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Aversive Associative Learning and Memory Formation by Pairing Two Chemicals in *Caenorhabditis elegans*

Mihiro Shibutani[1], **Suteera Vibulyaseck**[1], **Ichiro N. Maruyama**[1]

[1] Information Processing Biology Unit, Okinawa Institute of Science and Technology Graduate University

Corresponding Author

Ichiro N. Maruyama
ichi@oist.jp

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Abstract

The nematode *Caenorhabditis elegans* is an attractive model organism to study learning and memory at molecular and cellular levels because of the simplicity of its nervous system, whose chemical and electrical wiring diagrams were completely reconstructed from serial electron micrographs of thin sections. Here, we describe detailed protocols for the conditioning of *C. elegans* by massed and spaced training for the formation of short-term memory (STM) and long-term memory (LTM), respectively. By pairing 1-propanol and hydrochloric acid as conditioned and unconditioned stimuli, respectively, *C. elegans* was successfully trained to form aversive associative STM and LTM. While naïve animals were attracted to 1-propanol, the trained animals were no longer or very weakly attracted to 1-propanol. Like in other organisms such as *Aplysia* and *Drosophila*, "learning and memory genes" play essential roles in memory formation. Particularly, NMDA-type glutamate receptors, expressed in only six pairs of interneurons in *C. elegans*, are required for the formation of both STM and LTM, possibly as a coincidence factor. Therefore, the memory trace may reside among the interneurons.

Introduction

Learning and memory are vital for animals to survive and reproduce by efficiently navigating changing environments. *C. elegans* is an attractive model organism to study learning and memory at the molecular and cellular levels because of the simplicity of its nervous system, whose chemical and electrical wiring diagrams were completely reconstructed from serial electron micrographs of thin sections[1,2,3].

C. elegans learns to associate cultivation temperature with starvation and migrates away from its growth temperature with an aversive memory lasting for several hours[4,5]. Conditioning *C. elegans* with sodium chloride (NaCl) in the absence of food leads to a reduction in chemotaxis toward NaCl[6,7,8]. When paired with food, butanone attraction is enhanced as a result of appetitive learning[9,10,11]. Although these phenomena are interpreted as associative learning and memory[10,12], the distinction between associative learning and non-associative sensitization, habituation, and adaptation is not clear in the

C. elegans learning and memory paradigm[13,14]. Indeed, animals conditioned with butanone and food deprivation (aversive conditioning) showed depressed coupling of the butanone sensory neuron AWC^{ON} to target neurons by insulin signals from other neurons, including AIA interneurons, while animals conditioned with butanone and food (appetitive conditioning) showed enhanced coupling of AWC^{ON} to target neurons[15]. The insulin signaling causes gene expression changes induced by nuclear EGL-4 and other transcriptional regulators[16,17]. Thus, this aversive and appetitive learning and memory has analogies with non-associative habituation and sensitization, respectively, of presynaptic sensory neurons in the gill-withdrawal reflex in *Aplysia*[18,19].

By pairing two chemicals as the conditioned stimulus (CS) and unconditioned stimulus (US), we and others have developed protocols for the conditioning of *C. elegans* to form associative learning and memory without using food or starvation as the US[20,21,22,23]. In the present study, the protocols are modified to condition animals with 1-propanol and hydrochloric acid (HCl, pH 4.0) as the CS and US, respectively, for aversive learning and short-term memory (STM) and long-term memory (LTM). Naïve *C. elegans* is attracted by 1-propanol[24] and repelled by acid[25]. When conditioned with a mixture of 1-propanol and HCl (pH 4.0), *C. elegans* was no longer or very weakly attracted to 1-propanol.

