

Ca²⁺ Signaling via the Neuronal Calcium Sensor-1 Regulates Associative Learning and Memory in *C. elegans*

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Summary

On a radial temperature gradient, *C. elegans* worms migrate, after conditioning with food, toward their cultivation temperature and move along this isotherm. This experience-dependent behavior is called isothermal tracking (IT). Here we show that the neuron-specific calcium sensor-1 (NCS-1) is essential for optimal IT. *ncs-1* knockout animals show major defects in IT behavior, although their chemotactic, locomotor, and thermal avoidance behaviors are normal. The knockout phenotype can be rescued by reintroducing wild-type NCS-1 into the AIY interneuron, a key component of the thermotaxis network. A loss-of-function form of NCS-1 incapable of binding calcium does not restore IT, whereas NCS-1 overexpression enhances IT performance levels, accelerates learning (faster acquisition), and produces a memory with slower extinction. Thus, proper calcium signaling via NCS-1 defines a novel pathway essential for associative learning and memory.

Introduction

The neuronal calcium sensor-1 (NCS-1) belongs to the intracellular neuronal calcium sensor family of EF-hand calcium binding proteins (Braunewell and Gundelfinger, 1999) and is highly conserved throughout evolution, with

orthologs identified in yeast, *Drosophila*, *C. elegans*, avian birds, rodents, and humans (Nef, 1996). Its amino acid sequence is 100% conserved among vertebrates and shows only 25% divergence with *C. elegans* or 28% with yeast (De Castro et al., 1995). NCS-1 is neuron specific (Martone et al., 1999; Paterlini et al., 2000) and exhibits a high affinity for calcium of 300 nM (Cox et al., 1994), which is within the linear range of localized [Ca²⁺]_i fluctuations in neurons (Fontana and Blaustein, 1993; Yazejian et al., 2000). The yeast, but not vertebrate (Bartlett et al., 2000), NCS-1 protein has been shown to interact with phosphatidylinositol 4-OH kinase (Hendricks et al., 1999). The vertebrate recombinant NCS-1 has been reported to activate G protein receptor kinase 1 (De Castro et al., 1995; Iacovelli et al., 1999) to regulate evoked exocytosis in neuroendocrine cells (McFerran et al., 1998) and to substitute for calmodulin (Schaad et al., 1996). Indeed, calmodulin-dependent targets such as 3':5'-cyclic nucleotide phosphodiesterase, calcineurin, nitric oxide synthase enzymes (in vitro), or the *Paramecium* calmodulin-dependent potassium channel (in vivo) are directly activated by NCS-1 (Schaad et al., 1996). Moreover, overexpression of NCS-1 in the mouse hippocampus increases long-term potentiation (LTP) (P. N. et al., unpublished data), and overexpression of frequenin, the *Drosophila* ortholog of NCS-1, causes a chronic facilitation of transmitter release at the neuromuscular junction of flies and frogs through unknown mechanisms (Pongs et al., 1993; Rivosecchi et al., 1994; Olafsson et al., 1995). Importantly, the neuronal role of *ncs* genes in vivo has not been characterized by loss-of-function genetics in eukaryotes. To investigate the role of NCS-1 as a regulator of neuronal activity in vivo, we chose *C. elegans* as a model organism due to its simple nervous system and well-described neuronal circuitry with the ability to respond to diverse environmental stimuli such as touch, smell, taste, or temperature.

Results

The *Ce-ncs-1* gene, located on the left arm of chromosome X, encodes *Ce*-NCS-1, a small acidic protein composed of 192 amino acids (molecular mass of 22 kDa) that binds three calcium ions via four putative EF-hands (De Castro et al., 1995). Cellular distribution of *Ce*-NCS-1 was determined by light and immunofluorescence microscopy studies using a transgenic line (XA411) expressing the green fluorescent protein (GFP) under the control of the *Ce-ncs-1* promoter region (Figure 1). Confirmation of GFP staining and NCS-1-positive cells was obtained with antibodies against *Ce*-NCS-1 (data not shown). *Ce*-NCS-1 was predominantly expressed in sensory neurons (10 neuronal pairs: AWC, ASE, AWB, BAG, PHB, AWA, AFD, ADF, ASG, PHA). In addition, two pairs of interneurons (AVK, AIY), one motor neuron (RMG), and one muscle cell type (pm1) expressed *Ce*-NCS-1 (Table 1). Most of the NCS-1-expressing neurons were associated with two sensory organs, the head amphids and tail phasmids (Figures 1A and 1B). We ob-

