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Differential Evolutionary Rates of Neuronal Transcriptome in *Aplysia kurodai* and *Aplysia californica* as a Tool for Gene Mining

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Abstract: The marine mollusk *Aplysia* is a fascinating model organism for studying molecular mechanisms underlying learning and memory. However, evolutionary studies about *Aplysia* have been limited by the lack of its genomic information. Recently, large-scale expressed sequence tag (EST) databases have been acquired by sequencing cDNA libraries from *A. californica* and *A. kurodai*. The closeness between the two species allowed us to investigate rapidly evolving genes by comparing their orthologs. Using this method, we found that a subset of signal transduction genes in neurons showed rates of protein evolution higher than those of housekeeping genes. Moreover, we were also able to find several candidate genes that may be involved in learning and memory or synaptic plasticity among genes showing relatively higher K_a/K_s ratios. We also investigated the relationship between evolutionary rates and tissue distribution of *Aplysia* genes. They propose that the estimation of evolutionary rates cannot be a good marker to assess neuronal expression; however, it still can be an efficient way to narrow down the pool of candidate genes involved in neuronal functions for the further studies.

Keywords: *Aplysia*, EST, K_a/K_s, neuron, transcriptome

INTRODUCTION

The marine mollusk *Aplysia* is the one of the most important model organisms for studying learning and memory because its nervous system offers many advantages that could facilitate the discovery of molecular mechanisms, such as large cell size and simple nervous system (Bailey et al., 1996; Carew & Sahley, 1986; Kandel, 2001; Lee et al., 2008a). Because of these advantages, a large number of key molecules and molecular pathways that are important in learning and memory have been discovered extensively in *Aplysia* (Kandel, 1976; Lim et al., 1997).

Unlike the molecular and cellular approaches, however, few evolutionary approaches about neuronal genes of *Aplysia* have been taken until now. Lack of genomic information about *Aplysia* might be one of the important limiting factors that restrict this type of approach. Evolutionary approaches may provide useful information to find candidate genes for the studies of neuronal functions such as learning and memory or synaptic plasticity. Recently, two large-scale expressed sequence tag (EST) analyses for closely the related species *A. californica* and *A. kurodai* were completed (Moroz et al., 2006). Moroz, Kandel, and their colleagues sequenced over 200,000 ESTs and identified more than 65,000 nonredundant sequences from *A. californica* (Moroz et al., 2006). Kaang and his colleagues also sequenced 11,493 ESTs, which represented over 4859 nonredundant sequences from *A. kurodai* cDNA libraries derived from the central nervous system (CNS) (Lee et al., 2008b). These data from closely related species of *Aplysia* can provide additional insights into the biology and evolution of the molluskan nervous system and perhaps into neuronal-derived genes of vertebrate animals (Lee et al., 2008b; Moroz et al., 2006).

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Here, we subjected these two ESTs to a series of analyses to compare and evaluate the evolutionary rates of orthologous genes using the K_a/K_s ratio. First, we compared the rates of protein evolution between two subsets of selected neuronal derived genes in *Aplysia*. Based on these data, we also tried to find candidate genes for functional studies without prior knowledge. In addition, we investigated the tissue distributions of some genes to examine the relationship between the K_a/K_s ratio and differential neuronal expression.

MATERIALS AND METHODS

Orthologs Alignment and Calculation of K_a/K_s

For all experiments, two *Aplysia* EST databases were used as resources (Lee et al., 2008b; Moroz et al., 2006). *A. kurodai* contig_IDs are available at http://seahare.org, and *A. californica* contig_IDs are available at http://aplysia.uf-genome.org and http://aplysia.cu-genome.org. The orthologs alignment pipeline uses ClustalW sequence alignment tool (Thompson et al., 1994). As an input, it requires two orthologous mRNA sequences. It translates these mRNA sequences into protein sequences and chooses the longest open reading frame (ORF) with a start codon by a standard genetic code table. Because we performed the analysis using ESTs as resources (Lee et al., 2008b; Moroz et al., 2006), only well-aligned protein-coding sequences, which were manually inspected and verified to be translated in correct frames, were used for the analysis. Moreover, because PAML cannot perform well with short coding regions (Tzeng et al., 2004), we used only long singletons or contigs to overcome the limitation (cutoff threshold was 99 nucleotides encoding 33 amino acids including the start codon).

The K_a/K_s values were calculated by codeml program, implementing the method of Nei and Gojobori in PAML package (Yang, 1997). Orthologs were removed for K_a/K_s values over 10 due to many alignment errors or gaps.