Protocol

1. Recipes

1. NGM agar plates (step 2.1.)
    1. To prepare 6 cm NGM plates, dissolve 2.5 g of peptone, 3 g of NaCl, and 17 g of agar in 850 mL of doubly deionized H_2O (ddH_2O). Bring the total volume to 972 mL with ddH_2O.
    2. After autoclaving, cool down to ~65 °C and add 1 mL of 5 mg/mL cholesterol dissolved in ethanol, 1 mL each of 1 M $CaCl_2$ and 1 M $MgSO_4$, and 25 mL of 1 M potassium phosphate (pH 6.0). After mixing well, dispense 8 mL each to 6 cm (in diameter) Petri dishes.
    3. Keep the plates with lids on a bench at room temperature (RT) for 1 day, and then keep them in plasticware in a cold room until use.
2. Prepare Luria-Bertani (LB) medium (step 2.1.) by dissolving 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1 L of ddH_2O. Adjust the pH to 7.0 with 5 N NaOH (several drops) and sterilize by autoclaving.
    1. Prepare LB plates by adding 15 g of agar to LB medium. After autoclaving, cool down to ~60 °C and dispense 12 mL each to 9 cm (in diameter) Petri dishes. Keep plates in plasticware in a cold room until use.
3. To make an animal collector (step 2.4.), attach nylon mesh (30 µm mesh size) to the bottom of a clear acrylic cylindrical pipe (3.5 cm in length, 3 cm in external diameter, 2 mm in wall thickness) with glue.
4. To make 0.25% aqueous gelatin solution (step 2.4.), dissolve 0.25 g of gelatin in 100 mL of ddH_2O. Sterilize by autoclaving.
5. Chemotaxis assay plates (step 5.1.)
    1. To make agar plates for chemotaxis assay, dissolve 15 g of agar in 993 mL of ddH_2O by autoclaving, and cool down the solution to ~65 °C.

2. Then, add 5 mL of autoclaved 1 M potassium phosphate (pH 6.0), 1 mL of 1 M $CaCl_2$, and 1 mL of 1 M $MgSO_4$ to the agar solution. All of these solutions are separately sterilized by autoclaving.

3. Dispense 10 mL of the mixed solution to a 6 cm Petri dish. Place these plates with lids on a bench at RT for two days, and then put them on wet paper towels in plasticware at RT until use. These plates can be used for up to 10 days.

6. To make chemotaxis assay buffer (step 5.4.), mix 5 mL of 1 M potassium phosphate (pH 6.0), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 993 mL of ddH_2O. Separately sterilize all these solutions by autoclaving.

7. To make a 40 mL CS/US mixture solution (1% aqueous 1-propanol and HCl [pH 4.0]) (step 3.1. and step 4.1.), add 0.4 mL of absolute 1-propanol and 4 µL of 5 M HCl (0.1 mM at final concentration) to 39.6 mL of ddH_2O. Keep the solution at RT.

8. To make ddH_2O, treat tap water 2x with water purification systems (refer to **Table of Materials**).

9. Prepare a liquid culture (OD_{600} = ~0.7) of *Escherichia coli* OP50 by inoculating a fresh colony with a toothpick into 10 mL of LB medium and incubating at 37 °C for 7-8 h. Bacteria cultivated for a longer period can affect the outcome of conditioning, perhaps due to secondary metabolites.

2. Preparation of synchronized *C. elegans*

1. Using standard methods[26], cultivate animals on 6 cm NGM plates (step 1.1.). NGM plates are prepared by spreading 0.2 mL of an *E. coli* OP50 liquid culture in LB medium (see step 1.9.) and incubating at RT for no more than 24 h (old bacteria can affect the outcome of conditioning).

   NOTE: *C. elegans* learning and memory are extremely sensitive to mechanical, chemical, and temperature stresses. Therefore, it is highly recommended to cultivate animals, maintain all reagents including water, and perform all assays at RT between 17 °C and 20 °C. Physical and mechanical stimulation such as vortexing, rough pipetting, and centrifugation must be avoided. Fresh 1-propanol should be used every 3 months at the longest for an unknown reason. Importantly, animals must be cultivated with plenty of food as starvation can seriously affect the outcome of conditioning.

2. On day 1, pick and place five well-fed gravid animals (put more mutant animals that lay eggs slowly) on each of the four 6 cm NGM plates with a platinum worm picker, and let them lay ~50 eggs for 3 h at RT to obtain a synchronized population of adult animals. Stop egg laying by removing parent animals from the plates with a platinum worm picker.

   NOTE: Seeded plates should be kept at RT to minimize stress to the animals.

3. Cultivate the animals at RT for about 5 days, which is the time it takes for animals to reach their mature adult stage, not the young adult stage.

   NOTE: The cultivation period between 4.5 days and 5.5 days should be adjusted depending on the conditions since younger adult animals are more sensitive to the chemicals used for conditioning than mature adult animals (**Supplemental Figure 1**). After conditioning, younger adult animals may show lower chemotaxis index (C.I.) values.

4. Collect ~200 adult animals in an animal collector (see step 1.4.) by washing each plate with 1 mL of

0.25% aqueous gelatin (step 1.4.) This aqueous gelatin prevents adhesion of the animals to the surface of plastics such as pipette tips.