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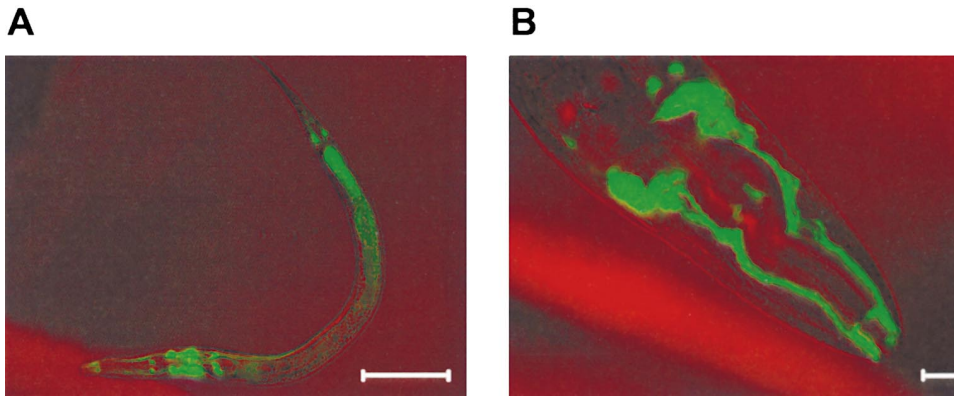


Figure 1. Expression Pattern of the *ncs-1::GFP* Reporter Gene

(A) *ncs-1* gene expression is observed in amphid, phasmid, nerve ring, and ventral nerve cord of L1 stage animals as GFP staining. Scale bar: 100 μ M.

(B) *ncs-1* gene expression in adult head showing GFP staining in amphid dendrites. Scale bar: 10 μ M

served a dendritic, axonal, and cell body subcellular distribution with Ce-NCS-1-specific antibodies (data not shown).

To investigate the functional role of Ce-NCS-1, knock-out (KO) animals were generated. An *ncs-1* Tc1 transposon insertion mutant line [*ncs-1(pk242::Tc1)*] was used to isolate a deletion derivative strain *ncs-1(qa401te)* (Figure 2A). The null *ncs-1* animals were viable, their developmental timing normal, although they were slightly dumpy (data not shown), and the NCS-1 protein was no longer present in these KO animals (Figure 3C). Since 8/10 pairs of NCS-1-positive neurons are known to be involved in chemotaxis and volatile odorant avoidance, several classes of odor responses were measured with the KO strain. Surprisingly, null *ncs-1* mutant animals behaved like wild-type worms (data not shown), suggesting that calcium signaling via NCS-1 is not involved in *C. elegans* odorant detection or that other calcium sensors in olfactory neurons can substitute or compensate for the lack of NCS-1.

As a cold-blooded animal, viable and fertile only within

a limited temperature range (12°C–26°C), *C. elegans* has efficient thermosensory behaviors, including thermal avoidance for protection against exposure to noxious temperature (Wittenburg and Baumeister, 1999) and thermotaxis for the perception of physiological (<0.1°C) changes in local temperature (Mori, 1999). Worms learn to associate a given temperature (the growth temperature) with the presence of food during a conditioning period (acquisition) of several hours (Hedgecock and Russell, 1975). This associative conditioning is reflected by a unique phenotype, the isothermal tracking (IT) behavior, which can be observed on unseeded plates with a radial gradient of temperature with a single animal migrating to the precise growth temperature ($\pm 0.2^\circ\text{C}$) (Hedgecock and Russell, 1975) and then moving isothermally. When the association is disrupted (by food exhaustion), the IT behavior is conserved for several hours (extinction period), and then a searching mode is activated, and the worms will cross isotherms randomly to seek food at other temperatures (Mori, 1999). But a change in temperature will not lead to a random search-

Table 1. NCS-1-Positive Cells and their Functions

Positive Cells	Function
Sensory neurons	
AWC (left, right)	Amphid neurons. Chemotaxis to volatile odorants (benzaldehyde, butanone, isoamyl alcohol)
ASE (L,R)	Amphid neurons. Chemotaxis to soluble compounds (Na ⁺ , Cl ⁻ , cAMP, biotin, lysine), egg laying
AWB (L,R)	Amphid neurons. Volatile avoidance
BAG (L,R)	Sensory neurons
PHB (L,R)	Phasmid neurons
AWA (L,R)	Amphid neurons. Chemotaxis to volatile odorants (diacetylene, pyrazine, 2,4,5-trimethylthiazol)
AFD (L,R)	Amphid neurons. Isothermal tracking behavior. Thermotaxis
ADF (L,R)	Amphid neurons. Dauer formation; chemotaxis to soluble compounds (minor)
ASG (L,R)	Amphid neurons. Dauer formation (minor); chemotaxis to soluble compounds (minor)
PHA (L,R)	Phasmid neurons
Interneurons	
AVK (L,R)	—
AIY (L,R)	Isothermal tracking behavior. Thermotaxis
Motor neuron	
RMG	Innervation of muscles in the head
Muscle cell	
pm1	Opening of the metastomal pharyngeal flaps