RT-PCR Analysis

For all experiments, mRNAs were isolated from five different kinds of tissues—central nervous system (CNS), buccal mass (BM), stomach (ST), gill (GL), and ovotestis (OT)—using TRIzol Reagent (Invitrogen) following the manufacturer's manual. Samples were then treated with RNase-free DNase I (Ambion) for 40 min to remove residual genomic DNA. cDNA was synthesized as described previously (Yim et al., 2006). The cDNA was amplified using specific primer sets (see Supplementary Table 4; online version only). PCR reaction consisted of one cycle of 95°C for 5 min, followed by 30 or 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. The final extension reaction was carried out at 72°C for 1 min.

Real-Time PCR

The cDNA described above was used for quantitative real-time PCR. PCR reactions were performed in the Thermal Cycler Dice Real Time System, TP800 (Takara) using SYBR Premix Ex *Taq*™ (Takara) and gene specific primer sets (see Supplementary Table 4; online version only). Amplification reaction consisted of one cycle of 95°C for 5 min, followed by 60 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. Data were collected during the extention phase at 72°C. S4 was used as an internal control. For the relative comparison of each mRNA, we analyzed C_T value using the $2^{-\triangle\triangle CT}$ method (Livak & Schmittgen, 2001).

Statistical Analysis

Data collected by real-time PCR were analyzed using a one-way analysis of variance (ANOVA) test. When significant differences in gene expression levels were found, post hoc comparisons were executed by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Differential Evolution of Neuronal Genes in *Aplysia*

In primates and rodents, the evolution of genes involved in various aspects of nervous system function is known to be faster than that of housekeeping genes that are involved in basic metabolic functions. Moreover, the evolutionary rates of these genes are significantly higher in primates than in rodents, consistent with the rapid evolution of brain structure and function (Dorus et al., 2004). However, it is not known that signal transduction genes in neurons show faster evolutionary rate in *Aplysia*, which has a relatively primitive nervous system. To investigate the rates of protein evolution based on sequences derived from the CNSs of two closely related *Aplysia* species, we compiled lists of molluskan homologs of mammalian genes involved in neuronal signaling as well as those of putative housekeeping genes. In the previous study, Dorus et al. selected genes that (i) play important roles in the nervous system, (ii) enriched in nervous system, or (iii) involved in nervous system disorders as "nervous system" genes (Dorus et al., 2004). As the first step to obtain the lists of signal transduction genes in neurons or housekeeping genes in *Aplysia*, we simply collected *Aplysia*

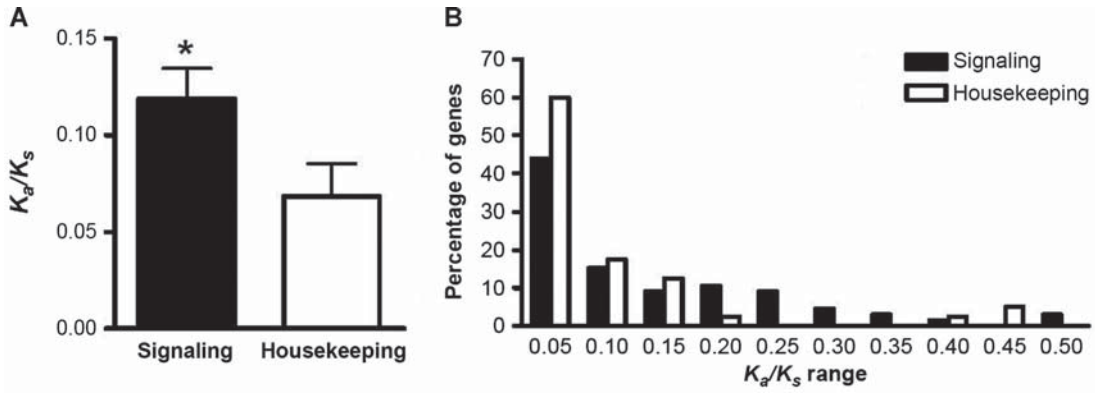

Figure 1. Faster evolution rate of the mammalian homologs of signal transduction genes in *Aplysia* neurons (see text for details). (**A**) Evolutionary rates of signal transduction genes and housekeeping genes in Aplysia. (**B**) The K_a/K_s distribution of the same two subsets of brain derived genes in *Aplysia*.

EST sequences homologous to those used in Dorus et al's study (Dorus et al., 2004). In addition, we used the GenBank databases to obtain homologous and orthologous sequences, which were previously cloned and characterized in *Aplysia californica* but do not exist either in *A. kurodai* or *A. californica* EST database. Finally, we excluded the homologous sequences that are not functionally characterized (e.g., unnamed protein product). In this way we obtained from both species of *Aplysia* 44 signal transduction genes in neurons as well as 31 putative housekeeping or basic metabolic genes (Supplementary Tables 1 and 2; online version only). It should be noted that we took a biased approach in the selection process of these two classes of genes and all the signal transduction genes in neurons are not preferentially expressed in the nervous system. The selection of two groups of genes was largely based on prior knowledge on gene functions in vertebrate homologs.