5. Wash the animals in the collector with ddH_2O (step 1.8.) by very gently moving the collector up and down 2x in ~10 mL of ddH_2O. Repeat this process 2x more (3x in total) with ~10 mL of ddH_2O each to prevent bacterial contamination.
   NOTE: Bacterial contamination seriously affects the chemotaxis of animals.

3. Mass training for short-term associative learning and memory

NOTE: See **Figure 1** for the massed training workflow.

1. Gently immerse the animal collector containing ~200 animals in 40 mL of a mixture of 1% 1-propanol and HCl (pH 4.0 after mixing with 1-propanol; see step 1.7.) in a crystallizing dish for ~1 s.
   NOTE: For the control animals, do the same but dip only in 1% aqueous 1-propanol. It would be better to treat animals with HCl (pH 4.0) only as another control.
2. Wash the animals in the collector by very gently immersing the collector 1x in 10 mL of ddH_2O in a well of a 6-well tissue culture plate.
   NOTE: This washing step must be very gentle and only be done 1x as extensive washing may prevent learning.
3. Repeat steps 3.1. and 3.2. 10x without interruption (inter-trial interval [ITI], 0 min).
   NOTE: Use fresh ddH_2O each time at RT.
4. Place the collector on an *E. coli* OP50 lawn on a 6 cm NGM plate for 10 min at RT in order for the animals to rest.
5. Wash the animals in the collector with ddH_2O by very gently moving the collector up and down 2x in ~10 mL of ddH_2O. Repeat this process 2x more (3x in total) with ~10 mL of ddH_2O each to prevent bacterial contamination.
6. Proceed to chemotaxis assay as described below (step 5).

4. Spaced training for long-term associative learning and memory

NOTE: See **Figure 2** for spaced training workflow.

1. Gently immerse an animal collector containing ~200 animals in 40 mL of a mixture of 1% 1-propanol and HCl (pH 4.0 after mixing with 1-propanol; see step 1.7.) in a crystallizing dish for ~1.0 s.
   NOTE: Do the same with 1% aqueous 1-propanol only as a control. It would be better to treat animals with HCl (pH 4.0) only as another control.
2. Wash the animals in the collector by very briefly immersing the collector 1x in 10 mL of ddH_2O in a well of a 6-well tissue culture plate.
   NOTE: This washing must be very brief as extensive washing may prevent learning.
3. Place the collector on an *E. coli* OP50 lawn on an NGM agar plate in a 6 cm (in diameter) Petri dish for 10 min at RT in order for the animals to rest.
   NOTE: This resting for 10 min as ITI is crucial for the animals to consolidate memories for the formation of LTM.
4. Repeat steps 4.1.-4.3. 10x.
5. Wash the animals in the collector with ddH_2O by very gently moving the collector up and down 2x in ~10 mL

of ddH_2O, which is kept at RT. Repeat this process 2x more (3x in total) with ~10 mL of ddH_2O each to prevent bacterial contamination.

