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Identification of three genes *C08F11.1*, *cec-1*, and *gtl-1* that contribute to learning in *Caenorhabditis elegans* using cDNA microarrays

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Abstract

Genes that are differentially expressed during repeated tap stimuli in *C. elegans* were screened with cDNA microarrays. Of the 9 up-regulated and 28 down-regulated genes identified, *in situ* hybridization results indicated that mRNAs of eight of the genes were detected in the head region. Inactivation of three genes (*C08F11.1*, *cec-1*, and *gtl-1*) by RNA-mediated interference (RNAi) treatments resulted in rapid recovery from the habituated state. These RNAi animals were also defective in behavioral plasticity produced when NaCl and starvation were used as paired stimuli. We detected their expressions of *cec-1* and *gtl-1* genes in sensory neurons and interneurons by using green fluorescent protein (GFP) constructs. These results provide direct evidence that the three identified genes contribute to learning in *C. elegans*. Based on distinct temporal and coordinated gene change accompanied by tap stimuli, relationship between gene expression and synaptic modulation is discussed.

Key words

learning, habituation, cDNA microarray, RNAi, *C. elegans*

Animals possess specific mechanisms to recognize and respond to mechanical stimulation in the environment. In *C. elegans*, the neurons forming anatomical synapses for mechanical stimulation such as touch or tap have been identified (1-3). The discovery of mutations in the mechanosensory system has contributed to the unraveling of the mechanism of mechanosensory transduction including ion channels and neurotransmission (4-6). The use of the molecular biology techniques over the last two decades has enabled molecular cloning of a number of genes encoding components necessary for mechanosensory transduction.

Rankin and coworkers originally noticed a habituation phenotype mediated by repetitive mechanical stimuli (3,7-9) and identified the neural circuit for tap-mediated stimuli (3,10).

In order to characterize the wide range of functional changes occurring during habituation, we have isolated mutants with abnormal habituation behavior (11).

Identification of the genes essential for habituation behavior may provide insight into the logic of neural integration for the habituation. So far, involvement of three genes (*eat-4*, *glr-1* and *dop-1*) in habituation behavior has been reported (12-14).

The current availability of cDNA microarrays containing sequences complementary to about one-half of the transcribed genes in *C. elegans* enables quantitative analysis of the transcript

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levels, particularly for genes whose transcription is influenced by tap stimulation (15-17).

To investigate the contribution of the identified genes to learning behavior, we adopted RNAi methods (18-20). Phenotypes of these RNAi animals reveal features suggesting novel pathways for habituation behavior.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Animals were grown at 20 ℃ unless otherwise noted. All strains were derivatives of Bristol strain N2 (21). The mutations used were *rrf-3* (*pk1426*), *gtl-1* (*ok375*), and *cec-1* (*ok1005*). For preparation of poly(A)$^+$ RNA, first-stage larva (L1) and adult hermaphrodites were synchronized by hypochlorite treatment.

Array analyses

Total RNA was prepared immediately after tap training from synchronously cultured wild-type N2 L1 and adult stage animals; poly(A)$^+$ RNA was purified by using Oligotex-dT30 (Roche Molecular Biochemicals) using the guanidine thiocyanate-acid-phenol extraction method.

Details on preparation of cDNA microarrays, hybridization, and determination of hybridization intensity are described elsewhere (15, 22). Five micrograms of poly(A)$^+$RNA prepared from wild-type N2 immediately after the tap stimulations were reverse transcribed using oligo(dT) primers and SuperScript II reverse transcriptase with the addition of Cy5-dCTP to generate Cy5-labeled probes. Poly(A)$^+$RNAs prepared from intact N2 were similarly used for the generation of Cy3-labeled probes. Equal amounts of the two probes were mixed and hybridized to a single array overnight at 42℃ .The hybridization experiments were done twice, and clones showing significantly identical hybridization results were picked. Clones showing identical differential expression accompanied by 180 times of 30-s ISI taps at the L1 and adult stage are shown in Table 1. Up(↑)- and down(↓)- regulated genes are expressed by arrows. Genes detected by *in situ* hybridization in head region are marked by circles(○) (Table I).