To measure the rates of protein evolution, we applied a common method (Hurst, 2002) in which we calculated the ratio (K_a/K_s) of the number of nonsynonymous substitutions per nonsynonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s). Although it is known that evolutionary rates of brain-specific genes are slower than those of other tissue-specific genes, they are still faster than those of housekeeping genes (Zhang & Li, 2004). We found that the average K_a/K_s ratio of selected signal transduction genes in neurons is significantly higher, by a factor of 2, than that of putative housekeeping genes (0.111 ± 0.019, $n = 44$; and 0.062 ± 0.017, $n = 31$, respectively, mean ± SEM; $p < .05$, Kolmogorov-Smirnov test; Figure 1A and B). The average K_a and K_s values of the signal transduction genes in neurons are 0.019 ± 0.004 and 0.209 ± 0.025, respectively. Because K_a/K_s values of signal transduction

Table 1. Top 15 *Aplysia* genes showing the highest K_a/K_s rate

Gene description	GI	*E*-value	K_a	K_s	K_a/K_s	HSP length$^+$
Vacuolar ATP synthase subunit e	68065343	5E–16	0.013	0.014	0.937	76
RAB2	288938	6E–62	0.068	0.140	0.488	141
Soluble acetylcholine receptor	17225107	5E–113	0.091	0.196	0.467	236
Hemocyanin	62679967	3E–81	0.063	0.136	0.466	158
Heart-type fatty acid–binding protein	17530523	2E–16	0.170	0.374	0.456	132
Glutathione *S*-transferase	8917596	6E–28	0.098	0.221	0.443	203
Cyclophylin isoform	94468464	5E–49	0.032	0.077	0.411	179
Dehydrogenases, short-chain family member (dhs-14)	17562906	1E–14	0.111	0.275	0.405	142
ATP synthase, mitochondrial F1 complex, alpha subunit	127798841	3E–106	0.006	0.015	0.400	266
ApCREB2	1123037	2E-120	0.027	0.070	0.386	228
Proline 4-hydroxylase	48735337	7E-80	0.032	0.087	0.366	260
MIP-related peptide precursor	8886135	2E-71	0.067	0.185	0.361	140
Zinc finger, HIT type 3	17389844	2E-19	0.063	0.183	0.346	148
Cct7-prov protein	50418287	2E-26	0.056	0.161	0.346	201
Translation initiation factor 5A	47085971	8E-58	0.017	0.054	0.322	151

Note.$^+$High score pairing length of translated amino acids sequences.

Table 2. Bottom 15 *Aplysia* genes showing the lowest K_a/K_s rates

Gene description	GI	*E*-value	K_a	K_s	K_a/K_s	HSP length[+]
Ribosomal protein S14	12083607	7E-65	0	0.0497	0	139
GTP-binding protein alpha-o subunit	9633	2E-83	0	0.0495	0	156
RHO_APLCA RAS-like GTP-binding protein RHO	132545	2E-95	0	0.0481	0	193
Unnamed protein product	67969593	1E-75	0	0.0478	0	245
40S ribosomal protein S15	20069100	8E-39	0	0.0474	0	78
Unnamed protein product	47230461	8E-21	0	0.0418	0	56
AGAP010957-PA	158287848	2E-79	0	0.0371	0	149
Guanine nucleotide regulatory protein beta subunit	312632	9E-103	0	0.0369	0	186
Ribosomal protein L12	22758902	4E-69	0	0.0325	0	158
Cnot4-prov protein	28278582	2E-43	0	0.0197	0	101
Y-box factor homolog (APY1)	1175568	5E-53	0	0.0192	0	165
Unnamed protein product	47228202	1E-41	0.0001	0.1492	0.0007	162
Splicing factor-like protein	51105084	2E-32	0.0001	0.1484	0.0007	73
Similar to Rps16 protein	50728374	1E-71	0.0001	0.1441	0.0007	146
Similar to glucocorticoid-induced gene 1	109464606	2E-14	0.0001	0.1404	0.0007	151

[+]High score pairing length of translated amino acids sequences.

genes in neurons were all <1.0, we can assume that positive selection pressure on these genes was not the major driving force of evolution (Hurst, 2002). Therefore, in large part these differences might be a result of stronger purifying selection constraints on the housekeeping genes than on the signal transduction genes in neurons in *Aplysia*. These values are also comparable with those presented in previous reports on humans and rodents (Duret & Mouchiroud, 2000; Zhang & Li, 2004). These data suggest that the comparative rates of protein evolution of the two subsets of neuronal-derived genes in *Aplysia* appear different. However, it should be noted that we examined only a limited number of genes that were selected by their predicted molecular functions and that the definition of neuronal-signaling-related gene can be argued as arbitrary.