6. Proceed to chemotaxis assay as described below (step 5.).

5. Chemotaxis assay

1. Prepare agar plates for chemotaxis assay in 6 cm plastic Petri dishes (see step 1.5.).
2. Transfer animals in the collector (step 2.5.), which is placed on a flat surface of a plastic Petri dish lid, to a 2 mL microcentrifuge tube with 1 mL of 0.25% aqueous gelatin using a sawed-off pipette tip with a >1 mm (inner diameter) opening.
   NOTE: It is important to use a sawed-off pipette tip to minimize shear stress on animals.
3. Remove the supernatant from the tube after the animals settle at the bottom of the tube by gravity for ~1 min (do not centrifuge).
4. Gently resuspend the animals in 1 mL of chemotaxis assay buffer (see step 1.6.) and let them settle down by gravity to the bottom of the tube for ~1 min (do not centrifuge). Remove as much supernatant as possible by pipetting.
5. Meantime, diagonally spot 4 µL each of 5% aqueous 1-propanol at two places and spot 4 µL each of ddH_2O at two other places in the same way, as shown in **Figure 3A**. For chemotaxis assay of mutants that have lower sensitivity to 1-propanol, spot higher concentrations of aqueous 1-propanol that result in ~0.6 chemotaxis index (C.I.) values of naïve mutants, as shown in **Supplemental Table 1**.
   NOTE: It is important to complete the spotting procedures as quickly as possible. Spot 5% aqueous 1-propanol since 1% aqueous 1-propanol is too weak to attract animals in the chemotaxis assay. In contrast, use 1% aqueous 1-propanol for conditioning since animals treated with higher concentrations of aqueous 1-propanol than 1% show lower C.I. values.
6. Spot 6 µL portions of the animal suspension in chemotaxis assay buffer (step 5.4.) containing ~60 animals at the center of three plates for chemotaxis assay using a sawed-off pipette tip with a ~1.0 mm (inner diameter) opening. Remove liquid as much as possible with a laboratory tissue wick without touching the animals and place a lid on the plate.
   NOTE: It is important to complete these procedures as quickly as possible.
7. Allow the animals to move freely on the plate for 10 min at RT, and then transfer the plate into a glass Petri dish on ice for 3 min to stop chemotaxis. Then, keep the plate in a refrigerator until counting the number of animals on the plate.
8. Count the number of animals in four sections, except for those in the center circle, under a stereomicroscope and calculate the chemotaxis index (C.I.) using the equation shown in **Figure 3B**. From the C.I. values, calculate learning index (L.I.) values as the difference between the C.I. value of the reference animals and the C.I. value of the conditioned animals ($L.I. = C.I._{reference} - C.I._{conditioned}$).
   NOTE: The C.I. value of the reference animals ($C.I._{reference}$) is the mean value of the C.I. values of animals conditioned with 1% aqueous 1-propanol only.

Representative Results

C. elegans was conditioned by massed training to form short-term aversive associative memory by pairing 1% aqueous 1-propanol and HCl (pH 4.0) as the CS and US, respectively. According to the protocol described above, synchronized animals were cultivated on a bench at an RT of 18 °C for 5 days and were very gently washed 2x with ddH_2O at an RT of 18 °C. Then, the animals were conditioned with a mixture of 1% aqueous 1-propanol and HCl (pH 4.0) for 1 s. We also trained animals with ddH_2O only, 1% aqueous 1-propanol only, and HCl (pH 4.0) only as references. After the conditioning, the animals were washed 1x with ddH_2O. We repeated the conditioning 10x without interruption (no ITIs). Successful conditioning was achieved by repeating the procedure more than 7x up to 10x. Conditioning more than 10x resulted in less efficient learning[21]. After the training, the animals rested on bacterial food for 10 min at RT (18 °C). After being washed with ddH_2O 3x, the animals were transferred to a microcentrifuge tube by suspending in 0.25% aqueous gelatin and settled down to the bottom by gravity. After removing the supernatant as much as possible, the animals were gently resuspended in chemotaxis assay buffer and then allowed to settle down to the bottom of the tube by gravity.

After removing as much supernatant as possible, the animal suspension was spotted on the center circle of a chemotaxis assay plate, which was kept at an RT of 18 °C, and then animals were allowed to freely move on the plate for 10 min at an RT of 18 °C. C.I. values were calculated using the equation shown in **Figure 3B**. As shown in **Figure 4A**, animals conditioned with the mixture of 1% 1-propanol and HCl were no longer attracted to 5% 1-propanol spotted on agar plates for chemotaxis assay, whereas naïve and reference animals were similarly attracted to 5% 1-propanol. After the massed training (step 3.), the memory was no longer observed within 3 h[20]. Furthermore, the memory formed by the massed training was sensitive to cold shock[20]. These results demonstrate that *C. elegans* successfully formed aversive STM by massed training.

Animals were also conditioned by spaced training 10x with a 10 min ITI between the training steps (step 4.). During the ITI, the collector with animals was placed on a bacterial lawn on a 6 cm NGM plate at an RT of 18 °C. Animals conditioned by the spaced training with a mixture of 1% aqueous 1-propanol and HCl (pH 4.0) were no longer attracted to 5% 1-propanol in comparison to animals treated with 1% 1-propanol only, HCl (pH 4.0) only, or ddH_2O only (**Figure 4B**). After the spaced training, the animals retained the memory for more than 12 h[20,21]. Moreover, the memory did not form when animals were treated with translation or transcription inhibitors and was resistant to cold shock[20,21]. Therefore, *C. elegans* successfully formed aversive LTM by spaced training.

