HT to induce LTF that is normally achieved by repeated five pulses of 5-HT in sensory to motor coculture (25). Similarly, the overexpression of Ap-eIF3e also converted STF to LTF. Moreover, when Ap-eIF3e expression was blocked by dsRNA, LTF induction by repeated pulses of 5-HT was impaired, suggesting that Ap-eIF3e is critically involved in consolidation of LTF. eIF3e/Int6 was initially characterized as a common integration site for the mouse mammary tumor virus (MMTV) in mouse mammary tumors (36). MMTV insertion into Int6 causes the production of a truncated mutant protein, which has the transforming activity, suggesting that normal Int6 functions as a tumor suppressor gene (37). Although it was identified as a subunit homolog of eIF3, which binds to 40S ribosomal subunit (38), it is not essential for translation and thought to play a regulatory role (39). Several lines of evidence suggest that eIF3e/Int6 is also involved in protein degradation by interacting with proteasome complexes (40, 41). Recently, eIF3e/Int6 was shown to be involved in activity-dependent internalization of voltage-gated calcium channel in cortical neuron (42). So far, the molecular function of Ap-eIF3e is unclear. Does Ap-eIF3e facilitate the translation of LTF-related proteins? Is it involved in the degradation of negative regulatory proteins during consolidation of LTF? These issues will be addressed in future experiments.

In summary, by constructing, annotating and comparatively analyzing *A. kurodai* ESTs with *A. californica* ESTs, our data provides a complementary resource to the *Aplysia* genome annotation. Moreover, we identified Ap-eIF3e as a positive regulator in 5-HT-induced LTF. Further functional studies of other differentially expressed genes found in the present study will provide insights to improve our understanding of the molecular mechanisms underlying learning and memory.

Materials and Methods

cDNA Library Construction. The total RNA was isolated from *A. kurodai* central nervous system (CNS) with TRIzol (Invitrogen), followed by the purification of poly (A)⁺ RNA (Oligotex mRNA midi kit; Qiagen). Five libraries were used in this study. A random-primed library was constructed by using a random primer (9mer) with a Takara cDNA synthesis kit (Takara) following the manufacturer's manual. cDNA inserts were flanked by a linker containing a *Bst*XI enzyme site and cloned into *Bst*XI-digested pYesTrp2 vector (Invitrogen). Full-length-enriched and 5'-end-enriched libraries were constructed as previously described (13, 15). Briefly, oligo-capping was performed after the purification of poly (A)⁺ RNA. After removing unligated 5'-oligo, cDNA was synthesized by using dT adapter-primer (full-length-enriched) or random adapter-primer

(5'-end-enriched) and amplified by PCR. PCR products were digested with *Sfi*I and cloned into *Dra*III-digested pME185-FL vector. The 5-HT-treated CNS library was constructed from mRNA, which was extracted from CNS of *in vivo* 5-HT-treated animals (250 μ M for 2 h). cDNA was synthesized by using oligo(dT) as a primer and adapted with *Eco*RI linker. Then, the cDNA was directionally cloned into Uni-ZAP XR vector (Stratagene). The buccal muscle library was constructed with mRNA from *Aplysia* buccal muscle by following the same procedure as the 5-HT-treated library using Uni-ZAP XR cloning system (Stratagene).

EST Sequencing, Assembly, and Annotation. Plasmid DNA was extracted from the libraries and sequenced by using a 3730xl DNA analyzer (Applied Biosystems). The sequencing primers used were 5'-TGGATGTTGCCCTTACTTCT-3' (full-length-enriched and 5'-end-enriched libraries) and 5'-GCGTGAATGTA-AGCGTGAC-3' (random-primed library), respectively. 5-HT-treated and buccal muscle libraries were sequenced with T3 primer. Base-calling and quality assessment were performed with phred (43, 44). Vector sequences were trimmed by using the sequence comparison program cross_match (45). EST clustering was performed by BLAST (18), and then clustered through a single-linkage method (46). Each clustered EST file was assembled by using the contig assembly program CAP3 (16). Gene ontology format files and gene association files of the Compugen gene ontology (17) were obtained from the GO database website (www.geneontology.org). We extracted information about the relationships of the GO terms through the hierarchical structure of gene ontology. To classify putative transcripts of *A. kurodai* into GO categories, these sequences were searched against the GO protein sets of Compugen's GenBank GO annotation by using BLAST.

