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군소의 신경관련 유전체 분석 및 기억 관련 유전자의 기능에 대한 연구

Analysis of neuronal transcriptome and functional studies of memory related-genes in *Aplysia*

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Analysis of neuronal transcriptome and functional studies of memory related-genes in *Aplysia*

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CONTENTS

Abstract

To study the molecular and cellular mechanism of learning and memory, marine mollusk *Aplysia* has been used for a long time. While extensive research has been performed, however, lack of its large size genomic database is the major limiting factor for investigation of the molecular mechanism of learning and memory in *Aplysia*. Especially, it has been established that activation of cAMP-PKA pathway is critical for memory consolidation in *Aplysia*. However, the 5-HT receptor which activates adenylyl cyclase has not been cloned. To overcome this limitation, high-throughput analysis based on the EST database of closely related species, *Aplysia kurodai* and *Aplysia californica*, was performed in the present study. Furthermore, a number of novel learning-related genes were cloned and characterized based on the high-throughput transcriptome analysis.

Firstly, some putative learning related genes were identified based on the 5-HT induced expression profile. It is well known that 5-HT not only activates several signal transduction pathways such as PKA and PKC, but also regulates expression of several learning-related genes in *Aplysia* neurons. To identify 5-HT regulated genes, microarray was performed as a primary screening and the hits were validated by quantitative RT-PCR analysis. Based on the screen, a number of candidate genes involved in the molecular mechanism of learning and memory were discovered.

Secondly, to investigate the more efficient way to identify learning-related genes, evolutionary analysis based on the expressed sequence tag (EST) databases of *A. kurodai* and *A. californica* was performed. Calculated evolutionary rate of each gene suggests that eliminating genes with extremely low evolutionary rates can be an effective way to find candidate genes for the learning and memory study, because many housekeeping genes show relatively low evolutionary rates. However,

the probability of neuronal expression of certain genes could not be predicted by calculating the K_a/K_s value of the genes.

Thirdly, the relationship between the AU-rich element(ARE) binding protein, ApAUF1, and the well-studied transcription factor, ApC/EBP was examined. Overexpressed ApAUF1 was localized to the cytosol and neurites. Data obtained from microarray and EST databases suggested that ApAUF1 may regulate ApC/EBP mRNA. ApC/EBP mRNA has several AREs in its 3'UTR, and the ApAUF1 is specifically bound to the region. These results indicated that the ApAUF1 may function as a negative regulator of ApC/EBP mRNA.

Finally, the 5-HT receptor which is positively coupled to adenylyl cyclase, was cloned based on the EST database and sequence analysis. The cloned receptor, 5-HT_{apAC1}, has strong homology to other invertebrate 5-HT_7 receptors. It is expressed in the sensory and motor neurons, and localized to plasma membrane and the synaptic region of sensory neurons.

5-HT_{apAC1} is specifically coupled to G_s, but not G_q, and also has an important role in the activation of the cAMP-PKA pathway by 5-HT treatment. Moreover, knock-down of the receptor blocks the induction of synaptic facilitation in non-depressed synapses as well as the reversal of synaptic depression called dishabituation in moderately depressed synapses.

Key words: *Aplysia*, synaptic facilitation, 5-HT, transcriptome, EST, gene mining, K_a/K_s, ApC/EBP, AU-rich element (ARE), ApAUF1, 5-HT_{apAC1}

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Chapter 1

Introduction

1. Background

Final goal of neuroscience is to understand the mechanism of the brain and the mind. Among these, memory is one of the most extensively studied brain functions. This may be not only because of the fact that it is relatively easy to measure quantitatively or qualitatively in a scientific experiment, but also because of the fact that learning and memory can be found in almost all animals. Learning and memory is one of the most basic mechanisms that can be found in animals with the simplest nervous system. However, even in studies in humans, which possess the most complex nervous system of all animals, the importance of knowledge about memory in understanding the brain and the mind cannot be reduced. Because memory is one of the most important building blocks of self-consciousness and self-consciousness is the most important brain function that makes a self-aware individual what he is. That is why the learning and memory study is critical to our understanding of the brain and the mind.

There are two forms of memory divided by its contents: explicit memory, which is about facts and events, and implicit memory, which is about certain skills such as riding a bicycle. Although different parts of the brain are involved in each form of the memory, it is known that the two forms share common molecular mechanisms (Kandel, 1989; Kandel et al., 2000).

Memory can also be divided into two categories by the duration of memory maintenance: one is short-term memory which persists from minutes to hours and the other is long-term memory which lasts days to even an entire lifespan. Moreover, it is well known that only post-translational modification of preexisting proteins is required to induce short-term memory, whereas, new mRNA and protein expression is needed to induce long-term memory (Kandel, 1989; Bailey et al., 1996; Kandel et al., 2000). Therefore, studying the molecular mechanism of

learning and memory is crucial to understanding the function of the brain and the mind.

Choosing an appropriate model organism is a very important step in studying some biological mechanisms. With its large neuron size and relatively simple nervous system, *Aplysia* is one of the most fascinating model organisms for studying the mechanism of memory (Kandel, 1976). The giant neuron of *Aplysia* is one of the biggest somatic cells in all animals, enabling many useful manipulations such as single neuron recording or microinjection of plasmids, RNAs or proteins (Kaang et al., 1993; Kaang, 1996). Moreover, it is easy to select neurons appropriate for specific experiment, because many of the neurons are physiologically identified (Kandel, 1976).

Mechanisms of short- and long-term memory in *Aplysia*

Sensitization of the gill or syphon-withdrawal reflex is known as examples of non-associative implicit memory (Carew and Sahley, 1986; Kandel, 1979). Sensitization is the mechanism by which an animal that is given a noxious stimulation learns the aversive stimulus and increases its size of response to a normal stimulation (Castellucci et al., 1986; Frost et al., 1985). In case of *Aplysia*, giving a single noxious electrical stimulus on its tail induces short-term sensitization, and repetitive noxious stimuli induce long-term sensitization (Pinsker et al., 1973).

Aplysia gill withdrawal response is mediated by three different neurons: sensory neuron, motor neuron, and heterosynaptic serotonergic interneuron. Noxious tail stimulation induces serotonin (5-HT) release from serotonergic interneurons, and the released 5-HT activates downstream pathways in presynaptic sensory neuron (Brunelli et al., 1976). Extensive studies about these events using the sensory-to-motor co-culture system proved that 5-HT induces short-term and long-term

synaptic facilitation by activating downstream kinases such as PKA, PKC, and MAPK (Kandel, 2001).

1) Molecular mechanisms of short-term synaptic facilitation in *Aplysia*

Short-term synaptic facilitation induced by a single electric tail shock is mimicked by a single pulse of 5-HT treatment in sensory-to-motor co-culture system (Rayport and Schacher, 1986). One pulse of 5-HT activates PKA and PKC by activating 5-HT receptors which are coupled to various G-proteins (Homayouni et al., 1997). Especially, in non-depressed synapses, activated PKA phosphorylates potassium channels and inhibits the potassium conductance. Decreased K^+ conductance increases membrane excitability and action potential duration, and finally leads to increment of synaptic transmission (Klein et al., 1982; Baxter and Byrne, 1990). In depressed synapse induced by repetitive sensory neuron stimulation, however, PKC becomes dominant to reverse of

the homosynaptic depression (Byrne and Kandel, 1996). Many scientists speculate that distinct serotonin receptors are needed to activate PKA and PKC. However, the serotonin receptor which can activate PKA through elevation of cAMP level has not yet been identified.

Until now, seven 5-HT receptor families have been cloned and identified in vertebrates. Among these, except the ionotropic receptor family 5-HT_3, all other 5-HT receptor families are G-protein coupled receptors (Watson and Arkinstall, 1994; Hoyer et al., 1994). Activation of 5-HT_1 and 5-HT_5 families inhibits adenylyl cyclase, activation of 5-HT_4, 5-HT_6 and 5-HT_7 families activates adenylyl cyclase, and 5-HT_2 receptors activates phospholipase C (PLC) (Watson and Arkinstall, 1994). Unlike to other animals, 5-HT receptors in *Aplysia* have not been cloned extensively. Excluding the partial clones or sequences, only four 5-HT receptors have been cloned, and two of them are negatively coupled to adenylyl cyclase (Angers et al., 1998; Barbas et al., 2002). And most other

studies on the *Aplysia* 5-HT receptors are based on pharmacological analysis (Cohen et al., 2003; Dumitriu et al., 2006). In this study, cloning and characterization of the first G_s-coupled 5-HT receptor in *Aplysia*, 5-HT_{apAC1}, were performed.

2) Molecular mechanisms of long-term synaptic facilitation in *Aplysia*

Unlike short-term memory, synthesis of new mRNA and protein is necessary to induce long-term memory (Barzilai et al., 1989; Kandel, 2001). Long-term synaptic facilitation induced by repetitive electric tail shock is mimicked by five pulses of 5-HT treatment in sensory-to-motor co-culture system (Castellucci et al., 1986). These repetitive stimulations activate cAMP-PKA pathway, and activated PKA recruits mitogen-activated protein kinase (MAPK) (Barzilai et al., 1989; Glanzman et al., 1990; Bailey et al., 1996). The activated MAPKs are transferred to the nucleus where they phosphorylate transcription factors including cAMP-

response element binding protein (CREB) (Kaang et al., 1993; Martin et al., 1997). These pathways activate CREB1 via PKA-mediated phosphorylation and release of its repressor, CREB2 (Martin et al., 1997; Bartsch et al., 1995). Activation of CREB1 then increases transcription of several immediate early genes (Figure 1). These immediate early genes are important to induce long-term facilitation in *Aplysia* sensory-to-motor synapses: a proteolytic enzyme, ApUch, causes persistent activation of PKA by degrading PKA-regulatory subunits (Hegde et al., 1997); a transcription activator, ApC/EBP, leads to transcription of many downstream genes (Alberini et al., 1994; Lee et al., 2001).

3) Function and regulation of ApC/EBP, a transcription factor important for memory consolidation

CCAAT/enhancer binding protein (C/EBP) is well known to interact with the CCAAT motif, which is located in the promoter region of various

kinds of genes (Agre et al., 1989). At least six subfamilies of C/EBP are known (C/EBP α, β, γ, δ, ε, and ζ) (Ramji and Foka, 2002; Cao et al., 1991). Moreover, highly conserved leucine-zipper domain that allows dimer formation and DNA binding was found in the C-terminal region of all C/EBPs (Nerlov and Ziff, 1994; Lekstrom-Himes and Xanthopoulos, 1998).

Among these, ApC/EBP, an *Aplysia* homolog of C/EBPβ, which binds to enhancer response element (ERE), is the most extensively studied to understand the molecular mechanism of learning and memory. By microinjecting ERE oligonucleotide or antisense oligonucleotide into sensory neurons of *Aplysia*, Alberini et al. revealed that ApC/EBP is a key molecule to consolidate 5-HT mediated long-term memory (Alberini et al., 1994). Moreover, Kaang and his colleagues showed that overexpression of ApC/EBP in sensory neurons can induce LTF by the only single pulse of 5-HT treatment (Lee et al., 2001).

Because induction of ApC/EBP expresses leads to expression of many late-response genes, its expression is temporally regulated in a precise manner. Expression of ApC/EBP is detected within 15 min after onset of 5-HT treatment, and decreases 4 h after 5-HT treatment (Alberini et al., 1994). It is comparable with mammalian C/EBP δ, which is highly unstable (Dearth and DeWille, 2003). Ubiquitin-proteasome pathway mediated degradation is suggested as a mechanism for instability of ApC/EBP protein (Yamamoto et al., 1999). While ApC/EBP mRNA is also unstable, however, the mechanism which regulates stability of the mRNA is not clearly discovered.

Recent studies have led to some clues about regulation of ApC/EBP mRNA stability. Firstly, ApC/EBP contains several AREs in its 3′UTR (Dearth and DeWille, 2003). Secondly, ApELAV, known as an ARE-binding protein, positively regulates stability of ApC/EBP by binding to the ARE region of the ApC/EBP 3′UTR (Yim et al., 2006). In this study, the role of

another ARE-binding protein, ApAUF1, was revealed contributing to our understanding of the mechanism of ApC/EBP mRNA decay.

Evolutionary approaches in other model animals

Many studies described above were performed to understand the molecular mechanism of learning and memory using *Aplysia* as a model animal, but comprehensive understanding of the mechanism remains elusive. This is partly because a lot of molecules and molecular pathways that play crucial roles in learning and memory have not been cloned and identified yet. Therefore, it is important to find the molecules involved in the molecular pathway of interest, a process called as gene mining or gene hunting (Dean, 2003; Li et al., 2004).

1) Studies on the evolutionary rates of housekeeping genes

One method to estimate the function of an undefined gene is expression profiling. If a gene express exclusively or highly enriched in certain tissue, then it is reasonable to assume that the gene has an important role in that tissue. On the other hand, genes that are necessary to maintain basic cellular activities and to successfully complete the cell cycle, which are called housekeeping genes (Warrington et al., 2000), are express ubiquitously in many of the tissues (Hsiao et al., 2001). Because the focus of this study is to find and characterize novel learning-related genes, trimming out housekeeping genes to narrow down the pool of learning-related gene candidates is very important.

Zhang and Li found an important clue to identifying housekeeping genes: difference in evolutionary rates between housekeeping genes and tissue-specific genes (Zhang and Li, 2004). They calculated average evolutionary rates by non-synonymous-to-synonymous substitution ratio (K_a/K_s) test (Nekrutenko et al., 2002). Nucleotide substitutions in protein-

coding regions can be divided into two categories: one is accompanied by a change in amino acid sequence (non-synonymous substitution), and the other is not (synonymous or silent substitution). Using the numbers of these substitutions, one can calculate K_a, the number of non-synonymous substitutions per non-synonymous site, and K_s, the number of synonymous substitutions per synonymous site(Nekrutenko et al., 2002). If the K_a/K_s value of a certain gene is relatively low, then one can hypothesize that the gene is under a stronger selection constraint (Hastings, 1996; Duret and Mouchiroud, 2000).

Zhang and Li demonstrated that housekeeping genes have lower K_a and K_a/K_s values compared with tissue-specific genes. These results suggest that housekeeping genes are under stronger selection constraint (Zhang and Li, 2004). Based on this information, a more efficient way to narrow down the pool of candidate genes for learning and memory was revealed in this study.

2) Studies on the evolutionary rates of nervous system-related genes

Evolutionary speed of nervous system-related genes has also been studied extensively. Dorus et al. especially focused on the genetic basis of the human brain evolution (Dorus et al., 2004). In primates and rodents, the evolutionary speed of nervous system-related genes is known to be faster than that of housekeeping genes. Moreover, evolutionary rates of these genes were significantly higher in primates than in rodents, comparable to the fast evolution of brain structure and function in primates (Dorus et al., 2004).

The idea of fast evolutionary speed of nervous system-related genes has been controversial. First, Dorus et al. used only 24 nervous system-related genes to compare the evolutionary rates between human and chimpanzee. Second, these 24 genes were manually compiled, so the list could be biased (Shi et al., 2006). Third, housekeeping gene may not be

a good control, as they are expected to have different evolutionary patterns (Duret and Mouchiroud, 2000; Zhang and Li, 2004). To overcome these limitations, unbiased approaches based on EST database and RT-PCR were used in addition to biased approaches modeled after the method of Dorus et al. However, due to the lack of functional information about the genes other and nervous system-related genes and housekeeping genes, housekeeping genes were still used as control.

3) Basic databases perform the evolutionary studies: large scale transcriptome databases

For a high-throughput evolutionary analysis described above, large-scale expressed sequence databases are necessary. One of the most extensively used transcriptome database is expressed sequence tag (EST). EST is obtained from one-shot sequencing of cloned cDNA, so it is a partial segment of full-length cDNA (Adams et al., 1991). With the

development of automated sequencing, EST has become a standard tool for gene discovery, similarity searching, and genome mapping (Adams et al., 1991; Velculescu et al., 1995).

Another well-known transcriptome database is serial analysis of gene expression (SAGE). Since EST can evaluate only a limited number of genes, SAGE was developed as the more detailed and rapid analyzing method (Velculescu et al., 1995). Since *Aplysia* whole genome sequencing was not completed by the time this study was performed, EST databases of closely related species *A. kurodai* and *A. californica* (Lee et al., 2008a; Moroz et al., 2006) were used extensively in this study.

Finally, this study could not have been completed without cooperation of other collaborators and their names are indicated at the foot of the figures when they contributed substantially. And Dr. Tae-Hyung Kim and Dr. Jong Bhak in KOBIC (Korean Bio Information Center) gave me a great help to annotate ESTs and evolutionary analysis.

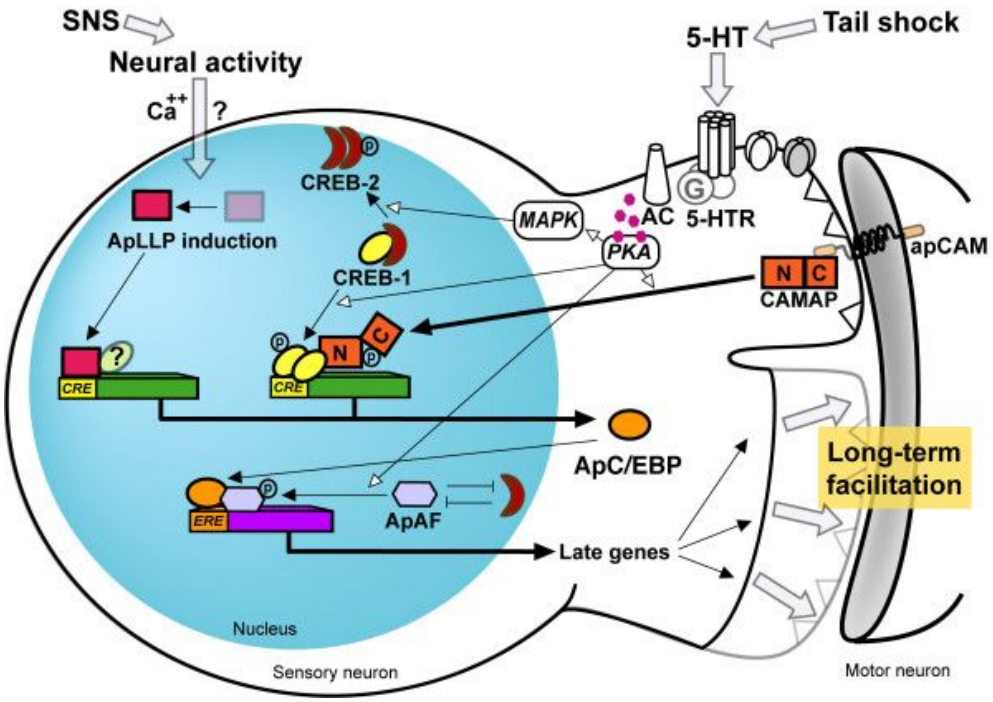


Figure 1. Molecular mechanism of long-term synaptic facilitation in the sensory neurons of *Aplysia* (Lee et al., 2008a).

2. Purpose of this study

Gene mining is one of the most important steps to study the molecular mechanism of learning and memory. However, this step is quite limited due to the absence of large scale genome analysis. In particular, the serotonin receptor, which is positively coupled to adenylyl cyclase, has not been identified. In order to expand our understanding of the molecular mechanism of learning and memory, many more learning-related genes have to be cloned and characterized.

In this study, many putative learning-related genes were mined based on large scale expression profiles and evolutionary analysis using *A. kurodai* and *A. californica* EST database. By performing microarray and real-time PCR, several novel 5-HT regulated genes were found. Moreover, to develop an efficient gene mining technique, evolutionary analysis was performed based on the EST library of two *Aplysia* species.

Furthermore, in another part of this study, some important learning related genes in *Aplysia* nervous system (5-HT_{apAC1} and ApAUF1) were cloned based on the analysis described above. To characterize expression profiles of these genes, *in-situ* hybridization, RT-PCR, Western blot, and immunocytochemistry were performed in cultured *Aplysia* neurons. Moreover, to understand the role of these genes in synaptic facilitation, gene expression was blocked by microinjection of dsRNA (5-HT_{apAC1}), or hybridized to *in-vitro* transcribed biotin-labeled probe (ApAUF1).

