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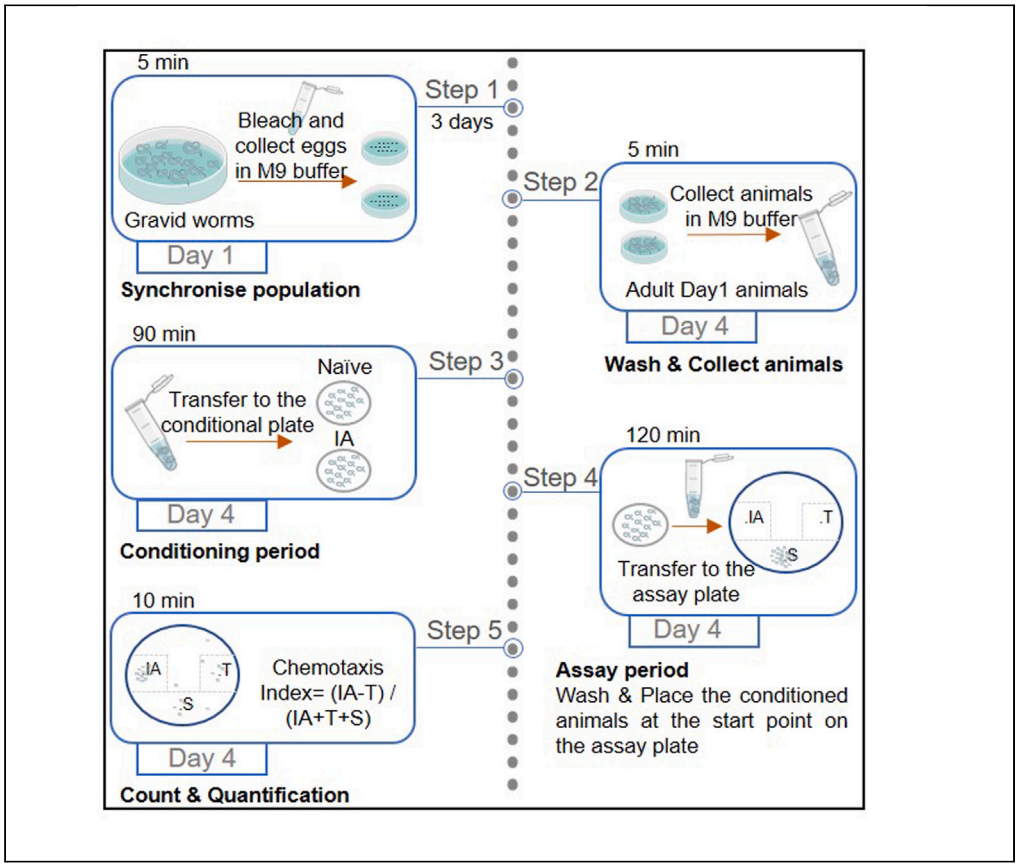
Protocol

Chemotaxis assay for evaluation of memory-like behavior in wild-type and Alzheimer's-disease-like *C. elegans* models



Here, we present an olfactory-dependent chemotaxis assay for evaluating changes in memory-like behavior in both wild-type and Alzheimer's-disease-like *C. elegans* models. We describe steps for synchronizing and preparing *C. elegans* populations and for performing isoamyl alcohol conditioning during starvation and chemotaxis assaying. We then detail counting and quantification procedures. This protocol is applicable to mechanistic exploration and drug screening in neurodegenerative diseases and brain aging.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Shu-qin Cao, He-Ling Wang, Konstantinos Palikaras, Nektarios Tavernarakis, Evandro Fei Fang

e.f.fang@medisin.uio.no

Highlights

In vivo chemotaxis-based memory to generate highly replicable data

Isoamyl alcohol (IA) used for conditioning during starvation and chemotaxis assaying

Applicable for examining multiple strains and treatment conditions simultaneously

A protocol that can be used for mechanistic studies of memory and brain aging

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Chemotaxis assay for evaluation of memory-like behavior in wild-type and Alzheimer's-disease-like *C. elegans* models

Shu-qin Cao,[1,6] He-Ling Wang,[1] Konstantinos Palikaras,[2] Nektarios Tavernarakis,[3,4] and Evandro Fei Fang[1,5,7,*]