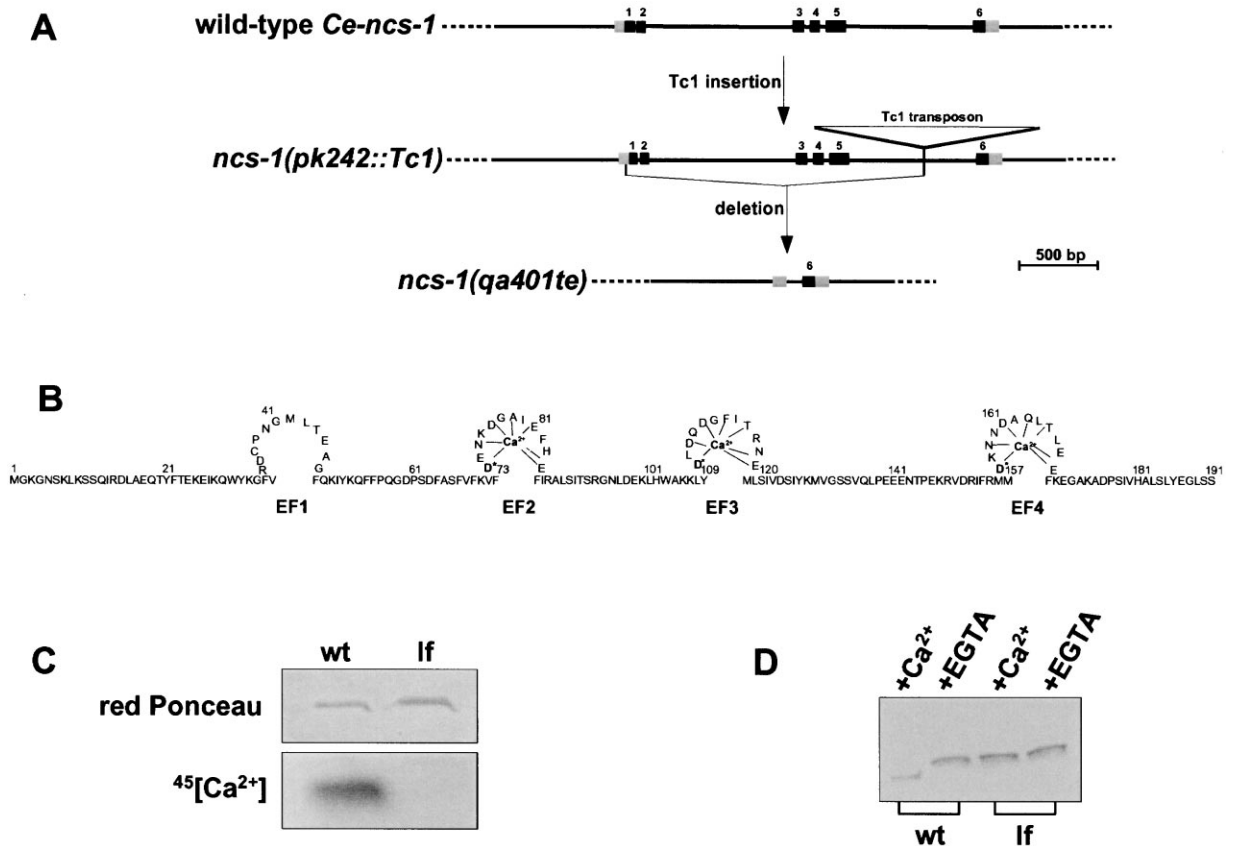


Figure 2. Ce-NCS-1: From Gene Structure to Calcium Sensor

(A) Physical maps of wild-type *Ce-ncs-1*, *ncs-1(pk242::Tc1)*, and null *ncs-1(qa401te)* deletion genes. Black boxes represent exons 1–6, and the gray boxes the 5' and 3' untranslated regions of the *ncs-1* gene. Scale bar: 500 bp.

(B) The NCS-1 protein contains 4 EF-hands (EF1–EF4), but the first binding site is degenerated and cannot bind Ca^{2+} (De Castro et al., 1995). The Asp positions D73, D109, and D157 are essential for calcium binding. Changing the three Asp residues (D*) into Ala inactivates Ca^{2+} binding (Putkey et al., 1989) (see below).

(C) The loss-of-function (lf) triple mutant was constructed by substituting the first Asp (D*) residue to an Ala of the three EF-hands EF2, 3, and 4.