Chapter 2

Neuronal Transcriptome Analysis

in *Aplysia kurodai*

Section I

Identification of Genes Related to Synaptic Plasticity Through Neuronal Transcriptome Analysis in *Aplysia kurodai*

Introduction

Marine mollusk *Aplysia* is one of the most fascinating model organisms to study molecular and cellular mechanisms of learning and memory because it has large neurons and a simple nervous system(Bailey et al., 1996; Carew and Sahley, 1986; Kandel, 2001). Due to these advantages, many of key molecules and molecular pathways with important roles in learning and memory have been extensively discovered in *Aplysia*. However, further molecular studies about memory-related genes in the *Aplysia* nervous system are highly limited due to lack of its genomic information. High throughput analysis based on the large genomic database may facilitate discovery of candidate genes that are critical in induction of learning and memory or synaptic plasticity.

Profiles of gene expression were firstly characterized by Moccia et al. using high-throughput gene analysis in *Aplysia* sensory neurons (Moccia et al., 2003). Next, the whole mitochondrial genome(Knudsen et al.,

2006) and a large scale ESTs of *A. californica* were sequenced and analyzed(Moroz et al., 2006). From the *A. californica* nervous system, more than 200,000 ESTs representing ~65,000 non-redundant sequences were sequenced. Among the non-redundant sequences they could annotate were ~4,900 genes (Moroz et al., 2006). Moreover, 11,493 ESTs were produced by sequencing several cDNA libraries generated from the nervous system of *A. kurodai* (Lee et al., 2008b). These ESTs were assembled in 4,859 non-redundant transcripts, and the maximum length was 3,200 bp (Lee et al., 2008b).

In this section, in order to find the genes regulated by 5-HT, a key neurotransmitter that induces synaptic facilitation, custom microarray composed of more than 7,000 cDNA clones were constructed and analyzed based on the EST databases from *A. kurodai* nervous system. To validate the 5-HT regulated genes predicted from microarray, real-time PCR was

performed. Finally, these procedures led to identification of five clones that are significantly up-regulated or down-regulated by 5-HT treatment.

Experimental procedures

1. *In-vivo* 5-HT Treatment of *Aplysia kurodai*

To screen for genes regulated by 5-HT in pleural ganglia, animals were *in-vivo* treated 250 μM of 5-HT. Two hours after onset of 5-HT treatment, animals were anesthetized and pleural ganglia were dissected and frozen using liquid nitrogen. Equal number of animals that were not exposed to 5-HT were used as control.

2. Microarray Hybridization, Scanning, and Analysis

cDNA microarray construction and hybridization were performed as described previously (Hegde et al., 2000). Pleural ganglia from three *in vivo* 5-HT treated and three control animals were isolated 2 h after the onset of 5-HT treatment. To obtain sufficient amount of RNA for the microarray hybridization, aRNAs were prepared from 2 μg of the total RNA purified from pleural ganglia (RiboAmp RNA amplification kit,

Arcturus). Six μg of aRNAs were labeled with Cy3 (control group) or Cy5 (5-HT group) by reverse transcription (SuperScript III reverse transcriptase, Invitrogen) and purified using Qiagen PCR purification columns, followed by hybridization to the *Aplysia* cDNA microarray which had been prehybridized with 0.1 mg/ml BSA. The slides were then washed, scanned (GenePix 4000B, Axon Instruments), and analyzed using Gene-Pix Pro and Acuity software (Axon Instruments). The results from two independent 5-HT treatments and hybridization analyses were averaged.

3. Annotation of ESTs Based on BLASTX

BLASTX was used to annotate the EST sequences that are regulated by 5-HT in microarray. Among the 27 5-HT-regulated ESTs, only 13 BLASTX-hit ESTs were analyzed by real-time PCR.

4. Real-time PCR

Dissected and frozen pleural ganglia described above were used for real-time PCR. RNA was purified using TRIzol Reagent (Invitrogen) following the manufacturer's manual. Purified RNAs were then treated with RNase-free DNase I (Ambion) for 40 min to remove residual genomic DNA. cDNA was synthesized using SuperScript Ⅲ reverse transcriptase (Invitrogen) with oligo dT primers (Invitrogen). Reactions were performed in the Thermal Cycler Dice Real Time System, TP800 (Takara) using SYBR Premix Ex Taq (Takara) and gene specific primer sets (Primer sequences are presented in table 1). Amplification reaction consisted of one cycle of 95℃ for 5 min, followed by 60 cycles of 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 30 s. S4 was used as an internal control. We analyzed C_T value using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Collected C_T values 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

1. Up- or Down-regulated Genes in Response to 5-HT

To identify genes with altered expression following by 5-HT treatment, cDNA microarrays were constructed. After filtering out low quality spots, 27 sequences were found to be up- or down-regulated in 5-HT-treated pleural ganglia (Table 1). To annotate these 27 sequences, BLASTX was used. Among these sequences, only thirteen clones showed significant matches. These results are presented in Table 2. The well-known immediate-early gene, ApC/EBP, was found in the list of up-regulated genes. However, alpha tubulin 2, which has a role in housekeeping, was also found in the same list. These results indicate that candidates from microarray analysis should be validated through a more sensitive and accurate method.

To validate the 5-HT regulated genes predicted from microarray, real-time PCR was performed. Thirteen BLASTX matched clones were

selected for further validation. qRT-PCR confirmed that four clones (ApC/EBP, matrilin, antistasin, and eIF3e) were significantly up-regulated and one clone (BAT1 homolog) was significantly down-regulated by *in-vivo* 5-HT treatment (Fig. 2).

Table 1. List of the clones that were up- or down-regulated by >2-fold 2 h after 5-HT treatment in microarray.

EST name	BLASTX result*	GenBank accession number	Ratio of medians (5-HT/ control)	Sense primer sequence	Antisense primer sequence	Short description of real-time PCR results†
Up-regulated clones						
5CAP092402_H04_760	Matrilin (AAN61407, 1.00E-33)	EY418286	5.377	ATCACCTTCCACCACACCTC	AGCCACATCATTCATCGTCA	Significant
5CAP090501-pMES_D10_142	Antistasin precursor (P38977, 2.00E-25)	EY417467	4.830	AGCATGGAAACGTCTTGGAC	AGAGCGAGGTTTGCATTCAC	Significant
5CAP031402_pMES_B12_1600	Cathepsin L-like cysteine proteinase precursor (AAQ22984, 5.00E-05)	EY417083	4.419	GAGACCAAAGTGGACGAGGA	CTTGAGCGCTTGTTGTGGTA	No change
5HTCNS062702-T3_D01_92	No hits	EY416228	3.582			
R5CAP031403_pMES_E11_756	No hits	EY394288	3.306			
5CAP090504-pMES_E03_435	Alpha tubulin 2 (AAM09674, 6.00E-48)	EY417749	2.984	CTCTCATCTCACGACGGACA	ATACCGTGCTCCAGGCAGTA	No change
5CAP090501-pMES_H12_192	No hits	EY417516	2.942			
5CAP092402_B10_694	LOC443610 protein (AAI28922, 7.00E-55)	EY418226	2.838	TACGCCGCCTATCCTTTATG	TACAGCCCTTGATCCCACTC	No change
C/EBP	ApC/EBP (AAA18286, 0)		2.594	TACTCTCAACCTTCCCTCAAGC	TGACAAATGAACAAAATGGACA	Significant
5CAP031402_pMES_D12_1621	ApCREB2 (AAA92437, 1.00E-25)	EY417103	2.548	AGCGTTTCTCTCCATACTCT	TGCAAGTCATCGACCTTAGTCT	No change
5CAP031402_pMES_B04_1592	No hits	EY417075	2.478			
5HTCNS122105-T3-C10-628	Eukaryotic translation initiation factor 3, subunit 6 (NP_001559, 5.00E-73)	EY416763	2.436	CGAGACATACGCCCAGTGTA	GCGATAGGCGAAAGAAACAG	Significant
5CAP092404_G12_948	No hits	EY418453	2.379			
5CAP090501-pMES_F08_164	hypothetical protein (XP_001176996, 6.00E-33)	EY417489	2.230	CTGGGCCATATTTTCACACC	GAGGCTTCACTCTTGGCAAC	No change
YesTrp090505-Bco3_05_B06_498	No hits	EY422272	2.173			
YesTrp090503-Bco3_03_D10_334	No hits		2.085			
YesTrp090503-Bco3_03_B08_308	No hits	EY422122	2.071			
Down-regulated clones						
5CAP101001_F09_1029	Kazal proteinase inhibitor (ABL74453, 2.00E-22)	EY418526	0.346	GGAAGCTGTGGTGATGGACT	CGGGAAGAGTGAAGGTCTTG	No change
5CAP120302-pMES_C06_1166	unnamed protein product (CAG08644, 2.00E-14)	EY419713	0.356	ATTTTTGCCTCCAACCTGTG	TCTGTCGGACGAATTCAAGA	Reverse trend
5CAP031401_pMES_A06_1493	No hits	EY416984	0.384			
5CAP092402_D03_711	No hits	EY418242	0.387			
5CAP120601_pMES_E03_2269	No hits	EY420078	0.393			
5CAP031403_pMES_B09_1682	No hits	EY417156	0.409			
5CAP092401_E01_625	No hits	EY418164	0.443			
5CAP031405_pMES_G09_1891	hCG1999844 (EAX04582, 0.013)	EY417337	0.460	TGCATTTATTTTCCCCCTCA	CATGCTTGGCATGAAAAAGA	No change
5CAP101001_G01_1033	No hits	EY418530	0.479			
5CAP101001_H07_1051	BAT1 homolog (AAQ13472, 2.00E-26)	EY418548	0.486	GCCGGAGAACTCTGACACAT	CGACCCTCGATGTAAGAGGA	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 $P > 0.10$.

(Microarray was done by Dr. Yong-Seok Lee, and result analysis was done in collaboration with Dr. Yong-Seok Lee)

Table 2. List of real-time PCR targets selected among clones up- or down-regulated >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	Antistasin (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 $P > 0.10$.

(Microarray analysis was done in collaboration with by Dr. Yong-Seok Lee)

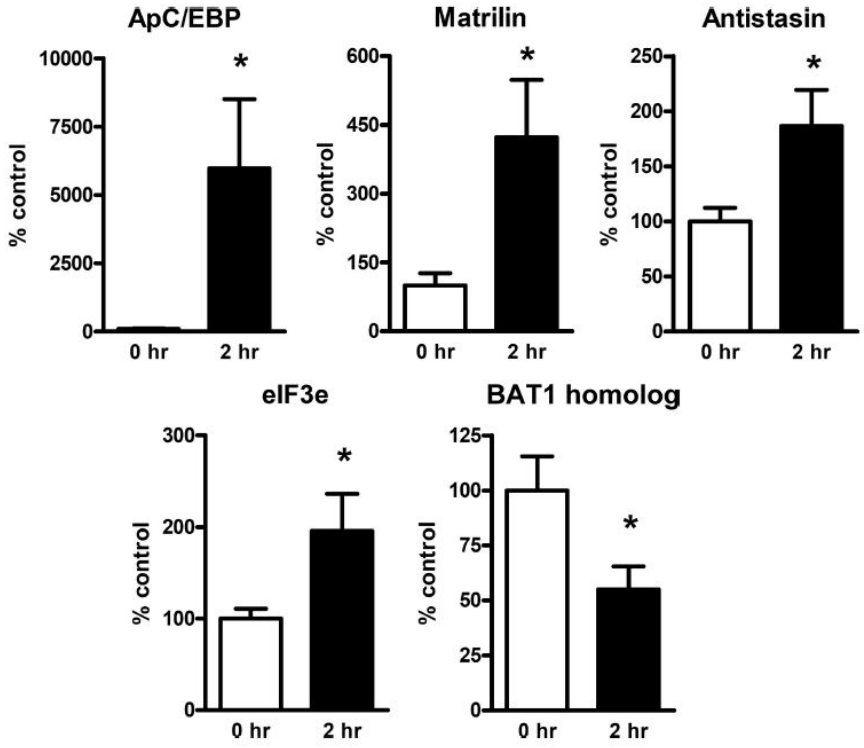


Figure 2. Real-time PCR confirmation of selected differentially expressed genes. The height of each bar indicates the average mRNA level ± SEM of each gene before and after 5-HT treatment. Asterisk indicates that $p < 0.05$ in Student's t-test.

Discussion

In this chapter, gene mining through cDNA microarray and real-time PCR analysis was described. To identify genes up- or down-regulated by 5-HT treatment, microarray was performed as a primary screening method based on the recently constructed *A. kurodai* EST database (Table 1 and 2). Microarray analysis focused on gene expression profile 2 h after the onset of 5-HT treatment, because this time point is known to be critical for the LTF induction in *Aplysia* (Montarolo et al., 1986; Alberini et al., 1994; Hegde et al., 1997; Barzilai et al., 1989). However, due to experimental conditions, it was necessary to validate hits from the microarray analysis; since microarray was performed as a primary screen, steps to achieve high stringency such as dye-swapping were not included. Following qRT-PCR confirmation, five 5-HT regulated genes were identified in total(Figure 2).

Among these, expression level of matrilin in 5-HT treated pleural ganglia was increased approximately five-fold compared to that in the control ganglia (Table 2 and Fig. 2). Matrilin, a member of fibril-forming extracellular matrix protein family, forms collagen-associating fibrillar or filamentous structures (Deak et al., 1999; Wagener et al., 2005). While the role of matrilin in the nervous system has yet to be identified, some reports suggest that extracellular matrix proteins can interact with ion channels and receptors, and play a pivotal role in induction of synaptic plasticity (Dityatev and Schachner, 2003). Another up-regulated gene, antistasin, may have a function in synaptic plasticity by directly or indirectly binding to membrane proteins or cytoskeletal proteins (Leadley et al., 2001). Finally, a down-regulated gene, BAT1 homolog, may regulate synaptic plasticity-related mRNAs as a splicing factor (Fleckner et al., 1997; Herold et al., 2003). Moreover, one of the up-regulated genes, Ap-eIF3e, was confirmed to be necessary to consolidate STF into LTF (Lee et al., 2008b).

The precise roles of these genes in LTF in *Aplysia* synapse still remains to be investigated.

Section Ⅱ

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

Introduction

Because of the advantages of *Aplysia* as a model animal for studying learning and memory, large number of key molecules and molecular pathways that are important in learning and memory have been discovered (Kandel, 1976; Schacher et al., 1988; Ghirardi et al., 1992; Alberini et al., 1994; Hegde et al., 1997; Lee et al., 2001; Lee et al., 2006; Lee et al., 2007). While speculating the function of candidate genes is especially important to study molecular mechanism of learning and memory, there are several examples of limitations (Dean, 2003).

One approach to overcome this limitation utilizes differential evolution rates of genes (Zhang and Li, 2004). It is known that housekeeping genes evolve more slowly and are under more robust selective pressures in comparison to tissue-specific genes (Zhang and Li, 2004). In primates and rodents, it is also known that the evolutionary speed of genes with various functions in the nervous system is faster than that of

housekeeping genes involved in basic metabolic functions. Moreover, consistent with the rapid evolution of brain structure and function, the evolutionary rates of genes related to signal transduction in neurons are significantly higher in primates than in rodents (Dorus et al., 2004). However, it is unknown whether or not genes related to signal transduction in neurons show faster evolutionary rate in *Aplysia*, due to the lack of a comprehensive database of its large genome.

Recently, two large-scale EST analyses for the closely related species *A. californica* and *A. kurodai* were completed by two groups (Moroz et al., 2006; Lee et al., 2008b). In this chapter, evolutionary rates of signal transduction gens and housekeeping genes in neurons were calculated based on the analysis using these EST databases. To measure the evolutionary speed of genes, K_a/K_s ratio, which is the number of non-synonymous substitutions per non-synonymous site (K_a) to the number of synonymous substitutions per synonymous site (K_s), (Hurst, 2002; Miyata

and Yasunaga, 1980; Desseyn, 2009) was used. The lists of candidate genes which may have crucial roles in inducing learning and memory or synaptic plasticity were found among the relatively fast-evolving genes. Next, the relationship between the K_a/K_s ratios and tissue expression pattern of *Aplysia* genes was revealed. It is assumed that the K_a/K_s ratios cannot work as a good marker to evaluate the neuronal expression of a certain unknown gene. However, it still can be an efficient way to narrow down the pool of candidate genes involved in learning or synaptic plasticity.

Experimental procedures

1. Ortholog Alignment and Calculation of K_a/K_s

For all experiments in this chapter, two *Aplysia* EST databases were used as resources (Moroz et al., 2006; Lee et al., 2008b). *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 ortholog alignment pipeline uses ClustalW sequence alignment tool (Thompson et al., 1994). Two orthologous mRNA sequences were entered as input and translated into protein sequences by the tool. The alignment tool then 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 with K_a/K_s values over 10 were removed due to many alignment errors or gaps.

2. Semi-quantitative RT-PCR Analysis

For all experiments in this chapter, mRNAs were isolated from five different 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; Lee et al., 2008b). The cDNA was amplified using specific primer

sets (see Table 3). PCR reaction was performed as described: one cycle of 95℃ for 5 min, followed by 30 or 35 cycles of 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 30 s. The final extension reaction was done at 72℃ for 1 min. PCR products were visualized on 2% agarose gel.

3. Real-time PCR

The cDNAs prepared as described above were also used for quantitative real-time 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 (Primer sequences are shown in table 3). Amplification reaction consisted of one cycle of 95℃ for 5 min, followed by 60 cycles of 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 30 s. As an internal control, S4 was used. We analyzed C_T value using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

4. Statistical Analysis

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

Table 3. Gene specific primer sequences used in this chapter.

