論文の内容の要旨

Genetic identification and functional analysis of CASY-1,
the homolog of Calsyntenin/Alcadein, involved in learning and memory in *Caenorhabditis elegans*

（線虫の学習・記憶におけるカルシンテニン/アルカデインホモログ
CASY-1 の遺伝学的同定と機能解析）

池田　大祐

(Introduction)

C. elegans is a well developed model organism for studying molecular mechanisms of learning and memory. Recent studies on the mechanisms of neural plasticity revealed common regulators of learning and memory in mammals and *C. elegans*. Therefore, identification of molecules essential for neural plasticity in *C. elegans* can provide important insights for understanding learning and memory in mammals, including humans.

C. elegans shows chemotaxis to a variety of chemoattractants 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, olfactory adaptation, and temperature learning. In salt chemotaxis learning, worms learn to avoid NaCl after experiencing a salt stimulus in association with starvation. Our laboratory recently reported that the insulin-like signaling pathway regulates this type of learning. However, the mechanism of this learning is not fully understood, and only a few molecular components are so far identified.

(Results)

To identify novel molecules involved in salt chemotaxis learning, I performed an EMS mutagenesis screen and obtained several candidate strains. Of these, JN401 showed the most severe defect. The mutant animals failed to avoid NaCl after conditioning with NaCl and starvation, while they showed normal chemotaxis when

unconditioned. I found that JN401 carries a missense mutation, *pe401*, in the gene B0034.3, which had been previously called *cdh-11*. I renamed this gene *casy-1* because of its orthology to calsyntenins/alcadeins. The learning defects of *pe401* mutants were restored by introduction of a cosmid (B0034) or a PCR fragment corresponding to the genomic region of *casy-1*, indicating that the impairment of *casy-1* function causes the learning defects. *casy-1* encodes the sole *C. elegans* ortholog of the mammalian calsyntenins/alcadeins. Calsyntenins/Alcadeins are type I transmembrane proteins highly conserved in metazoa. They have two tandem cadherin domains as well as an LG/LNS domain in the ectodomains, i.e. extracellular regions. These proteins are highly expressed in mammalian brain. However, their physiological roles and molecular functions in the nervous system are mostly unknown.

To characterize the molecular function of this gene further, deletion alleles were obtained and incorporated in subsequent analyses. Among them, *tm718* and *hd41* are putative null alleles because both carry a deletion in the N-terminal region of *casy-1*, which results in a frame shift. All these mutants exhibited strong learning defects similar to those in *pe401* mutants.

Some mutants defective in salt chemotaxis learning also exhibit defects in other types of learning or sensory processing. For example, mutants of *hen-1*, encoding an LDL-receptor-like secretory protein, and *ins-1*, encoding an insulin-like peptide, show defects in salt chemotaxis learning and temperature learning. Gain-of-function mutations of *egl-30* Gq alpha cause defects in salt chemotaxis learning and olfactory adaptation. These observations imply that overlapping molecular mechanisms operate in salt chemotaxis learning and other forms of learning. To investigate the possible role of *casy-1* in these types of learning, the mutants were tested for olfactory adaptation and temperature learning. In both assays, the *casy-1* mutants exhibited significant defects. In contrast, *casy-1* mutants neither showed large defects in their response to cultivation temperature nor benzaldehyde, as long as the worms were not pretreated under starvation. I also found that *casy-1* is important for another type of information processing: sensory integration. In a paradigm for sensory integration, the fraction of worms that cross an aversive barrier to reach an attractive odorant is scored. *hen-1* mutants show abnormal sensory integration in addition to the defects in learning. As is the case with *hen-1* mutants, *casy-1* mutants showed defects in this sensory processing whereas the mutants normally respond to diacetyl or Cu^{2+} when presented separately. These results suggest that CASY-1 is not essential for the primary sensory transduction, but is important for higher-order information processing.

CASY-1 could be either required for neural development or for post-developmental neural functions to support salt chemotaxis learning. I tested these possibilities using a heat-inducible *hsp16.2* promoter. Learning defects of the *casy-1* mutants were strongly rescued when a functional *casy-1* was transiently expressed in the adult stage, indicating that *casy-1* is required in the mature neural circuit.