In situ hybridization

The detailed protocol for whole-mount *in situ* hybridization is given at the web site

http://nematode. lab. nig. ac. jp/method/insitu_embryo. html

RNAi experiments

Double-stranded RNA interference (RNAi) is an effective method for disrupting expression of specific genes in *C. elegans*. Both the microinjection (18, 23) and the bacterial feeding methods (25, 26) were adopted for the RNAi experiments. Details on the microinjection method are described elsewhere (23). The efficacy of RNAi treatment in neuronal cells has been confirmed (24). The RNAi experiments using the bacterial feeding method (25, 26) were performed as follows. Plasmids for dsRNA production in *E. coli*: HT115 (DE3) were derivatives of vector pPD129.36 (27). These L4 hermaphrodites of the *rrf-3* (*pk1426*) strain were placed onto NGM plates containing 100 μg/ml ampicillin and 0.4 mM IPTG seeded bacteria expressing dsRNA and were incubated for approximately 24 hr (20).

Then, three progeny were independently plated onto plates with the same growth conditions and kept for 24 hr. The L1 progeny from these animals were transferred to fresh feeding plates and were used for analysis at the young adult stage.

Behavioral assays

Responses to tap stimulations were tested by repeatedly applying a mechanical stimulus at designated interstimulus intervals (ISIs) (28, 29). The magnitude of each response was quantified by tracing the path of backward movement. The distance was expressed as reversal magnitude, a value relative to the body length of the animal. Details on the methods used for tap stimulations are given elsewhere (11, 29, 30). To test for recovery from habituation, animals were given 40 tap stimuli at 30-s ISI. After one hour, the magnitude of the response to tap was measured. The extent of recovery was calculated from average magnitude of 5 initial responses.

Chemotaxis towards NaCl was assayed as given (31) with some modifications. The animals were washed, placed on a conditioning plate (10 mM MOPS-

Table I A selection of genes that respond to the tap protocol

Clone	Gene	Expression	*in situ* Head positive	Description
yk38b11	*msp-81*	↑		major sperm protein
yk308e3	*msp-49*	↑		major sperm protein
yk411e7	*msp-19*	↑		major sperm protein
yk366f8	*msp-3*	↑		major sperm protein
yk150c2	*B0410.3*	↑		unknown
yk544c6	*C08F11.1*	↑	O	plasmodium falciparum hypothetical protein
yk116d10	*cec-1*	↑	O	chromo domain containing protein
yk576c10	*Y64H9A*	↑		cornichon like protein
yk355c12	*C04E12.3*	↑		unknown
yk44g5	*C24A8.4*	↓	O	protein kinase
yk334g2	*amx-2*	↓		flavin containing amine oxidoreductase
yk435f9	*F01E11.1*	↓		UDP-glucoronosyl and UDP-glucosyl transferase
yk366f6	*nas-15*	↓		zinc metalloprotease
yk334g2	*T21H8.1*	↓		amine oxidase
yk41g10	*vab-1*	↓		tyrosine-protein kinase receptor
yk363b5	*F35C11.5*	↓		phospholipase A2
yk396f8	*scd-2*	↓		serine/threonine protein kinase
yk383f11	*ama-1*	↓		RNA polymerase II
yk543d1	*T05B4.3*	↓		secretory protein
yk76c2	*qui-1*	↓		WD-40 domain
yk286g7	*F53B6.2*	↓		thrombospondin type 1 domain
yk369f3	*gcp-1*	↓		gut-specific cysteine protease
yk373f8	*B0507.1*	↓	O	EGF-like domain
yk208g12	*C28H8.3*	↓		putative helicase
yk245b12	*T22F7.1*	↓		synaptic vesicle transporter like protein
yk308e9	*gtl-1*	↓	O	TRP channel family
yk459e12	*M01E5.3*	↓		putative protein, with a coiled coil-4 domain
yk386d6	*eat-1*	↓	O	LIM domains
yk289g7	*T06d8.3*	↓		lipid phosphate phosphatase
yk272h9	*C49G7.4*	↓	O	secretory protein
yk350d10	*Y37A1C.1a*	↓		amino acid permease
yk46b12	*C34F6.1*	↓		serine-proteinase inhibitor
yk239c3	*F57F4.4*	↓		EGF like domain
yk529d4	*Y47D3B.6*	↓		EB module containing serine protease inhibitor
yk544e12	*unc-52*	↓	O	heparan sulfate proteoglycan core protein (HSPG2)
yk506f12	*pqn-74*	↓		Q/N-rich domain like protein
yk470d6	*pha-4*	↓		fork head/HNF-3-related transcription factor

Clones showing identical differential expression accompanied by 180 times of 30-s ISI taps at the L1 and adult stages are indicated. Information on the hybridization density of each cDNA clone is available at http://www.geocities.jp/rhosonolab/

NH_4 [pH 7.2], 50 mM NaCl, 3% agar) or a mock-conditioned plate (10 mM MOPS- NH_4 [pH 7.2], 3% agar) and incubated at 20 ℃ for 4 hr. The animals were again collected and chemotaxis was assayed by placing them at the center of a 6-cm plate with chemotaxis agar (10 mM MOPS-NH_4 [pH 7.2], 3% agar) on which a salt gradient had been formed for 19-23 hr by placing an agar plug containing 50 mM NaCl at one end of the plate. After 30 min, the number of animals was counted and the index was calculated as (A-B)/(A+B) where A was the number of animals on the NaCl side of the plate and B was the number of animals on the opposite side. To disregard the effect of differences in mobility, animals that remained at the center were not counted (32).

Real-time quantitative RT-PCR

To further confirm the reliability of the array data, the mRNA levels of the three genes were quantified by real-time quantitative RT-PCR. Actin mRNA was used as an internal control. Aliquots of cDNA (50, 75, and 100 ng) from intact and tap-stimulated animals

were amplified in 25 μl reactions containing 150 nM of each primer and 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems). PCR assays and the quantitation of the PCR products were performed with ABI PRISM 7500 (Applied Biosystems).

Expression constructs and generation of transgenic animals

To construct reporter plasmids, potential promoter regions of the three genes were amplified with cosmid clones; 2715 bp (+1075~ − 1649) for *gtl-1*; 5297 bp (+3382~ − 1915) for *cec-1*; and 918 bp (+751~ − 167) for *C08F11.1*. The amplified PCR fragments were cloned into pPD95.67 (provided by A. Fire) to create GFP fusions.

To generate transgenic lines expressing the these genes, germline transformation was performed as described previously (33). Cells expressing GFP reporter genes were identified (34).

RESULTS

Detection of genes by differential hybridization using cDNA microarrays

Recovery from habituation in *C. elegans* is dependent on interstimulus interval (35). When given 180 taps at 30-s ISIs, animals retained the habituated state at least four hours (data not shown). We therefore screened genes accompanied by habituation after 180 taps at 30-s ISIs by using cDNA microarrays that cover about one-half of the *C. elegans* genes (15-17). We isolated about 4 μg of two sets of independently prepared mRNA from L1 animals cultured with and without 180 taps at 30-s ISIs. We compared the hybridization density with the mRNA as a probe and found 248 genes that were up- and down-regulated during the tap stimulations. We then examined the expression of mRNA derived from animals at the young adult stage. Genes showing altered expression concordantly in both developmental stages are summarized in Table 1. Nine clones showed increased hybridization density and 28 clones showed decreased hybridization density. The spatial expression patterns of the genes identified using cDNA microarrays were determined by whole-mount *in situ* hybridization. The results revealed that the eight genes were primarily expressed in the head region (Fig. 1). To confirm the reliability of the array data on the *C08F11.1*, *cec-1* and *gtl-1* genes, we further examined the expression of the mRNA derived from animals at the young adult stage by real-time quantitative polymerase chain reaction with reverse transcription (RT-PCR) (Fig. 2). These patterns in three transcripts were consistent with the results obtained by the cDNA microarray analyses.

RNAi analysis of identified genes

The roles of the eight identified genes that are specifically expressed in head neurons were examined by RNA-mediated interference (RNAi). RNAi can be achieved either by injection of double-stranded RNA (dsRNA) into hermaphrodites (18, 19, 36) or by feeding the worms with *E. coli* that produce an appropriate dsRNA (26, 27). We tested the eight genes with both RNAi methods.

We compared the habituation and retention in the *rrf-3* (RNAi) animals with the phenotypes of intact animals. We confirmed that both phenotypes are indistinguishable between *rrf-3* and wild-type animals (data not shown). We were unable to find any abnormalities in either habituation or its retention with the *eat-1*(RNAi), *B0507.1*(RNAi), *C24A8.4*(RNAi), *C49G7.4*(RNAi), and *unc-52*(RNAi) animals (data not shown). However, we found that the *C08F11.1*(RNAi), *cec-1*(RNAi), and *gtl-1*(RNAi) animals showed rapid recovery from the habituated state (Fig. 3). We reached the same conclusion with both RNAi methods, although we present only the results of the bacterial feeding RNAi method. The results indicate that the three identified genes contribute to recovery from habituation. Of the three RNAi animals, *C08F11.1*(RNAi) recovered from habituation most rapidly. We then tested the effect of adding either two of the three types of dsRNAs. These animals exhibited normal habituation but recovered rapidly from habituation (Fig. 3). It was noticed that the extent of recovery did not correspond to *C08F11.1*(RNAi) but to *gtl-1*(RNAi) or *cec-1*(RNAi). Thus, it is probable that these genes contribute mutually rather than independently to the retention of habituation.

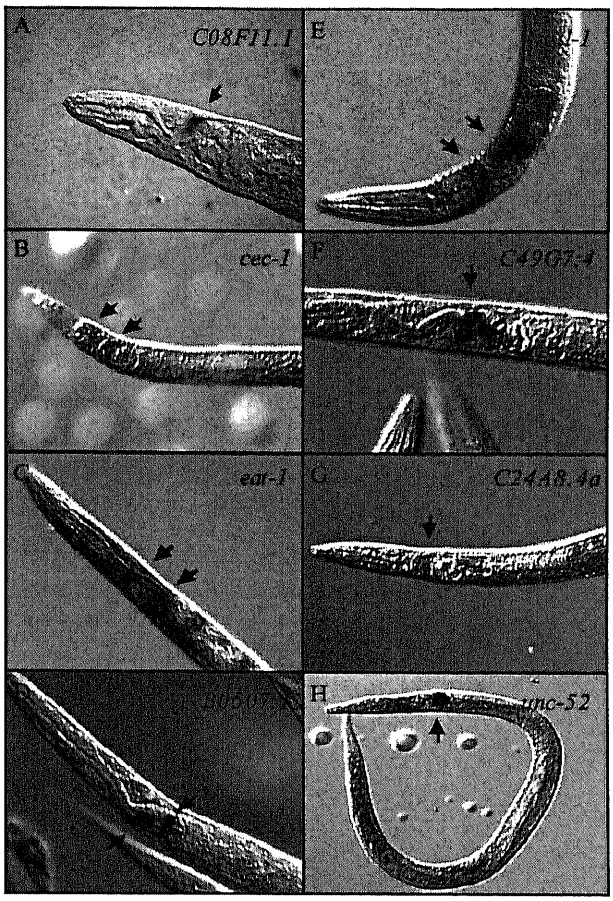

Fig. 1. Whole-mount *in situ* hybridization showing expression at the head region of genes detected by cDNA microarrays. Animals were hybridized with an antisense digoxygenin-single stranded RNA probe and detected with alkaline phosphatase (Materials and Methods). Regions with that positive signals by *in situ* hybridization are marked by arrows. (A) *C08Fll.I*, (B) *cec-1*, (C) *eat-1*, (D) *B0507.1*, (E) *gtl-1*, (F) *C49G7.4*, (G) *C24A8.4a*, (H) *unc-52*.

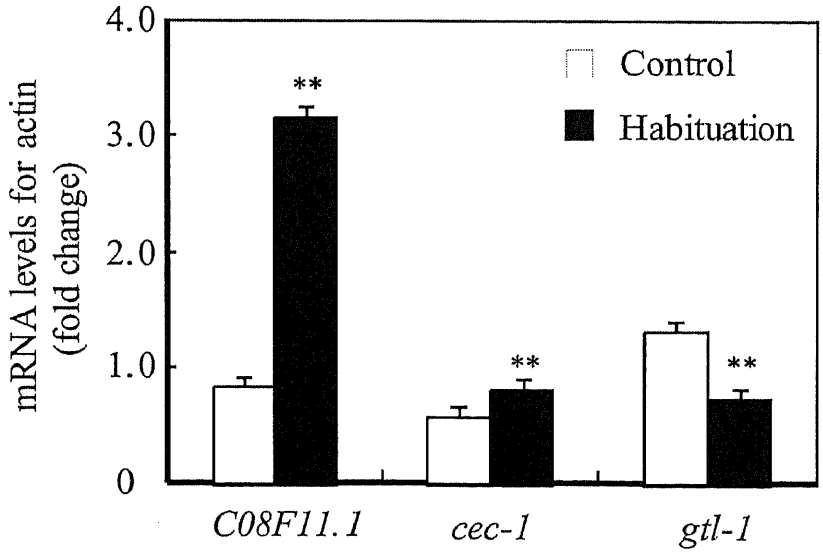

Fig. 2. mRNA levels of young adult hermaphrodites (n= 5) before (□) and after (■) 180 tap stimuli at 30-s ISIs. The ratio shown is of the three endogenous gene messages to the endogenous *act-1* message in wild-type animals. The means of the ratios from 20 independent PCR experiments performed at least twice are shown with SD. Independently performed real-time RT-PCR experiments showed similar relative differences. Asterisks indicate that the mRNA levels are significantly different between control and habituation: *C08F11.1* ($p<0.001$), *cec-1* ($p<0.01$), and *gtl-1* ($p<0.001$).

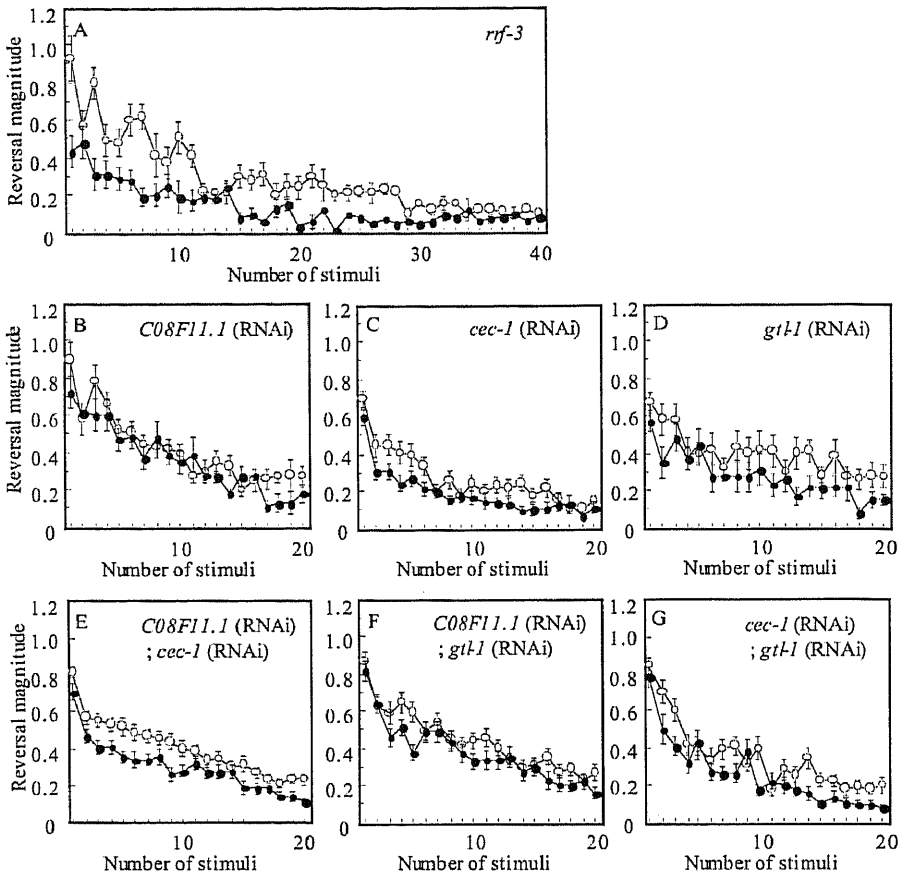

Fig. 3. Habituation (○) and recovery from habituation (●) after RNAi treatment. All animals received 40 tap stimuli at 30-s ISIs. After one hour, they were given recovery tests. (A) Control and (B to G) RNAi treatments. (A) *rrf-3* (n=23), (B) *C08F11.1* (RNAi) (n=33), (C) *cec-1* (RNAi) (n=32), (D) *gtl-1* (RNAi) (n=29), (E) *C08F11.1* (RNAi); *cec-1* (RNAi) (n=69), (F) *C08F11.1* (RNAi); *gtl-1* (RNAi) (n=60), and (G) *cec-1* (RNAi); *gtl-1* (RNAi) (n =39).

Chemotaxis and associative learning phenotypes

We then examined the effects of the identified genes on chemotaxis and associative learning in RNAi animals. The wild-type and *rrf-3* (*pk1426*) animals showed chemotaxis towards NaCl but avoided NaCl after conditioning with NaCl in the absence of food (31, 32). The *C08F11.1*(RNAi) animals showed normal chemotaxis towards NaCl and avoided NaCl after starvation (Fig. 4). However, the *cec-1*(RNAi) and *gtl-1*(RNAi) animals did not avoid NaCl after such conditioning, although they showed normal chemotaxis towards NaCl after mock conditioning. These results indicate that the *cec-1*(RNAi) and *gtl-1*(RNAi) animals have defects in the behavioral plasticity of chemotaxis towards NaCl.

Recently, we obtained *gtl-1* (*ok375*) and *cec-1* (*ok1005*) mutants. We compared these two types of behaviors of two mutants with those of RNAi animals. Both mutants showed the rapid recovery from habituation and the abnormal response for NaCl after starvation (data not shown), similar to those of RNAi animals.

Neurons expressing the *C08F11.1*, *cec-1* and *gtl-1* genes

We made their GFP reporter constructs to identify the neuronal cells exressed functionally because of the difficulty from the results of *in situ* hybridization. The *C08F11.1*::*gfp* reporter gene was expressed in the excretory system (37) consisting of an excretory cell and a gland cell (Fig. 5A-I). *C08F11.1* was also expressed in the amphid sheath cells, AMshL and AmshR, and in one of the excretory cells. By immunostaining with anti-*Cec-1* antibodies, Agosteni et al., (38) found that the protein was present in all somatic cells. From the expression of the *cec-1*::*gfp*

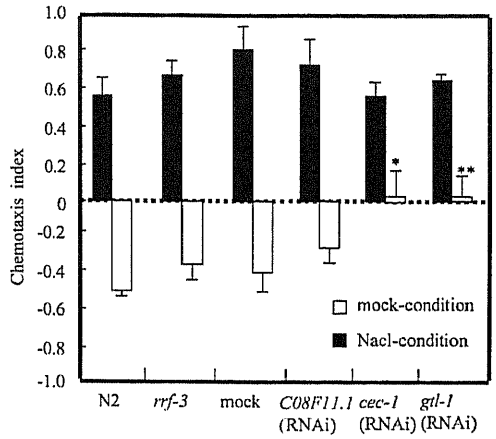

Fig. 4. Chemotaxis toward NaCl of animals conditioned without food and without NaCl (mock-conditioned) or without food and with NaCl (conditioned). Adult 50-100 hermaphrodites were washed and placed on a bacteria-free NGM plate with (□) or without (■) NaCl for 4 hrs and assayed for chemotaxis to NaCl. Values are means ± S.E. (n =5). Asterisks indicate that the chemotaxis index of the mutants *cec-1* ($p<0.05$) and *gtl-1* ($p<0.01$) is significantly different from that of the wild-type under the same conditions.

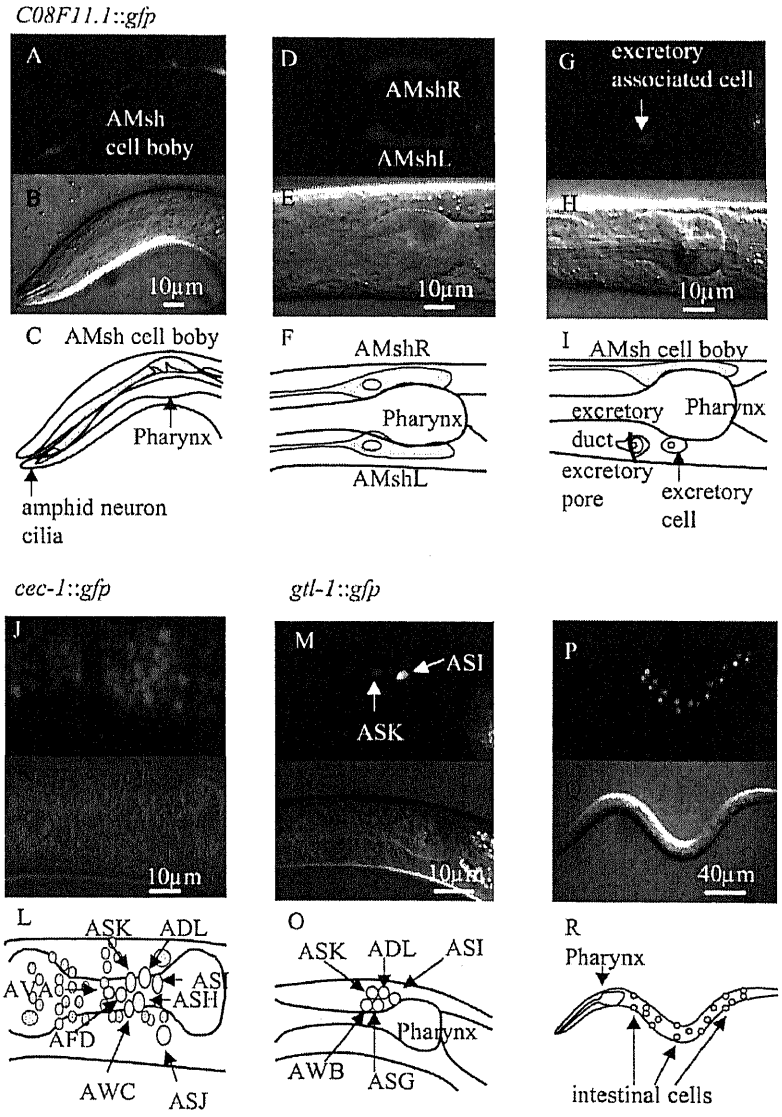

Fig. 5. Expression of the *C08F11.1::gfp* (panel A-I), the *cec-1::gfp* (J-L), and the *gtl-1::gfp* (M-R) fusion genes at the late larval stage. Fluorescence (A, D, G, J, M and P) and differential interference contrast (DIC) (B, E, H, K, N and Q).

reporter gene construct, we confirmed that the *cec-1* gene was expressed in almost all somatic cells. We further found that the expression of *cec-1*::*gfp* was especially strong in neurons forming the head ganglion and the lumbar ganglion in the tail (Fig. 5J-L). In addition to expression in intestinal cells, *gtl-1*::*gfp* fusion was expressed in the ASK and ASI sensory neurons (Fig. 5M-R).

DISCUSSION

cDNA microarray technology is advantageous for investigating expression profiles in large-scale analyses. Using microarray technology, we have generated a catalogue of genes responsible for tap-mediated habituation. Because the expression patterns of genes is greatly dependent on the developmental stage, it is desirable to search at different developmental stages to detect the genes essential for learning behaviors. We therefore isolated mRNA from two different stages, although the tap response is slightly different in the L1 and young adult stages (39, 40). Habituated animals that received 40 taps at 30-s ISIs recovered from habituation within three hours. To prolong the habituated state, the animals were administered 180 taps at 30-s ISIs. We identified 248 genes from the L1 animals and 215 genes from the young adult animals (http://www.geocities.jp/rhosonolab/). Only about 10% of the detected genes in both developmental stages were identical (Table 1).

Of these expressed genes, we noticed eight genes that were specifically expressed in the head region. We further focused our attention on the three identified genes (*C08F11.1*, *cec-1*, and *gtl-1*) that were associated with rapid recovery from habituation in the RNAi experiments. RNA levels from these genes are altered during tap stimulations, and the syntheses of the gene products are probably influenced after the tap stimulations. However, in the identified genes, *gtl-1* is down-regulated and the other two genes (*cec-1* and *C08F11.1*) are up-regulated (Table 1 and Fig. 2). Irrespective of the differences of expression, the phenotypes of habituation behavior in the RNAi-treated animals are similar to each other. Over-expression of the two up-regulated genes during tap stimulations will provide further information on the relationship between habituation behavior and accompanying gene expression.

The *C08F11.1*(RNAi), *cec-1*(RNAi), and *gtl-1*(RNAi) animals recovered from habituation more rapidly than do intact animals. In addition to exposure to *C08F11.1*(RNAi), animals exposed to *cec-1*(RNAi) or *gtl-1*(RNAi) showed phenotypes of *cec-1*(RNAi) or *gtl-1*(RNAi) animals, respectively. These results suggest that the three genes do not function on an independent pathway but probably function on a related pathway concerned with habituation and recovery from habituation. Also, we found that the *cec-1*(RNAi) and *gtl-1*(RNAi) animals were abnormal in the behavioral assay induced by a pair of stimuli: NaCl and starvation, indicating that two identified genes are involved in both non-associative and associative learning behaviors tested.

The function of the *C08F11.1* gene is unknown other than that it contains an amino terminal signal sequence and a potential transmembrane domain at the carboxy terminal. However, it should be noted that the protein has significant homology (43%) with a G-protein receptor. The gene was expressed in non-neuronal sheath cells and excretory cells. The sheath cells surround the dendrites of secretory neurons and secrete a matrix around the chemosensory cilia (41). The excretory duct cell is one of two vital components of an osmoregulatory system. It is thought to be a conduit for the excretion of excess fluid from large excretory cells in the exterior of an animal (37). From these properties, the *C08F11.1* gene may contribute indirectly to the retention of habituation. The *gtl-1* gene encodes a potential calcium channel protein. The protein has a significant homology with TRP7 (a novel putative Ca^{2+} channel protein), which is highly expressed in the brain (42). The *gtl-1* gene is also expressed in amphidial neurons ASK and ASI. Both ASK and ASI sensory neurons provide direct synaptic input to the AIA interneurons that constitute a potential neural circuit for the interaction of NaCl and starvation signals. As are ASE and ASG, ASI is important in chemotaxis including chemotaxis towards NaCl. Therefore, it is likely that both ASK and ASI affect associative learning. However, the two types of sensory neurons contribute to habituation behavior. It is likely that the weak expression of *gtl-1* in the tap circuit is essential for the habituation

behavior. The *cec-1* gene encodes a nuclear and chromatin-associated protein (38). The chromodomain is a phylogenetically conserved sequence motif that was identified as a region of homology between the *Drosophila* receptor protein Pc (43) and the heterochromatin constitutive protein HPI (44). Pc is a repressor protein that is necessary to maintain the inactive transcriptional state of developmental regulatory genes. Therefore, it seems likely that CEC-1 functions as a transcriptional repressor of a multimeric complex, which associates with specific target sites on the chromosomes. Interestingly, we found that the *cec-1* gene is expressed in almost all somatic cells but more strongly in head ganglia. Since the gene probably contributes to gene regulation, the decreased CEC-1 activity caused by RNAi might block learning behavior. The neural circuit in tap stimulation has been reported by Rankin et al (10). A *cec-1*::*gfp* fusion was expressed in interneurons (AVA and AVD) underlying tap response, but other two genes were not expressed, indicating that their promoter regions used were not sufficient or their fluorescent signals might be relatively low. For uncovering functional significance of these genes, we will need to make and analyze transgenic worms in which their gene expressions are regulated by neural specific promoters.

Retention of habituation in *C. elegans* is probably brought out by down-regulation of synaptic transmission between neurons constituting the mechanical tap circuit. Changes in chemotactic ability toward NaCl during conditioning with NaCl and starvation are probably integrated by a neural circuit, which has not yet been identified. We revealed that a potential Ca^{2+} channel encoded by *gtl-1* and a transcriptional regulator encoded by *cec-1* are involved in the above two types of learning behaviors. Further detailed investigations are essential for determining the precise roles of these genes on learning behavior.

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cDNA マイクロアレイを用いた線虫*Caenorhabditis elegans*学習遺伝子 — *C08F11.1, cec-1, gtl-1* — 同定

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要　旨

動物は一般に環境の機械刺激を認識し、反応する能力がある。線虫*C.elegans*ではこうした機械刺激を認識する神経回路網が同定されている。この神経回路網で働く遺伝子探索を目的として線虫*C. elegans*に繰り返しタップ刺激を与えることにより発現が変化する遺伝子をcDNAマイクロアレイを用いてスクリーニングした。その結果37遺伝子が同定され、そのうち9遺伝子はup-regulateし、28遺伝子はdown-regulateしていた。そのうち8遺伝子は*in situ*ハイブリダイゼーションで頭部神経系での発現が認められた。RNAi法でこれらの遺伝子をノックダウンすると*C08F11.1*, *cec-1*及び*gtl-1*の3遺伝子で慣れ学習異常が観察された。*C. elegans*はNaClに正の化学走性を示す。しかし、高濃度NaCl存在下で飢餓状態に置くと逆にNaClを忌避するようになる。同定された3遺伝子をRNAi法でノックダウンするとNaCl/飢餓に伴う連合学習でも忌避記憶保持能が劣る異常が観察された。こうしたことから同定された3遺伝子は学習関連遺伝子であると結論された。

*C08F11.1*遺伝子はGタンパク質受容体ホモログ、*gtl-1*はTRPM型チャネルタンパク質を、また*cec-1*はクロマチン親和性タンパク質をコードしていた。現在これら3遺伝子の学習への寄与について調べている。