Evaluation of the long-term memory for thermosensation regulated by neuronal calcium sensor-1 in *Caenorhabditis elegans*

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Abstract: Objective To evaluate whether the thermotaxis tracking model is suitable for assessing long-term memory (LTM) in the nematode *Caenorhabditis elegans*. **Methods** Animals were trained at 20 °C overnight in presence of food. The percentage of animals performing isothermal tracking (IT) behavior was measured at different time intervals after the training. **Results** The percentage of animals performing IT behavior, the numbers of body bends inside and outside the training temperature, and the expression patterns of AFD and AIY neurons were similar to those in control animals at 36 and 48 h after training; whereas when extending to 60, 72, and 84 h, locomotory behavior defects were observed in the assayed animals, suggesting that this thermal tracking model is feasible for analyzing LTM at 36 and 48 h after training. Moreover, the percentage of animals performing IT behavior was reduced at 18, 36, and 48 h after training in neuronal calcium sensor-1 gene (*nsc-1*) mutant animals compared with that in wild-type N2 animals. In addition, exposure to plumbum (Pb) significantly repressed the LTM at 18, 36, and 48 h after training in both wild-type N2 and *nsc-1* mutant animals. **Conclusion** The thermotaxis tracking model is suitable for evaluating the LTM regulated by NCS-1, and can be employed for elucidating regulatory functions of specific genes or effects of stimuli on memory in *C. elegans*.

Keywords: long-term memory; thermosensation; neuronal calcium sensor-1; *Caenorhabditis elegans*

1 Introduction

Memory is a most important mechanism according which animals perform behaviors to adapt to various environments and survive. It is used as a mental working space for performing a task. The term memory can be generally defined as encoding, storage and retrieval of learnt knowledge. Memory can be simply classified into short-term memory (STM), intermediate-term memory (ITM), and long-term memory (LTM). Over the past 30 years, there is a great deal of convergence in mechanisms of plasticity; and the molecular basis

of memory seems to be highly conserved across phylogeny^[1].

The nematode *Caenorhabditis elegans* has become an ideal organism model to unravel the complex memory process for several key features: the simple nervous system consisting of 302 neurons, subtle synaptic connections, and the complete wiring diagram covering chemical and electrical connections^[2]. The entire genome of *C. elegans* has been mapped and sequenced as well, allowing us use forward or reverse genetic techniques to elucidate the molecular mechanisms of memory. Three simple forms of memory in *C. elegans*, memory for thermosensation, memory for chemosensation, and memory for mechanosensation, have been studied extensively.

Thermotaxis is a particularly sophisticated, experience-dependent behavior in *C. elegans*; and this behavior in spatial thermal gradients is governed by worms' memory of

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previous cultivation temperature and food conditioning^[3]. When animals are placed on a thermo-gradient plate, they will isothermally track the memorized temperature consistent with the previous culture temperature, which gives a robust association between the temperature (conditional stimulus; CS) being in memory and nutritional state (unconditional stimulus; US)^[3]. Neuronal calcium sensor-1 (NCS-1), which is present in the AFD and AIY neurons, is essential for optimal isothermal tracking (IT) and is associated with learning and memory^[3].

Under appropriate training condition, *C. elegans* can show a long-lasting memory (> 24 h) for mechanosensation habituation^[1]. Nevertheless, this model described by Gomez *et al.*^[3] has not been investigated for its possible value in assessment of LTM. In the present study, we examined the possibility of using this thermotaxis tracking model to evaluate LTM.

2 Materials and methods

2.1 Chemicals All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The plumbum (Pb) concentration (50 $\mu\text{mol/L}$) were selected as previously described^[4].

2.2 Strains and culture conditions Wild-type N2 worms, *ncs-1(qa406)* mutant worms, and transgenic markers (*Pgcy-8::GFP*, labeling the sensory neuron AFD^[8]; and *Pttx-3::RFP*, labeling the interneuron AIY^[9]) were provided by the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. Worms were cultured and manipulated using standard methods^[5] and all strains were maintained at 20 °C. Gravid animals were washed off from plates into centrifuge tubes and lysed with a bleaching mixture (0.45 mol/L NaOH, 2% HOCl). Age-synchronous populations of larva (L4-stage) were obtained by collection^[6]. The L4-stage larva animals were washed with double-distilled water twice, followed by K medium once (50 mmol/L NaCl, 30 mmol/L KCl, 10 mmol/L NaOAc, pH 5.5).