Gene Description	Contig/EST	Sense primer sequence	Antisense primer sequence	Size (bp)
vacuolar ATP synthase subunit H	CL122Contig1	5'-CCCACCTCCTTCATTAGCAA-3'	5'-ATGTGTAACAACGCGGTGAA-3'	158
soluble acetylcholine receptor	CL5Contig4	5'-GCTGGGCAGGAATAAACAAA-3	5'-TGGGATCCTAACGAGTACGG-3'	166
haemocyanin	5HTCNS081202-T3_A11_182	5'-CTGGGCAGTTTCACCAACTT-3'	5'-GTTTACTCGTCGGCCACTGT-3'	173
heart-type fatty-acid binding protein	CL991Contig1	5'-ACCAGGTGTCGGAGACAAAC-3'	5'-ACATTTCCATCCTCCACTGC-3'	169
cyclophylin isoform	CL758Contig1	5'-CCAACATAGCCGTAGCCATT-3'	5'-CTTAGCTGTGGCTGTGTCCA-3'	195
dehydrogenases,short chain family member (dhs-14)	pME_02-C01-pME-S	5'-CGCCACAGATGCTAAAGTGA-3'	5'-ATGTTGCCTTTGGTTTTTGC-3'	222
ATP synthase, mitochondrial F1 complex, alpha subunit	CL217Contig1	5'-AGGAGCCTATGCAGACAGGA-3'	5'-TCTCGTCTCCAGCATCATTG-3'	119
ApCREB2	CL135Contig1	5'-GCAGGTAGCCCCTGTCATAA-3'	5'-CTTCAGCACTGCCAATTGAA-3'	179
proline 4-hydroxylase	CL11Contig1	5'-CACTGGCAACCTTGAGGAAT-3'	5'-GTCCGCCGATATTGTCAACT-3'	170
MIP-related peptide precursor	5CAP120301-pMES_A12_1067	5'-TGATGCTAAGGTGGACACGA-3'	5'-TCCAGAGCCAATTCTTCAGC-3'	162
Zinc finger, HIT type 3	pME_01-E04-pME-S	5'-GACGAGTGAAACTGGGTGGT-3'	5'-ACCAGGTTCTCCATCATTGC-3'	154
Cct7-prov protein	5CAP092404_D10_910	5'-ACATCCGTTACGCTTCTTGC-3'	5'-TCTCCAGCAGTTCCTCAGT-3'	187
translation initiation factor 5A	CL97Contig1	5'-GGTATCAAGAACGCCAAGGA-3'	5'-CAATCAGCTAGCGAGCACAG-3'	236

unnamed protein product	pME_08-F06-pME-S	5'-GACGAGTGAAACTGGGTGGT-3'	5'-CCAGGTTCTTCCATCATTGCT-3'	154
ENSANGP00000012700	CL12Contig1	5'-ATTACGCGGATACGGAAGTG-3	5'-GATGGTGCCATCTCCATCTT-3'	172
unnamed protein product	5CAP120606_ pMES_D07_2718	5'-ATGACACGATGGCTGTTCAA-3'	5'-AAGTCCCACAGAAACGCTTG-3'	187
Cnot4-prov protein	CL911Contig1	5'-GGGAGAGTTCCTCAGGTTCC-3'	5'-TGCCATTGACGGTACCACTA-3'	153
ribosomal protein L12	5HTCNS081201-T3_D06_129	5'-AAAATTGGCCCTCTTGGTCT-3'	5'-CTCTGATGATCAAGGCAGCA-3'	160
40S ribosomal protein S15	CL481Contig1	5'-CGTGGCTTGAAGAGAAAACC-3'	5'-CGTGCTTGACAGGCTTGTAG-3'	247
ribosomal protein S14	CL908Contig1	5'-AGCAACAAGAGCAGGTGGTT-3'	5'-TGCCAACATAGCAGCGTAAG-3'	203
YBOXH_APLCA Y-box factor homolog	CL19Contig1	5'-GACGGTGGGTACTACGGCTA-3'	5'-TCGTTGCATTTGAGCAAGTC-3'	236
guanine nucleotide regulatory protein beta subunit	CL934Contig1	5'-CCTCCGGGTTCTCATTTGTA-3'	5'-GGGAGATTGGTAGTCGGTCA-3'	232
RHO_APLCA RAS-like GTP-binding protein RHO	CL179Contig1	5'-ACAGCGGGACAAGAGGACTA-3'	5'-GTACGTTGGGGCAGAAGTGT-3'	154
GTP-binding protein alpha-o subunit	S_YESTrp121201-BCO3_H06_85	5'-GATCTCTGCCCACCAACATC-3'	5'-TGTGGGTGGACTCAGGTGTA-3'	229
unnamed protein product	5CAP1127_ pMES_H12_3334	5'-GTCCGGCAGAAGATCAAGAG-3'	5'-GAACAGGCTTTTCCACTTCG-3'	178
similar to 40S ribosomal protein S16	YesTrp090505-Bco3_05_F07_547	5'-GTTGGGCAAGGAGAGATTTG-3'	5'-CAGCGATCTGTGTACTGGA-3'	187
similar to glucocorticoid induced gene 1	pME_07-G05-pME-S	5'-TGCCGATTACTCAAGGGAAC-3'	5'-CATTGTTTGCTGCCATTTTG-3'	238

(This work was done in collaboration with Dr. Yong-Seok. Lee)

Results

1. Collecting the Lists of Neuronal Signal Transduction Genes and Housekeeping Genes in *Aplysia*.

In primates and rodents, it is known that evolutionary rates of genes involved in nervous system function are greater than those of housekeeping genes involved in basic metabolic functions (Dorus et al., 2004). However, it is not known whether signal transduction genes in neurons show faster evolutionary rates in *Aplysia*, which has a relatively primitive nervous system. To measure the rates of protein evolution based on the sequences originated from the CNSs of two closely related *Aplysia* species, lists of *Aplysia* homologs of mammalian genes involved in neuronal signaling as well as those of putative housekeeping genes were complifed. In the first step to find these lists, *Aplysia* EST sequences homologous to those used in the study of Dorus et al. were collected (Dorus et al., 2004). For the second step, the GenBank databases were used to obtain

homologous and orthologous sequences which had been previously cloned and characterized in *Aplysia californica* but do not exist in either *A. kurodai* or *A. californica* EST database. Finally, homologous sequences that have not been functionally characterized (e.g., unnamed protein product) were excluded from the lists. In this way, 44 signal transduction genes in neurons as well as 31 putative housekeeping or basic metabolic genes were obtained from two closely related *Aplysia* species (Tables 4 and 5).

2. Differential Evolutionary Rates of Neuronal Genes in *Aplysia*

To measure the rates of protein evolution in *Aplysia*, K_a/K_s ratio was used. It was found that the average K_a/K_s ratio of selected signal transduction genes in neurons is significantly higher than that of putative housekeeping genes, by a factor of 2 (0.111 ± 0.019, n=44; and 0.062 ± 0.017, n=31, respectively, mean ± SEM; $p < 0.05$, Kolmogorov–Smirnov test; Figure 3A and B). It is assumed that positive selection pressure on

these genes was not the major driving force of evolution because K_a/K_s ratios of signal transduction genes in neurons were all < 1.0 (Hurst, 2002). This suggests that there might be stronger purifying selection constraints on the housekeeping genes than on the signal transduction genes in neurons in *Aplysia* (Zhang and Li, 2004).

3. Unbiased Evaluation of the Evolutionary Rates of *Aplysia* Orthologs

For the next step, based on the biased approach which revealed that signal transduction genes in neurons show faster evolutionary rates (Figure 3), new candidate genes for studies of learning and memory or synaptic plasticity were identified simply by measuring the K_a/K_s rates without any prior knowledge about the genes. These unbiased evaluations were performed based on the total collection of *Aplysia* orthologs without any categorization to the K_a/K_s analysis. Only well-aligned, BLASTX-matched 410 orthologous sequences were used for this analysis. To

calculate the K_a/K_s values of total *Aplysia* ESTs, the same method described in Figure 3 was used. The average K_a/K_s value of these genes was 0.093 ± 0.005, which was between those of the selected signal transduction genes in neurons (0.111 ± 0.019) and the housekeeping genes (0.062 ± 0.017).

The 15 *Aplysia* genes which showed the highest K_a/K_s rates are listed in Table 6. From this list, it was found some candidate genes for functional studies about learning and memory or synaptic plasticity such as RAB2, soluble acetylcholine receptor, and ApCREB2. Furthermore, the bottom 15 *Aplysia* genes showing the lowest K_a/K_s rates were listed in Table 7. This list included four ribosomal proteins that can be considered housekeeping genes. Moreover, from the list of bottom 15 genes, there was no interesting candidate gene to investigate for its function on learning and memory or synaptic plasticity based on gene description. These evolutionary analyses based on bioinformatics proposed that trimming out

the genes which have relatively low evolutionary rates can be an effective way to narrow down the pool of candidate genes for the learning and memory or synaptic plasticity study in *Aplysia* nervous system.

4. Tissue Distribution of Fast- and Slow-Evolving Genes

It is known that there is a correlation between gene expression and amino acid sequence divergence at least in humans and rodents (Zhang and Li, 2004). Therefore the hypothesis – 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 – was tested by investigating the tissue distribution of selected genes. To measure the tissue distribution of the genes, semi-quantitative RT-PCR and real-time PCR were done using the cDNA derived from the total RNA of five different

kinds of tissues as templates: central nervous system (CNS), buccal mass (BM), stomach (ST), gill (GL), and ovotestis (OT).

Among the 13 genes that showed the highest K_a/K_s values, two genes (soluble acetylcholine receptor, heart-type fatty acid-binding protein) were highly expressed significantly in the CNS, and one gene (MIP) was expressed only in the CNS (Figure 4 A and B). RT-PCR and real-time PCR analysis were also performed on the 14 genes that showed lowest K_a/K_s values. Unlike the 13 genes with highest K_a/K_s values, these genes had the tendency to exhibit less variable expression levels across the five tissues. However, no strong relationship was found between K_a/K_s values and differential tissue expressions. Among the 14 genes that showed lowest K_a/K_s values, 2 genes (ENSANGP00000012700 and GTP-binding protein alpha-o subunit) showed significantly higher expression in the CNS and no gene was expressed exclusively in the CNS (Figure 5A and

B). These data suggest that evolutionary rate of some genes cannot be an effective marker to estimate neuronal expression in *Aplysia*.

Table 4. Putative nervous system-related genes used for K_a/K_s analysis

Contig_IDs (*A. kurodai*)	Contig_IDs (*A. californica*)	*Ka*	*Ks*	*Ka/Ks*	GI	Description	E_value
YesTrp127_Eco3_F01_2245	APL_all_052305.2238.C1	0.0682	0.1396	0.4883	288938	RAB2	7.00E-104
CL5Cont1g4	APL_all_052305.373.C5	0.0912	0.1955	0.4667	17225107	soluble acetylcholine receptor	3.00E-134
CL419Cont1g1	APL_all_052305.495.C3	0.0358	0.0929	0.385	1123037	ApCREB2	6.00E-138
YESTrp120604_ECO3_E02_1947	APL_all_052305.3781.C1	0.028	0.0904	0.3099	110755907	PREDICTED: similar to RasGEF domain family, member 1B	2.00E-82
5HTCNS081201-T3_G02_155	APL_all_052305.5181.C1	0.0139	0.0531	0.2623	4176498	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	2.00E-49
CL58Cont1g1	APL_all_052305.9731.C1	0.0124	0.051	0.2427	19526818	solute carrier family 25, member 3 precursor	1.00E-104
5CAP120303-pMES_A07_1228	CNSN01-F-059848-501	0.0749	0.3092	0.2423	17826925	putative oxidoreductase	4.00E-17
5CAP090501-pMES_H09_189	APL_all_052305.21.CB2	0.0535	0.2243	0.2385	66524874	PREDICTED: similar to tubulin alpha-1 chain	9.00E-108
5CAP120604_pMES_B07_2511	CNSN01-F-100824-501	0.0631	0.2685	0.2351	24431113	matrilin	7.00E-21
CL672Cont1g1	APL_all_052305.4691.C3	0.0258	0.1188	0.2169	1750212	carboxypeptidase E-1	1.00E-168
YesTrp090505-Eco3_05_B02_494	APL_all_052305.2689.C1	0.0166	0.0839	0.1972	38678736	PKA type II regulatory subunit	5.00E-67
CL338Cont1g1	APL_all_052305.1.C985	0.0089	0.0515	0.1731	13516974	Lys-conopressin preprohormone	1.00E-90
pME_02-A08-pME-S	APL_all_052305.12859.C2	0.0055	0.0388	0.1419	30959104	nervous system adducin	5.00E-90

CL451Contig1	APL_all_052305.3302.C1	0.0211	0.1697	0.1242	3347852	sialic acid-binding lectin 2	2.00E-54
R5CAP120604_pMES_H07_1392	APL_all_052305.5336.C1	0.0074	0.0601	0.1229	27924412	Rap1b-prov protein	5.00E-79
YesTrp1127_Bco3_F02_3213	CNSN01-F-070647-501	0.092	0.7747	0.1187	18255296	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9	7.00E-19
5CAP120304-pMES_F03_1349	APL_all_052305.17813.C1	0.0226	0.1967	0.1151	110760264	PREDICTED: similar to Serine/threonine-protein kinase 3 isoform 1	3.00E-72
CL311Contig1	APL_all_052305.3360.C1	0.0112	0.1339	0.0834	1927213	ubiquitin carboxyl-terminal hydrolase	8.00E-122
CL249Contig1	APL_all_052305.159.C5	0.0249	0.3078	0.0809	62512144	Synaptotagmin-1 (Synaptotagmin I) (p65)	1.00E-158
CL85Contig1	APL_all_052305.510.C2	0.0104	0.1433	0.0723	262054	calreticulin	2.00E-174
R5CAP031402_pMES_F10_673	APL_all_052305.10689.C1	0.0074	0.107	0.0689	545835	phospholipase C beta 4, PLC beta 4 {N-terminal}	3.00E-41
CL102Contig1	APL_all_052305.5469.C1	0.0152	0.22	0.0689	27695167	Sdcbp-prov protein	7.00E-86
YesTrp1127_Bco3_C01_2914	APL_all_052305.8492.C2	0.0035	0.0552	0.0643	39795764	Protein phosphatase 2A, catalytic subunit, alpha isoform	3.00E-147
CL296Contig1	CNSN27-F-040847-501	0.0094	0.1621	0.0581	66509032	PREDICTED: similar to Proteasome 26S subunit subunit 4 ATPase CG5289-PA isoform 1	2.00E-39

YESTrp120601_BC03_G03_1726	APL_all_052305.15801.C1	0.0245	0.4511	0.0542	38174397	Synaptosomal-associated protein, 29kDa	5.00E-28
pME_08-E03-pME-S	APL_all_052305.10808.C1	0.0114	0.2542	0.045	21388656	twitchin	2.00E-81
YESTrp120604_BC03_G12_1974	APL_all_052305.1260.C1	0.0142	0.3975	0.0356	74001211	PREDICTED: similar to roundabout 1 isoform a	1.00E-23
YesTrp1127_Bco3_H07_2487	APL_all_052305.151.C4	0.0089	0.2937	0.0304	773571	neurofilament protein NF70	2.00E-78
CL981Contig1	APL_all_052305.195.C2	0.0093	0.3584	0.0259	194293947	amyloid precursor protein	8.00E-37
CL60Contig1	APL_all_052305.257.C1	0.0016	0.0732	0.0224	46048903	voltage-dependent anion channel 2	7.00E-118
5HTCNS122104-T3-A01-525	APL_all_052305.16420.C1	0.0045	0.2049	0.0219	164519043	protease, serine 27 precursor	1.00E-19
CL541Contig1	APL_all_052305.16107.C1	0.0023	0.1219	0.0192	32306513	phosphodiesterase 4D isoform 2	6.00E-121
YESTrp120605_BC03_G12_2056	APL_all_052305.1912.C2	0.0036	0.2178	0.0164	29747932	Mtap4 protein	3.00E-27
YESTrp120602_BC03_A01_1744	APL_all_052305.7472.C2	0.0021	0.1371	0.0156	14190057	potassium channel protein Shal 1.h	5.00E-56
CL422Contig1	APL_all_052305.1973.C5	0.0095	0.6309	0.0151	201085693	serotonin transporter	2.00E-117
CL1Contig27	APL_all_052305.21.CB1	0.0048	0.5934	0.008	20069089	alpha tubulin 2	0
CL9Contig1	APL_all_052305.1.C273	0.001	0.1531	0.0065	30088884	beta tubulin	0
CL1Contig5	APL_all_052305.21.CB3	0.001	0.2938	0.0035	20069087	alpha tubulin 1	0
CL12Contig1	APL_all_052305.103.C8	0	0.0371	0.001	5572	Calmodulin	2.00E-78
YesTrp090502-Bco3_02_H10_286	APL_all_052305.17002.C1	0.0003	0.3002	0.001	29122671	matrix metalloproteinase	2.00E-48

YesTrp1127_Bco3_D07_2291	APL_all_052305.283.C2	0.0003	0.1224	0.001	45384260	nucleoside diphosphate kinase B		1.00E-57
S_YESTrp121201-BCO3_H06_85	APL_all_052305.5584.C1	0	0.0495	0.001	9633	GTP-binding protein alpha-o subunit		2.00E-161
YesTrp1127_Bco3_F10_3181	APL_all_052305.6345.C1	0.0002	0.1856	0.001	54398904	pannexin 3		3.00E-09
CL73Contig2	APL_all_052305.79.C3	0.0001	0.0902	0.001	28630243	guanine nucleotide-binding protein		3.00E-161

(This work was done in collaboration with Dr. Yong-Seok Lee)

Table 5. Putative housekeeping genes used for K_a/K_s analysis.

Contig_IDs (*A. kurodai*)	Contig_IDs (*A. californica*)	*Ka*	*Ks*	*Ka/Ks*	GI	Description	E_value
CL217Contig1	APL_all_052305.1104.C1	0.0061	0.0152	0.4003	127798841	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, cardiac muscle	3.00E-106
CL87Contig1	APL_all_052305.11669.C1	0.0108	0.0275	0.391	66549728	PREDICTED: similar to ADP ribosylation factor 75F CG8385-PB, isoform B isoform 1	9.00E-92
CL36Contig1	APL_all_052305.466.C1	0.0183	0.1592	0.1147	47076115	mu class glutathione S-transferase	1.00E-67
5HTCNS081203-T3_C04_290	APL_all_052305.1104.C3	0.0088	0.0779	0.1127	32766606	Atp5a1 protein	2.00E-144
CL769Contig1	APL_all_052305.225.C1	0.0128	0.1203	0.1061	22758894	ribosomal protein L9	2.00E-69
CL890Contig1	APL_all_052305.3765.C2	0.0034	0.033	0.1029	41054585	eukaryotic translation initiation factor 5	3.00E-62
CL408Contig1	APL_all_052305.1.CB72	0.0072	0.0801	0.0904	49619095	NOP56	2.00E-162
CL982Contig1	APL_all_052305.244.CB1	0.0067	0.0864	0.0773	37723970	QM protein	4.00E-108
YBSTrp120302-BC03_B08_744	APL_all_052305.969.C1	0.02	0.3114	0.0642	19111573	enolase	2.00E-80
CL510Contig1	APL_all_052305.1704.C1	0.0096	0.1491	0.0641	13991680	proteasome subunit N3	2.00E-91
CL30Contig1	APL_all_052305.366.C11	0.0089	0.1602	0.0555	11602727	putative fructose-bisphosphate-aldolase	1.00E-90

5HTCNS122105-T3-F06-653	APL_all_052305.170.C2	0.0041	0.0754	0.0547	164519006	dynein light chain LC8-type 2	2.00E-46
CL471Contig1	APL_all_052305.951.C1	0.009	0.196	0.0457	22758866	ribosomal protein L23a	8.00E-62
CL683Contig1	APL_all_052305.1558.C1	0.0029	0.0717	0.0405	22758880	ribosomal protein S11	6.00E-66
CL945Contig1	APL_all_052305.1578.C2	0.0062	0.2016	0.031	47607474	light organ C8 alpha proteasome subunit	8.00E-99
CL676Contig1	APL_all_052305.16.C1	0.0028	0.0972	0.0293	22758856	ribosomal protein L19	1.00E-56
CL364Contig1	APL_all_052305.643.C1	0.0056	0.1985	0.028	27763677	eukaryotic translation initiation factor 2 alpha subunit	1.00E-105
CL94Contig1	APL_all_052305.502.C1	0.0041	0.1483	0.0276	56377786	glyceraldehyde-3-phosphate dehydrogenase (GAPDH) homologue	6.00E-142
CL16Contig2	APL_all_052305.340.C2	0.0025	0.1158	0.0213	42494887	heat shock protein 70	0
CL143Contig1	APL_all_052305.0.C2	0.002	0.1101	0.0178	12620237	ribosomal protein S6	1.00E-118
CL936Contig1	APL_all_052305.91.C1	0.0023	0.1445	0.0158	15718687	ribosomal protein S3	5.00E-110
CL140Contig1	APL_all_052305.36.C2	0.0019	0.1254	0.0151	22758868	ribosomal protein S4	4.00E-112
5CAP120606_pMES_E11_2731	APL_all_052305.917.C2	0.002	0.2042	0.0097	66509714	PREDICTED: similar to Rpt3 CG16916-PA	0
CL128Contig1	APL_all_052305.425.C1	0.0012	0.1358	0.0086	49903495	Polyadenylate-binding protein 1	7.00E-172
CL1Contig29	APL_all_052305.881.C1	0.0001	0.067	0.001	28174920	Rpl17 protein	2.00E-68
CL147Contig1	APL_all_052305.1.CB3	0.0002	0.1727	0.001	27503244	H3f3b-prov protein	3.00E-69

							Tyrosine 3-	
S_5CAP0122-PMES_C02_90	APL_all_052305.1132.C1	0.0001	0.0518	0.001	27882500	monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide b		4.00E-41
CL179Contig1	APL_all_052305.540.C2	0	0.0481	0.001	132545	RAS-like GTP-binding protein RHO		4.00E-95
CL567Contig1	APL_all_052305.5716.C1	0.0001	0.0845	0.001	18253043	60S acidic ribosomal protein P1		9.00E-22
YesTrp032303-BCO3_C02_1181	CNSN01-C-007381-501	0.0001	0.0766	0.001	66560175	PREDICTED: similar to Mig-2-like CG5588-PB, isoform B		2.00E-90
5CAF1127_pMES_A07_3767	PEG001-C-006604-501	0.0001	0.0796	0.001	33089110	thioredoxin peroxidase		2.00E-85

(This work was done in collaboration with Dr. Yong-Seok Lee)

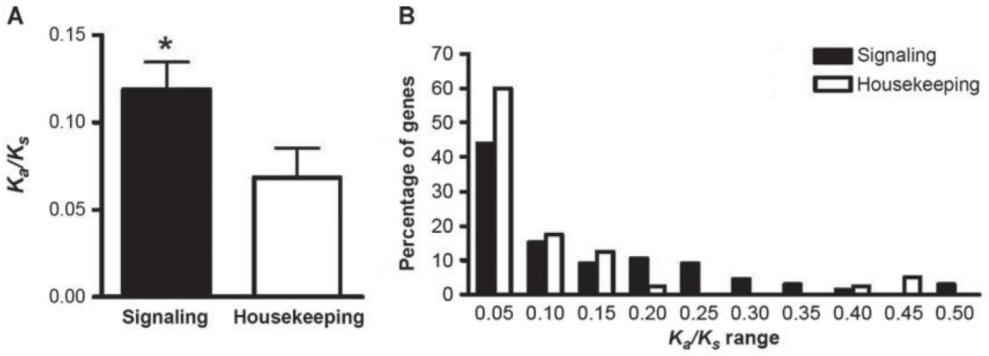


Figure 3. Faster evolutionary rate of mammalian homologs of neuronal signal transduction genes in *Aplysia*.