Unbiased Evaluation of the Evolutionary Rates of *Aplysia* Orthologs

We next subjected the total collection of *Aplysia* orthologs without any categorization to the K_a/K_s analysis. We only used well-aligned, BLASTX-matched 410 orthologous sequences for this analysis (Supplementary Table 3; online version only). To calculate the K_a/K_s value of total *Aplysia* ESTs, we used the same method as we used in Figure 1 to measure the K_a/K_s values of signal transduction genes in neurons and housekeeping genes. The average K_a/K_s of these genes was 0.093 ± 0.005, which was between the values of the selected signal transduction genes in neurons (0.111 ± 0.019) and the housekeeping metabolism-related genes (0.062 ± 0.017). Because *A. kurodai* ESTs were originally collected from the central ganglia, all the orthologs can be considered to have derived from neurons, glia, and components of connective tissues and circulatory systems (Lee et al., 2008b).

Because our biased approach revealed that signal transduction genes in neurons show faster evolutionary rates, we tried to find candidate genes for the studies of learning and memory or synaptic plasticity simply by measuring the evolutionary rates without a priori knowledge about those genes (Supplementary Table 3; online version only). Table 1 demonstrates the top 15 *Aplysia* genes that showed the highest K_a/K_s rates. Some of them (RAB2, soluble acetylcholine receptor, and ApCREB2) had already been used for the K_a/K_s calculation as signal transduction genes in neurons (Supplementary Table 3; online version only). From this list, we could find some candidate genes for functional studies about learning and memory or modulation of the neuronal function. Of these, a small guanosine triphosphate (GTP)-binding protein RAB2 is known to play a role in neuronal adhesion and neurite growth in dissociated rat midbrain neurons (Ayala et al., 1990). The vacuolar adenosine triphosphate (ATP) synthase (v-ATPase), which we identified in *Aplysia*, is an important proton pump that acidifies a wide variety of intracellular and some extracellular compartments (Nishi & Forgac, 2002). In the nervous system, v-ATPase is involved in vesicle exocytosis (Hiesinger et al., 2005) and in loading synaptic vesicles with neurotransmitters (Amara & Kuhar, 1993). However, v-ATPase expression is not limited to the nervous system. It is abundant in all secretory tissues such as salivary glands and components of the digestive tract as well as in epithelial structures (Nelson & Harvey, 1999). Another example of gene mining in this study was proline 4-hydroxylase. This enzyme catalyzes the formation of 4-hydroxyproline in collagens and more than 10 additional proteins with collagen-like sequences. It also negatively regulates the stability of several