[1]Department of Clinical Molecular Biology, University of Oslo and Akershus University Hospital, Lørenskog, Norway

[2]Department of Physiology, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

[3]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, 70013 Heraklion, Greece

[4]Department of Basic Sciences, Faculty of Medicine, University of Crete, 70013 Heraklion, Greece

[5]The Norwegian Centre on Healthy Ageing (NO-Age), Oslo, Norway

[6]Technical contact: shuqin.cao@medisin.uio.no

[7]Lead contact

*Correspondence: e.f.fang@medisin.uio.no
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SUMMARY

Here, we present an olfactory-dependent chemotaxis assay for evaluating changes in memory-like behavior in both wild-type and Alzheimer's-disease-like *C. elegans* models. We describe steps for synchronizing and preparing *C. elegans* populations and for performing isoamyl alcohol conditioning during starvation and chemotaxis assaying. We then detail counting and quantification procedures. This protocol is applicable to mechanistic exploration and drug screening in neurodegenerative diseases and brain aging.

BEFORE YOU BEGIN

The olfactory-dependent chemotaxis assay using *Caenorhabditis elegans* (*C. elegans*) has been developed and used by many labs, including Colbert and Bargmann,[1] Saeki, Yamamoto and Iino,[2] as well as Voglis and Tavernarakis.[3] This protocol has been modified and used routinely in the Tavernarakis, Palikaras, and Fang laboratories.

Memory impairment is one of the major pathological features of Alzheimer's disease (AD). Our previous studies confirmed memory impairment in both Amyloid-beta (Aβ) and tau *C. elegans* models of AD, and we have identified drug candidates for memory improvement using the isoamyl alcohol (IA)-based chemotaxis assay in worms.[4–6] The following protocol includes detailed procedures for the performance of an IA-based chemotaxis assay; the suggested experimental times for each step are based on the times used in the below-mentioned protocol. This protocol includes two *C. elegans* strains (the N2 wild type/WT, and an AD worm strain CK12 with Tau pathology) under two treatment conditions (Vehicle/Veh, and a testing drug nicotinamide riboside/NR), and thus comprises 4 groups, namely N2 (Veh), N2 (NR), CK12 (Veh), and CK12 (NR). The time used on each step should be adjusted based on the total group numbers used in each step of the experiment.

Preparation of nematode growth medium (NGM) plates

Timing: 1–2 h

This step explains in detail how to prepare various NGM plates including maintenance, drug, conditioning, and assaying plates, which will be used for the following steps in this protocol.

1. Prepare liquid NGM (the recipe in the materials and equipment section).
   a. Add 25 mL NGM per large-size maintenance plates.
   b. Add 8 mL NGM per medium-size drug containing plates.
   c. Add 8 mL NGM per medium-size conditioning plates.
   d. Add 18 mL NGM per large-size assaying plates.

⚠ CRITICAL: The large-size maintenance plates can be stored at 4°C for up to 1 month. Seed OP50 (1 mL) 3 days before use and leave at 20°C–22°C. The test drug plate preparation should be based on characteristics such as solubility and stability of the drug being tested. There are different ways of adding the drug to the NMG plates. A drug (either hydrophilic or hydrophobic) which is stable in aqueous solution and at 55°C can be mixed into the autoclaved liquid NGM directly before pouring the NGM plates (cooled down to around 55°C); these plates can be stored in 4°C and it is recommended that they be used within a week (again depending on the stability of the drug). For a hydrophilic drug with low stability in NGM plates, it is recommended that the drug be added by evenly spreading it over the top of the NGM plates (with food lawn) 12–24 h before use. For a hydrophobic drug with low stability in NGM plates but that is stable at 55°C, it can be mixed into the autoclaved liquid NGM directly before pouring the NGM plates (cooled down to around 55°C); after adding OP50, these plates should be used as quickly as possible. The memory experiment (conditioning and assaying) plates (steps 1c and 1d) must be prepared 72 h before the assay time and kept at 20°C–22°C, ensuring that all plates are properly dried and exhibiting a similar level of moisture; no OP50 should be added to these plates. For one biological experiment, four plates are needed per group: two medium-sized plates for conditioning and two large sized plates for assaying. To reduce variation in each experiment, it is necessary to use the same batch of agar mixture to prepare both conditioning and assay plates.