(D) Ce-NCS-1 is a calcium sensor. Calcium bound wild-type NCS-1 displayed a greater electrophoretic mobility than the apo form, whereas lf-NCS-1 mobility was not affected by the presence (+Ca²⁺) or absence (+EGTA) of free calcium. It suggests that Ca^{2+} induces an allosteric change in the conformation and probably activity of Ce-NCS-1.

ing mode but rather a slow reacquisition of the association between food and the new temperature. As Ce-NCS-1 was found in AFD and AIY, two neurons of the thermotaxis neural circuit, *ncs-1(qa401te)* KO worms were tested for IT behavior at 20°C (measurement as percentage of worms performing isothermal tracks at 20°C). IT recordings of single worms were visualized after 90 min on testing plates as shown in Figure 3A. *Ce-ncs-1* KO animals were abnormal, showing a significant difference in behavior when compared with wild-type (WT) animals (Figure 3B). Many WT animals (75% ± 8%; n = 94) exhibited normal IT behavior, whereas only 31% ± 9% of *ncs-1(qa401te)* mutants (n = 96) performed normally. The majority of the KO animals showed irregular IT behaviors and, based on previous descriptions of thermotaxis phenotypes by Mori and Oshima (1995), were classified into five categories: 31% were cryophilic, 27% athermotactic, 6% thermophilic, 5% showed intermediate behavior (mixed athermotactic and normal phenotypes), and 31% were normal. The

overall IT defects of the *ncs-1* mutants (mostly athermotactic and cryophilic) were similar to the phenotypes observed with laser-killed AFD (athermotactic and cryophilic) or AIY (mostly cryophilic) animals, or with *ttx-3* (mostly cryophilic) mutants (Hobert et al., 1997), but were clearly different from AIZ (mostly thermophilic) laser-killed animals (Mori and Ohshima, 1995).

We also tested the thermal avoidance behavior of the *ncs-1* knockout strain upon exposure to a noxious temperature. Noxious temperature causes a withdrawal reflex that differs significantly from thermotaxis behavior, involves different neurons, and is influenced by mutations in distinct genes (Wittenburg and Baumeister, 1999). The behavior of the *ncs-1(qa401te)* mutant did not differ from that of wild-type worms in this assay (data not shown).

To ensure that the diminution of IT behavior with the KO mutant was due to the absence of Ce-NCS-1, we performed a germline rescue of the KO strain using either a 7 kb genomic fragment transgene containing the