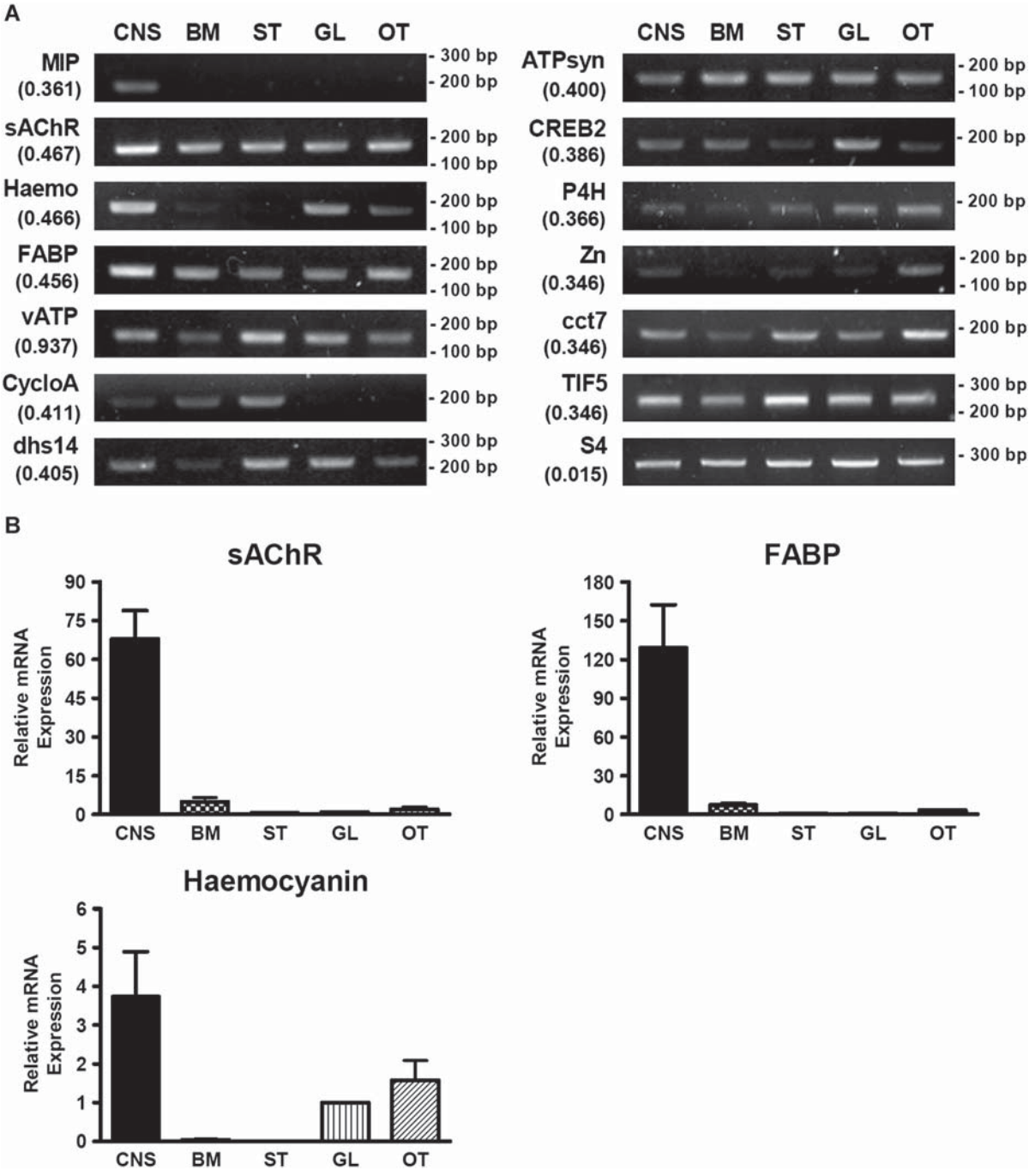

Figure 2. Differential expression levels of the genes showing the highest K_a/K_s values. (**A**) RT-PCR results on the 13 *Aplysia* genes showing highest evolutionary rates. Soluble acetylcholine receptor, hemocyanin, heart-type fatty acid–binding protein, and MIP were highly expressed in the central nervous system. One of these genes (MIP) was expressed only in CNS. K_a/K_s ratio for each gene is indicated in parenthesis (**B**) Expression levels of three genes (soluble acetylcholine receptor, hemocyanin, and heart-type fatty acid–binding protein) were confirmed by real-time PCR. Two genes (soluble acetylcholine receptor and heart-type fatty acid–binding protein) showed significantly higher expression levels in the CNS when compared to the other tissues ($p < .05$, ANOVA and Tukey's multiple comparison test). Hemocyanin was also significantly enriched in the CNS except when compared to the OT ($p > .05$, ANOVA and Tukey's multiple comparison test). CNS, central nervous system; BM, buccal mass; ST, stomach; GL, gill; OT, ovotestis; vATP, vacuolar ATP synthase subunit e; sAChR, soluble acetylcholine receptor; Haemo, hemocyanin; FABP, heart-type fatty acid–binding protein; CycloA, cyclophylin isoform; dhs14, dehydrogenase, short-chain family member; ATPsyn, ATP synthase, mitochondrial F1 cmplex alpha subunit; CREB2, ApCREB2; P4H, proline 4-hydroxylase; MIP, MIP-related protein precursor; Zn, zinc finger, HIT type 3; cct7, cct7-prov protein; TIF5, transcription initiation factor 5A.

proteins that have critical roles in adaptation to hypoxic or oxidative stress (Kivirikko & Myllyharju, 1998; Siddiq et al., 2005). The transcript of proline 4-hydroxylase can be specific for connective tissues that support molluskan ganglia. Interestingly, this protein homolog has been suggested as a target for neuroprotection in the central nervous system of mammals (Siddiq et al., 2005). As the final example, MIP-related peptide precursor has been identified in *Aplysia* and has been reported to operate in the neural circuits that initiate feeding (Fujisawa et al., 1999) and gill-siphon withdrawal (Moroz et al., 2006). Furthermore, we also looked at the bottom 15 *Aplysia* genes showing low K_a/K_s rates (Table 2). We found that there were four ribosomal proteins that could be considered as housekeeping genes involved in basic protein synthesis. Moreover, we could not find any interesting candidate gene for functional studies on learning and memory or modulation of the neuronal function based on gene description among bottom 15 genes. These bioinformatical analyses suggest that trimming out the genes that have relatively low K_a/K_s ratio can be an effective way to narrow down the pool of candidate genes for the functional studies of learning and memory or modulation of the neuronal function in *Aplysia*.