I 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 as well as in other tissues such as intestine and gonadal sheath cells. I noticed strong GFP signals in many head neurons, including ASE salt sensing neurons. Previously, our group demonstrated that components of the PI3K (phosphatidyl inositol 3-kinase) pathway function in the ASER (right ASE) neuron for salt chemotaxis learning. I determined the neuron(s) in which *casy-1* is required for salt chemotaxis learning by cell-specific rescue experiments and found that the expression of *casy-1* solely in the ASER neuron is sufficient to rescue the defects. In contrast, the defects were not rescued by expression of *casy-1* in other neurons. These data suggest that *casy-1* acts mainly in the

ASER sensory neuron in the mature neural circuit for salt chemotaxis learning.

Because both CASY-1 and the components of the PI3K pathway act in the ASER neuron for salt chemotaxis learning, I examined the genetic relationship between these genes. In *daf-18* PTEN mutants, which have elevated levels of the PI3K signaling, naive animals show reduced chemotaxis to NaCl compared to the wild type. I found that the *casy-1* mutation suppresses the reduced chemotaxis of *daf-18*. This result suggests that *casy-1* acts either downstream of or in parallel to *daf-18*. I 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. The learning defect of *casy-1; ins-1* double mutant was more severe than that of either single mutant. These results suggest that *casy-1* acts in parallel to the insulin-like signaling pathway.

Calsyntenins/Alcadeins are cleaved by unknown secretases in the extracellular region. By analogy, CASY-1 might also be proteolytically cleaved. I obtained a result consistent with this possibility by observing the localization of N-terminal and C-terminal GFP fusion proteins, NtGFP::CASY-1 and CASY-1::CtGFP, respectively. GFP fluorescence was observed in coelomocytes, only for NtGFP::CASY-1, but not for CASY-1::CtGFP. Coelomocytes are macrophage-like scavenger cells present in the pseudocoelomic cavity that take up various compounds from the body cavity fluid. 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 of CASY-1, respectively, and were expressed in head neurons (Figure A, RYV). Only the signal of mRFP, tagged to the ectodomain, was detected in coelomocytes. mRFP signals in coelomocytes were observed even in normally cultured naive animals. I made similar observations when the fusion protein was expressed in the ASER neuron alone, though the fluorescence was weaker in this case. In contrast, both mRFP and Venus signals were observed in coelomocytes for secreted forms of CASY-1. When mRFP was fused to the N-terminus of the protein lacking the whole extracellular region, it was not detected in coelomocytes (Figure A, RYV(ΔNt)). Therefore, I conclude that in head neurons, including ASER, the ectodomain is cleaved from the full length CASY-1 and released into the body cavity.

Regarding the processing of CASY-1, I attempted to determine which part of the protein is important for salt chemotaxis learning. I found that the ectodomain, even without transmembrane and cytoplasmic regions, is enough to rescue the learning defects of the *casy-1* mutants (Figure A, RYV(ΔCt), RYV800 and RYV700), while the fragments that lack the ectodomain did not rescue the defect (Figure A, RYV(ΔNt) and CtV). I also found that the ectodomain lacking the signal peptide failed to rescue (Figure A, RYV700(ΔSP)), suggesting that only the released fragments modulate learning. Next, I performed a functional domain mapping of the ectodomain. I expected that the LG/LNS domain is essential for learning because two alleles that lead to the impairment of the extracellular region around the LNS domain cause defects in learning. LNS domains are often found in extracellular proteins and are implicated in interaction with a variety of cellular receptors or ligands. As expected, deletion of LNS domain abolished the functionality of CASY-1 (Figure A, RYV(ΔLNS)). Furthermore, several shorter forms of the ectodomains, even though they spare the LNS domain, could not rescue the defective phenotype (Figure A, RYV600, RYV530 and RYV331-700). These results suggest that the flanking regions of LNS domain are also required for salt chemotaxis learning. The partial rescue observed in the cadherin domain-deleted fragments implies that the cadherin domains may have accessory but non-essential roles (Figure A, RYV(ΔCads)).