We also examined the effects of mutations in "learning and memory genes" on the formation of STM and LTM. The *crh-1* gene encodes the ubiquitous transcription factor cAMP-response element-binding protein (CREB), *glr-1* and *nmr-1* encode α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA)-type and *N*-methyl-D-aspartate (NMDA)-type glutamate receptor subunits, respectively, and *stau-1* encodes the double-stranded RNA-binding protein Staufen isoform. These genes play essential roles in classical conditioning in *C. elegans*, *Drosophila*, *Aplysia*, and mice. Using a mixture of 1% aqueous 1-propanol and HCl (pH 4.0), the formation of STM and LTM was dependent on all the genes (**Figures 5A,B**).

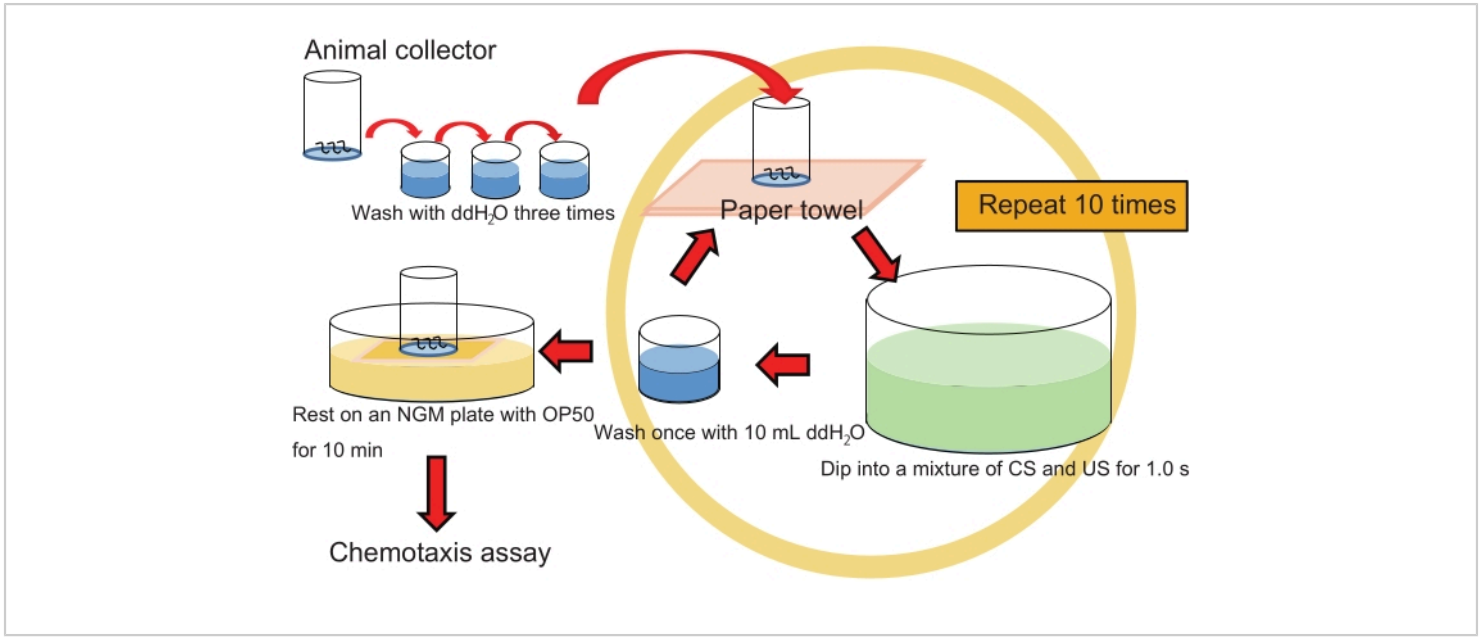

Figure 1: Experimental schematic of massed training. Please click here to view a larger version of this figure.