Tissue Distribution of Fast- and Slow-Evolving Genes

To further explore the implications of this observation, we investigated the tissue distributions of these genes. It is known that there is a correlation between gene expression and amino acid sequence divergence at least in humans and rodents (Zhang & Li, 2004). And we can speculate that there is a higher probability that the gene may play a significant biological role in a specific tissue where it is highly expressed, although the tissue-specific enrichment of a gene does not necessitate an important function within that tissue. To examine the tissue distributions of the 13 genes showing the highest K_a/K_s, reverse transcriptase–polymerase chain reaction (RT-PCR) analysis was performed using total RNAs from five different kinds of tissues as templates: central nervous system (CNS), buccal mass (BM), stomach (ST), gill (GL), and ovotestis (OT). Among these 13 genes, 4 (soluble acetylcholine receptor, hemocyanin, heart-type fatty acid–binding protein, and *Mytilus* inhibitory peptide (MIP) showed higher expression levels in the central nervous system than in the other tissues. The expression levels of three genes (soluble acetylcholine receptor, hemocyanin, and heart-type fatty acid–binding protein) were confirmed by real-time PCR (Figure 2A and B). Similarly, RT-PCR analysis was also performed on the 14 genes that showed the lowest K_a/K_s values. Unlike the 13 genes with the highest K_a/K_s values, these genes with low K_a/K_s values tended to show less variable expression levels across the five tissues. This might be because some housekeeping genes such as ribosomal proteins were contained in the list of genes with low K_a/K_s values. However, we could not find any strong relationship between K_a/K_s values and differential tissue expressions. Compared to the genes that exhibited the highest K_a/K_s values, the four specific genes enriched in the CNS, two genes (ENSANGP00000012700 and GTP-binding protein alpha-o subunit) showed higher expression levels in the CNS among the 14 putative low rated genes. These results were also confirmed by real-time PCR (Figure 3A and B). These data suggest that evolutionary rate of some genes cannot be an effective marker to estimate the neuronal expression in *Aplysia*.

CONCLUSION

Among the species of *Aplysia, A. californica* and *A. kurodai* are the most extensively studied. However, the comparative studies between these two species are very limited: for example, behavioral and ecological niche of *A. kurodai* and *A. californica* have not been systematically investigated. In the present study, we investigated the molecular and genetic diversity between these two species.

Comparing the K_a/K_s ratio using *A. kurodai* and *A. californica* EST databases, we discovered that the evolutionary rates of a group of selected nervous system–related and signal transduction–associated genes were higher than those of putative housekeeping genes (Lee et al., 2008b; Moroz et al., 2006). Although we examined a limited number of genes, we confirmed that signal transduction genes in neurons, basically defined by Dorus et al. (Dorus et al., 2004), showed faster evolutionary rates than those of housekeeping genes in *Aplysia*. We were also able to find candidates for further functional studies simply by measuring the evolutionary rates in the group of genes with higher K_a/K_s ratios without *a priori* knowledge about those genes. We suggest that trimming out the genes that have very low K_a/K_s ratio can be an efficient way to narrow down the pool of candidate genes involved in learning and memory or modulation of the neuronal functions. We also tested whether the evolutionary rate of some genes can be used as an effective marker to estimate neuronal expression. Although we found that evolutionary rate is not an effective marker to estimate neuronal expression, we suggested differential tissue expression profiles of some genes. These expression profiles, however, still help us identify candidate genes that have important roles in *Aplysia* CNS.