(Discussion)

From above observations, I propose a model in which the ectodomain of CASY-1 containing the LNS domain is released from sensory neurons and modulates learning (Figure B). 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 the 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 they are expressed in the sensory neuron, ASER, in salt chemotaxis learning. Hence, the released peptide is likely to act in an autocrine or paracrine fashion (Figure B).

Mammalian calsyntenins/alcadeins can physically interact with APP (amyloid precursor protein) via scaffold proteins, and both are coordinately metabolized in neurons. Calsyntenins/alcadeins and APP also show similar localization in neuritic plaques of patients with Alzheimer's diseases (AD). Interestingly, a recent study suggests that a SNP in the human calsyntenin-2 locus shows strong genetic linkage with memory performance. However, there is no direct evidence that this gene regulates learning and memory. Thus, my findings provide the first evidence that the calsyntenin/alcadein family is essential for learning and memory in *vivo*.

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

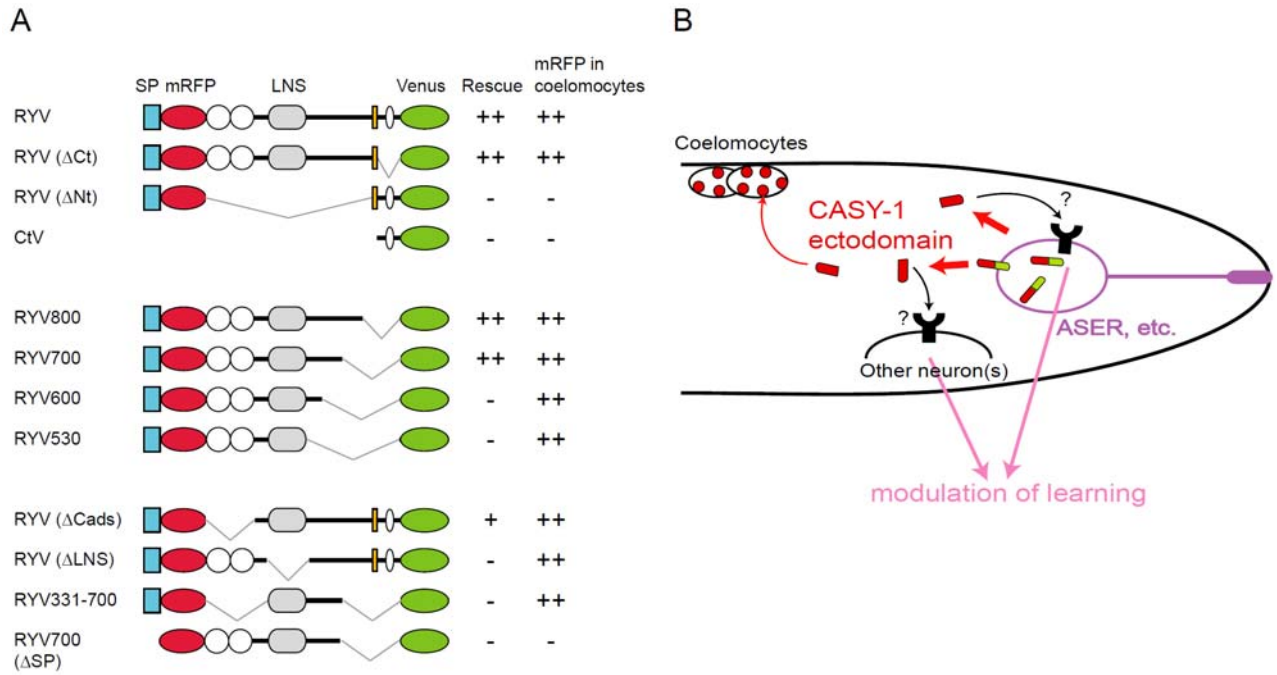

Figure A. Functional domain mapping of *casy-1*

Schematic depiction of deletion constructs, summary of the results of rescue experiments, and localization of mRFP signals in coelomocytes.

Figure B. A model for CASY-1 function

CASY-1 expressed in the sensory neurons including ASER is constitutively cleaved and the ectodomain is released. Released ectodomain acts on either the neuron itself or other nearby targets and modulates learning. It is also endocytosed by the scavenger cell, coelomocyte.