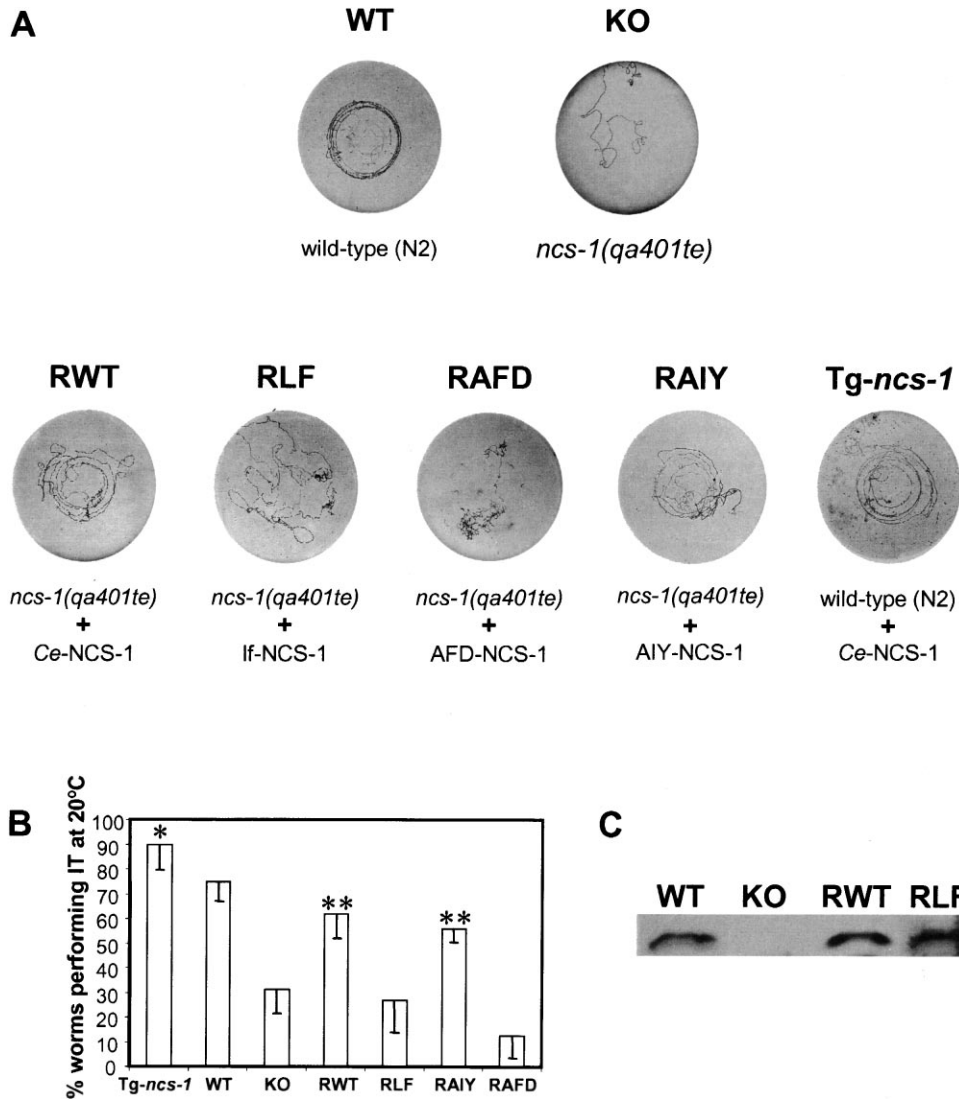


Figure 3. Ca^{2+} Signaling via NCS-1 in the AIY Interneuron Is Essential for Isothermal Tracking Behavior

(A) Individual isothermal tracking (IT) records. Photographs of normal or disrupted isothermal behavior tracks of wild-type (WT), *ncs-1(qa401te)* knockout (KO), rescued *ncs-1(qa401te)* with wild-type *ncs-1* (RWT), or with loss-of-function *ncs-1* (RLF), or with AFD neuron-specific promoter (RAFD) driving *ncs-1* expression, or with AIY neuron-specific promoter (RAIY) driving *ncs-1* expression, and wild-type plus transgenic *ncs-1* (Tg-*ncs-1*) individual worms are shown. Thermotaxis assays were performed as described in Mori and Ohshima (1995) (see Experimental Procedures). (B) Percentage (group performance) of worms performing IT behavior after overnight feeding at 20°C. Each data point represents 4–10 independent assays using ~10–20 animals per assay. At least 2–3 different lines were generated for each transgene construct. The chi-square distribution and t test were used to determine the significance of IT behavior performance between the different strains. The p value (asterisk, ≤ 0.02) indicates a significant difference between Tg-*ncs-1* animals as compared to wild-type worms. The p values (double asterisk, ≤ 0.002) represent significant differences of performance between KO animals and RWT or RAIY worms. For these experiments, standard deviations range from 7% to 14%. A trace is considered as isothermal if more than half of the trace length left on the agar surface by a single animal is circular or present an arc of circle near the isotherm of the growth temperature.

(C) Ce-NCS-1 protein levels in the various WT, KO, RWT, RLF strains or lines. Western blot analysis using Ce-NCS-1 polyclonal antibodies and 80 μ g of total protein extract reveals the presence of the NCS-1 calcium sensor in the wild-type strain (WT), in the NCS-1 rescued wild-type lines (RWT), and in the rescue loss-of-function lines (RLF). Note the absence of NCS-1 in the knockout strain (KO).

entire *ncs-1* genomic coding region plus ~3 kb of its 5' upstream genomic sequence (data not shown) or a PCR fragment containing the *ncs-1* cDNA coding region plus ~3 kb of the 5' upstream genomic sequence (lines RWT; Figures 3A and 3B). Both transgenes were able to rescue the *ncs-1* mutant defective phenotype, resulting in restored IT behavior in 62% \pm 9% of animals ($n = 92$, $p = 0.00001$).

To test whether the function of Ce-NCS-1 was calcium dependent, we generated a mutated form of NCS-1 unable to bind calcium (loss-of-function or If-NCS-1). Replacement of the crucial Asp residues of the three EF-hand calcium binding sites (positions 73, 109, and 157; Figure 2B) with Ala prevented both Ca^{2+} binding (Figure 2C) and Ca^{2+} -dependent conformational shift of If-NCS-1 (Figure 2D). Lines obtained with the If-*ncs-1* transgene

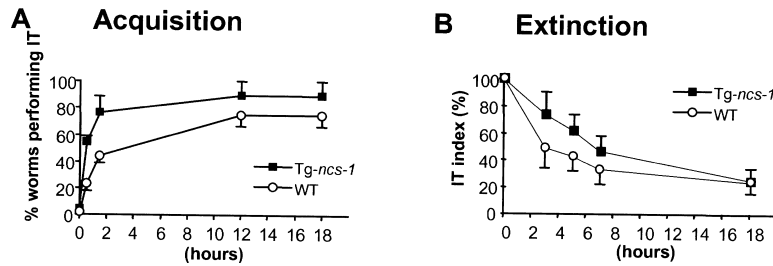


Figure 4. Faster Acquisition (Learning) and Longer Retention (Memory) for NCS-1 Over-expressing Worms

(A) The acquisition of the association of food at a given temperature was determined for wild-type (WT) and overexpressing NCS-1 (*Tg-ncs-1*) worms by measuring the percentage of worms performing IT behavior at 20°C. Briefly, worms were grown on seeded plates at 25°C for at least 12 hr, and then shifted individually to a seeded plate at 20°C for different time intervals.