(A) Evolutionary rates of signal transduction genes and housekeeping genes in *Aplysia*. The height of each bar corresponds to the average K_a/K_s ratio ± SEM of each subset of the genes. Asterisk indicates that $p < 0.05$ in Kolmogorov–Smirnov test.

(B) The K_a/K_s distribution of the same two subsets of brain derived genes in *Aplysia*. The height of each bar represents percentage of each subset of genes in each K_a/K_s range.

(This work was done in collaboration with Dr. Yong-Seok Lee)

Table 6. Top 15 *Aplysia* genes showing the highest K_a/K_s ratio.

Gene Description	GI	*E*-value	*Ka*	*Ks*	*Ka/Ks*	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
Haemocyanin	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

+High score pairing length of translated amino acids sequences

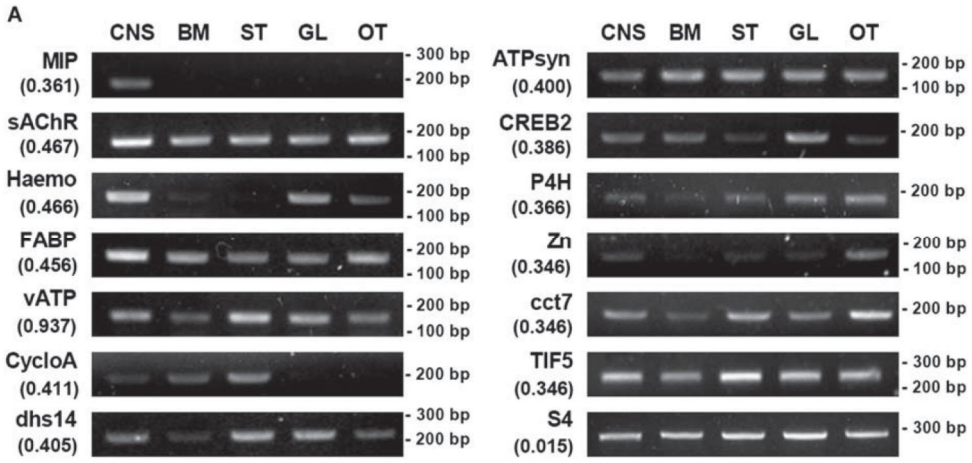
(This work was done in collaboration with Dr. Yong-Seok Lee)

Table 7. Bottom 15 *Aplysia* genes showing the lowest K_a/K_s ratio.

Gene Description	GI	*E*-value	*Ka*	*Ks*	*Ka/Ks*	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

(This work was done in collaboration with Dr. Yong-Seok Lee)


A
CNS BM ST GL OT
MIP (0.361)
- 300 bp
- 200 bp
sAChR (0.467)
- 200 bp
- 100 bp
Haemo (0.466)
- 200 bp
- 100 bp
FABP (0.456)
- 200 bp
- 100 bp
vATP (0.937)
- 200 bp
- 100 bp
CycloA (0.411)
- 200 bp
dhs14 (0.405)
- 300 bp
- 200 bp
CNS BM ST GL OT
ATPsyn (0.400)
- 200 bp
- 100 bp
CREB2 (0.386)
- 200 bp
P4H (0.366)
- 200 bp
Zn (0.346)
- 200 bp
- 100 bp
cct7 (0.346)
- 200 bp
TIF5 (0.346)
- 300 bp
- 200 bp
S4 (0.015)
- 300 bp

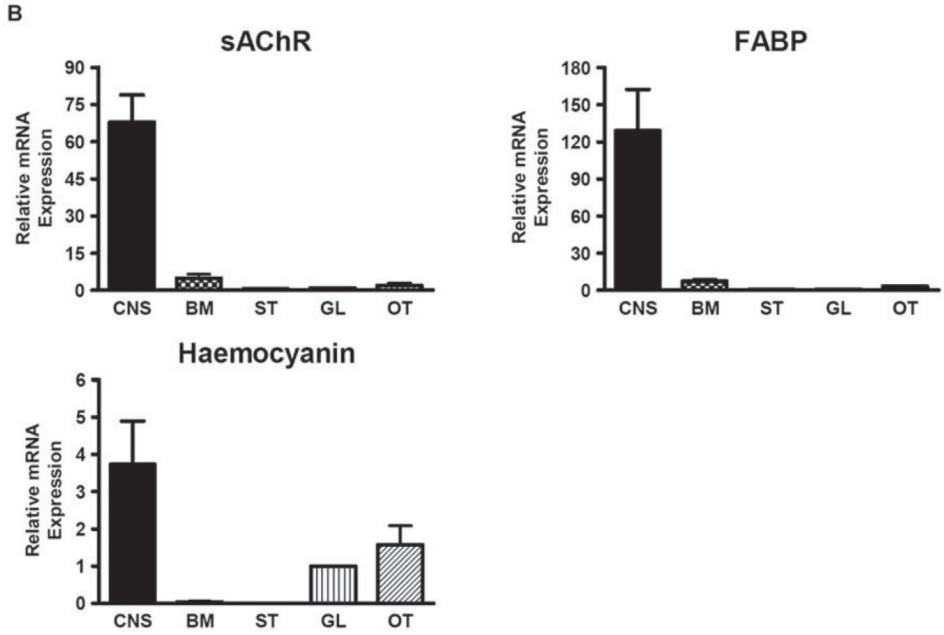

B
sAChR
Relative mRNA Expression
90
75
60
45
30
15
0
CNS
BM
ST
GL
OT
FABP
Relative mRNA Expression
180
150
120
90
60
30
0
CNS
BM
ST
GL
OT
Haemocyanin
Relative mRNA Expression
6
5
4
3
2
1
0
CNS
BM
ST
GL
OT

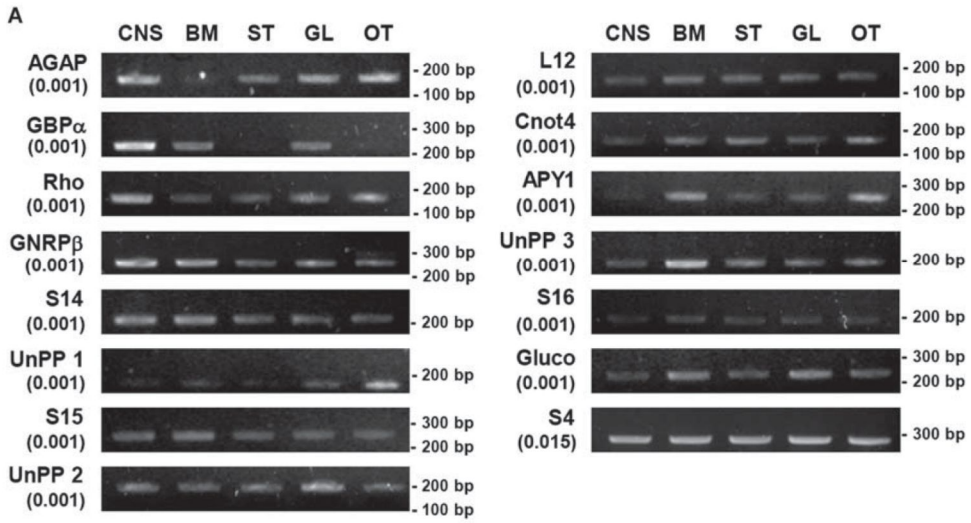
Figure 4. Differential tissue expression levels of genes with the highest K_a/K_s values.

(A) RT-PCR results of the 13 *Aplysia* genes showed the highest K_a/K_s values. K_a/K_s value for each gene is indicated in parenthesis.

(B) Tissue expression levels of the three genes were confirmed by real-time PCR. The height of each bar represents cDNA level normalized against that of gill (Numbers were represented in arbitrary unit). $p < 0.05$ in ANOVA and Tukey's multiple comparison tests.

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 complex 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.


A
CNS BM ST GL OT
AGAP (0.001)
- 200 bp
- 100 bp
GBPα (0.001)
- 300 bp
- 200 bp
Rho (0.001)
- 200 bp
- 100 bp
GNRPβ (0.001)
- 300 bp
- 200 bp
S14 (0.001)
- 200 bp
UnPP 1 (0.001)
- 200 bp
S15 (0.001)
- 300 bp
- 200 bp
UnPP 2 (0.001)
- 200 bp
- 100 bp
CNS BM ST GL OT
L12 (0.001)
- 200 bp
- 100 bp
Cnot4 (0.001)
- 200 bp
- 100 bp
APY1 (0.001)
- 300 bp
- 200 bp
UnPP 3 (0.001)
- 200 bp
S16 (0.001)
- 200 bp
Gluco (0.001)
- 300 bp
- 200 bp
S4 (0.015)
- 300 bp

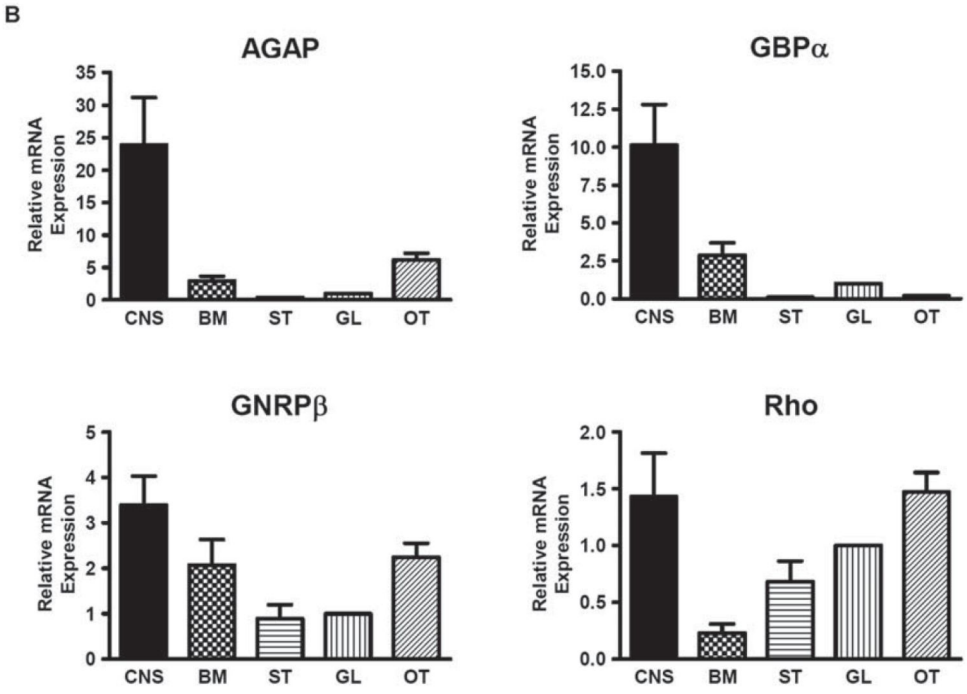

B
AGAP
GBPα
GNRPβ
Rho
Relative mRNA Expression
CNS
BM
ST
GL
OT

Figure 5. Differential tissue expression levels of genes with the lowest K_a/K_s values.

(A) RT-PCR results of the 14 *Aplysia* genes showing the lowest evolutionary rates. K_a/K_s value for each gene is indicated in parenthesis.

(B) Tissue expression levels of four genes were confirmed by real-time PCR. The height of each bar represents cDNA level normalized against that of gill (Numbers were represented in arbitrary unit). $p < 0.05$ in ANOVA and Tukey's multiple comparison tests.

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.

Discussion

In this chapter, molecular and genetic diversity between two closely related species, *A. kurodai* and *A. californica*, was described. By comparing the evolutionary rates though calculating K_a/K_s ratio using *A. kurodai* and *A. californica* EST databases (Lee et al., 2008b; Moroz et al., 2006), it was found that evolutionary rates of a group of selected nervous system related genes were higher than those of putative housekeeping genes (see Table 4, 5 and Figure 3). This was a biased approach because the selection of these two classes of genes was largely based on prior knowledge such as study of Dorus et al. and GenBank databases (Dorus et al., 2004). Since calculated K_a/K_s values of signal transduction genes in *Aplysia* neurons were all < 1.0, it was speculated that positive selection pressure on these genes was not the major driving force of evolution (Hurst, 2002; Zhang and Li, 2004). Therefore, these differences may largely due to stronger evolutionary constraints on the housekeeping genes

than on the signal transduction genes in *Aplysia* neurons. Although relatively small number of genes were used in this chapter, the results were comparable to the results from other studies performed in mammals (Dorus et al., 2004; Zhang and Li, 2004; Duret and Mouchiroud, 2000). However, it is important to note the arbitrary nature of the selection of neuronal signaling-related genes based on their predicted molecular function.

Next, we investigated whether measuring the evolutionary rates by calculating K_a/K_s ratio without any prior knowledge about these genes can be an efficient way to find candidate genes for functional studies. From the top 15 *Aplysia* genes that showed the highest K_a/K_s ratio (Table 6), a lot of interesting candidate genes for studying learning and memory or synaptic plasticity of *Aplysia* neurons could be found. Of these, it is known that small guanosine triphosphate (GTP)-binding protein RAB2 has a pivotal role in adhesion to another neuron and outgrowth of neurites in dissociated

rat midbrain neurons (Ayala et al., 1990). Another gene, vacuolar adenosine triphosphate (ATP) synthase (v-ATPase), is known as a proton pump that acidifies large parts of intracellular and some extracellular compartments (Nishi and Forgac, 2002). Moreover, it is involved in loading of neurotransmitters to the synaptic vesicles (Amara and Kuhar, 1993) and vesicle exocytosis (Hiesinger et al., 2005). The other candidate gene, proline 4-hydroxylase, catalyzes the formation of 4-hydroxyproline in collagens and collagen-like sequences (Kivirikko and Myllyharju, 1998). It has also been shown to reduce the stability of several proteins associated with adaptation to hypoxic or oxidative stress (Siddiq et al., 2005). Finally, MIP-related peptide precursor has been identified in *Aplysia* and reported to operate in the neural circuits that initiate feeding (Fujisawa et al., 1999) and gill-siphon withdrawal reflex (Moroz et al., 2006).

Furthermore, the bottom 15 *Aplysia* genes that showed the lowest K_a/K_s rates were also listed (Table 7). Interestingly, there are four

ribosomal proteins that could be considered as housekeeping genes. Moreover, there is no interesting candidate for functional studies on learning and memory or synaptic plasticity based on the description of these genes. These data suggest that trimming out the genes that have extremely low K_a/K_s ratios can be an efficient way to narrow down the pool of candidate genes for the functional studies of *Aplysia* neurons.

Finally, tissue distributions of selected genes (See Table 6 and 7) were investigated to confirm the hypothesis 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 (Figure 4 and 5). By RT-PCR and real-time PCR, tissue distribution of the 13 genes showing the highest K_a/K_s values and 14 genes showing the lowest K_a/K_s values were measured (Table 6 and 7). There was some tendency that shows less variable expression across tissues and some

putative housekeeping genes such as ribosomal proteins were found among the genes that showed lowest K_a/K_s values. However, no significant correlation between neuronal gene expression and K_a/K_s value was detected (Figure 4 and 5). These data suggest that evolutionary rate of some genes cannot be an effective marker to estimate neuronal expression in *Aplysia.*

Chapter 3

Functional studies on memory-related genes in *Aplysia kurodai*

Section I

Molecular Characterization of AU-rich Element-Binding Protein, ApAUF1, in *Aplysia kurodai*.

Introduction

Serotonin (5-HT) is known as a key neurotransmitter not only for induce STF but also induce LTF (Abel and Kandel, 1998; Bailey et al., 1996; Kandel, 2001). In the early phase of LTF induction, it is critical that the expression of immediate early gene such as ApC/EBP (Alberini et al., 1994; Lee et al., 2001; Lee et al., 2006), kinesin heavy chane (KHC) (Puthanveettil et al., 2008), and ubiquitin C-terminal hydrolase (ApUch) (Hegde et al., 1997). Especially, ApC/EBP is a transcription factor which has an important role to express other late-response genes and, therefore, it is known as a molecular switch for the induction of long-term memory (Alberini et al., 1995; Lee et al., 2001). Expression of ApC/EBP is tightly regulated because regulating the amount of late-response genes is very critical to induce LTF. For example, increment of ApC/EBP is detected within 15 min after 5-HT treated, and it's expression level is returned to baseline within 4 hr after onset of 5-HT stimulation (Alberini et al., 1994).

It is known that ApC/EBP is degraded by ubiquitin-proteasome pathway, however, degradation mechanism of ApC/EBP mRNA is not clearly understood yet (Yamamoto et al., 1999). Since ApC/EBP mRNA has AREs which is important element to mRNA destabilization in its 3'UTR, its stability may be regulated by ARE binding proteins such as ELAV (Yim et al., 2006).

In order to find the negative regulators of ApC/EBP, Lee and his colleagues performed the microarray analysis using the pleural ganglia were isolated 4 h after the onset of 5-HT treatment (Lee et al., 2008b). Interestingly, only ApAUF1 mRNA was increased 4 h after the 5-HT treatment (Lee et al., unpublished). Moreover, AUF1 is one of the AU-rich element(ARE)-binding protein, and it is known as a destabilizing factor for c-Myc (DeMaria and Brewer, 1996). Since ApC/EBP has AREs on its C-terminus, it could be speculated that ApAUF1 may work as ApC/EBP

mRNA destabilizing molecule. To test this hypothesis, expression profile of ApAUF1 and its binding to ApC/EBP mRNA were revealed in this chapter.

Experimental procedures

1. *In-vivo* 5-HT Treatment to *Aplysia kurodai*

To monitor the changes of ApAUF1 5-HT treatment in *Aplysia* ganglia, 250 μM of 5-HT were *in-vivo* treated to animals. Two hours after onset of 5-HT treatment, animals were anesthetized and abdominal central ganglia were dissected and frozen using liquid nitrogen. The animals which were not exposed to 5-HT were used as control.

2. Semi-quantitative RT-PCR

Total RNAs were extracted from tissues using TRIzol Reagent (Invitrogen). The cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) with oligo dTs (Invitrogen) as a primer. These cDNAs were used as templates for PCRs for ApAUF1 (AUF1 sense, 5'-TGGATCCCAAGAGGTTGAAG-3'; AUF1 antisense, 5'-GGATGATAGCCTCCATGAGC-3) and S4 (S4 sense, 5'-

GACCCTCTGGTGAAGGTGAA-3' ; S4 antisense, 5' -TGGACAGCTTCACACCTTTG-3'). Amplification was carried out for 28-35 cycles (94 ℃ 15 s; 60 ℃ 15 s; 72 ℃ 30 s). PCR products were visualized on a 2% agarose gel.

3. Western blot and Immunocytochemistry

Western blot and immunocytochemistry were performed as described previously (Kim et al., 2006; Lee et al., 2007). To detect endogenous ApAUF1 by western blot, *in-vivo* 5-HT treated ganglia were lysed by RIPA buffer and immobilized on the nitrocellulose membrane. Lab-maid antibody against ApAUF1 was used as primary antibody in Western blot.

To detect overexpressed pNEXδ-3×FLAG-ApAUF1, cultured sensory neurons microinjected with pNEXδ-3×FLAG-ApAUF1 and pNEXδ-EGFP were fixed by the fixative solution containing 4%(w/v) PFA

and 30%(w/v) sucrose in PBS. After 30 min of fixation, cells were rinsed with PBS, and then permeabilized with 0.5% Triton X-100 in PBS if needed. (Thompson et al., 2004). To detect the FLAG epitope, mFLAG M2 antibody (Sigma) was used as a primary antibody.