Microarray and qRT-PCR. The construction and hybridization of the cDNA microarray were performed as described previously (47). Please see *SI Materials and Methods* for the details.

Cloning and Microinjection of Ap-eIF3e cDNA. cDNA showing a high homology to eIF3e (5HTCNS122105-T3-C10-628) was rescued from an *A. kurodai* 5-HT-treated cDNA library. The 5'-end of the cDNA including the start codon was obtained by performing 5'-RACE using the SMART-RACE cDNA amplification kit (Clontech). The full-length Ap-eIF3e CDS was amplified by PCR using *A. kurodai* pleural ganglia cDNA as templates. The amplified CDS was subcloned into a *Hind*III-*Xba*I site of expression vector pNEX δ (4). To generate dsRNA, a *Xho*I-*Sac*I fragment of 5HTCNS122105-T3-C10-628 (\approx 800 bp) was subcloned into pLitmus28i (New England Biolabs). Sense and antisense RNAs were synthesized by *in vitro* transcription of linearized template DNAs using T7 RNA polymerase (Ambion) and annealed. Microinjection was primarily conducted as described previously (4). The DNA (pNEX δ -Ap-eIF3e, 500 ng/ μ L) or dsRNA (500 ng/ μ L) was mixed with a reporter construct pNEX δ -EGFP (500 ng/ μ L) in the injection solution (10 mM Tris-Cl; 100 mM NaCl; and 0.1% fast green; pH 7.3) immediately before the injection.

Sensory-to-Motor Coculture and Electrophysiology. Sensory-to-motor neuron coculture of *A. kurodai* and electrophysiological recording of 5-HT-induced long-term facilitation were performed as described previously by Lee and colleagues (25). The percent change in EPSP amplitude of basal synaptic transmission was measured by comparing EPSP amplitude at 24 h after DNA or dsRNA microinjection with initial EPSP amplitude. To induce long-term facilitation in sensory-to-motor synapse, five pulses of 5-HT (10 μ M) were treated for 5 min at 15-min intervals. The resultant percent change in EPSP amplitude was calculated by comparing EPSP amplitude at 24 h after 5-HT treatment to that before 5-HT treatment.

ACKNOWLEDGMENTS. This work was supported by the National Creative Research Initiative Program of the Korean Ministry of Science and Technology and the Marine and Extreme Genome Research Center Program, Ministry of Marine Affairs and Fisheries, Republic of Korea. Korean Bio Information Center was supported by the Korean Ministry of Science and Technology under grant number M10407010001-04N0701-00110 and by the Ministry of Information and Communication, Korea, under the Korea Agency Digital Opportunity and Promotion support program (06-121). Y.-S.L., S.-L.C., and D.-J.J. are supported by BK21 fellowships. L.M. is supported by National Institutes of Health, National Science Foundation, McKnight Brain Research Foundation, and University of Florida opportunity funds. E.K. is supported by the Howard Hughes Medical Institute.

- Bailey CH, Bartsch D, Kandel ER (1996) Toward a molecular definition of long-term memory storage. *Proc Natl Acad Sci USA* 93:13445–13452.
- Carew TJ, Sahley CL (1986) Invertebrate learning and memory: From behavior to molecules. *Annu Rev Neurosci* 9:435–487.

- Kandel ER (2001) The molecular biology of memory storage: A dialogue between genes and synapses. *Science* 294:1030–1038.
- Kaang BK (1996) Parameters influencing ectopic gene expression in *Aplysia* neurons. *Neurosci Lett* 221:29–32.