Note: For clarity, the dishes used in this protocol consist of 92 × 16 mm transparent dishes with ventilation cams, (termed the 'large sized' petri dishes), 60 × 15 mm transparent dishes with ventilation cams (termed 'Medium sized' petri dishes), and 35 × 11 mm transparent dishes with ventilation cams (termed 'Small sized' petri dishes). Plates must be prepared well in advance of the start time for these experiments. For drug plates (step 1b), if the testing drug is added to the medium plate during the pouring stage, to secure the best conditions for the testing drug, we suggest storing the plates at 4°C for up to 1 week. Three days before the experiment, add 200 μL of OP50 (1×10^{11} cfu/mL),[7] and leave at 20°C–22°C for three days. If no testing drug is pre-added to the medium plate, it can be stored at 4°C and should be used within a week. 72 h before the experiment, add 200 μL of OP50 (1×10^{11} cfu/mL), and leave at 20°C–22°C. 24 h before the experiment, add the drug to the plate. For this demonstrative case, nicotinamide riboside (NR, stock concentration 500 mM, detailed below) is used. To prepare a 2 mM NR plate, we add 32 μL of NR stock solution (500 mM) + 168 μL ddH_2O to help with even spreading on the medium-size NGM plate (8 mL) with OP50 lawn. In conditions such as limited availability of testing drug and to perform RNAi-related experiments, a small-size plate (with 3–4 mL of NGM added) could be used.

Preparation of OP50

Timing: 1–2 days

These steps describe preparation of *E. Coli* OP50 in liquid Luria broth (LB), and detection of concentration. The liquid OP50 with optimized concentration will be used to feed *C. elegans*.

2. Inoculate an individual colony of *E. Coli* OP50 in 3 mL liquid Luria broth (LB) and culture in a 37°C shaker (200 rpm) for 6 h.
3. Transfer to a baffled shake flask containing 500 mL LB, and culture in a 37°C shaker (200 rpm) 16–18 h.
4. Detect and optimize the concentration of OP50 using OD600 measurement.

Note: OP50 with a concentration of 1×10^{11} cfu/mL may be used during *ad libitum* feeding conditions.[7] OP50 should be stored at 4°C and used within 1 month.

Preparation of gravid adults for synchronized cultures

Timing: 3–4 h

These steps describe utilization of gravid adults to lay eggs and get enough progeny for synchronized population of *C. elegans* for further memory experiments.

5. Pick and place 15 to 30 gravid adults on a large-size maintenance plate and allow them to lay eggs for 3–4 h.
6. Remove the adults and cultivate the progeny in a 20°C incubator for 3–4 days before bleaching.

Note: The aim of these steps is to get 400–800 gravid worms (enough for 4 experimental groups; if more groups are needed, more egg plates should be prepared), which will provide enough fertilized eggs for experiments. It is critical to ensure that enough OP50 is provided to avoid starvation of the worms as starvation generates trans-generational effects via epigenetic modifications, leading to the production of inaccurate data. If the worm cohorts become starved, overcrowded, or contaminated (e.g., bacterial or fungal contamination), these worm plates should be excluded from the assay. For clarity, our internal definition of overcrowding is that worms occupy over 30% of the whole plate area; worm overcrowding affects their development and health.[8]

If the starved worms are used for propagation of experimental worms, make sure to maintain these starved worms for three generations in an *ad libitum* food condition to 'clean out' epigenetic changes and bring their condition back to 'normal'; 4th generation worms can once again be used for propagation of experimental worms. Also, remember to check whether or not a significant proportion of worms in the mixed population are gravid before bleaching. Some strains are developmentally delayed and will not have enough fertilized eggs after only 3 days' growth at 20°C; accordingly, a longer (for developmentally delayed worm strains) or shorter (for developmentally accelerated worm strains) waiting period should be applied to ensure an appropriate number of gravid worms are available before bleaching to collect eggs for population synchronization.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Agar	Merck (Sigma-Aldrich)	A1296
Bacto-Peptone	Gibco™ (Thermo Fisher Scientific)	211677
Bacto-Tryptome	Gibco™ (Thermo Fisher Scientific)	211699
Calcium chloride ($CaCl_2$, MW 110.98)	Merck (Sigma-Aldrich)	C1016