4. RNA-protein pull-down assay

This experiment performed as describe elsewhere (Mastushita-Sakai et al., 2010) with small modifications. Biotin-labeled RNA was prepared by *in-vitro* transcription with T7 RNA polymerase (Promega). A 3×FLAG-tagged ApAUF1-overexpressing 293T cell was lysed by using lysis/binding buffer containing 50 mM Tris · HCl at pH 7.6, 150 mM NaCl, 5% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1 mM DTT, 0.2 mg/mL heparin, 0.2 mg/mL yeast tRNA, 0.25% (w/v) BSA, protease inhibitor cocktail, protein phosphatase inhibitor cocktail (Roche), and 40 U/mL RNasin (Promega). Eight micrograms of biotinylated RNA were mixed with 200

μg (0.2 mg/mL) of pre-cleared cell lysate and incubated for 1 h at 4 ℃. After pulling down with NeutraAvidin Agarose Resin (Thermo Scientific), Western blot was performed by using an mFLAG-m2 antibody (1:2,000; Sigma).

5. Microinjection of Plasmids or dsRNA into *Aplysia* Sensory Neurons

p3×FLAG-ApAUF1 (500 ng/μL) was mixed with the reporter construct pNEXδ-EGFP (500 ng/μL) and dissolved in the injection solution (Tris-Cl, 10 mM; KCl, 100 mM; fast green, 0.1%; pH 7.3). These solutions were microinjected into *Aplysia* cultured sensory neurons as previously described (Kaang, 1996; Kaang et al., 1992). Three or 4 days after culture, plasmids were microinjected into the cells. Cells were incubated 1 or 1.5 days after microinjection before use.

Results

1. Expression Profiles of ApAUF1 in *Aplysia* Sensory Neurons.

In order to monitor the expression, immunocytochemistry analysis using cultured *Aplysia* sensory neurons were performed. Overexpressed 3×FLAG-ApAUF1 was localized mainly in the somatic region, near the plasma membrane and perinucleus region and also detected in the neurites (Figure 6). This is not consistent to the data from mammalian AUF1, which is mainly localized in the nucleus (Zhang et al., 1993).

2. Confirm the Expression Level Change of ApAUF1 after 5-HT Treatment.

To clone the AUF1 which is the homolog of mammalian AUF1, *A. kurodai* EST database were used. One clone (EY418173.1) showed high homology to mammalian AUF1, and it also showed increment of mRNA expression from microarray analysis (Lee et al., 2008b). To reveal the increment of ApAUF1, western blot was performed. However, expression

level of ApAUF1 was not increased or decreased by *in-vivo* 5-HT treatment (Figure 7).

3. Association of ApAUF1 with ApC/EBP 3'UTR.

AUF1 is known to interact with many ARE-containing mRNAs such as c-myc, c-fos, and TNF-α (Chen and Shyu, 1995). To confirm whether the ApAUF1 binds to ApC/EBP AREs, RNA-protein pull-down assay was done using two biotin-labeled probe; one is putative AREs deleted form (ΔARE), and another is not deleted form (wild-type, WT)(Figure 8A). Two biotin-labeled probes were incubated with 3×FLAG-ApAUF1 overexpressed HEK293T cell lysates, and then RNA-protein complex were pulled down and analyzed. Wild-type probe was bound to overexpressed 3×FLAG-ApAUF1, however, ΔARE mutant probe could not bind to 3×FLAG-ApAUF1 (Figure 8B). These results

indicate that the AREs of ApC/EBP are required for the interaction with ApAUF1.

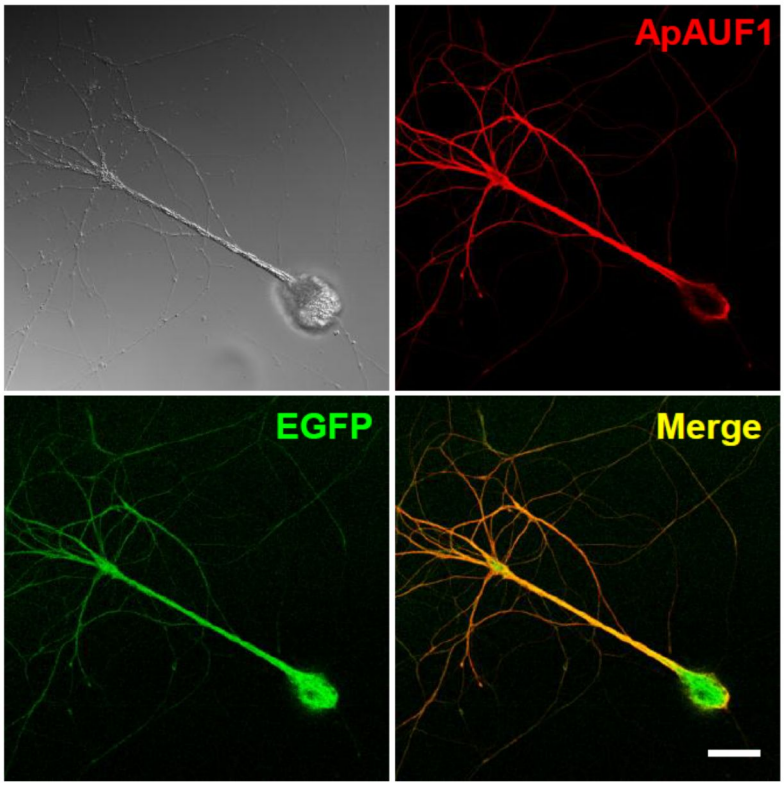

ApAUF1
EGFP
Merge

Figure 6. Expression of ApAUF1 in the *Aplysia* nervous system.

The subcellular localization of overexpressed 3×FLAG-ApAUF1. 3×FLAG-ApAUF1 was indicated as red, co-injected injection marker, EGFP, indicated as green. Scale bar, 50 μM.

(This work was done in collaboration with Jae-Hoon Shim)

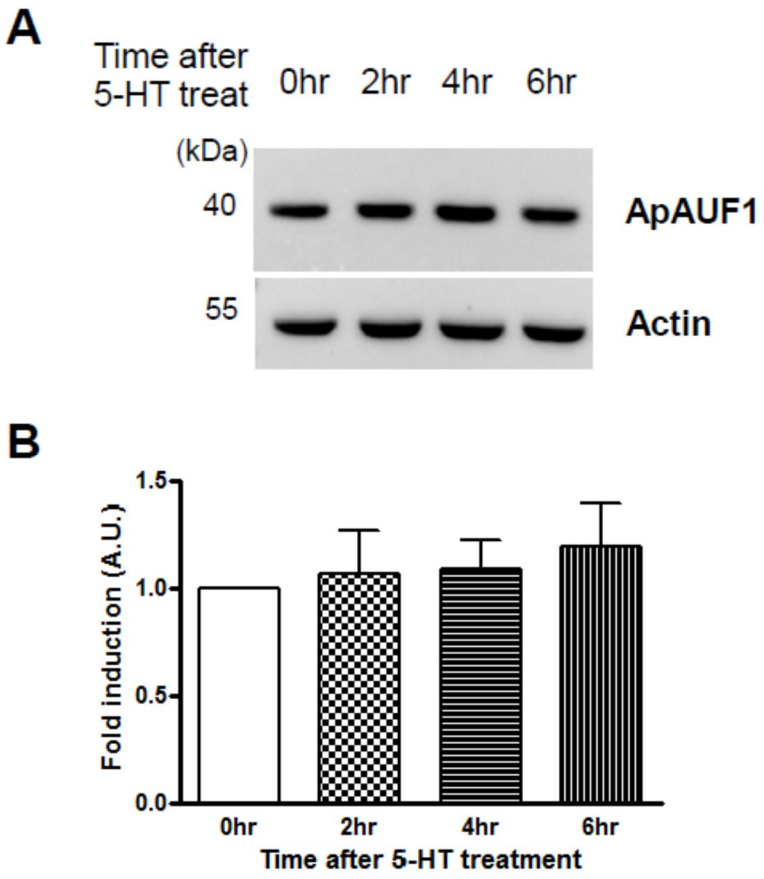

A
Time after
5-HT treat
0hr
2hr
4hr
6hr
(kDa)
40
ApAUF1
55
Actin
B
Fold induction (A.U.)
1.5
1.0
0.5
0.0
0hr
2hr
4hr
6hr
Time after 5-HT treatment

Figure 7. Increase in protein expression level of ApAUF1 by 5-HT treatment.

(A) Representative Western blot of ApAUF1 expression level.

(B) The height of each bars indicates that the normalized protein level ± SEM. Band intensity of each ApAUF1 lane was normalized by band intensity of each actin lane.

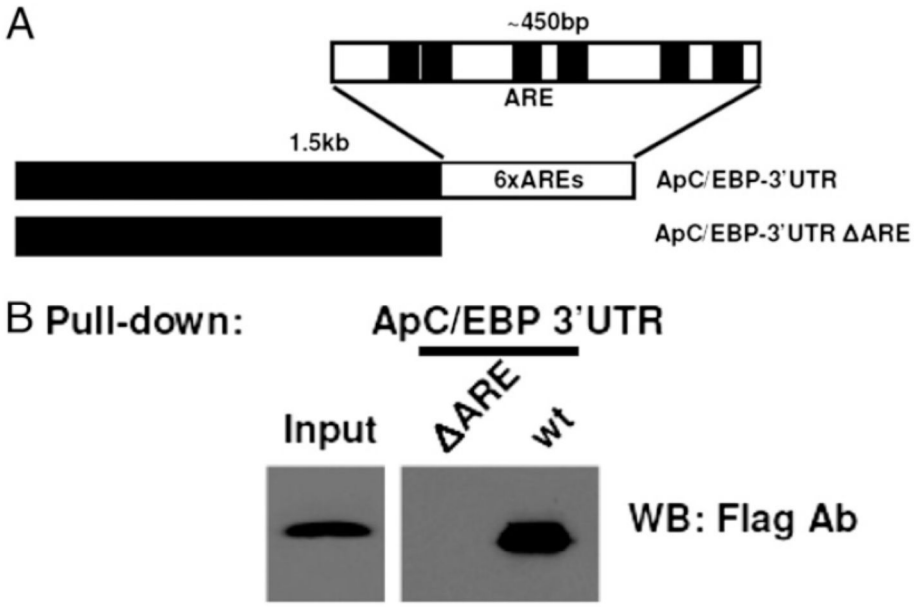

A
~450bp
ARE
1.5kb
6xAREs
ApC/EBP-3'UTR
ApC/EBP-3'UTR ΔARE
B Pull-down:
ApC/EBP 3'UTR
Input
ΔARE
wt
WB: Flag Ab

Figure 8. Binding of ApAUF1 to the AREs of ApC/EBP 3′UTR.

(A) Schematic figure of reporter RNAs. In case of ⊿ARE, 444 bp containing six AREs of ApC/EBP 3'UTR were deleted.

(B) RNA binding specificity of ApAUF1. Using mFLAG-m2 antibody, RNA-protein complex were detected. Wild-type probe but not ⊿ARE probe was bound to overexpressed 3×FLAG-ApAUF1.

Discussion

In this chapter, molecular characterization of ARE-binding protein ApAUF1 was performed. Unexpectedly, overexpressed 3×FLAG-ApAUF1 was localized in the perinucleolar region, and neurites (Figure 12). It could not exclude the possibility of overexpression artifact, however, similar results also revealed in endogenous ApAUF1 (Lee et al., 2012). Interestingly, mammalian AUF1 showed different localization pattern with that of ApAUF1 (Zhang et al., 1993), however, the mechanism that makes the differences is remain to be understand.

Next, expression profile of ApAUF1 after 5-HT treatment was monitored (Figure 7). Since ApAUF1 mRNA was increased at 4 h after onset of 5-HT treatment (Lee et al., 2008b), it is expected that the protein level of ApAUF1 is increased in the similar time point. However, it is confirmed that ApAUF1 did not increased by 5-HT treatment through Western blot analysis (Figure 7).

Finally, RNA-protein pull-down assay was performed to investigate whether ApAUF1 is bound to the AREs of ApC/EBP 3'UTR or not. It is well known that not only the protein level of ApC/EBP, but also the mRNA level of ApC/EBP are rapidly down-regulated during memory consolidation (Yamamoto et al., 1999; Alberini et al., 1994), however, the mechanism which regulates ApC/EBP mRNA is not discovered yet. Since mammalian AUF1 is known as mRNA destabilizing protein (DeMaria and Brewer, 1996), the result that ApAUF1, the homolog of mammalian AUF1, could bind to the ApC/EBP 3'UTR (Figure 8) suggests the possibility that ApAUF1 is involved in the post-transcriptional regulation of ApC/EBP mRNA.

Section Ⅱ

Cloning and Characterization of Serotonin Receptor That Positively Coupled to Adenylyl Cyclase Involved in Learning-related Heterosynaptic Facilitation in *Aplysia kurodai*.

Introduction

Serotonin (5-HT) is a well-known key neurotransmitter that modulates short-term and long-term synaptic plasticity in *Aplysia* (Kandel, 2001; Montarolo et al., 1986; Klein et al., 1982; Barbas et al., 2003). 5-HT stimulates several downstream pathways including PKA (Schacher et al., 1988; Kaang et al., 1993; Mercer et al., 1991; Abrams et al., 1984), PKC (Byrne and Kandel, 1996; Sugita et al., 1994) and MAPKs (Guan et al., 2003; Martin et al., 1997) through 5-HTRs which have seven transmembrane domains (Watson and Arkinstall, 1994). Among these signaling pathways about learning and memory, it suggested that adenylyl cyclase-cAMP-PKA pathway is the most important for learning and memory storage (Kandel, 2001). Although there were a lot of efforts to identify the starting molecule of this molecular pathway, however, 5-HT receptor which positively coupled to adenylyl cyclase was not yet identified in *Aplysia* (Schlenstedt et al., 2006; Angers et al., 1998).

In vertebrates, total seven 5-HT receptor families have been cloned and identified; only 5-HT_4, 5-HT_6 and 5-HT_7 positively coupled to adenylyl cyclase, and others do not (Hoyer et al., 2002; Watson and Arkinstall, 1994). In this chapter, using *Aplysia* EST databases and degenerative PCR method, the first 5-HT receptor which positively coupled to adenylyl cyclase was cloned. Following the molecular function of the receptor, it was entitled as 5-HT_{apAC1}. As expected from many other researches (Dale et al., 1987; Baxter and Byrne, 1990), this receptor activates adenylyl cyclase-cAMP-PKA pathway but not PKC pathway, and has an essential function to induce synaptic plasticity in *Aplysia* synapses.

Experimental procedures

1. Molecular Cloning of 5-HT_{apAC1} cDNA

Molecular cloning of 5-HT_{apAC1} was performed in two different ways. Firstly, one sequence (PEG003-C-228120-501) was found to be highly homologous with other invertebrate 5-HT_7 receptors from the *Aplysia* EST database (http://seahare.org). PCR reactions were performed using three different primers (5-HTR sense 1, 5'-TGGGATACGCCAATTCTTTC-3'; 5-HTR sense 2, 5'-CTCAGGACCAGC AACACAGT -3'; 5-HTR antisense 1, 5'-GCAAGTGAAGTAAAGAGGAGA TCCA-3'). Amplification was carried out for 3 min at 94 ℃ (one cycle), followed by 35 cycles of 15 s at 94 ℃, 15 s at 60 ℃, 1 min at 72 ℃, and a final extension of 5 min at 72 ℃. Nested PCR was done in the same way. By the PCR reaction, small fragment containing 444bp, named 5-HTR #7 was obtained. Using this fragment as an antisense probe, *Aplysia kurodai* cDNA library which contains approximately 2.0 × 10^5 clones to obtain the

full-length ORF, and the single positive signal was detected (Lee et al., 2008b). The obtained clones were analyzed by DNA sequencing, it was confirmed that they contained single entire ORF (5-HT_{apAC1}).

Secondly, degenerative PCR was performed using two degenerative PCR primers. Sense primer is designed based on the highly conserved amino acid sequence, NPXXY, which is located in the fifth transmembrane domains. And antisense primer is designed based on the QIYATL motif found in the seventh transmembrane domains of invertebrate 5-HT_7 receptors such as *Helisoma* and *Drosophila* (5HT sense new 1, 5'-CARATITAYGCMACICTA-3'; 5HTR antisense new 1, 5'-TGYRTADATIAYIGGRTT-3').

Amplification was carried out for 3 min at 94 ℃ (one cycle), followed by 35 cycles of 15 s at 94 ℃, 15 s at 60 ℃, 1.5 min at 68 ℃, and a final extension of 5 min at 68 ℃. Through the PCR reaction, a DNA fragment of 519 bp was obtained. The fragment was showed ~ 80% homology with the

Helisoma trivolvis 5-HT_7 receptor. Using this fragment as a probe, *Aplysia kurodai* cDNA library was screened to acquire the entire ORF, and single positive signal was detected (Lee et al., 2008b). The obtained clones were analyzed by DNA sequencing, it was confirmed that they contained the same entire ORF (5-HT_{apAC1}). The sequence of 5-HT_{apAC1} was subcloned into *Hin*dIII/*Kpn*I-digested pNEXδ (Kaang et al., 1993) to create pNEXδ-5-HT_{apAC1}.

2. Phylogenetic Analysis of Cloned 5-HT_{apAC1}

Phylogenetic analysis was performed using ClustalW software using DNA and amino acid sequence of cloned 5-HT_{apAC1} and other receptors.

3. Single Cell RT-PCR

Single cell RT-PCR protocol was modified from the methods described previously (Dulac and Axel, 1995; Li et al., 2009). Cultured sensory or LSF motor neurons were harvested with RNA*later* (Ambion), and then transferred to 22 μL of ice-chilled lysis buffer in PCR tubes, containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 0.5 % Nonidet P-40, and 20 U RNasin (Promega). The L7 motor neuron was directly pulled from the desheathed abdominal ganglion and transferred to the lysis buffer. After lysis (70 ℃ for 5 min), DNase I treatment was performed at 37 ℃ for 10 min, by adding DNase treated solution containing 5 μL RNase-free DNase I (Ambion), 4 μL (10 U / μL) of DNase I buffer (Ambion), and 5 μL RNase-free water (Ambion). To terminate the DNase reaction, 2 μL of EDTA was added and then heated at 37 ℃ for 10 min. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) with oligo dT or random hexamers or gene-specific primers (Invitrogen). A control reaction was performed without adding the reverse

transcriptase. The cDNA was used as templates for PCR for 5-HT_{apAC1} (5-HTR scRT sense 1, 5'-ACGGCTCCCTGTATGTCAAC-3'; 5-HTR scRT antisense 1, 5'- CCAGATGTCACAGACGATGG-3'), and sensorin (senso-sense, 5'-AACAGAAACAGTCTTTCCCC-3'; senso-antisense, 5'-TCTTGACTCACCAACTGCC-3'). Template were amplified using Ex-Taq (Takara) for 40 cycles (94 ℃ 15 s; 60 ℃ 30 s; 72 ℃ 30 s) except for 5-HT_{apAC1}. In the case of 5-HT_{apAC1}, amplification was carried out for 35 cycles and followed by 35 cycles of nested PCR (5-HTR sense 2, 5'-CAAACGGCACAGACCTGTTC-3'; 5-HTR antisense 2, 5'-AACCGCTTCACTATGGCAAC-3'). PCR products were visualized on a 2% agarose gel.

4. Semi-quantitative RT-PCR

Total RNAs were extracted from tissues using TRIzol Reagent (Invitrogen). The cDNA was synthesized by SuperScript III reverse

transcriptase (Invitrogen) with oligo dTs or random hexamers (Invitrogen) as a primer. These cDNAs were used as templates for PCRs for 5-HT_{apAC1} (5-HTR-RT-sense, 5'-TGGGATACGCCAATTCTTTC-3'; 5-HTR-RT-antisense, 5'-GCAAGTGAAGTAAAGAAGAGATCCA- 3') and S4 (S4 sense, 5'-GACCCTCTGGTGAAGGTGAA-3'; S4 antisense, 5'-TGGACAGCTTCACACCTTTG-3'). Amplification was carried out for 28-35 cycles (94 ℃ 15 s; 60 ℃ 15 s; 72 ℃ 30 s). PCR products were visualized on a 2% agarose gel.

5. *In-situ* Hybridiazation in *Aplysia* Ganglia or Cultured Neurons

In-situ hybridization was performed as described previously (Kim et al., 2006). Partial fragment of 5-HT_{apAC1} (# 7 probe, 444 bp from 1,178th nucleotide from the initial ATG for Figure 11C; 3icl probe, 297 bp from 754th nucleotide from the initial ATG for Figure 11D and Figure 11) was used as a probe. DIG-labeled probes were made by in vitro

transcription using a DIG RNA labeling mix (Roche). The DIG-labeled probe was detected using an anti-DIG antibody (Roche), followed by development with NBT/BCIP (Roche).