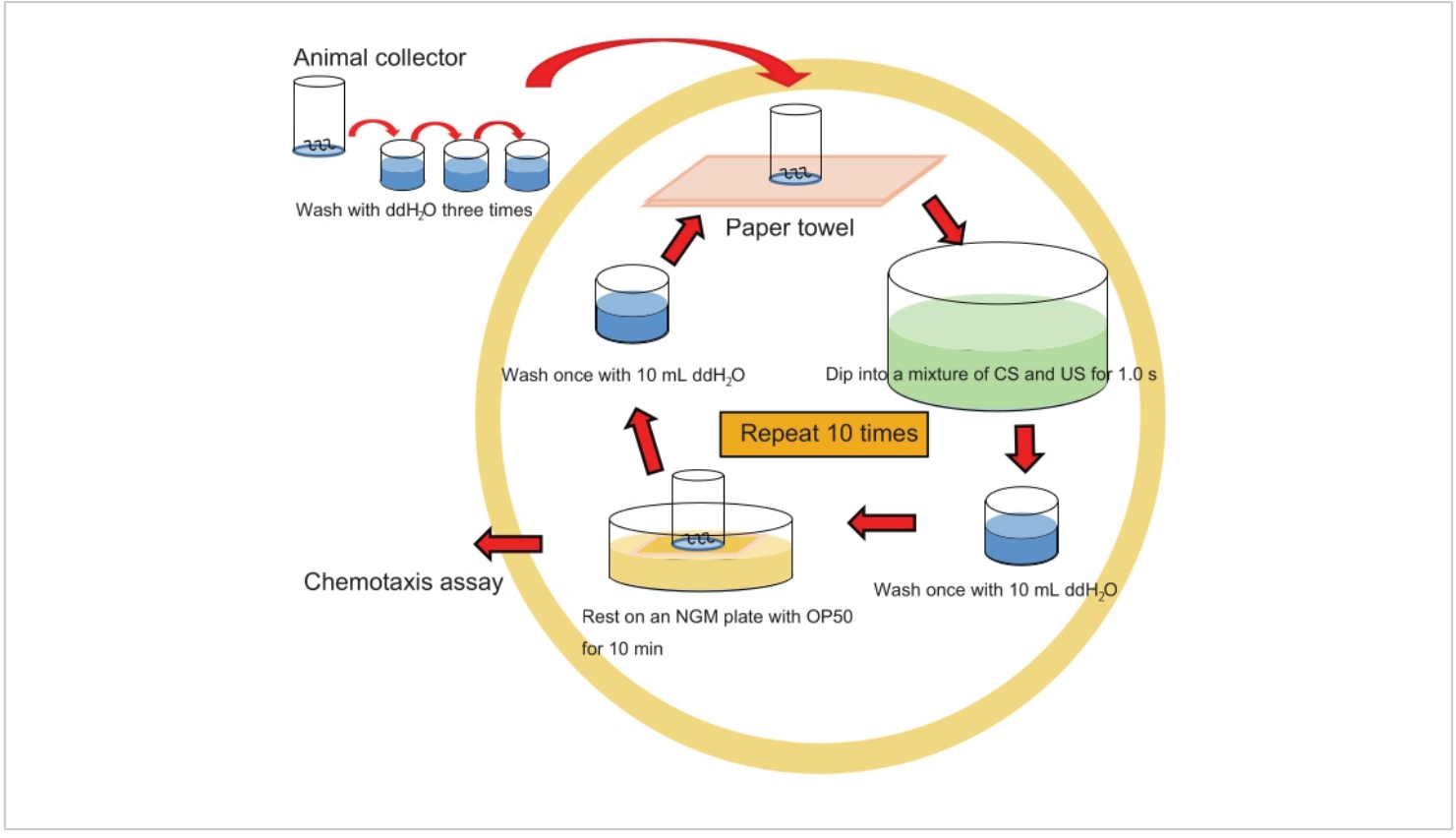

Figure 2: Experimental schematic of spaced training. Please click here to view a larger version of this figure.

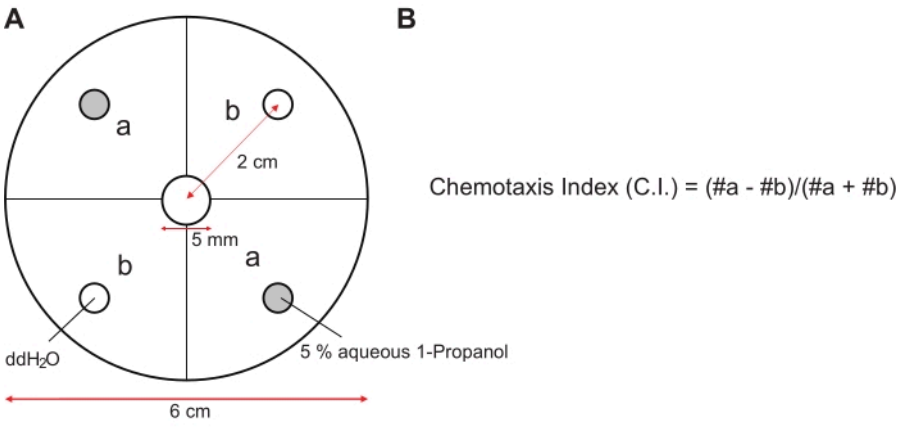

Figure 3: Chemotaxis assay and chemotaxis index. (**A**) Schematic representation of a chemotaxis assay plate. Petri dishes (6 cm in diameter) were separated into four areas as shown, and 4 µL each of 5% aqueous 1-propanol or ddH_2O were diagonally spotted at two places each, 2 cm away from the center. (**B**) Chemotaxis index values were calculated from the equation shown by counting the number of animals in areas "a" and "b" after the completion of the chemotaxis. Please click here to view a larger version of this figure.