Pb is a neurotoxic metal, and its damages on cognitive and memory are largely formed in exposed animals^[10]. To examine whether this thermotaxis tracking model is suitable for assessing the memory defects induced by a specific stimuli, we investigated the percentages of animals performing IT behavior in wild-type N2 worms and *ncs-1(qa406)* mutants exposed to Pb (50 $\mu\text{mol/L}$). The exposure was performed in 12-well sterile tissue culture plates and carried out

for 6 h at 20 °C in absence of food.

2.3 Thermotaxis tracking behavior assay The memory assay was performed basically as previously described^[3]. As shown in Fig. 1A, a cohort of synchronized young adult animals were incubated in nematode growth medium (NGM) plates at 20 °C overnight in presence of food (the training, unconditioned stimuli), and then washed twice with M9 buffer and transferred to an unseeded plate (conditioned stimuli) at 20 °C. After training, animals (approximately 50 worms) were individually deposited on a 9-cm petri dish containing 9-mL NGM for different time intervals (18, 36, 48, 60, 72, and 84 h). A frozen acetic-containing vial was placed on the center of the plate, and the plate was incubated at 26 °C for 90 min in constant 60% humidity to create a radial temperature gradient from 17 °C to 25 °C. The percentage of animals performing IT behavior were determined by the removal tracks of animals left on the agar surface upon the plate. All of the assays were replicated more than three times.

2.4 Body bends inside and outside the training temperature

The number of body bends was counted as previously described^[7]. Animals were picked onto another plate and scored for the number of body bends in an interval of 20 s. A body bend was counted as a change in the direction of the part of the animals corresponding to the posterior bulb of the pharynx along the *y* axis, assuming that the animal was traveling along the *x* axis. In the memory assay model, animals within the 20 °C region at the 18, 36, and 48 h time points were picked out for assay of body bends inside the training temperature. Animals were directly picked out from the training or Pb exposure plates for assay of body bends outside the training temperature. Fifteen animals were examined per treatment.

2.5 Fluorescence images Fluorescence images of neurons in the control and exposed adult animals were captured by a Zeiss Axiocam MRm camera equipped with a Zeiss Axioplan 2 Imaging System, with a $\times 40$ objective for cell body observation and a $\times 60$ objective for synapse observation.

2.6 Statistical analysis All data were analyzed by SPSS 13.0 software and expressed as mean $\pm$ SD. Student *t*-test was performed between control and treated or mutant animals. The probability levels less than 0.05 were considered statistically significant.

3 Results

3.1 Evaluation of LTM in the thermotaxis tracking model

Gomez *et al.*^[3] has studied IT behavior of worms at different time intervals (< 18 h) after training. To evaluate the possibility of using this thermotaxis tracking model to evaluate LTM, we analyzed the percentages of animals performing IT behavior at 36, 48, 60, 72, and 84 h after training. Compared with that at 18 h after training, the percentage of wild-type N2 animals performing IT behavior did not decrease at 36 and 48 h; whereas when extending to 60, 72, and 84 h, the percentage decreased significantly ($P < 0.01$). Especially at 84 h, the percentage of animals performing IT behavior was very low, suggesting that the thermotaxis tracking model was not suitable for evaluating LTM 84 h later after the training. (Fig. 1)

We next examined the locomotion behaviors. As shown in Fig. 2, the number of body bends outside the assay model was reduced significantly at 84 h after training ($P < 0.05$ vs 18 h). In addition, the number of body bends inside the training temperature in the memory assay model were also decreased significantly at 60, 72 ($P < 0.05$ vs 18 h), and 84 h ($P < 0.01$ vs 18 h) after training. These results indicated that the thermotaxis tracking model would be suitable for evaluating LTM at 36 h and 48 h post-training.