6. Transfection, Western Blot, and Immunocytochemistry

Western blot and immunocytochemistry were performed as described previously (Kim et al., 2006; Lee et al., 2007). The entire coding region of 5-HT_{apAC1} was subcloned into pFLAG-CMV2 or p3×FLAG7.1 (Sigma). To express 5-HT_{apAC1} in *Aplysia* neurons, FLAG-5-HT_{apAC1} was subcloned into pNEXδ (Kaang et al., 1993). These constructs were expressed in HEK293T cells with or without the expression marker pmCherry-N1 (Clontech) using Lipofectamine 2000 (Invitrogen) and were incubated for 1.5 days before experiments. In *Aplysia* sensory neurons, pNEXδ-FLAG-5-HT_{apAC1} and pNEX3-synaptophysin-EGFP were microinjected as described below. To detect the FLAG epitope, mFLAG M2

antibody (Sigma) was used as a primary antibody and anti-mouse Cy3 (red), or anti-mouse Alexa488 (green) was used as a secondary antibody.

7. Heterologous Expression and Electrophysiology in *Xenopus* oocytes

cRNA preparation and microinjection into *Xenopus* oocytes was performed as described previously (Lee et al., 1999). cRNAs for 5-HTapAC1, Ap oa1 (Chang et al., 2000), mouse 5-HT_{2C} (previously termed 5-HT_{1C}) (Lubbert et al., 1987), and human CFTR (Bear et al., 1991) were prepared by in vitro transcription using a MEGAscript kit (Ambion). The cRNAs were qualified by electrophoresis and microinjected into oocytes. Two-electrode voltage clamp was performed with a GeneClamp 500 amplifier (Axon Instruments). The voltage electrode and the current electrode, with the resistances of 1-5 MΩ, were filled with 3M KCl. The oocytes were placed in a chamber perfused with Ca^{2+}-free ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM Hepes, pH 7.5. The holding

potential was set at −70 mV. The data were collected on a chart recorder and stored on video cassettes through Digidata (Instrutech) for later analysis.

8. Double-stranded RNA Synthesis

cDNA containing a partial ORF (281 bp, 1178th to the stop codon) and 3'UTR (163 bp) of 5-HT_{apAC1} was subcloned into pLitmus28i. The construct was linearized and then used as a template for *in-vitro* transcription. Using the MEGAscript RNA*i* kit (Ambion), each strand of RNA was transcribed and then annealed followed by manufacturer's manual. Double-stranded firefly luciferase (dsLuci) was used as a control.

9. Intracellular cAMP Level Measurement Using FRET

To measure the change of the intracellular cAMP level, fluorescence resonance energy transfer (FRET) was used. The construct

which was used to FRET was a generous gift from Changjoon Justin Lee (Korea Institute of Science and Technology, Korea), and the analysis was performed as described previously (Park et al., 2005). Cultured and microinjected *Aplysia* sensory neurons were incubated for 2 days. We performed FRET imaging on a Zeiss (LSM510) confocal laser scanning microscope. For excitation, 458 nm wavelength was used, and fluorescence emission was collected with BP 480–520 IR (for CFP) and LP 530 (for YFP) emission filters. For the measurement of the fluorescence emission, we selected three regions of interest (ROI) with diameters of 5 μm. The selection was made on the region of the axon hillock because axon hillocks showed the smallest morphological change after 5-HT treatment. Because the binding of cAMP to CFP-Epac(δDEP/CD)-YFP induces a conformational change, reduced FRET indicates the degree of intracellular cAMP elevation. Decreased FRET was converted into an elevated cyan fluorescence protein (CFP)/yellow fluorescent protein (YFP) emission ratio.

Measurements were made every 10 s for 600 s. After nine measurements to get the baseline ratio, a 100 μM stock solution of 5-HT in L15/ASW was applied to meet the final concentration of 10 μM. To minimize mechanical artifacts from the application, we applied the stock solution on the edge of the culture plate. In this treatment condition, the drug would have had to diffuse through the bath media to the cell. This technique may account for the time delay between the drug treatment and cAMP increase. Nine measurements, which were made from three ROIs for 30 s, were averaged for statistical analysis.

10. Microinjection of Plasmids or dsRNA into *Aplysia* Sensory Neurons

Various DNA constructs (500 ng/μL) or dsRNA (500 ng/μL) was mixed with the reporter construct pNEXδ-EGFP (500 ng/μL) and dissolved in the injection solution (Tris-Cl, 10 mM; KCl, 100 mM; fast green, 0.1%; pH 7.3). These solutions were microinjected into *Aplysia*

cultured sensory neurons as previously described (Kaang, 1996; Kaang et al., 1992). Three or 4 days after culture, dsRNAs were microinjected into the cells. Cells were incubated 1 or 1.5 days after microinjection.

11. *Aplysia* Cell Culture and Electrophysiology

Sensory cell culture and sensory-to-motor co-culture were performed as described previously (Schacher and Proshansky, 1983; Kim et al., 2006). Briefly, sensory neurons were dissociated from sensory clusters of pleural ganglia, and LFS motor neurons were used for sensory-to-motor co-culture. Membrane excitability and spike broadening were measured in the sensory neurons of ventrocaudal clusters from pleural ganglia as described elsewhere (Chang et al., 2000). Sensory neurons were used to experiment only if their resting potential was between −30 to −45 mV when impaled. To measure the membrane excitability, 0.1-0.5 nA of a current pulse (500 msec) was injected to induce a single action potential.

Five minutes after 5-HT treatment, the same amount of current was injected, and the number of action potentials was counted. To measure the spike broadening, a 0.2-0.8 nA of current pulse was delivered into the cells for 15 msec. The spike duration was measured at the time from the peak of the action potential to 25% of the peak. Spike broadening was measured 2 min after the membrane excitability measurement. EPSP recordings in sensory-to-motor co-culture were performed as previously reported (Lee et al., 2001; Ghirardi et al., 1992). To induce moderate synaptic depression, five electrical stimuli (ISI: 20 sec) were applied to the presynaptic sensory neuron. Ten micromolar of 5-HT (Sigma) was bath-applied for those experiments. For Figure 14A, 10 μM of 5-HT was bath-applied for 1 min, followed by 4 min of washout using bath solution (1 mL/min). The synapses that initial EPSP was under 2 mV were not used for analysis.

12. Statistical Analysis

The results are represented as mean ± SEM. When comparing two groups, the unpaired Student's *t*-test was used. To compare three or more groups, one-way ANOVA was used. If any significant between groups was found, then Newman-Keuls post-hoc test was followed.

Results

1. Molecular Cloning of 5-HT_{apAC1} from the *Aplysia kurodai* Nervous System.

In order to clone genes encoding the 5-HT receptors which are positively coupled to adenylyl cyclase in *A. kurodai*, short sequence originated from *Aplysia* EST database and degenerative primers based on cloned type 7 5-HTR sequences came from *Drosophila* and *Apis* were used. From the *Aplysia* EST database, one sequence which showed high homology to other invertebrate 5-HT type 7 receptors was found. Through the repeated PCR reactions, 444-bp PCR product was obtained. Using this fragment as a probe, full-length 5-HT_{apAC1} cDNA which have 1,458 bp ORF was obtained (Figure 9B).

In order do the degenerative PCR, two degenerative primers were used. On the basis of the amino acid sequences of the fifth and seventh transmembrane domains of invertebrate 5-HT_7 receptors, sense and antisense primers were designed. In addition to the highly conserved amino

acids sequences, NPXXY, it was found that extraordinarily conserved QIYATL motif which is only found in invertebrate 5-HT_7 receptors (Figure 9A). Through the PCR reaction using these degenerative primers, 519-bp PCR product which have >80% sequence homology with the *Helisoma trivolvis* 5-HT_7 receptor was obtained. Using the fragment as a probe, the same full-length 5-HT_{apAC1} cDNA was obtained (Figure 9B). Cloned 5-HT_{apAC1} encodes 485 amino acids with a predicted molecular weight of 54 kDa.

2. Phylogenetic Analysis of Cloned Receptor, 5-HT_{apAC1}.

Using ClustalW software, mRNA sequence of 5-HT_{apAC1} was compared with other 5-HT receptors such as *Drosophila*, *Apis*, *Helisoma* and so on (Figure 10A). 5-HT_{apAC1} was closely linked to 5-HT_7 receptors of pond snail, honeybee and fruit fly. It indicates that 5-HT_{apAC1} was closely related to other invertebrate 5-HT_7 receptors. These results

indicated that the cloned 5-HT_{apAC1} is classed as the 5-HT_7 family, which is positively coupled to adenylyl cyclase (Figure 10B).

3. Tissue Distribution of 5-HT_{apAC1}.

In order to monitor the tissue expression pattern of clone receptor, 5-HT_{apAC1}, RT-PCR and *in-situ* hybridization were performed. 5-HT_{apAC1} was highly expressed in abdominal ganglia, and moderately expressed in buccal ganglia, central ganglia containing pleural-pedal ganglia, and gill. 5-HT_{apAC1} was also slightly expressed in buccal mass, however, it was not detected in ovotestis (Figure 11A). There results were consistent with Drummond et al.'s study using the [3H]LSD binding assay (Drummond et al., 1980). To further understand the expression profile of 5-HT_{apAC1}, single cell RT-PCR analysis was performed (Figure 11B). It clearly showed 5-HT_{apAC1} was expressed not only in sensory neurons, but also in L7 and LFS motor neurons.

In-situ hybridization analysis revealed that 5-HT_{apAC1} was widely expressed throughout nervous system of *Aplysia*, not only the sensory clusters of pleural and abdominal ganglia, but also the L7 motor neurons located in abdominal ganglia (Figure 11C). Expression of 5-HT_{apAC1} in sensory neurons came from pleural ganglia and LFS motor neurons was also verified by cell *in-situ* hybridization using sensory-to-motor co-culture (Figure 11D).

4. Cellular Localization of 5-HT_{apAC1}.

To examine the subcellular localization of 5-HT_{apAC1}, N-terminal FLAG-tagged 5-HT_{apAC1} (FLAG-5-HT_{apAC1}) was transiently expressed in HEK293T cells. Through western blot and immunocytochemistry, expression and localization of the receptor was revealed (Figure 12A and B). Overexpressed FLAG-5-HT_{apAC1} was detected at the plasma membrane of 293T cells (Figure 12B). Overexpressed FLAG-5-HT_{apAC1}

was also detected in plasma membrane of *Aplysia* sensory neurons (Figure 12C, left panel). Moreover, FLAG-5-HT_{apAC1} was co-localized with synaptophysin-EGFP at neurites and varicosities in sensory neurons (Figure 12C, right panel). It indicates that cloned receptor, 5-HT_{apAC1}, is expressed in synaptic region of sensory neurons.

5. G-protein Coupling Specificity of 5-HT_{apAC1}.

To confirm the G-protein coupling specificity of 5-HT_{apAC1}, *Xenopus* oocytes with or without cystic fibrosis transmembrane conductance regulator (CFTR) were used. One of the well-studied chloride channels, CFTR, has been used to confirm that a receptor can activate G_s protein or not because CFTR is activated by PKA (Lee et al., 1999; Chang et al., 2000).

Treatment of 1 μM 5-HT induced significant CFTR current (320 $\pm$ 123.8 nA, n=8) in 5-HT_{apAC1} and CFTR injected oocytes (Figure 13A).

As a positive control, Ap oa_1, octopamine receptor which is positively coupled to adenylyl cyclase (Chang et al., 2000), was co-injected with CFTR. Treatment of 1 μM of octopamine also induced significant CFTR current (565.0 ± 104.1 nA, n=8) in the oocyte(Figure 13B).

It is also known that *Xenopus* oocytes have an endogenous fast-activating chloride channel which is activated by Ca^{2+} after activation of G_q (Lubbert et al., 1987). Microinjection of Gq-coupled receptor, $5\text{-}HT_{2c}$, into oocytes showed typical agonist-activated transient inward current by 5-HT treatment (835.0 ± 35.4 nA, n=2) (Figure 13C2). However, $5\text{-}HT_{apAC1}$ cRNA-injected oocytes did not show any transient chloride current by 5-HT treatment (Figure 13C1). These results indicate that $5\text{-}HT_{apAC1}$ is positively coupled to G_s, but not to G_q.

6. $5\text{-}HT_{apAC1}$ Modulates cAMP in Response to 5-HT.

In order to investigate the endogenous role of 5-HT_{apAC1} in sensory neurons, doubled stranded (ds) RNA of 5-HT_{apAC1} (ds5-HT_{apAC1}) was generated (Lee et al., 2001). Cell *in-situ* hybridization analysis revealed that microinjected ds5-HT_{apAC1} could knock down endogenous 5-HT_{apAC1} (Figure 14). As a negative control, dsLuci was co-injected with EGFP as an expression marker. Endogenous 5-HT_{apAC1} mRNA level was suppressed to almost 50% by ds5-HT_{apAC1} microinjection (relative % of 5-HT_{apAC1} mRNA intensity: dsLuci, 100.0 ± 7.2, n=10; ds5-HT_{apAC1}, 59.2 ± 8.7, n=18; $P < 0.01$, Student's *t*-test) (Figure 14).

To monitor the role of 5-HT_{apAC1} on the cAMP production in response to 5-HT in *Aplysia* sensory neurons, cAMP level was indirectly measured by Epac-based FRET sensor, CFP-Epac(δDEP-CD)-YFP (Figure 12). Epac is a well-known cAMP activated guanine nucleotide exchange factor (GEF) (DiPilato et al., 2004). When the cAMP binds to Epac, molecular conformation of Epac is changed. Conformational change of

Epac changes the CFP/YFP ratio, which is indicator of cAMP level (Ponsioen et al., 2004). Level of cAMP was increased in dsLuci-injected sensory neurons, however, but not ds5-HT_{apAC1}-injected sensory neurons by 10 μM 5-HT treatment (Figure 15). These results suggest that 5-HT_{apAC1} has an important role to produce cAMP by 5-HT in *Aplysia* sensory neurons.

7. 5-HT_{apAC1} Modulates Membrane Excitability and Spike Duration.

It is well known that activation of adenylyl cyclase modulate K^+ currents in order to increase membrane excitability and spike duration that are important to induce STF in *Aplysia* sensory neurons (Byrne and Kandel, 1996; Chang et al., 2000; Baxter and Byrne, 1990; Goldsmith and Abrams, 1992). To investigate whether 5-HT_{apAC1} is critically involved in membrane excitability increase and spike broadening, ds5-HT_{apAC1} was microinjected into cultured sensory neurons. Knock-down of endogenous

5-HT_{apAC1} significantly blocked the increment of membrane excitability after 5-min treatment of 10 μM 5-HT (number of spikes: dsLuci, 13.5 ± 1.3, n=4 vs. ds5-HT_{apAC1}, 5.7 ± 1.4, n=14; P < 0.05, Student's *t*-test) (Figure 16A). Broadening of spike duration was also blocked by ds5-HT_{apAC1} microinjection (% increase of spike duration in normal ASW: dsLuci, 39.7 ± 15.7, n=5 vs. ds5-HT_{apAC1}, 11.1 ± 4.3, n=14; *P* < 0.05, Student's *t*- test) (Figure 16B). To monitor the effect of 5-HT_{apAC1} activation on the S-type K^+ channel mediated spike broadening, the spikes were recorded in the media containing 100 mM TEA and 20 μM nifedipine. In the presence of these two drugs, every K^+ current other than the 5-HT-sensitive S-type K^+ currents are blocked (Goldsmith and Abrams, 1992; Baxter and Byrne, 1989). Knocking-down of 5-HT_{apAC1} under these conditions completely blocked the spike broadening (in TEA/nifedipine-ASW: % increase of spike duration: dsLuci, 100.5 ± 27.0, n=6 vs. ds5-HT_{apAC1}, 0.2 ± 9.5, n=5; P < 0.05, Student's *t*-test) (Figure 16C). These

results demonstrate that 5-HT_{apAC1} is necessary to activate the cAMP-PKA pathway and to increase spike duration and excitability of membrane.

8. Necessity of 5-HT_{apAC1} in Synaptic Facilitation.

It is well known that cAMP-PKA pathway is critical to induce synaptic facilitation (Kandel, 2001). To investigate the role of 5-HT_{apAC1} in synaptic facilitation in non-depressed synapses, ds5-HT_{apAC1} was microinjected into presynaptic sensory neurons of a sensory-to-motor synapses. Microinjection of ds5-HT_{apAC1} did not showed any effect on the basal synaptic transmission (% change of EPSP: dsLuci, 105.6 ± 53.0, n=3 vs. ds5-HT_{apAC1}, 109.8 ± 31.1, n=3; P = 0.9477, Student's *t*-test). Two kinds of experimental conditions were used to monitor the effects of 5-HT_{apAC1} knock-down on the short-term synaptic facilitation. First, 5-HT (10 μM) was treated for 1 min and then washed out (Ghirardi et al., 1992; Hawkins et al., 2006). In this experimental condition, knock-down of

5-HT_{apAC1} completely blocked short-term synaptic facilitation (%change of EPSP: dsLuci, 85.0 ± 23.9, n=7; vs. ds5-HT_{apAC1}, 7.7 ± 16.8, n=9; $P < 0.05$, Student-s *t*-test) (Figure. 17A). Knock-down of 5-HT_{apAC1} was also significantly but not completely blocked short-term synaptic facilitation in second experimental condition which cells were exposed to 5-HT for 5 min (% change of EPSP: dsLuci, 97.1 ± 17.5, n=10 vs. ds5-HT_{apAC1}, 31.8±19.7, n=12; $P < 0.05$, Student's *t*-test) (Figure 17B).

Moreover, synaptic facilitation is state-dependent. In non-depressed synapse, PKA acts as a major modulator to induce synaptic facilitation, whereas activity of PKC becomes dominant in depressed synapse (Byrne and Kandel, 1996; Chang et al., 2000; Ghirardi et al., 1992; Braha et al., 1990). To depress the sensory-to-motor synapses, sensory neurons were repeatedly stimulated. It was improved that the 1×5-HT-mediated reversal of synaptic depression was significantly lower in ds5-HT_{apAC1}-injected cells than that of dsLuci-injected cells (% change of

EPSP: dsLuci, 270.3 ± 64.2, n=7 vs. ds5-HT_{apAC1}, 76.2 ± 32.7, n=7; P < 0.05, Student's *t*-test) in moderately depressed synapses (Figure 18). These results indicate that the cloned receptor, 5-HT_{apAC1}, has a critical role in activation of cAMP-PKA pathway which is important for induction of short-term facilitation at non-depressed synapses as well as the reversal of homosynaptic depression at moderately depressed synapses.