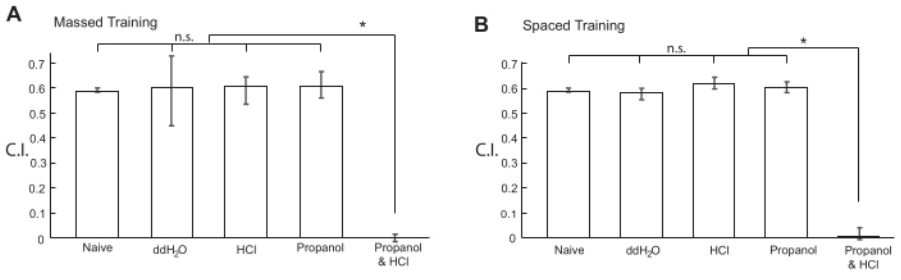

Figure 4: Chemotaxis index values of animals conditioned with chemicals. Synchronized wild-type N2 animals were conditioned with chemicals indicated by (**A**) massed training 10x or (**B**) spaced training 10x. Flowcharts of the massed and spaced training protocols used are shown in **Figure 1** and **Figure 2**, respectively. After the conditioning, the animals were free to move for 10 min on a 6 cm agar plate for chemotaxis assay at an RT of 18 °C. C.I. values were calculated using the equation shown in **Figure 3B**. Data for this figure are provided in **Supplemental Table 1**. Data from the naïve animals were replotted in both figure panels. Bar plot shows the 1st quartile, median, and 3rd quartile. Asterisks (*P < 0.05) indicate statistically significant differences determined by one-way ANOVA followed by Dunnett's multiple comparison test. Please click here to view a larger version of this figure.

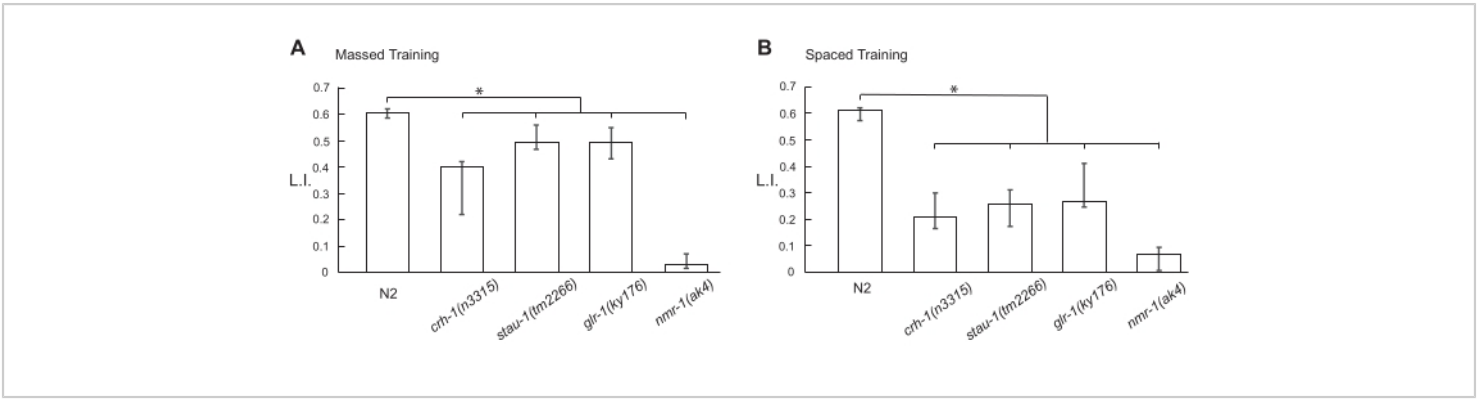

Figure 5: Learning index values of conditioned mutant animals. Synchronized wild-type N2 and mutant animals indicated were conditioned with a mixture of 1% aqueous 1-propanol and HCl (pH 4.0) by (**A**) massed training 10x or (**B**) spaced training 10x. Flowcharts of the massed and spaced training protocols used are shown in **Figure 1** and **Figure 2**, respectively. After the conditioning, the animals were free to move for 10 min on a 6 cm agar plate for chemotaxis assay at an RT of 18 °C. Data for this figure are provided in **Supplemental Table 2**. Bar plot shows the 1st quartile, median, and 3rd quartile. Asterisks (*P < 0.05) indicate statistically significant differences determined by one-way ANOVA followed by Dunnett's multiple comparison test. Please click here to view a larger version of this figure.