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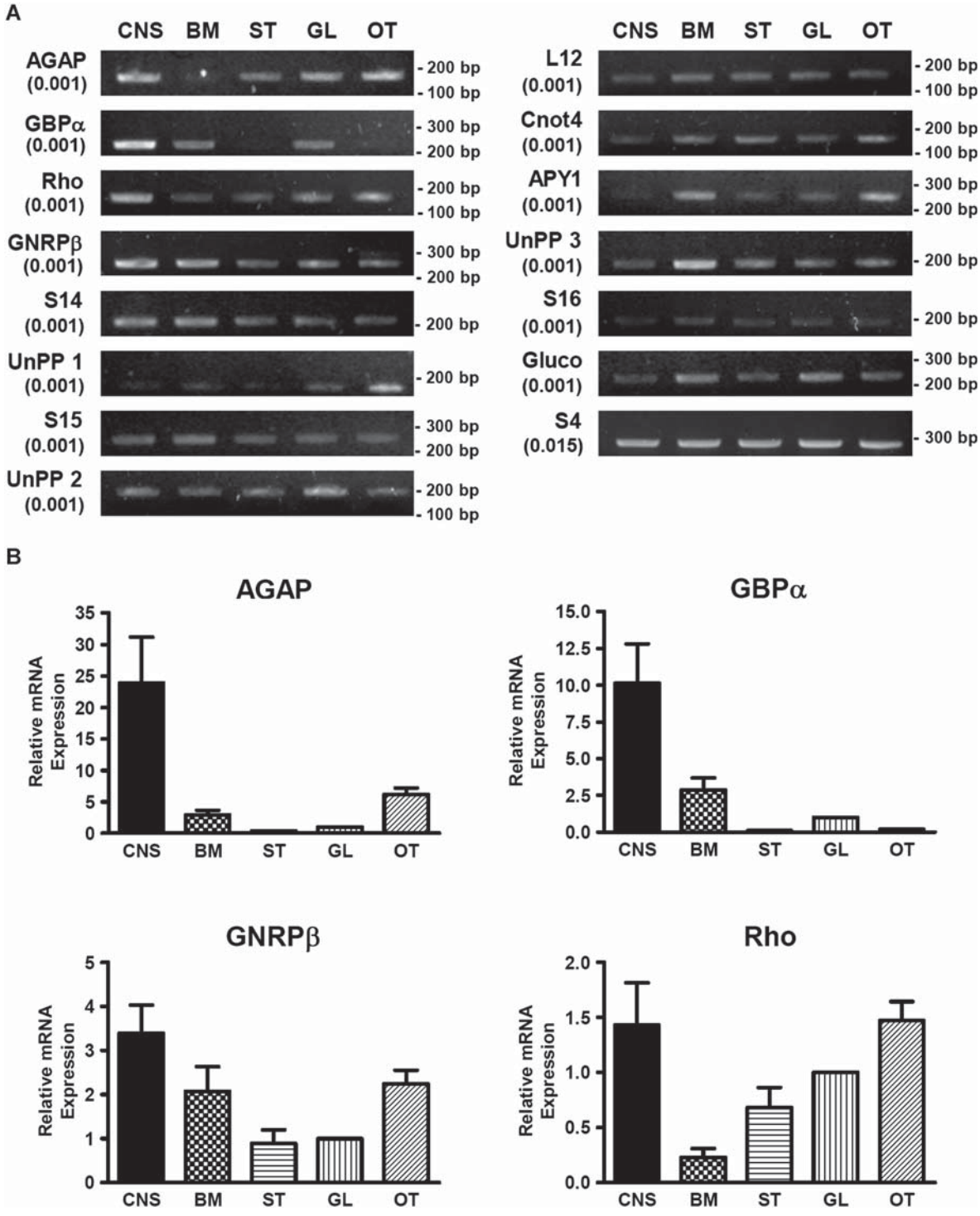

Figure 3. Differential expression levels of the genes showing the lowest K_a/K_s values. (A) RT-PCR results on the 14 *Aplysia* genes showing the lowest evolutionary rates. ENSANGP00000012700 and GTP-binding protein alpha-o subunit were highly expressed in the central nervous system. K_a/K_s ratio for each gene is indicated in parenthesis. (B) Expression levels of four genes (AGAP010957-PA, GTP-binding protein alpha-o subunit, guanine nucleotide regulatory protein beta subunit, and RHO_APLCA RAS-like GTP-binding protein RHO) were confirmed by real-time PCR. Two genes (AGAP010957-PA and GTP-binding protein alpha-o subunit) showed significantly higher expression levels in the CNS when compared to the other tissues ($p < .05$, ANOVA and Tukey's multiple comparison test). GNRPb was also significantly enriched in the CNS compared to the ST and GL, and Rho showed significantly higher expression in CNS than in BM ($p < .05$, ANOVA and Tukey's multiple comparison test). AGAP, AGAP010957-PA; GBPα, GTP-binding protein alpha-o subunit; Rho, RHO_APLCA RAS-like GTP-binding protein RHO; GNRPβ, guanine nucleotide regulatory protein beta subunit; S14, ribosomal protein S14; UnPP, unnamed protein product; S15, 40S ribosomal protein S15; L12, ribosomal protein L12; Cnot4, Cnot4-prov protein; APY1, YBOXH_APLCA Y-box factor homolog; S16, similar to Rps16 protein; Gluco, similar to glucocorticoid-induced gene 1.