For both strains, the maximal levels of IT behavior (absolute values) were reached after pairing the conditioning stimuli for at least 12 hr. Fifty percent of the maximum level was reached after 68 min for WT worms, and after only 28 min with *Tg-ncs-1* worms. As we scored half-maximal acquisition instead of the relative IT index (see definition below), the experiment was internally controlled for increased performance for each strain.

(B) The extinction of this association (food at 20°C) was determined for wild-type (WT) and overexpressing NCS-1 (*Tg-ncs-1*) worms. Briefly, worms were grown at 20°C in presence of food for at least 18 hr, washed at 20°C, and transferred to unseeded plate at 20°C for different time intervals. Normalized IT values (IT index) were used to correct for the increased performance of *Tg-ncs-1* worms after conditioning and to only consider extinction of trained animals. One hundred percent correspond to the mean performance achieved after 18 hr at 20°C (see Figure 4A for absolute values). Half maximal extinction was obtained after 3 hr with WT worms, whereas *Tg-ncs-1* worms had a prolonged retention and reached half-maximal extinction after about 7 hr.

(RLF) were assayed for IT behavior (Figures 3A and 3B) and showed a defective IT phenotype ($27\% \pm 13\%$, $n = 78$), despite the expression of the *lf-NCS-1* mutated protein (Figure 3C). This indicates that normal IT behavior is calcium dependent and requires a functional, calcium binding NCS-1 sensor.

To determine which cells require NCS-1, we performed a mosaic rescue of the KO animals using AFD (*gcy-8* [Yu et al., 1997]) or AIY (*ttx-3* [Hobert et al., 1997]) specific promoters driving the expression of *ncs-1*. We observed a rescued IT behavior ($56\% \pm 5\%$) with the *ttx-3::NCS-1* construct (RAIY animals, $n = 50$, $p = 0.002$), at a level similar to the rescue observed in RWT animals (Figures 3A and 3B). No rescue ($12.5\% \pm 9\%$) in IT behavior was obtained with the *gcy-8::NCS-1* construct (RAFD animals, $n = 40$) (Figures 3A and 3B). These data strongly suggest that for normal IT behavior, NCS-1

function is required in the AIY but not AFD or any other neurons.

Does an increased level of NCS-1 affect the IT behavior of WT animals? After generating transgenic lines overexpressing NCS-1 (*Tg-ncs-1*) using the *ncs-1* cDNA under the control of the *ncs-1* promoter (presence of the construct determined by PCR), we measured the effect in thermotaxis. Figure 3B shows remarkably that NCS-1 overexpression significantly ($p = 0.018$) increases IT thermotaxis performance ($90\% \pm 10\%$, $n = 70$) as compared to WT animal behavior ($75\% \pm 8\%$). These results demonstrate that the level of NCS-1 activity can determine the efficiency of IT performance and establish that NCS-1 is likely to be essential to the behavior and not merely permissive for IT.

To further characterize *Tg-ncs-1* worms, we studied their IT behavior performance in greater details and

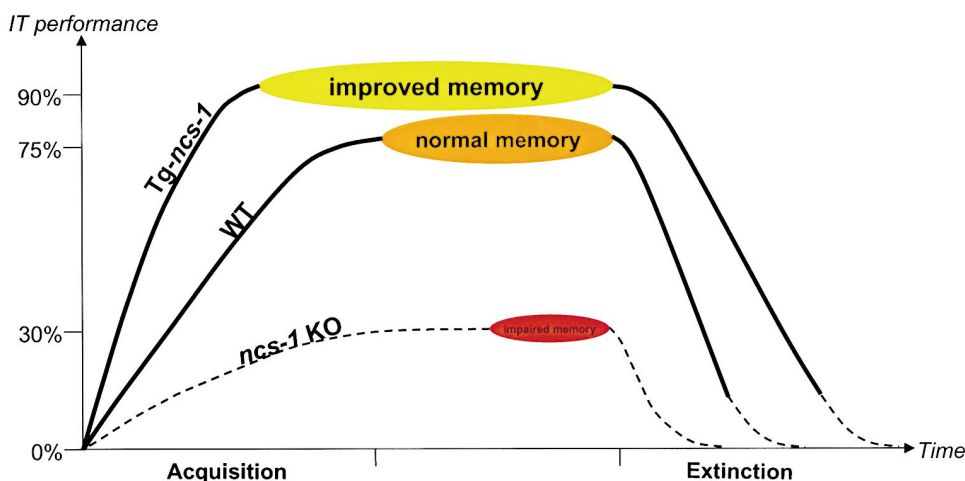


Figure 5. Regulation of Associative Learning and Memory by NCS-1

The schematic view indicates that the amount of NCS-1 directly regulates IT behavior. The absence of the neuronal calcium sensor-1 (*ncs-1* KO) impedes the majority of worms from performing isothermal tracking behavior, whereas its presence (WT) allows it. Overexpression of NCS-1 (*Tg-ncs-1*) enhances performance levels, accelerates learning, and produces a memory with slower extinction. Slower extinction might reflect increased responsiveness of the AIY integrative neurons to $[Ca^{2+}]_i$ stimuli. The amount of NCS-1 in the AIY neurons and the strength of Ca^{2+} stimulation are linked together to modulate associative learning and memory in *C. elegans*. The dotted lines represent hypothetical IT responses.