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cholesterol ($C_{27}H_{46}O$, MW 386.65)	Merck(Sigma-Aldrich)	C8667
Dipotassium hydrogen phosphate (K_2HPO_4, MW 174.18)	Merck (Sigma-Aldrich)	P3786
Disodium hydrogen phosphate (Na_2HPO_4, MW 141.96)	Merck (Sigma-Aldrich)	1.06585
Isoamyl alcohol ($C_5H_{12}O$, MW 88.15)	Merck (Sigma-Aldrich)	8.22255
Magnesium sulfate ($MgSO_4$, MW 120.37)	Merck (Sigma-Aldrich)	M7506
Nicotinamide riboside chloride (NR, MW 290.70)	NIAGEN (ChromaDex)	40C910-19237-21
Potassium dihydrogen phosphate (KH_2PO_4, MW 136.09)	Merck (Sigma-Aldrich)	1.04871
Sodium azide (NaN_3, MW 65.01)	Merck (Sigma-Aldrich)	S2002
Sodium chloride (NaCl, MW 58.44)	Merck (Sigma-Aldrich)	S9888
Sodium hydroxide (NaOH, MW 39.40)	Merck (Sigma-Aldrich)	106498
Sodium hypochlorite (NaOCl)	Sigma Aldrich	1056142500
Ethanol (C_2H_5OH)	Merck (Sigma-Aldrich)	1009802500
Yeast Extract	Merck (Sigma-Aldrich)	8013-01-2
Experimental models: Organisms/strains		
C. elegans: Strain N2: wild-type Bristol isolate (hermaphrodite)	Caenorhabditis Genetics Center	https://cgc.umn.edu/
C. elegans: Strain CK12: ls[paex-3tau4R1N(P301L); pmyo-2GFP] (hermaphrodite)	Steven Zuryn Lab	http://zurynlab.com/
Software and algorithms		
Excel	Microsoft Excel	https://www.microsoft.com/en-us/microsoft-365/excel
GraphPad Prism	GraphPad	https://www.graphpad.com/
Other		
Medium-size Petri dish, 60 × 15 mm, transparent, with ventilation cams	Sarstedt	82.1194.500
Large-size Petri dish, 92 × 16 mm, transparent, with ventilation cams	Sarstedt	82.1473.001
1.5 mL microcentrifuge tubes	VWR	525-0990
10 µL pipette filter tips	Starlab	S1121-2710-C
200 µL pipette filter tips	Starlab	S1120-8710-C
1000 µL pipette filter tips	Starlab	S1126-7710-C
P10 pipette	Eppendorf	3123000020
P200 pipette	Eppendorf	3123000055
P1000 pipette	Eppendorf	3123000063
Glass pipette	Corning	7095D-5X
Parafilm M all-purpose laboratory film	Amcor	PM 992, PM996
0.2 µm syringe filter	Corning	CLS431219
Syringe	Merck	Z116866
15 mL conical centrifuge tubes	Falcon	10773501
50 mL conical centrifuge tubes	Falcon	10203001
10 mL serological pipettes	Fisherbrand	11879181
25 mL serological pipettes	Fisherbrand	11839660
Pipette controller	Integra	Pipetboy acu2
Baffled shake flask	Merck	F0402
1 L laboratory bottle	Merck	BR122562
Kimwipes™ disposable wipers	Merck	Z188956
Stereozoom microscope	Zeiss	Stemi 508
Cooled incubator	PHCbi	MIR-554
Multitron incubator shaker	INFORS HT	–

MATERIALS AND EQUIPMENT

K buffer (pH6)		
Reagent	Final concentration	Amount
Potassium dihydrogen phosphate	0.75 M	102 g
Dipotassium hydrogen phosphate	0.33 M	58 g
ddH_2O	N/A	1 L
Sterilize by autoclaving. Autoclaved K buffer can be stored at 20°C–22°C for 6 months.		