5. Kandel ER (1976) in *Cellular basis of behavior* (W.H. Freeman and company, New York).
6. Lim CS, Chung DY, Kaang BK (1997) Partial anatomical and physiological characterization and dissociated cell culture of the nervous system of the marine mollusc *Aplysia kurodai*. *Mol Cells* 7:399–407.
7. Moccia R, et al. (2003) An unbiased cDNA library prepared from isolated *Aplysia* sensory neuron processes is enriched for cytoskeletal and translational mRNAs. *J Neurosci* 23:9409–9417.
8. Knudsen B, Kohn AB, Nahir B, McFadden CS, Moroz LL (2006) Complete DNA sequence of the mitochondrial genome of the sea-slug, *Aplysia californica*: Conservation of the gene order in *Euthyneura*. *Mol Phylogenet Evol* 38:459–469.
9. Moroz LL, et al. (2006) Neuronal transcriptome of *Aplysia*: Neuronal compartments and circuitry. *Cell* 127:1453–1467.
10. Montarolo PG, et al. (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* 234:1249–1254.
11. Alberini CM, Ghirardi M, Metz R, Kandel ER (1994) *C/EBP* is an immediate-early gene required for the consolidation of long-term facilitation in *Aplysia*. *Cell* 76: 1099–1114.
12. Hegde AN, et al. (1997) Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. *Cell* 89:115–126.
13. Suzuki Y, Sugano S (2003) Construction of a full-length enriched and a 5'-end enriched cDNA library using the oligo-capping method. *Methods Mol Biol* 221:73–91.
14. Suzuki Y, Yoshitomo-Nakagawa K, Maruyama K, Suyama A, Sugano S (1997) Construction and characterization of a full length-enriched and a 5'-end-enriched cDNA library. *Gene* 200:149–156.
15. Suzuki Y, Sugano S (2001) Construction of full-length-enriched cDNA libraries. The oligo-capping method. *Methods Mol Biol* 175:143–153.
16. Huang X, Madan A (1999) CAP3: A DNA sequence assembly program. *Genome Res* 9:868–877.
17. Xie H, et al. (2002) Large-scale protein annotation through gene ontology. *Genome Res* 12:785–794.
18. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
19. Burks EA, Bezerra PP, Le H, Gallie DR, Browning KS (2001) Plant initiation factor 3 subunit composition resembles mammalian initiation factor 3 and has a novel subunit. *J Biol Chem* 276:2122–2131.
20. Hofmann K, Bucher P (1998) The PCI domain: A common theme in three multiprotein complexes. *Trends Biochem Sci* 23:204–205.
21. Kriegstein AR, Castellucci V, Kandel ER (1974) Metamorphosis of *Aplysia californica* in laboratory culture. *Proc Natl Acad Sci USA* 71:3654–3658.
22. Kandel ER (1979) in *Behavioral Biology of Aplysia: A Contribution to the Comparative Study of Opisthobranch Molluscs* (W. H. Freeman, San Francisco).
23. Han JH, Lim CS, Lee YS, Kandel ER, Kaang BK (2004) Role of *Aplysia* cell adhesion molecules during 5-HT-induced long-term functional and structural changes. *Learn Mem* 11:421–435.
24. Chang DJ, et al. (2000) Activation of a heterologously expressed octopamine receptor coupled only to adenylyl cyclase produces all the features of presynaptic facilitation in *Aplysia* sensory neurons. *Proc Natl Acad Sci USA* 97:1829–1834.
25. Lee JA, et al. (2001) Overexpression of and RNA interference with the CCAAT enhancer-binding protein on long-term facilitation of *Aplysia* sensory to motor synapses. *Learn Mem* 8:220–226.
26. Lee JA, Kim H, Lee YS, Kaang BK (2003) Overexpression and RNA interference of Ap-cyclic AMP-response element binding protein-2, a repressor of long-term facilitation, in *Aplysia kurodai* sensory-to-motor synapses. *Neurosci Lett* 337:9–12.