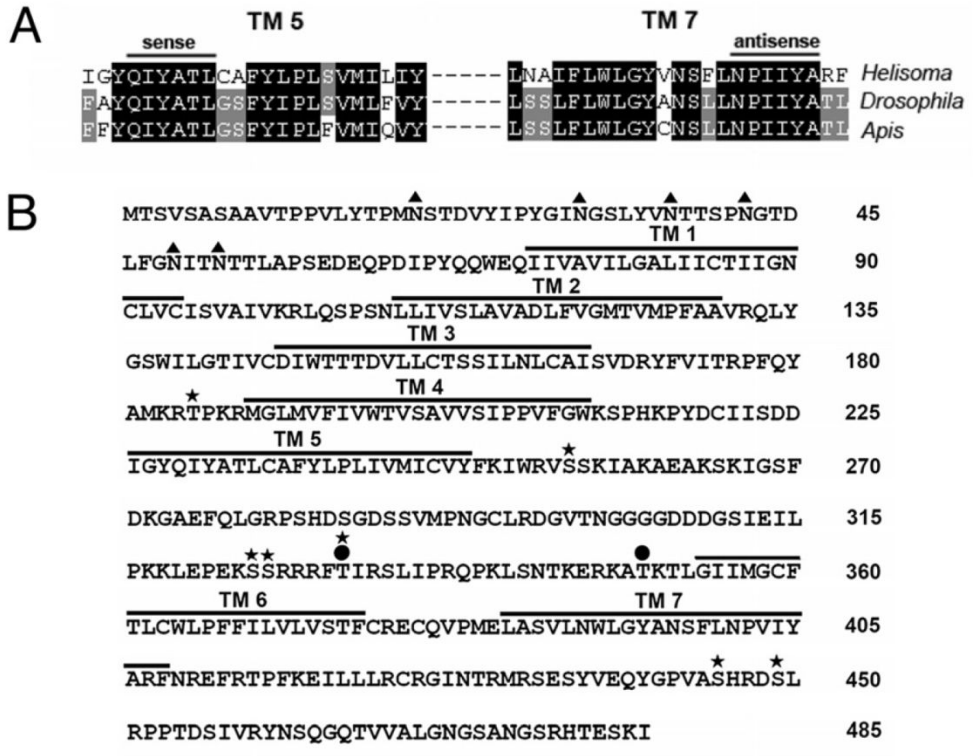

A
TM 5
TM 7
sense
antisense
IGYQIYATLCAFYLPLSVMILIY-----LNAIFLWLGYVNSFLNPIIYARF Helisoma
FAYQIYATLGSFYIPLSVMLFVY-----LSSLFLWLGYANSLLNPIIYATL Drosophila
FFYQIYATLGSFYIPLFVMIQVY-----LSSLFLWLGYCNSLLNPIIYATL Apis
B
MTSVSASAAVTPPVLYTPMNSTDVYIPYGINGSLYVNTTSPNGTD 45
TM 1
LFGNITNTTLAPSEDEQPDIPYQQWEQIIVAVILGALIICTIIGN 90
TM 2
CLVCISVAIVKRLQSPSNLLIVSLAVADLFVGMTVMPFAAVRQLY 135
TM 3
GSWILGTIVCDIWTTTDVLLCTSSILNLCAISVDRYFVITRPFQY 180
TM 4
AMKRTPKRMGLMVFIVWTVSAVVSIPPVFGWKSPHKPYDCIISDD 225
TM 5
IGYQIYATLCAFYLPLIVMICVYFKIWRVSSKIAKAEAKSKIGSF 270
DKGAEFQLGRPSHDSGDSSVMPNGCLRDGVTNGGGGDDDGSIEIL 315
PKKLEPEKSSRRRFTIRSLIPRQPKLSNTKERKATKTLGIIMGCF 360
TM 6
TM 7
TLCWLPFFILVLVSTFCRECQVPMELASVLNWLGYANSFLNPVIY 405
ARFNREFRTPFKEILLLRCRGINTRMRSESYVEQYGPVASHRDSL 450
RPPTDSIVRYNSQGQTVVALGNGSANGSRHTESKI 485

Figure 9. Molecular cloning of 5-HT_{apAC1}.

(A) Multiple sequence alignments of TM5 and TM7 of invertebrate 5-HT_7 receptors. Highly conserved QIYATL and NPXXY motif were used to make degenerative primers.

(B) Deduced amino acid sequence of 5-HT_{apAC1}. Seven transmembrane (TM) domains are indicated and numbered. Expected phosphorylation sites for PKA (●), PKC (★), and N-glycosylation sites (▲) are indicated.

(This work was done in collaboration with Dr. Yong-Seok Lee)

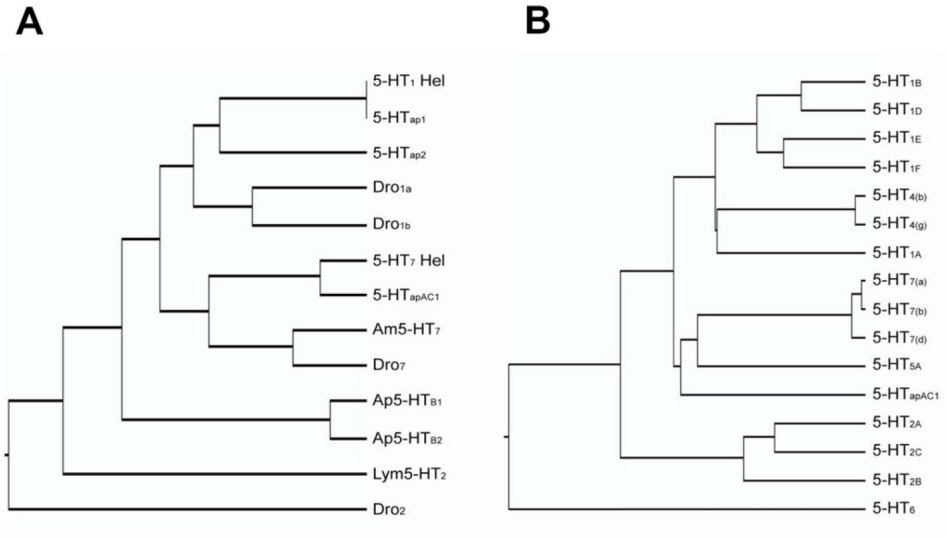


Figure 10. Phylogenetic analysis of 5-HT_{apAC1}.

(A) 5-HT_{apAC1} was analyzed with other invertebrate 5-HT receptors.

(B) 5-HT_{apAC1} was analyzed with other vertebrate 5-HT receptors.

(This work was done in collaboration with Dr. Yong-Seok Lee)

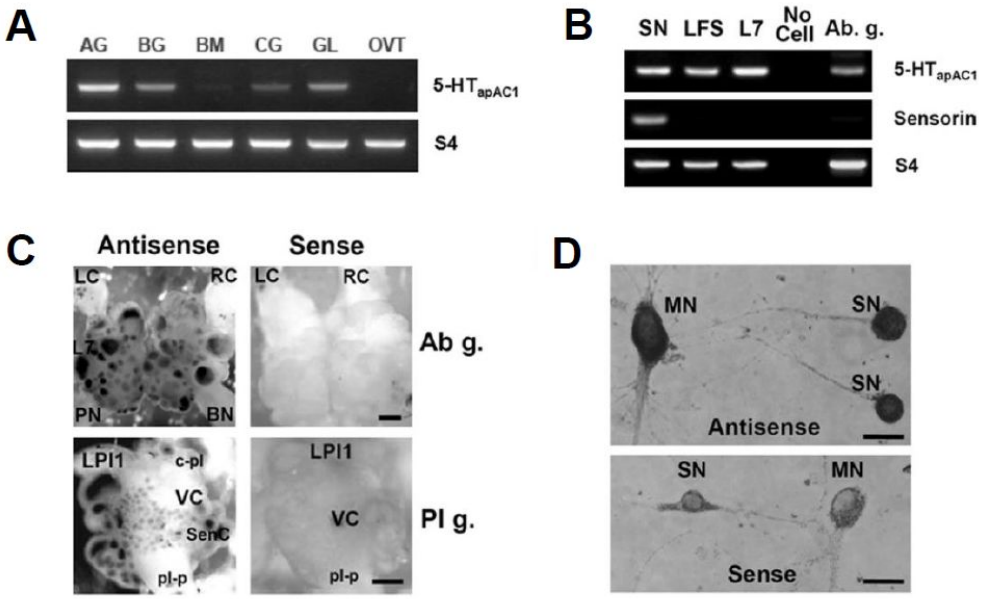

A
AG
BG
BM
CG
GL
OVT
5-HTapAC1
S4
B
SN
LFS
L7
No Cell
Ab. g.
5-HTapAC1
Sensorin
S4
C
Antisense
Sense
LC
RC
LZ
PN
BN
Ab g.
LPl1
c-pl
VC
SenC
pl-p
Pl g.
D
MN
SN
Antisense
Sense

Figure 11. Tissue distribution of 5-HT_{apAC1}.

(A) Multiple tissue RT-PCR analysis. AG, abdominalganglia; BG, buccal ganglia; BM, buccal mass; CG, central ganglia including cerebral, pleural and pedal ganglia; GL, gill; OVT, ovotestis.

(B) Single cell RT-PCR analysis of 5-HT_{apAC1}. Sensorin which is exclusively expressed in sensory neuron was used as control. No cell lane was used to confirm the contamination or non-specific amplification of PCR reaction. SN, sensory neuron; LSF, LSF motor neuron; L7, L7 motor neuron; Ab. g., total RNA from the abdominal ganglion.

(C) Whole mount *In-situ* hybridization of 5-HT_{apAC1} mRNA in sensory clusters. Dorsal abdominal ganglia and left pleural ganglia were shown. LC, left pleuro-abdominal connective; RC, right pleuro-abdominal connective; PN, pericardial nerve; BN, branchial nerve; L7, L7 motor neuron; LPI1, left pleural giant neuron; SenC, sensory cluster; c-pl,

cerebro-pleural connective; pl-p, pleuropedal connective. Upper scale bars indicates 500 μm, lower scale bars indicates 200 μm.

(D) Cell *in-situ* hybridization of 5-HT_{apAC1} mRNA using sensory-to-motor co-culture. Sense probe was used to confirm the non-specific hybridization. Scale bars indicate that 50 μm.

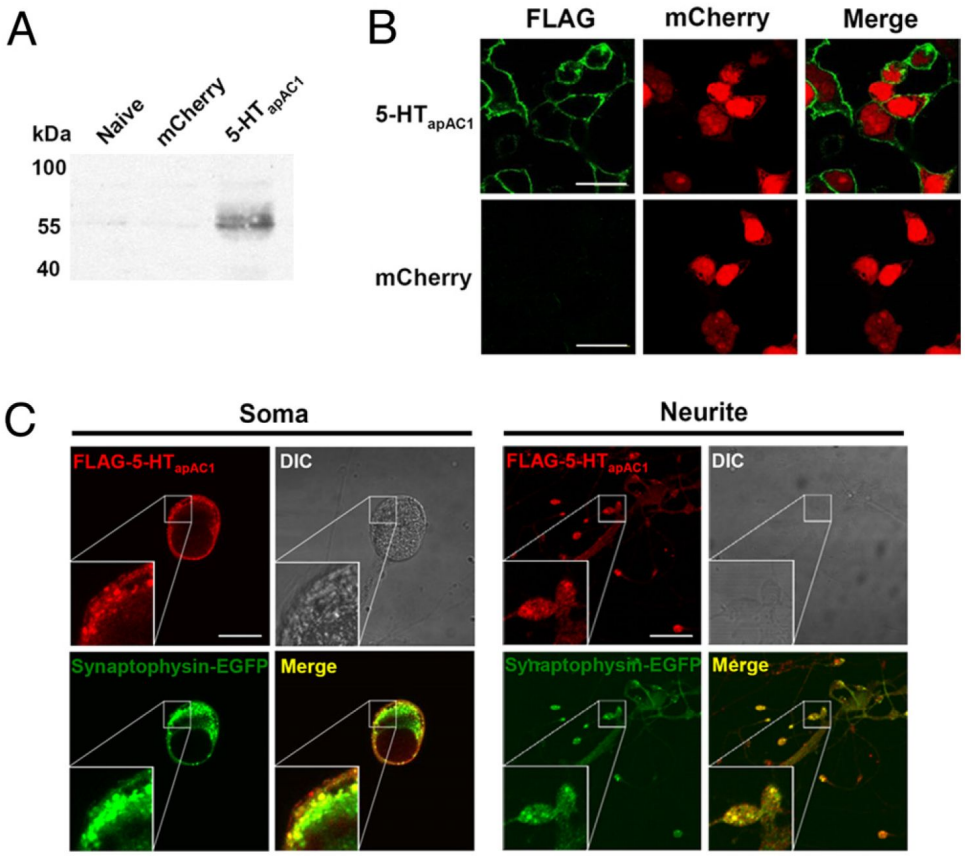

A
kDa
Naive
mCherry
5-HT apAC1
100
55
40
B
FLAG
mCherry
Merge
5-HT apAC1
mCherry
C
Soma
Neurite
FLAG-5-HT apAC1
DIC
Synaptophysin-EGFP
Merge

Figure 12. Subcellular localization of 5-HT_{apAC1}.

(A) Expression of 5-HT_{apAC1} in HEK293T cells was demonstrated through the Western blot.

(B) Expression pattern of 5-HT_{apAC1} in HEK293T cells was confirmed by Immunocytochemistry. FLAG-5-HT_{apAC1} indicated as green color. Co-transfected overexpression marker, mCherry-N1 (red), was diffusely distributed in the cytosol.

(C) Co-localization of overexpressed FLAG-5-HT_{apAC1} (red) and synaptophysin-EGFP (green) in sensory-to-motor co-culture. Scale bars indicates 30 μm. Parts of the plasma membrane and synaptic bouton were magnified in the small box, respectively.

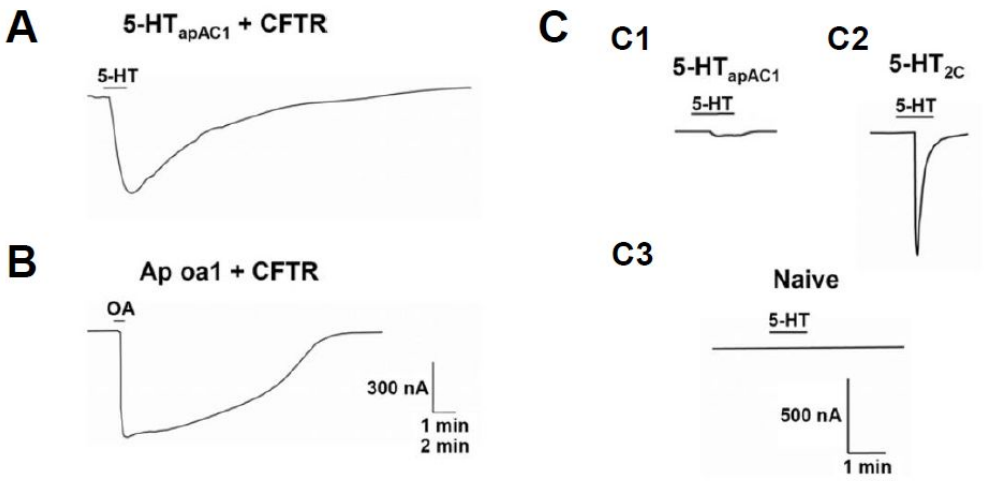

A
5-HT$_{apAC1}$ + CFTR
5-HT
B
Ap oa1 + CFTR
OA
300 nA
1 min
2 min
C
C1
5-HT$_{apAC1}$
5-HT
C2
5-HT$_{2C}$
5-HT
C3
Naive
5-HT
500 nA
1 min

Figure 13. G-protein coupling specificity of 5-HT_{apAC1}.

(A, B) CFTR cRNA (2.5 ng) was co-injected with 5-HT_{apAC1} (2.5 ng) or Ap oa1 cRNA (2.5 ng), respectively. Vertical scale bar indicates that 300 nA. Horizontal scale bar indicates 1 min for A and 2 min for B respectively.

(C) Following cRNAs were microinjected into the oocytes: 2.5 ng of 5-HT_{apAC1} (D1), 100 pg of 5-HT2c cRNA (D2), and distilled water (D3). Scale bars, 500 nA and 1 min.

(This work was done in collaboration with Dr. Yong-Seok Lee)

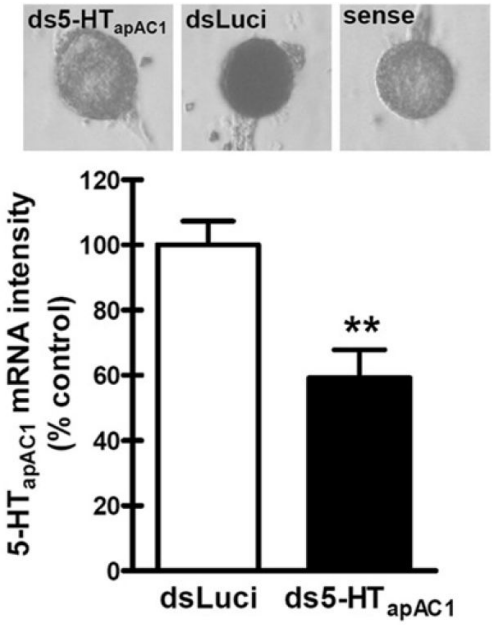


Figure 14. Knock-down of endogenous 5-HT_{apAC1} by dsRNA microinjection.

Upper panels are representative sample images showing endogenous 5-HT_{apAC1} mRNA intensity. The histogram shows the averaged mRNA intensity ± SEM. Data were normalized by the mean value of dsLuci. **, $P < 0.01$.

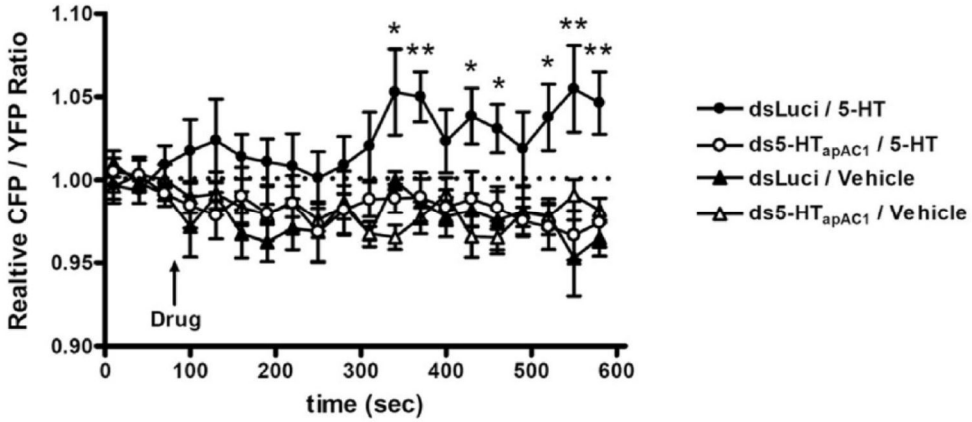

Realtive CFP / YFP Ratio
1.10
1.05
1.00
0.95
0.90
0
100
200
300
400
500
600
time (sec)
Drug
*
**
*
*
*
**
**
dsLuci / 5-HT
ds5-HTapAC1 / 5-HT
dsLuci / Vehicle
ds5-HTapAC1 / Vehicle

Figure 15. Epac-based FRET signal in *Aplysia* sensory neurons.

Intracellular cAMP level was measured by relative CFP/YFP ratio. 10 μM 5-HT or same volume of vehicle was treated to sensory neurons. One-way ANOVA followed by a Newman-Keuls post-hoc test was performed for each time point. Asterisks indicate significant difference between dsLuci/5-HT and ds5-HT_{apAC1}/5-HT. *, $P < 0.05$; **, $P < 0.01$.

(This work was done in collaboration with Dr. Seung-Hee Lee and Nuribalhae Lee)

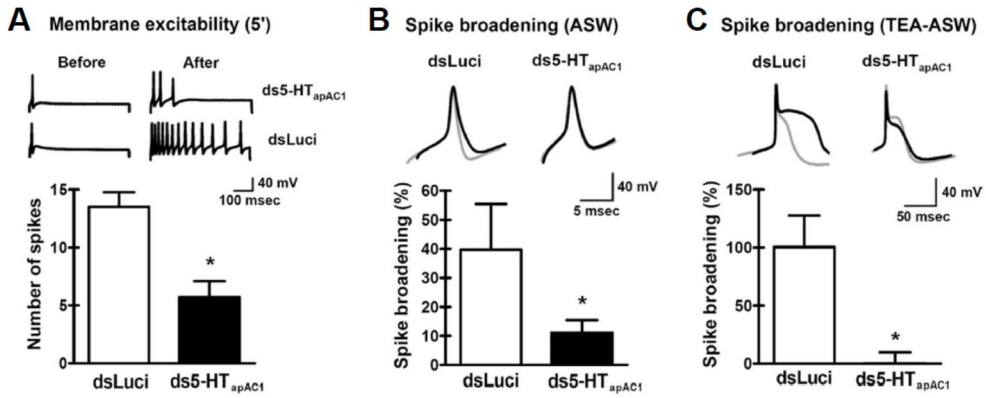

A
Membrane excitability (5')
Before
After
ds5-HT apAC1
dsLuci
40 mV
100 msec
Number of spikes
15
10
5
0
*
dsLuci
ds5-HT apAC1
B
Spike broadening (ASW)
dsLuci
ds5-HT apAC1
40 mV
5 msec
Spike broadening (%)
60
50
40
30
20
10
0
*
dsLuci
ds5-HT apAC1
C
Spike broadening (TEA-ASW)
dsLuci
ds5-HT apAC1
40 mV
50 msec
Spike broadening (%)
150
100
50
0
*
dsLuci
ds5-HT apAC1

Figure 16. Impairment of 5-HT mediated amplification of membrane excitability and broadening of spikes by 5-HT_{apAC1} knock-down.