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REFERENCES

Amara, S. G., & Kuhar, M. J. (1993). Neurotransmitter transporters: Recent progress. *Annu Rev Neurosci, 16,* 73–93.

Ayala, J., Touchot, N., Zahraoui, A., Tavitian, A., & Prochiantz, A. (1990). The product of rab2, a small GTP binding protein, increases neuronal adhesion, and neurite growth in vitro. *Neuron, 4,* 797–805.

Bailey, C. H., Bartsch, D., & Kandel, E. R. (1996). Toward a molecular definition of long-term memory storage. *Proc Natl Acad Sci U S A, 93,* 13445–13452.

Carew, T. J., & Sahley, C. L. (1986). Invertebrate learning and memory: From behavior to molecules. *Annu Rev Neurosci, 9,* 435–487.

Dorus, S., Vallender, E. J., Evans, P. D., Anderson, J. R., Gilbert, S. L., Mahowald, M., et al. (2004). Accelerated evolution of nervous system genes in the origin of Homo sapiens. *Cell, 119,* 1027–1040.

Duret, L., & Mouchiroud, D. (2000). Determinants of substitution rates in mammalian genes: Expression pattern affects selection intensity but not mutation rate. *Mol Biol Evol, 17,* 68–74.

Fujisawa, Y., Furukawa, Y., Ohta, S., Ellis, T. A., Dembrow, N. C., Li, L., et al. (1999). The *Aplysia mytilus* inhibitory peptide-related peptides: Identification, cloning, processing, distribution, and action. *J Neurosci, 19,* 9618–9634.

Hiesinger, P. R., Fayyazuddin, A., Mehta, S. Q., Rosenmund, T., Schulze, K. L., Zhai, R. G., et al. (2005). The v-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in *Drosophila*. *Cell, 121,* 607–620.

Hurst, L. D. (2002). The K_a/K_s ratio: Diagnosing the form of sequence evolution. *Trends Genet, 18,* 486.

Kandel, E. R. (1976). *Cellular basis of behavior*. New York: W.H. Freeman and Company.

Kandel, E. R. (2001). The molecular biology of memory storage: A dialogue between genes and synapses. *Science, 294,* 1030–1038.

Supplementary material available online

Table showing collated results

Kivirikko, K. I., & Myllyharju, J. (1998). Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biol, 16,* 357–368.

Lee, Y. S., Bailey, C. H., Kandel, E. R., & Kaang, B. K. (2008a). Transcriptional regulation of long-term memory in the marine snail *Aplysia*. *Mol Brain, 1,* 3.

Lee, Y. S., Choi, S. L., Kim, T. H., Lee, J. A., Kim, H. K., Kim, H., et al. (2008b). Transcriptome analysis and identification of regulators for long-term plasticity in *Aplysia kurodai*. *Proc Natl Acad Sci U S A, 105,* 18602–18607.

Lim, C. S., Chung, D. Y., & Kaang, B. K. (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.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta c(t)) method. *Methods, 25,* 402–408.

Moroz, L. L., Edwards, J. R., Puthanveettil, S. V., Kohn, A. B., Ha, T., Heyland, A., et al. (2006). Neuronal transcriptome of aplysia: Neuronal compartments and circuitry. *Cell, 127,* 1453–1467.

Nelson, N., & Harvey, W. R. (1999). Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol Rev, 79*, 361–385.

Nishi, T., & Forgac, M. (2002). The vacuolar (H+)-ATPases—Nature's most versatile proton pumps. *Nat Rev Mol Cell Biol, 3,* 94–103.

Siddiq, A., Ayoub, I. A., Chavez, J. C., Aminova, L., Shah, S., LaManna, J. C., et al. (2005). Hypoxia-inducible factor prolyl 4-hydroxylase inhibition. A target for neuroprotection in the central nervous system. *J Biol Chem, 280,* 41732–41743.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res, 22,* 4673–4680.

Tzeng, Y. H., Pan, R., & Li, W. H. (2004). Comparison of three methods for estimating rates of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol, 21,* 2290–2298.

Yang, Z. (1997). PAML: A program package for phylogenetic analysis by maximum likelihood. *Comput Appl Biosci 13,* 555–556.

Yim, S. J., Lee, Y. S., Lee, J. A., Chang, D. J., Han, J. H., Kim, H., et al. (2006). Regulation of ApC/EBP mRNA by the Aplysia AU-rich element-binding protein, ApELAV, and its effects on 5-hydroxytryptamine-induced long-term facilitation. *J Neurochem, 98,* 420–429.

Zhang, L., & Li, W. H. (2004). Mammalian housekeeping genes evolve more slowly than tissue-specific genes. *Mol Biol Evol, 21,* 236–239.