We further investigated the expression pattern changes of AFD and AIY neurons at 36 and 48 h after training. Thermotaxis of *C. elegans* is controlled by the sensory neuron

Fig. 1 Evaluation of long-term memory (LTM) in the thermotaxis tracking model. A: Model diagram for memory assay. STM, short-term memory. B: Comparison of the percentages of animals performing isothermal tracking (IT) at different time intervals after training. Data are expressed as mean $\pm$ SD. ** $P < 0.01$ vs 18 h. C: Dynamic changes of the percentages of animals performing IT behavior after training.

Fig. 2 Body bends of animals at different time intervals after training. Inside represents the body bends inside the training temperature in the memory assay model. Outside represents the body bends outside the memory assay model. Data are expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$ vs 18 h inside. # $P < 0.05$ vs 18 h outside.

AFD and the interneuron AIY^[8]. As shown in Fig. 3, the fluorescent intensities of AFD and AIY cell bodies were not changed at 36 and 48 h after training compared with control. Moreover, fluorescent intensities and distribution of synapses in AFD neurons did not change until 36 and 48 h. These results indicated the normal thermotaxis of worms at 36 and 48 h after training, and further confirmed the feasibility of using thermotaxis tracking model in evaluating the LTM at 36 and 48 h after training.

3.2 Mutation of NCS-1 suppressed LTM in the thermotaxis tracking model In the thermotaxis tracking model, the enhancer NCS-1 improved memory (< 18 h) via longer

Fig. 3 Expression patterns of AFD and AIY neurons of animals inside the training temperature at different time intervals after training. The left and right panels show the entire neuron of AFD or AIY, respectively. The circled regions indicate the cell bodies of neurons. L, left neuron; R, right neuron. The middle panel shows the synapses of AFD neuron. The arrowheads indicate the cell bodies. Con, control. Scale bar, 10 μ m.

Fig. 4 Mutation of NCS-1 results in the long-term memory (LTM) defects. A: Comparison of the percentages of animals performing isothermal tracking (IT) in wild-type N2 and *ncs-1 (qa406)* animals at different time intervals after training. Data are expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. B: Body bends of *ncs-1 (qa406)* animals at different time intervals after training. Inside represents the body bends inside the training temperature in the memory assay model. Outside represents the body bends outside the memory assay model.

Fig. 5 Exposure to Pb significantly suppresses the long-term memory (LTM) at different time intervals after training in wild-type N2 and *ncs-1 (qa406)* animals. Data are expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$ vs wild-type N₂.

retention, suggesting the essential role of NCS-1 in controlling memory behaviors^[3]. Similarly, the percentages of animals performing IT behavior at 18, 36, and 48 h post-training were reduced by 18.6%, 31.7%, and 44.4%, respectively, in *ncs-1* mutant animals compared with that in wild-type N2 animals (Fig. 4). The *ncs-1(qa406)* mutants did not have obvious body bend defects at 18, 36, and 48 h after training. Therefore, the proper calcium signaling via NCS-1 also defines a pathway essential for LTM behaviors in the thermotaxis tracking model.

3.3 Exposure to Pb significantly repressed LTM at different time intervals after training As shown in Fig. 5, exposure to Pb (50 $\mu\text{mol/L}$) repressed the percentages of animals performing IT behavior in both wild-type N2 and *ncs-1(qa406)* animals at 18, 36, and 48 h after training. Moreover, the LTM defects induced by Pb (50 $\mu\text{mol/L}$) exposure became more severe than those in *ncs-1(qa406)* animals at 36 and 48 h after training. The locomotion behaviors in wild-type N2 and *ncs-1(qa406)* animals were normal after they exposed to Pb (50 $\mu\text{mol/L}$) for 6 h (data not shown). Our data suggested that this thermotaxis tracking model can be employed for evaluating the memory defects induced by a specific stimuli.

4 Discussion

The thermotaxis tracking model was first described by Gomez *et al.*^[3]. In that model, they evaluated the associative learning and memory based on the thermotaxis properties of *C. elegans*. The behavior of worms on spatial thermal gradients is governed by a memory of its previous cultivation temperature^[11]. *C. elegans* can compare the ambient temperature with a memory of its previous cultivation temperature to enact different strategies to navigate thermal gradients, which contributes to their ability to compensate for prolonged changes in temperatures of their environments^[11,12]. Especially, in our present model, the thermal tracking behaviors are simply controlled by the functions of AFD/AIY synapses, and the AIY interneuron serves as an integrator of food and temperature inputs^[3]. Therefore, evaluation of the feasibility for this model in analyzing the LTM memory will be useful for exploring it as a classical model system in elucidating the cellular and molecular mechanisms of memory in *C. elegans*.