(A) Upper panels are examples of action potentials (APs) recorded in sensory neurons before and after 5-HT treatment (10 μM, 5min). The height of bar represents mean number of spikes ± SEM. Scale bar, 100 msec and 40 mV. *, $P < 0.05$.

(B, C) Upper panels are examples of single AP recorded in sensory neurons before and after 5-HT treatment (10 μM, 5min). To isolate S-type K^+ current, TEA (100 mM) and nifedipine (20 μM) was used in panel C. The height of bars corresponds to the mean % of spike broadening ± SEM. Scale bar, 5 msec and 40 mV for panel B, 50 msec, 40mV for panel C, respectively. *, $P < 0.05$.

(This work was done in collaboration with Dr. Seung-Hee Lee)

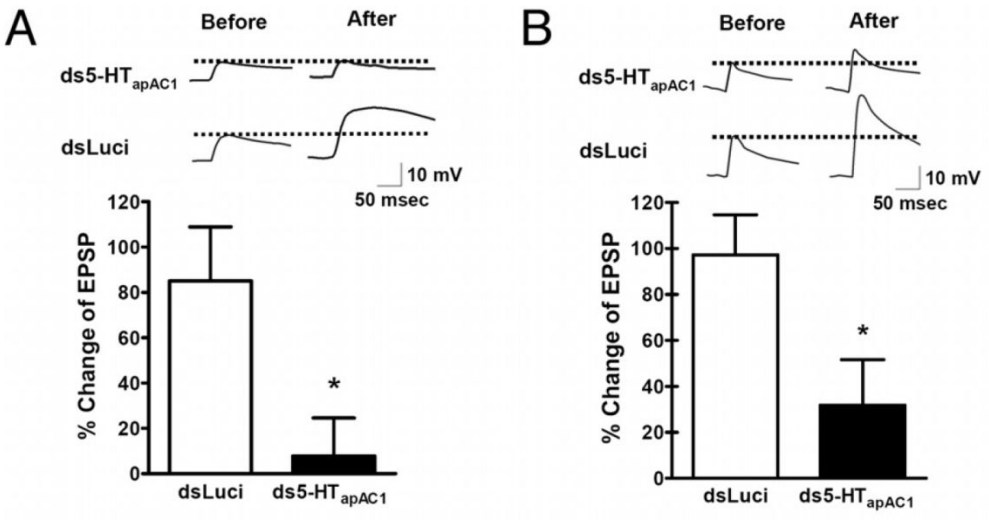

A
Before
After
ds5-HT apAC1
dsLuci
10 mV
50 msec
% Change of EPSP
120
100
80
60
40
20
0
*
dsLuci
ds5-HT apAC1
B
Before
After
ds5-HT apAC1
dsLuci
10 mV
50 msec
% Change of EPSP
120
100
80
60
40
20
0
*
dsLuci
ds5-HT apAC1

Figure 17. Impairment of 5-HT induced short-term facilitation by 5-HT_{apAC1} knock-down.

(A) Short-term facilitation induced by 5-HT (10 μM, 1 min) treatment followed by 4 min of washout.

(B) Short-term facilitation induced by longer exposure of 5-HT (10 μM, 5 min). Upper panels are representative trances of EPSPs recorded in LFS motor neurons after electrical sensory neuron stimulation. The height of bars corresponds to the mean % change of EPSP ± SEM. Scale bar, 50 msec, 10 mV. *, $P < 0.05$.

(This work was done in collaboration with Dr. Seung-Hee Lee)

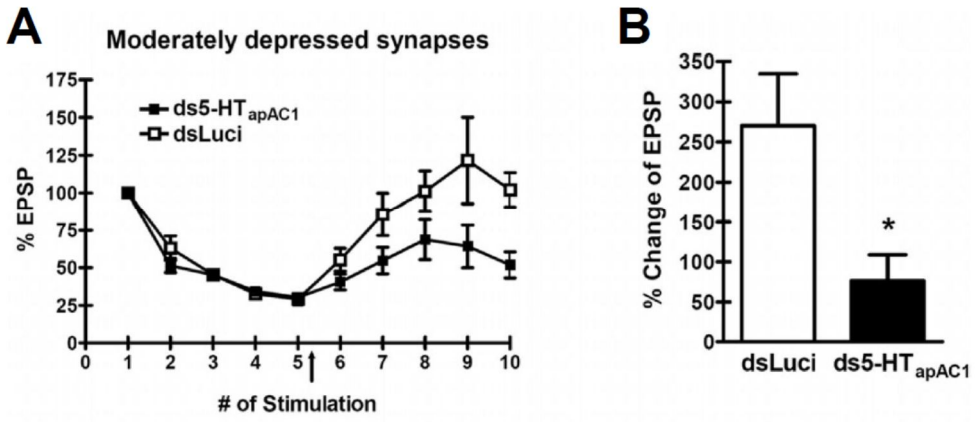

A
Moderately depressed synapses
ds5-HT apAC1
dsLuci
% EPSP
175
150
125
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10
# of Stimulation
B
% Change of EPSP
350
300
250
200
150
100
50
0
*
dsLuci
ds5-HT apAC1

Figure 18. Partial impairment of 5-HT induced reversal of synaptic depression in moderately depressed synapses by knock-down of 5-HT_{apAC1}.

(A) Changes of EPSPs measured by repeated sensory neurons stimulation (ISI = 20 sec). 5-HT (10 μM) was treated right after 5^{th} stimulation.

(B) The height of each bars indicates that the mean % change of EPSP ± SEM. % change of EPSP was measured as (mean EPSP at the 9^{th} and 10^{th} stimuli) - (mean EPSP at the 4^{th} and 5^{th} stimuli)/(mean EPSP at the 4^{th} and 5^{th} stimuli) × 100 (%). *, $P < 0.05$.

(This work was done in collaboration with Dr. Seung-Hee Lee)

Discussion

In this chapter, the first serotonin receptor which is positively coupled to adenylyl cylase was cloned and characterized in *Aplysia kurodai*. The cloned receptor named as 5-HT_{apAC1}, considering that the receptor is positively coupled to adenylyl cyclase. However, it is not exclude the possibilities that the existence of other receptor subtypes which is positively coupled to adenylyl cyclase in *Aplysia*.

To clone the serotonin receptor, *Aplysia* EST databases and degenerative PCR method were used. Extensive amino acid sequence analysis revealed that invertebrate 5-HT_7 receptors share a unique motif, QIYATL, in their fifth transmembrane domain (see Figure 9A). The function of the unique motif is not yet discovered. Moreover, among the highly homologous biogenic amine receptors, such as dopamine, octopamine, or other 5-HT receptors cloned in *Aplysia*, the QIYATL motif is not found. The deduced amino acid sequences of cloned receptor, 5-HT_{apAC1}, showed

specific components of typical G protein-coupled receptor, such as seven transmembrane domains, the tripeptide DRY (Asp-169-Arg-170-Tyr-171) which is important for G protein coupling (Moro et al., 1993), and the NPXXY motif (Asn-401-Pro-402-Tyr-405) has a role for the receptor desensitization and internalization (Barak et al., 1994) (Figure 9B). Putative PDZ-binding motif was also found in the C-terminus of 5-HT_{apAC1}, suggests that the receptor may bind to the scaffolding proteins to form a signaling complex (Figure 9B).

For the next step, RT-PCR and *in-situ* hybridization were performed to monitor the expression profile of the clone receptor, 5-HT_{apAC1}. As expected, 5-HT_{apAC1} was expressed in sensory neurons of sensory clusters; however, it is also expressed in the motor neurons such as L7 and LFS (Figure 11). Function of the 5-HT and 5-HT receptors in the motor neurons on the synaptic facilitation is not well understand, so it could be interesting to study the role of 5-HT_{apAC1} in the motor neurons.

To understand the coupling specificity of 5-HT_{apAC1}, *Xenopus* oocytes were used (Figure 13). Chloride currents were measured in the oocytes which were microinjected CFTR and 5-HT_{apAC1} cRNA (Figure 10A). Moreover, the typical fast chloride currents were observed in the oocytes which were microinjected 5-HT_{2c}, but not 5-HT_{apAC1} (Figure 13C). These results were indicated that 5-HT_{apAC1} is specifically coupled to G_s, but not to G_q.

Finally, endogenous function of 5-HT_{apAC1} was revealed by knock-down of the expression of 5-HT_{apAC1} using RNA interference technique. The region used to make ds5-HT_{apAC1} was the first obtained partial clone of 5-HT_{apAC1} by nested PCR, and this region is also used to screen the *Aplysia* cDNA library to acquire the full-length ORF. Moreover, in the entire *Aplysia* EST databases (Lee et al., 2008b; Moroz et al., 2006), this region has the only match (PEG003-C-228120-501) which is considered

as an *A. californica* homolog of 5-HT_{apAC1}. Although the possibility of off-target effects of dsRNA could not be excluded, the information suggests that ds5-HT_{apAC1} may specifically knock down 5-HT_{apAC1}.

Lack of the specific antibody against 5-HT_{apAC1}, efficiency of ds5-HT_{apAC1} was revealed by *in-situ* hybridization (Figure 14). Interestingly, knock-down of 5-HT_{apAC1} was not that effective (~ 40% reduction in mRNA levels), it was enough to block the 5-HT induced synaptic responses significantly (Figure 15-18). The knock-down efficiency of dsRNA which is measured by amount of the protein remains to be tested. If the protein expression is also reduced partially, it might suggest that there could be a certain expression threshold to activate downstream pathway.

By knocking-down the expression of 5-HT_{apAC1}, it is found that 5-HT_{apAC1} is critical to increment of membrane excitability, broadening of spike, and facilitation of non-depressed synapses (Figure 15-17). Especially, the data which knocking down of 5-HT_{apAC1} could reverse only

the half of the synaptic depression in moderately depressed synapses where PKA and PKC is correspondingly involved (Figure 18) was consistent with earlier studies (Byrne and Kandel, 1996; Ghirardi et al., 1992).

Cloning of 5-HT_{apAC1} is very important because it reminds to us about existence of several unsolved important questions: molecular mechanism of synaptic facilitation in *Aplysia*. Is there some more receptors or receptor families which are positively coupled to adenylyl cyclase? Is the receptor desensitized or internalized when it exposed long time by 5-HT or in the depressed synapses? If so, what is the mechanism for such down-regulation? In the depressed synapses, which receptors do the important role on the reversal of depression? Which proteins are bound to the receptor? What is the effect of the receptor activation in the motor neurons? These questions should be solved by further molecular, cellular, and physiological studies.

Chapter 4

Conclusion

Conclusion

Ⅰ. Making a pool of candidate genes to study for learning and memory by high-throughput analysis, and characterizing the genes which has an important role in learning and memory was the major goal of this study. For the first step, 5-HT regulated genes were mined through cDNA microarray and real-time PCR analysis. Concisely, four clones: matrilin, antistasin, Ap-eIF3e, and BAT homolog were newly found that significantly up-regulated or down-regulated by 5-HT treatment.

Moreover, to narrow down the pool of candidate genes to study learning and memory, the way to select the housekeeping genes using the calculated evolutionary ratio was verified. Calculating the K_a/K_s value using the selected set of nervous system-related genes and housekeeping genes revealed that that evolutionary speed of nervous system-related genes was faster than that of housekeeping genes. Using real-time PCR reaction, it was also confirmed that measuring the evolutionary rates by calculating

K_a/K_s ratio without any prior knowledge about the genes can be an efficient way to find candidate genes for functional studies. Through these approaches, four genes: RAB2, v-ATPase, proline 4-hydroxylase, and MIP-related peptide precursor were proved to be enriched in neurons. The fact that Ap-eIF3e, one of the selected clone obtained from the candidate gene pool described above, was proved to be important to induce long-term synaptic facilitation (Lee et al., 2008a), suggests that one can find a candidate genes to study the mechanism of learning and memory using these gene mining technique.

Ⅱ. For the next step, specific genes which have a critical role to induce synaptic facilitation were cloned and characterized based on the knowledge described above. In order to find candidate genes, microarray and EST database were extensively investigated.

Firstly, in order to find the negative regulation mechanism of ApC/EBP mRNA, ApAUF1 was characterized. Since ApC/EBP mRNA is highly unstable and it has AREs on its 3'UTR, an ARE-binding protein, ApAUF1, could be expected as a negative regulator of ApC/EBP mRNA. Using the RNA-protein pull-down assay, it was confirmed that ApAUF1 could bind specifically to the AREs of ApC/EBP mRNA. This result speculates that ApAUF1 can work as a negative post-transcriptional regulator of ApC/EBP mRNA.

It is well known that the importance of 5-HT induced signaling pathway to induce synaptic facilitation, however, Gs-coupled 5-HT receptor in *Aplysia* sensory neuron is not identified. In order to find the first serotonin receptor which is positively coupled to adenylyl cyclase, EST databases and degenerative PCR method were used. The cloned receptor, 5-HT_{apAC1}, has high homology to the invertebrate 5-HT_7 receptors. Moreover, it was found that 5-HT_{apAC1} expressed in sensory

and motor neurons, and localized to the plasma membrane and synaptic region of the neurites in the sensory neurons. Finally, knocking-down 5-HT_{apAC1} blocked induction of 5-HT mediated short-term facilitation. These results indicated that 5-HT_{apAC1} is a lost missing link of 5-HT mediated synaptic facilitation mechanism study.

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국문초록

바다달팽이 군소는 학습과 기억의 분자적 및 세포적 메커니즘을 연구하는 데 오랜 기간 사용되어 왔다. 수많은 연구들이 진행되어 왔음에도 불구하고, 대규모 유전체 정보의 부재로 인해 군소에서의 학습과 기억의 분자적 연구는 상당 부분 제한되어 왔다. 특히 세로토닌에 의한 cAMP-PKA 신호전달 경로의 활성화가 학습과 기억에 매우 중요하다는 사실은 잘 알려져 있다. 하지만 adenylyl cyclase 와 양성적으로 연결된 세로토닌 수용체의 존재는 아직 밝혀진 바가 없다. 이러한 제한을 극복하기 위하여, 본 연구에서는 두 근연종 *A. kurodai* 와 *A. californica* 에서 얻어진 발현체 데이터베이스를 이용하여 대단위 분석을 실시하였다. 더 나아가, 이러한 대단위 분석을 바탕으로 학습과 기억에 관련된 새로운 유전자들을 동정하고 분석할 수 있었다.

처음으로, 세로토닌에 의한 유전자 발현 변화를 바탕으로 하여 몇몇 기억관련 유전자 후보들을 발굴하였다. 세로토닌이 PKA 나 PKC 와 같은 신호전달경로를 활성화할 뿐 아니라, 수많은 기억관련 유전자의 조절에 관여한다는 사실은 잘 알려져 있다. 따라서 마이크로어레이와 실시간 중합효소연쇄반응(PCR)을 이용하여 세로토닌에 의해 조절되는 유전자들을 확인하였다. 이를 통해 학습과 기억의 분자적 메커니즘을 연구하기 위한 수많은 후보 유전자들을 발굴할 수 있었다.

둘째로, 기억관련 유전자 발굴에 더 효율적인 방법을 찾기 위해 *A. kurodai* 와 *A. californica* 발현체 데이터베이스를 바탕으로 진화적 분석이 이루어졌다. 수많은 Housekeeping 유전자들이 낮은 진화 속도를 보이므로, 극단적으로 낮은 진화 속도를 보이는 유전자들을 배제하는 것이 학습과 기억에 관련된 유전자를 찾는데 효율적이라는 사실이 유전자 진화 속도 분석을 통해 확인되었다. 하지만 특정 유전자가 신경세포에 발현될지 여부는 진화적 속도를 측정하는 방식을 통해 예상할 수 없었다.

셋째로, AU-rich element 결합 단백질인 ApAUF1 과 잘 알려진 전사인자인 ApC/EBP 간의 관계를 확인하였다. 과발현된 ApAUF1 은 세포질과 신경돌기에 위치하는 것으로 확인되었다. 마이크로어레이 분석과 EST 데이터베이스를 통해 ApC/EBP 를 조절할 것으로 예상되는 ApAUF1 을 동정하였다. ApC/EBP mRNA 는 3' UTR 에 여러 개의 ARE 를 가지고 있으며, ApAUF1 은 이들에 특이적으로 결합함이 확인되었다. 이러한 결과들은 ApAUF1 이 ApC/EBP mRNA 를 조절하는 역할을 할 수 있음을 시사한다.

마지막으로, EST 데이터베이스 및 유전자 서열 분석을 통해 adenylyl cyclase 와 양성적으로 연결된 세로토닌 수용체를 동정하였다. 동정된 수용체 5-HT_{apAC1} 은 다른 무척추동물의 7 형 세로토닌 수용체와 강한 상동성을 보였다. 그 수용체는 감각뉴런뿐 아니라 운동뉴런에도 발현되었으며, 감각뉴런의 세포막과 시냅스 부위에 위치한다는 것이 확인되었다. 5-HT_{apAC1} 는 G_q 가 아닌 G_s 단백질과만 특이적으로 연결되어 있으며, 세로토닌에 의한

cAMP-PKA 신호전달경로의 활성화에 중요한 역할을 하였다. 더 나아가, 동정된 수용체의 발현 억제는 비-억제된 시냅스에서의 시냅스 촉진 및 중간 정도 억제된 시냅스에서의 탈-습관화를 저해한다는 것이 확인되었다.

주요어: 군소, 시냅스 가소성, 세로토닌, 발현체, 유전자 발굴, 진화적 속도, ApC/EBP, AU-rich element (ARE), ApAUF1, 5-HT_{apAC1}

감사의 글

잡히지 않을 듯 멀게만 느껴지던 졸업을 앞둔 지금, 고마운 얼굴들이 참 많이도 떠오릅니다. 제가 지금 이 자리에 설 수 있었던 이유는 때로는 자상하게, 때로는 엄하게 많은 것을 가르쳐주셨던 선배님과 선생님, 그리고 부족함 많은 저를 불평 없이 도와주었던 후배님 덕분입니다. 또한 공동연구를 통해 부족함을 보완해주었던 KOBIC 의 연구원 여러분들, 필요할 때 상부상조해왔던 주위의 여러 연구실의 동료 과학자 분들께도 고개숙여 감사의 인사를 드립니다.

울고 웃고, 배우고 또 가르치며 보내왔던 관악에서의 10 년. 주마등처럼 스쳐가는 많은 사건들 중 거의 모두를 함께 했던 신경생물학 실험실은 아마도 영원히 잊지 못하겠지요. 기쁨과 슬픔, 애정과 분노, 만족과 실망, 수많은 기억과 추억들로 가득한 이 곳은 앞으로도 제 마음의 북극성으로 남아 있을 것입니다.

설레는 마음으로 첫 시작을 했던 2003 년의 봄을 떠올려 봅니다. 새로운 각오를 다지며 두 번째 시작을 했던 2007 년의 봄도 떠올려 봅니다. 두 번의 새로운 시작이 있었음에도, 그간의 세월을 충분히 잘 살아왔는가를 자문하면 부끄럽기 그지없습니다. 이제 박사 졸업을 하고, 세 번째의 새로운 출발을 하겠지요. 잘 했기 때문에 따낸 박사학위가 아니라, 앞으로 잘 하라고 주어진 박사학위로 생각하고 감사한 마음으로 다시 한 번 걸어가겠습니다.

길다면 길고 짧다면 짧은 세월을 보내며 아무 것도 모른 채 의욕만으로 가득 차 있던 한 과학도가 이제 한 사람의 독립적인

과학자가 되었습니다. 학위 과정의 끝은 끝이 아니라 끝없는 학문의 길을 걸어가는 시작일 뿐임을 항상 가슴에 새기고 겸허한 마음으로 한발한발 걸어가겠습니다. 정해진 이정표도 없고 앞도 잘 보이지 않는 칠흑같이 어두운 길이라 할지라도, 끝이 없는 길임을, 그리하여 길을 걷다 그 위에서 생을 마칠 것임을 알지라도, 그 길에서 만나게 될 것들에 대한 호기심과 희망을 등불삼아 기꺼이 걸어가겠습니다.

처음은 한 작은 인간의 발자국일 뿐이지만, 그 발자국을 따라 한 사람이, 두 사람이, 그리고 많은 사람이 걷다 보면 모두에게 기쁨을 줄 수 있는 큰 길이 되리라 믿습니다.

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