Supplemental Figure 1: Young adult animals are sensitive to chemical treatment. Day 4 and day 5 wild-type N2 animals after hatching were massed-trained 10x with HCl, pH 4.0, without interruption and were then assayed for chemotaxis to 5% aqueous 1-propanol. Bars are means ± S.E.M. (n = 19). Asterisks (*P < 0.05) indicate statistically significant differences determined by two-way ANOVA followed by the Tukey-Kramer post-hoc test. Please click here to download this File.

Supplemental Table 1: Data corresponding to Figure 4. Please click here to download this Table.

Supplemental Table 2: Data corresponding to Figure 5. Please click here to download this Table.

Discussion

In the present study, all the reagents were kept at an RT of ~18 °C on average, and animals were cultivated on a bench at the RT to avoid stress to the animals. Furthermore, all the experimental procedures were carried out at the RT. Animals were initially cultivated in an incubator at 20 °C and then conditioned on a bench at ~24 °C using reagents at the RT. Under these conditions, the outcomes of the conditioning were very variable. At the low RT, *C. elegans* grows slowly and should be cultivated longer than at 20 °C until the animals reach the mature adulthood stage, as younger adult animals are more sensitive to the chemicals used for conditioning than mature adult animals and may show lower C.I. values.

The most critical step for successful conditioning is the washing of animals with ddH_2O immediately after each chemical treatment. Therefore, mechanical and temperature stresses should be minimized by using sawed-off pipet tips, keeping reagents at RT, and very gently washing the animals by very slowly moving the animal collector up and down in ddH_2O. Thorough washing of the animals each time

after conditioning may affect learning and memory. The conditions of the chemotaxis assay plates also severely affect the outcomes. Too dry or too wet plates prevent smooth locomotion of the animals. Plates were prepared as described in step 1.; a good plate is one for which the 4 µL spots of ddH_2O or 5% aqueous 1-propanol are completely absorbed by the agar in approximately 5 min after spotting. As described above, animal ages are also critical for successful conditioning. Young adult animals are sensitive to mechanical and chemical treatment, resulting in variable outcomes, although very aged animals may not be suitable for conditioning either.

The shelf life of 1-propanol depends on brands and lots and is less than 3 months at RT. When the C.I. values of naïve animals get worse, it would be recommended to use fresh 1-propanol for the conditioning and chemotaxis assay.

The formation of memory by massed training was not affected by the treatment of animals with translation inhibitors (cycloheximide and anisomycin) and a transcription inhibitor (actinomycin D), while the formation of memory by the spaced training was markedly inhibited by the inhibitors[20,21]. Furthermore, the former memory decayed by cold shock, while the latter was retained for a longer period than the former and was resistant to cold shock. These results demonstrate that the former is STM and the latter is LTM, respectively[20,21]. However, the memory formed by the massed training may consist of STM and middle-term (intermediate-term) memory since STM is weakly dependent upon the CREB transcription factor (**Figure 5A**). This is consistent with the result that the STM was retained for more than 1 h[20,21]. The formation of both STM and LTM is highly dependent on *nmr-1*, which is expressed only in six pairs of neurons (AVA, AVD, AVE, RIM, AVG, and PVC) in *C. elegans*[27,28]. In these neurons, therefore, NMDA receptors may act as a molecular coincidence detector of 1% aqueous 1-propanol and HCl (pH 4.0) signals for synaptic plasticity, where the synaptic strengthening required for both STM and LTM can result from coincidental firing of pre- and post-synaptic neurons[29,30,31,32,33]. Therefore, the aversive associative memory may form among the interneurons.

The methods described in the present study should be applicable for appetitive olfactory learning and short-term and long-term associative memory using 1-nonanol as the CS and potassium chloride as the US[21]. It is interesting to compare the neuronal circuits that are involved in the formation of appetitive and aversive memories.

Disclosures

The authors declare no conflicts of interest.

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