In the present study, we provided evidence to indicate the feasibility of this thermal tracking model in analyzing LTM

at 36 and 48 h after training. The time intervals of 60, 72, and 84 h after training would cause the locomotion behavior defects in assayed animals (Fig. 2), although we could still record obvious percentages of animals performing IT behavior at 60 and 72 h after training (Fig. 1). In contrast, the percentages of animals performing IT behavior, number of body bends inside and outside the assay model, and expression patterns of AFD and AIY neurons in animals at 36 and 48 h after training were all similar to those in control animals (Fig. 1-3). Recent physiological data have shown that patterns of AFD neuronal activity are closely correlated with the previous temperature, indicating that the memory of cultivation temperature may be encoded in patterns of AFD neurons^[13-15]. The LTM persisted by *C. elegans* is similar to that (48 h, LTM) in snail^[16]. With appropriate training parameters, *C. elegans* can also show a long-lasting form of memory (> 24 h) for mechanosensation^[1].

Moreover, we observed that mutation of NCS-1 suppressed the LTM in the thermotaxis tracking model. NCS-1 belongs to the intracellular neuronal calcium sensor family of EF-hand calcium binding proteins^[17]. Loss-of-function of NCS-1 does not restore IT, whereas NCS-1 overexpression enhances IT performance levels, and produces a memory (< 18 h) with slower extinction^[3]. NCS-1 can have a pre-synaptic role at the AIY interneuron synapses with AIZ and RIA or a postsynaptic function at the AFD/AIY synapses^[3]. Therefore, the proper calcium signaling encoded by NCS-1 may also define a pathway essential for LTM behaviors in the thermotaxis tracking model. In addition, these results suggest that the thermotaxis tracking model is suitable for examining the function of a specific gene in regulating memory.

We further provided evidence that this thermotaxis tracking model can be employed for evaluating the memory defects induced by a specific stimuli by analyzing the memory behaviors in wild-type N2 and *ncs-1(qa406)* animals exposed to Pb (50 $\mu\text{mol/L}$). Pb is a neurotoxic metal, and its damages on cognitive and memory are observed in many other exposed animals^[10,18].

Together, these results suggest that the thermotaxis tracking model is suitable for evaluation of the LTM regulated by NCS-1 in *C. elegans*. This thermotaxis tracking model can be employed for examining the function of a specific gene or stimuli in regulating or affecting memory behaviors.

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对秀丽线虫模型 NCS-1 调控的温度感知长时程记忆的评价研究

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摘要: 目的 评价线虫温度趋向性模型是否适用于长时程记忆的研究。方法 在 20℃ 含有新鲜食物过夜培养的训练条件下, 考察在不同时间点执行温度趋向性(isothermal tracking, IT)行为的虫体百分比。结果 训练后 36 与 48 h, 线虫的 IT 百分数、温度趋向性模型内外的头部摆动频率、以及 AFD 和 AIY 神经元的表达模式与对照组相似, 而训练后 60、72 和 84 h 的线虫则表现出运动行为上的缺陷, 表明温度趋向性模型适合于在训练 36 和 48 h 后的线虫中进行长时程记忆的考察。此外, 与野生型 N2 相比, 神经元钙传感蛋白(neuronal calcium sensor-1, NCS-1)的突变能够降低训练后 18、36 和 48 h 的虫体的 IT 百分数。而铅暴露可以显著抑制训练后 18、36 和 48 h 野生型和 *ncs-1* 突变体动物在不同时间点下的 IT 百分数。结论 温度趋向性模型适用于评价秀丽线虫中 NCS-1 参与调控的温度感知长时程记忆, 且该模型可用于针对线虫中参与调节或影响记忆行为的某一特定基因或刺激信号功能的揭示。

关键词: 长时程记忆; 温度感知; 神经元钙传感蛋白; 秀丽线虫