M9 buffer		
Reagent	Final concentration	Amount
Potassium dihydrogen phosphate	22 mM	3 g
Disodium hydrogen phosphate	42.3 mM	6 g
Sodium chloride	85.5 mM	5 g
ddH_2O	N/A	1 L
1 M Magnesium sulfate	0.1%	1 mL
Sterilize by autoclaving. Cool down the autoclaved M9 buffer to 20°C–22°C and add 1 mL of 1 M $MgSO_4$. The prepared M9 buffer can be stored at 20°C–22°C for 12 months.		

LB broth (pH7)		
Reagent	Final concentration	Amount
Bacto-Tryptone	1% w/v	10 g
Yeast extract	0.5% w/v	5 g
Sodium Chloride	0.17 M	10 g
ddH_2O	N/A	1 L
Follow a standard recipe.[9] Sterilize by autoclaving, and store at 4°C for up to 3 months.		

NGM for agar plates		
Reagent	Final concentration	Amount
Agar	1.7% w/v	17 g
Bacto-Peptone	0.25% w/v	2.5 g
Sodium chloride	0.3% w/v	3 g
ddH_2O	N/A	1 L
Add additional ingredients aseptically (for 1 L)		
1 M Calcium chloride	1 mM	1 mL
5 mg/mL Cholesterol (in ethanol)	5 µg / mL	1 mL
1 M Magnesium sulfate	1 mM	1 mL
K buffer (pH 6)	2.5%	25 mL
Sterilize by autoclaving and let cool to 55°C in a water bath for fresh use. Autoclaved NGM can be storedat 4°C for 3 months. Then, defrost the NGM solution by heating 15–20 min using microwave when needed, and cool down to 55°C in the water bath. Add additional ingredients in liquid NGM at 55°C before pour into plates.		

Alkaline hypochlorite (Bleaching) solution		
Reagent	Final concentration	Amount
5% Sodium hypochlorite	1%	2 mL
5 M Sodium hydroxide	0.5 M	1 mL
ddH_2O	N/A	7 mL
Store in 20°C–22°C and use within 2 months.		

- 20 mM Sodium azide: dissolve 13 mg of sodium azide in 10 mL ddH_2O.

The prepared sodium azide solution may be stored for up to 2 months at 20°C–22°C.

- 500 mM nicotinamide riboside (NR): dissolve 145 mg of NR in 5 mL ddH_2O.

Filter for sterilization. Fresh prepare or aliquot (0.5 mL) for storage. If not using immediately, the aliquoted NR solution must be stored at −20°C and used within 1 month. Avoid freezing and thawing more than 2 times.

- 1 M Calcium chloride: dissolve 5.55 g of calcium chloride in 50 mL ddH_2O.

Sterilize by autoclaving after preparation. The sterilized calcium chloride solution must be stored at 20°C–22°C and used within 6 months.

- 5 mg/mL Cholesterol solution: dissolve 250 mg of cholesterol in 50 mL 95% ethanol.

The Cholesterol solution must be stored in 20°C–22°C and used within 6 months.

- 1 M Magnesium sulfate: dissolve 6.02 g of magnesium sulfate in 50 mL ddH_2O.

Sterilize by autoclaving. The autoclaved solution must be stored at 20°C–22°C and usedwithin 6 months.

- 5 M Sodium hydroxide: dissolve 9.85 g of sodium hydroxide in 50 mL ddH_2O.

The sodium hydroxide solution must be storedat 20°C–22°C and used within 2 months.

STEP-BY-STEP METHOD DETAILS

Synchronize populations and place on designated conditional plates

Timing: 10–30 min

These steps include collection of eggs by bleaching of 400–800 gravid worms from each strain, and then placing 150–200 eggs on each designated conditional plate (with/without drugs) to generate synchronized populations. This is a crucial step for memory assays, and minimizes age-dependent variability.

1. Collect *ad libitum*-fed animals of mixed population (with 400–800 gravid worms) from the large-size worm maintenance NGM plate(s) using 1.5 mL M9 buffer.
2. Transfer them to a 1.5 mL tube (pre-labeled with group name).
3. Remove the supernatant after the majority of adult worms have sunk to the bottom of the tube due to gravity (normally within a minute).
4. