CASY-1, an ortholog of calsyntenins/alcadeins, is essential for learning in Caenorhabditis elegans

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Calsyntenins/alcadeins are type I transmembrane proteins with two extracellular cadherin domains highly expressed in mammalian brain. They form a tripartite complex with X11/X11L and APP (amyloid precursor protein) and are proteolytically processed in a similar fashion to APP. Although a genetic association of calsyntenin-2 with human memory performance has recently been reported, physiological roles and molecular functions of the protein in the nervous system are poorly understood. Here, we show that CASY-1, the Caenorhabditis elegans ortholog of calsyntenins/alcadeins, is essential for multiple types of learning. Through a genetic screen, we found that casy-1 mutants show defects in salt chemotaxis learning. casy-1 mutants also show defects in temperature learning, olfactory adaptation, and integration of two sensory signals. casy-1 is widely expressed in the nervous system. Expression of casy-1 in a single sensory neuron and at the post-developmental stage is sufficient for its function in salt chemotaxis learning. The fluorescent protein-tagged ectodomain of CASY-1 is released from neurons. Moreover, functional domain analyses revealed that both cytoplasmic and transmembrane domains of this protein are dispensable, whereas the ectodomain, which contains the LG/LNS-like domain, is critically required for learning. These results suggest that learning is modulated by the released ectodomain of CASY-1.

Learning and memory is a fundamental process by which animals adapt to the changing environment. A wealth of knowledge has been obtained about the underlying mechanisms of learning and memory, but much remains to be elucidated. Previous studies have revealed regulators of learning and memory conserved between mammals and Caenorhabditis elegans (1–7). Therefore, the analysis of genes found in C. elegans can provide important insights into the mechanisms of learning and memory in mammals, including humans.

C. elegans shows chemotaxis to a variety of chemotactants and thermotaxis to cultivation temperature when cultured under well-fed conditions. In contrast, they show reduced response to or even avoidance of these sensory cues after exposure to continuous sensory stimulation under severe conditions such as starvation. Several such learning paradigms have been reported, for example, salt chemotaxis learning (4, 8), olfactory adaptation (9), and temperature learning (10).

In salt chemotaxis learning, worms learn to avoid NaCl after experiencing a salt stimulus in association with starvation. We have reported that the insulin/Pi3K (phosphatidyl inositol 3-kinase) signaling pathway, Go (GOA-1) and Gq (EGL-30) pathway, and HEN-1, a secretory protein with an LDL motif, regulate salt chemotaxis learning (4, 11, 12). The insulin-like signaling pathway and HEN-1 also regulate temperature learning (5, 12). HEN-1 has an additional function in the information processing, integration of attractive and aversive sensory signals (12). These studies and others provided important progress toward understanding the molecular mechanisms of learning and sensory processing, but further progress is needed for the full understanding of the processes.

In the current study, we identified casy-1, an ortholog of calsyntenins/alcadeins, through a screening for mutants with altered phenotypes in salt chemotaxis learning. Calsyntenins (also called alcadeins or Alcs) are cadherin-type I transmembrane proteins originally found in mammals. They are highly expressed in the central nervous system (13, 14). Biochemical studies revealed that calsyntenins/alcadeins can associate with the scaffold protein X11/X11L, which in turn associates with APP, resulting in the formation of a tripartite complex in the brain (15). Calsyntenins and APP are coordinately metabolized in neurons (16). The extracellular region of calsytenin-1 is released into the synaptic cleft, whereas the intracellular region, which can bind Ca2⁺, is internalized (13, 16). Furthermore, interaction between calsyntenin-1 and kinesin-1 blocked transport of APP-containing vesicles and increased β-amyloid generation (17). Despite these pieces of biochemical information, little is known about physiological functions of calsyntenins in vivo. We found that C. elegans casy-1 was expressed widely in the nervous system. The expression of the wild-type CASY-1 protein in a single sensory neuron can rescue the learning defects. casy-1 mutants show large defects in multiple types of learning. Furthermore, we performed functional domain mapping and found that the released ectodomain of CASY-1 can rescue the defects. We propose that CASY-1 acts as a precursor of the learning-modulating neurohormone, and this modulatory mechanism may be conserved in mammals.

Results

Identification of casy-1, a Calsytenin/Alcadein Homolog, as a Learning-Related Gene. We previously reported that C. elegans learns to avoid NaCl after being exposed to NaCl under starvation conditions (salt chemotaxis learning) (4, 8). To identify unknown molecules involved in salt chemotaxis learning, we performed an EMS mutagenesis screen (18). We screened for mutants that failed to avoid NaCl after conditioning in a buffer containing NaCl and obtained several candidate strains. Of these, we further characterized the JN401 mutant, which showed defective learning when conditioned in NaCl-containing buffer [Fig. 1A, casy-1(pe401)], see below]. We obtained similar results in the plate-conditioning method [supporting information (SI) Fig. S1]. Chemotaxis of the mutants to various
that the impairment of casy-1 carry a deletion in the N-terminal region of obtained and incorporated in subsequent analyses (Fig. 1).

They have two tandem cadherin domains and an LG/LNS domain membrane proteins highly conserved in metazoa (Fig. S2) (19). **

** carrying only the transformation marker in A mutants. Asterisks represent significant differences from control animals (Δ). We also found that casy-1 is important for another type of information processing: sensory integration. In a paradigm for testing sensory integration, worms must cross an aversive barrier of Cu(II) to reach an attractive odorant diacetyl (12). casy-1 mutants showed defects in this type of sensory processing (Fig. 2C), whereas these mutants normally responded to diacetyl or Cu(II) when presented separately (Fig. S3B and C). These results suggest that CASY-1 is not essential for primary sensory transduction, but is important for a variety of higher-order information processing.

**Temporal and Spatial Requirement of CASY-1 in Salt Chemotaxis Learning.** CASY-1 is a nonclassical cadherin-like protein. Various cadherin-like proteins are expressed and have functions in the nervous system. For example, classic neural cadherins, including N-cadherin, play a role in the developmental organization of the brain (20, 21) and synapse formation (22). A recent report showed that N-cadherin also regulates memory formation in the adult brain (23). To investigate whether CASY-1 could be required either for neural development or for postdevelopmental neural functions underlying salt chemotaxis learning, we performed rescue experiments by transient gene expression. Learning defects of the casy-1(tm718) mutants were strongly rescued when functional casy-1 was transiently expressed by a heat-inducible hsp16,2 promoter in the adult stage (Fig. S4A). This result indicates that casy-1 acts in the mature neural circuit.

Mammalian calsyntenins are widely expressed in the central nervous system (13–15). Given that CASY-1 is involved in behavioral plasticity, casy-1 is expected to be expressed in the nervous system. We determined the expression patterns of casy-1 using a green fluorescent protein reporter driven by the authentic casy-1 promoter. Fluorescent signals were observed throughout the nervous system and in other tissues such as intestine and gonadal sheath cells (Fig. S3B). We noticed strong GFP signals in many head neurons, including most amphid sensory neurons (Fig. 3C). The expression was observed from the embryonic stage. We also investigated the intracellular localization of CASY-1 by using fluorescent protein-tagged CASY-1 (see below). We found that CASY-1 is mainly localized to cell bodies of neurons, especially plasma membranes and intracellular membranes (Fig. S4). Localization of the protein in the neuronal processes was not evident but occasionally observed.

To identify neuron(s) in which CASY-1 acts in salt chemotaxis learning, we expressed casy-1 cDNA by several neuron-specific promoters in casy-1 mutants and examined whether each of them a frame shift. All these mutants exhibited strong learning defects similar to those in pe401 mutants (Fig. 1D).

**Multiple Types of Learning Defects in casy-1 Mutants.** Some mutants defective in salt chemotaxis learning also exhibit defects in other types of learning or sensory processing. To investigate the possible roles of casy-1 in these types of learning, the mutants were tested for olfactory adaptation and temperature learning. In olfactory adaptation, worms were soaked in a buffer containing the odorant benzaldehyde, and then they were tested for chemotaxis to benzaldehyde (9). After preexposure to benzaldehyde, wild-type animals displayed aversive responses to benzaldehyde, whereas casy-1 mutants continued to show a strong chemotaxis to benzaldehyde (Fig. 2A). In temperature learning, worms were cultured at 20°C without food for 0–4 h, and temperature preference was assayed on a thermal gradient plate (10). Whereas wild-type animals gradually lost the preference for 20°C, casy-1 mutants retained the temperature preference after conditioning without food for 4 h (Fig. 2B). These results suggest that casy-1 is required for multiple forms of learning. On the other hand, if casy-1 mutants were not pretreated under starvation, they did not show large defects in their response to benzaldehyde (Fig. S3A), or cultivation temperature (Fig. 2B, time = 0). We also found that casy-1 is important for another type of information processing: sensory integration. In a paradigm for testing sensory integration, worms must cross an aversive barrier of Cu(II) to reach an attractive odorant diacetyl (12). casy-1 mutants showed defects in this type of sensory processing (Fig. 2C), whereas these mutants normally responded to diacetyl or Cu(II) when presented separately (Fig. S3B and C). These results suggest that CASY-1 is not essential for primary sensory transduction, but is important for a variety of higher-order information processing.
can rescue the learning defects of the mutants. We found that the expression of casy-1 solely in the ASER neuron is sufficient to rescue the defects (Fig. 3D). In contrast, the defects were not rescued by expression of casy-1 in other neurons. These results indicate that CASY-1 acts in the ASER neuron in salt chemotaxis learning, the same site as where the PI3K pathway acts (4).