compared it with WT worms. We determined the time needed for the acquisition (learning) and determined the extinction period (memory) of the associative information (presence of food at the temperature of 20°C). For acquisition experiments (Figure 4A), the worms were grown for at least 12 hr in presence of food at 25°C, and then were shifted individually for different time intervals onto a seeded plate at 20°C, and their IT behavior at 20°C was determined. As shown in Figure 4A, WT worms needed about 68 min to reach 50% of their maximal performance level, whereas *Tg-ncs-1* worms reached their 50% level after only 24 min. Overexpressing NCS-1 worms were therefore 2–3 times faster than the WT to learn the novel conditioning paradigm (food at 20°C). For both strains, a maximal level of performance was already reached after about 12 hr. For extinction experiments (Figure 4B), the worms were grown on seeded plates at 20°C for at least 18 hr, and then individual young adult worms were washed at 20°C, transferred onto unseeded plates at 20°C for different time intervals, and their IT behavior at 20°C was determined. As shown in Figure 4B, trained WT worms needed about 3 hr to lose 50% of their maximal performance level, whereas *Tg-ncs-1* worms lost 50% of their maximal level only after about 7 hr. Therefore, the extinction period of the associative paradigm (food at 20°C) was prolonged for at least twice as long with the NCS-1-overexpressing worms as compared to WT worms. For both strains, the return to a baseline level was achieved after about 18 hr. Together, these data indicated that an elevated amount of the NCS-1 calcium sensor protein enhances not only performance, but also learning and memory functions, via faster acquisition and longer retention (Figure 5).

Discussion

Reinforcement via faster acquisition together with higher final performance, not surprisingly, leads to memories that are more persistent, and therefore are consistent with a longer retention period (Milner et al., 1998). However, if extinction is also a learning mechanism, then *Tg-ncs-1* worms need more time to react to the absence of one conditioning stimulus (i.e., food) that is likely to be linked to the level of $[Ca^{2+}]_i$ signaling (see Figures 5 and 6).

Our loss-of-function and mosaic rescue data clearly demonstrate that the presence and amount of the calcium sensor NCS-1 in AIY neurons plays a central role in influencing Ca^{2+} -dependent associative learning in *C. elegans* as demonstrated by its direct regulatory effects on IT behavior. We have been able to determine that calcium signaling or binding by NCS-1 is critical for this activity and that the NCS-1 signaling pathway is essential for performing IT behavior. As shown on the schematic diagram (Figure 6), NCS-1 could have a pre-synaptic role at the AIY interneuron synapses with AIZ and RIA or a postsynaptic function at the AFD/AIY synapses. The presynaptic activity of NCS-1 is supported by preliminary data indicating that increased levels of NCS-1 in the mouse hippocampus enhance LTP via a presynaptic facilitation (P. N. et al., unpublished data). Similarly, the increase of IT behavior observed when

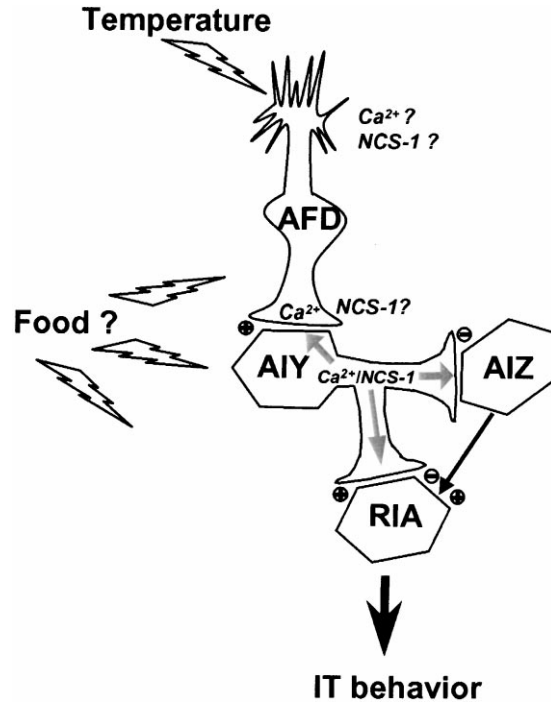


Figure 6. Model for a Pre- or Postsynaptic Role of the Neuronal Calcium Sensor-1

NCS-1 is present in the AFD and AIY neurons, either at the dendritic or axonal terminals, and its function in the AIY neurons is essential for IT behavior. In this model, the AIY interneuron serves as an integrator of food and temperature inputs, and the NCS-1 calcium sensor transduces calcium signals and regulates synaptic strength between AIY/AIZ and AIY/RIA cells at the presynaptic location or between AFD/AIY neurons in a postsynaptic location. The plus or minus sign indicates the presence of an excitatory or inhibitory synapse.