27. Barzilai A, Kennedy TE, Sweatt JD, Kandel ER (1989) 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in *Aplysia* sensory neurons. *Neuron* 2:1577–1586.
28. Noel F, Nunez-Regueiro M, Cook R, Byrne JH, Eskin A (1993) Long-term changes in synthesis of intermediate filament protein, actin and other proteins in pleural sensory neurons of *Aplysia* produced by an in vitro analogue of sensitization training. *Brain Res Mol Brain Res* 19:203–210.
29. Liu QR, et al. (1997) A developmental gene (Tolloid/BMP-1) is regulated in *Aplysia* neurons by treatments that induce long-term sensitization. *J Neurosci* 17:755–764.
30. Deak F, Wagener R, Kiss I, Paulsson M (1999) The matrilins: A novel family of oligomeric extracellular matrix proteins. *Matrix Biol* 18:55–64.
31. Wagener R, et al. (2005) The matrilins—adaptor proteins in the extracellular matrix. *FEBS Lett* 579:3323–3329.
32. Dityatev A, Schachner M (2003) Extracellular matrix molecules and synaptic plasticity. *Nat Rev Neurosci* 4:456–468.
33. Leadley RJ Jr, Chi L, Porcari AR (2001) Non-hemostatic activity of coagulation factor Xa: Potential implications for various diseases. *Curr Opin Pharmacol* 1:169–175.
34. Fleckner J, Zhang M, Valcarcel J, Green MR (1997) U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction. *Genes Dev* 11:1864–1872.
35. Herold A, Teixeira L, Izaurralde E (2003) Genome-wide analysis of nuclear mRNA export pathways in *Drosophila*. *EMBO J* 22:2472–2483.
36. Marchetti A, et al. (1995) Int-6, a highly conserved, widely expressed gene, is mutated by mouse mammary tumor virus in mammary preneoplasia. *J Virol* 69:1932–1938.
37. Rasmussen SB, Kordon E, Callahan R, Smith GH (2001) Evidence for the transforming activity of a truncated Int6 gene in vitro. *Oncogene* 20:5291–5301.
38. Asano K, Merrick WC, Hershey JW (1997) The translation initiation factor eIF3-p48 subunit is encoded by int-6, a site of frequent integration by the mouse mammary tumor virus genome. *J Biol Chem* 272:23477–23480.
39. Bandyopadhyay A, Matsumoto T, Maitra U (2000) Fission yeast Int6 is not essential for global translation initiation, but deletion of int6(+) causes hypersensitivity to caffeine and affects spore formation. *Mol Biol Cell* 11:4005–4018.
40. Yen HC, Gordon C, Chang EC (2003) Schizosaccharomyces pombe Int6 and Ras homologs regulate cell division and mitotic fidelity via the proteasome. *Cell* 112:207–217.
41. von Arnim AG, Chamovitz DA (2003) Protein homeostasis: A degrading role for Int6/eIF3e. *Curr Biol* 13:R323–325.
42. Green EM, Barrett CF, Bultynck G, Shamah SM, Dolmetsch RE (2007) The tumor suppressor eIF3e mediates calcium-dependent internalization of the L-type calcium channel CaV1.2. *Neuron* 55:615–632.
43. Ewing B, Green P (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8:186–194.
44. Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8:175–185.
45. Gordon D, Abajian C, Green P (1998) Consed: A graphical tool for sequence finishing. *Genome Res* 8:195–202.
46. Pertea G, et al. (2003) TIGR Gene Indices clustering tools (TGICL): A software system for fast clustering of large EST datasets. *Bioinformatics* 19:651–652.
47. Hegde P, et al. (2000) A concise guide to cDNA microarray analysis. *Biotechniques* 29:548–556.