Genetic Relationship Between casy-1 and the Insulin-Like Signaling Pathway. Because both CASY-1 and the components of the PI3K pathway act in the ASER neuron for salt chemotaxis learning, we examined the genetic relationship between these genes. In daf-18 PTEN mutants, which have elevated level of the PI3K signaling, naïve animals show reduced chemotaxis to NaCl compared with the wild type (4). We therefore tested the phenotypes of casy-1; daf-18 double mutants and found that the casy-1 mutation suppresses the reduced chemotaxis of daf-18 (Fig. S5A). This result suggests that casy-1 acts either downstream of or in parallel to daf-18.

We next investigated the genetic interaction between casy-1 and another component of the insulin/PI3K pathway, ins-1. ins-1 acts upstream of the PI3K pathway in salt chemotaxis learning (4). We constructed a double mutant of casy-1 and ins-1 and then tested it for salt chemotaxis learning. The learning defect of casy-1; ins-1 double mutants was more severe than that of either single mutant (Fig. S5B). These results suggest that casy-1 acts in parallel to the insulin-like signaling pathway.

Release of CASY-1 Ectodomain from Neurons. Many transmembrane proteins have been shown to undergo ectodomain shedding, the release of the extracellular domain by proteolysis (24). It has been shown that calsyntenins/alcadeins are cleaved in the extracellular region (13, 16). By analogy, we reasoned that CASY-1 might also be proteolytically cleaved. Our observation of the localization of N- and C-terminal GFP fusion proteins, NtGFP;CASY-1 and CASY-1::CtGFP, respectively, is consistent with this possibility. GFP fluorescence was observed in coelomocytes only for NtGFP::CASY-1 but not for CASY-1::CtGFP (data not shown). Coelomocytes are macrophage-like scavenger cells present in the pseudocoelomic cavity that take up various compounds from the body cavity fluid (25). Because these two constructs share the same transcriptional regulatory sequences, distinct localization patterns suggest a proteolytic cleavage of the CASY-1 protein. To confirm this hypothesis, fluorescent markers, mRFP and Venus, were simultaneously fused to the N terminus and C terminus, respectively, of CASY-1 (Fig. 4G, RYV) and were expressed in head neurons. Only the signal of mRFP, tagged to the ectodomain, was detected in coelomocytes (Fig. 4A–C). mRFP signals in coelomocytes were observed even in normally cultured naïve animals. We made similar observations when the fusion protein was expressed in the ASER neuron alone, although the fluorescence was weaker in this case (data not shown). Therefore, we concluded that in head neurons including ASER, the ectodomain is cleaved from the full-length CASY-1 and released into the body cavity.

Functional Domain Mapping of CASY-1. Regarding the processing of CASY-1, we attempted to determine which part of the protein is important for salt chemotaxis learning (Fig. 4G). The cytoplasmic region of mammalian calsyntenin-1 binds Ca\(^{2+}\), and it was proposed that calsyntenins may be involved in proteolysis-dependent modulation of synaptic functions through its Ca\(^{2+}\)-binding capacity (13, 26). Based on this idea, the cytoplasmic region of CASY-1 may be essential for learning in C. elegans. On the other hand, our observation that the mutations of the extracellular domain lead to impairment of learning suggests the importance of the ectodomain (Fig. 1C and D, pe401 and hd33).

We found that the fragment lacking the cytoplasmic region can rescue the learning defects of the casy-1 mutants (Fig. 4G and Fig. S6A, RYV(ΔCt)), whereas the fragments that lack the ectodomain did not rescue the defect (Fig. 4G and Fig. S6A, RYV(ΔN) and CvT). These results indicate that the cytoplasmic acidic region is dispensable at least for salt chemotaxis learning. We further shortened the remaining region of the protein and tested whether the truncated proteins are functional. Unexpectedly, we found that the ectodomain alone, even without the transmembrane region, could rescue the learning defects of the casy-1 mutants (Fig. 4G and Fig. S6A, RYV800 and RYV700). In these constructs, both mRFP and Venus signals were observed in coelomocytes, suggesting that the whole protein is secreted (Fig. 4D–F, RYV700). We also found that the ectodomain lacking the signal peptide failed to rescue [Fig. 4G, RYV700(ΔSP)], suggesting that only the secreted fragments support learning.

When shorter forms of the ectodomain were tested, they did not rescue the defect (Fig. 4G, RYV600 and RYV530). These
results suggest that the central part of the extracellular region is important for learning. We expected that the LG/LNS domain is essential for learning because two mutant alleles that lead to the impairment of the extracellular region around the LNS domain cause defects in learning (Fig. 1 C and D, pe401 and hd33). LNS domains are often found in extracellular proteins and are implicated in interaction with a variety of cellular receptors or ligands (27, 28). As expected, deletion of the LNS domain abolished the functionality of CASY-1 [Fig. 4G, RYV(ΔLNS)]. Because LNS domain by itself is not enough to rescue the defects (Fig. 4G, RYV600, RYV530, and RYV331–700), we concluded that LNS domain and its flanking regions are necessary for modulating learning. Slight but nonsignificant rescue observed in the cadherin domain-deleted fragments implies that the cadherin domains may have accessory roles [Fig. 4G and Fig. S6B, RYV(ΔCads)].

Discussion

Involvement of casy-1 in Multiple Forms of Learning. We demonstrated that CASY-1 is essential for multiple forms of learning, including salt chemotaxis learning, olfactory adaptation, and temperature learning (Figs. 1 and 2). Several genes are known to be variously involved in these types of learning. Loss-of-
function mutations of goa-1 Goα and gain-of-function mutations of egl-30 Gqα lead to defects in salt chemotaxis learning and olfactory adaptation (4, 11). ins-1 mutants and hen-1 mutants show defects in salt chemotaxis learning and temperature learning (4, 5, 12). hen-1 mutants show normal olfactory adaptation, but have an additional phenotype, the defect in integration of two sensory signals (12), a phenotype shared by casy-1 mutants. On the other hand, ins-1 mutants are normal in the integration assay (5). These observations suggest that different forms of learning and sensory integration in C. elegans depend on overlapping molecular mechanisms. We propose that casy-1 constitutes another essential molecular pathway, which modulates all these forms of learning and sensory integration.

Ectodomain Shedding and Regulation of Learning and Memory. Although most neural cadherin-like molecules are required at the developmental stage, our data strongly suggest that CASY-1 acts in the mature neural circuit, because transient expression of CASY-1 in the adult stage is sufficient to rescue the learning defects of casy-1 mutants (Fig. 3A). Neuron-specific rescue experiments suggest that CASY-1 is required in the salt-sensing ASER neuron for salt chemotaxis learning (Fig. 3D). We have shown evidence that CASY-1 is cleaved and released from neurons like its mammalian homologs, and the ectodomain of CASY-1 is sufficient for salt chemotaxis learning (Fig. 4). Altogether, we propose a model in which the ectodomain of CASY-1 containing the LNS domain is released from sensory neurons and modulates learning (Fig. 5).

If the ectodomain of CASY-1 acts as a signaling molecule, which neuron(s) and which molecules are receptors of CASY-1? The observation that mRFP signal was observed even in coelomocytes suggests that the ectodomain of CASY-1 could circulate through most of the body cavity. However, CASY-1 is functional only when it is expressed in the sensory neuron, ASER, in salt chemotaxis learning (Fig. 3D). This observation suggests that the CASY-1 ectodomain is functional only within a short distance and acts locally in an autocrine or paracrine fashion rather than an endocrine fashion in salt chemotaxis learning (Fig. 5).

Given the necessity of the LNS domain in learning, the putative target may be a receptor for the LNS domain. The LNS domain is present in many membrane proteins or secreted proteins and has a variety of targets (27, 28). Biochemical approaches using the minimal rescuing construct will be necessary to identify the binding partners.

Relationship Between Calsyntenin and Alzheimer’s Disease and/or Memory. Mammalian calsyntenins/alcadeins can physically interact with APP via scaffold proteins (15), and both are coordinately metabolized in neurons (16). Calsyntenins/alcadeins and APP also show similar localization in neuritic plaques of patients with Alzheimer’s disease (AD) (15). Interestingly, a recent study suggests that a SNP in the human calsyntenin-2 locus shows a strong association (genetic linkage) with memory performance (29). However, there is no direct evidence that this gene regulates learning and memory. Thus, our findings provide the first evidence that the calsyntenin/alcadein family is essential for learning and memory in vivo.

The possible role of calsyntenin-1 in learning and memory was suggested by Vogt et al. who first reported a study on calsyntenin-1 (13). They found that the cytoplasmic acidic region can bind Ca2+ and proposed that calsyntenin-1 may link extracellular proteolysis in the synaptic cleft and postsynaptic Ca2+ signaling (13). Therefore, our observations that the extracellular region of CASY-1 alone is sufficient for learning and that the cytoplasmic region is entirely dispensable were rather unexpected. Interestingly, sAPP, the ectodomain of APP, was recently shown to be sufficient to mediate the physiological functions of APP, including those for learning (30). Similarly, the ectodomain of APL-1, a C. elegans APP homolog, is sufficient to rescue the lethality of apl-1 mutants (31). Because calsyntenin and APP form a tripartite complex via the scaffold protein X11/X1L and their proteolytic processing is coregulated (16), it may turn out that the cleaved ectodomains of calsyntenin and APP act in concert or in parallel to modulate learning and memory.

For the functional analysis of the calsyntenin family, lack of redundancy within the family genes and facility of transgenic rescue experiments are strong advantages of C. elegans. Our findings may lead to the discovery of mechanisms of human memory and previously unknown relationships between calsyntenins and dementia or learning disability in AD patients.

Materials and Methods

Strains and Culture. C. elegans strains were cultivated at 20°C under standard conditions (18). The Escherichia coli strain OP50 was the food source in temperature learning assays, whereas NA22 was used in the other behavioral assays. Strains used in this study were wild-type Bristol N2, can-1(pe401), casy-1(tm718), casy-1(n533), casy-1(n441) II, ins-1(nr2091) IV, daf-18(e1375), and daf-18(lf198) IV.

EMS Mutagenesis, Mutant Screens, and Positional Cloning of casy-1. EMS mutagenesis was performed as described (18). We collected F2 animals attracted to NaCl after prolonged incubation in NaCl-conditioning buffer without food. We repeated this selection for six generations. After establishing clonal strains from individual candidates, the learning phenotype of each strain was confirmed. Linkage of the learning defect of JN401 to LG II was determined by ordinary genetic mapping. Snap-SNPs were used to map the mutation to a central 110-kb region of LG II. After genome sequencing, we found a missense mutation, pe401, in the gene B0304.3, which was previously called cdh-11. We confirmed that B0034.3 is the responsible gene for the learning-defective phenotype by rescue experiments.

Salt Chemotaxis and Salt Chemotaxis Learning Assays. The salt chemotaxis assays and salt chemotaxis learning assays were performed as described (4) with some modifications. Except for Fig. 1B and Fig. S5A, we performed chemotaxis assays on 6-cm assay plates [5 mM potassium phosphate (pH 6.0), 1 mM CaCl2, 1 mM MgSO4, 2% agar], on which a salt gradient was formed overnight by using an agar plug containing 50 mM NaCl. Washed animals were placed on assay plates and incubated for 15 min at 23°C. Chemotaxis index was calculated as described (4). In Fig. 1B and Fig. S5A, we used 9-cm assay plates, and the assay time was 30 min. In these assays, agar plug included 100 mM NaCl in Fig. S5A, whereas several different concentrations of human calsyntenin-1 were used in Fig. 1B. For salt chemotaxis learning assays, we mainly used the “liquid conditioning method,” in which collected adult animals were transferred into conditioning buffer [5 mM potassium phosphate (pH 6.0), 1 mM CaCl2, 1 mM MgSO4] with 20 mM NaCl (NaCl-conditioning).
or without NaCl (mock-conditioning) and were incubated at 22°C for 1 h. All behavioral assays were independently performed at least four times.

Other behavioral assays, heat shock experiments, plasmid constructions, germ-line transformation, and statistic analyses are described in SI Text.

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