NCS-1 is overexpressed (*Tg-ncs-1* animals) may reflect a state where the AIY presynaptic terminals are maximally stimulated. The observation of a presynaptic effect on overexpression of NCS-1 at the neuromuscular junction of flies and frogs (Rivosecchi et al., 1994; Olafsson et al., 1995) supports this hypothesis and suggests a conserved function for NCS-1 through evolution. However, the mechanisms by which NCS-1 regulates neurotransmitter release and/or synaptic plasticity are not yet known. Clearly, the overall level of IT performance for the *ncs-1* KO animals is still significant (30%), suggesting that other calcium sensors must be present.

Could the AIY interneuron serve as an integrator of food and temperature inputs in the form of Ca^{2+} signals provided by the AFD and surrounding cells? These signals, detected by the neuronal calcium sensor NCS-1, could be transmitted to further downstream targets such as 3':5'-cyclic nucleotide phosphodiesterase, calcineurin, nitric oxide synthase, potassium channels, or phosphatidylinositol 4-OH kinase via mechanisms that could influence AIY synaptic strength. Ca^{2+} signaling via NCS-1 therefore defines a novel pathway for the regulation of synaptic efficacy.

Together, these thermotaxis-enhanced or -deficient NCS-1 strains provide valuable tools to study synaptic plasticity at the molecular, cellular, and network levels

using live animals, as well as a model that might well help to understand conserved functions such as long-term memory and associative learning across species.

Experimental Procedures

Ce-*ncs-1* Gene Expression

The *ncs-1::GFP* reporter gene was constructed by subcloning a *ncs-1* 3100 bp promoter into the GFP expression vector pPD95.75 (from A. Fire labs, Carnegie Institution of Washington, Department of Embryology, Baltimore, MD). Transgenic worms were generated as previously described (Mello et al., 1991). The marker *rol-6* (plasmid pRF4) and the *ncs-1::GFP* construct were coinjected into gonads of hermaphrodite animals. Aligning GFP fluorescence images with differential interference Nomarski images allowed the identification of *ncs-1::GFP*-positive cells (see Table 1).

Preparation of the *ncs-1* Knockout Strain

A homozygous mutant *ncs-1(pk242)* with a Tc1 insertion located at position 5231 relative to the *ncs-1* gene fragment was obtained by PCR screening of a Tc1 insertion library from R. Plasterk (Holland). Deletion derivatives were obtained as described in (Plasterk, 1995). A strain missing the genomic DNA region between exon 1 and 5 of the *ncs-1* gene was isolated (this deletion removed the first initiator ATG). This initial homozygous strain named XA401 *ncs-1(qa401te)* was backcrossed five times with N2 wild-type animals (final name XA406).

Mutagenesis

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

Calcium Overlay

⁴⁵[Ca²⁺]-radioactive binding is readily detected with wild-type (WT) NCS-1, but not with the loss-of-function (lf) Ce-NCS-1. A protein control with Red Ponceau staining is shown. Five micrograms of recombinant purified WT NCS-1 or lf NCS-1 was run by electrophoresis on a 10% SDS-PAGE gel, blotted onto nitrocellulose membrane, and incubated with ⁴⁵[Ca²⁺] followed by several washes, and were visualized by autoradiography for 48 hr (Maruyama et al., 1984).

Electrophoretic Mobility of NCS-1

Five micrograms of purified WT NCS-1 or lf NCS-1 were subjected to electrophoresis on 10% SDS-PAGE in the presence of 5 mM CaCl₂ or 2 mM EGTA. Proteins were stained for visualization with Coomassie blue (Geiser et al., 1991).

Thermotaxis Tracking Behavior Assay

Briefly, 20–30 worms were grown overnight at a constant temperature of 20°C (the conditioned stimulus) in presence of a fresh lawn of the bacteria strain OP50 (the unconditioned stimulus) on a 6 cm petri dish filled with a medium (NGM) consisting of 1.7% agar, 0.25% bacto peptone, 50 mM NaCl, 25 mM potassium phosphate, pH 6.0. Young adults were then transferred on to a fresh plate devoid of bacteria for 2 min. Individual worms were then deposited on a 9 cm petri dish containing 9 ml of NGM. A radial gradient of temperature was created by placing a vial containing frozen acetic acid on the bottom of the plate and incubating the plate at 26°C for 90 min in presence of a constant humidity of 60%. Upon removal of the animal from the plate, tracks left on the agar surface were photographed.

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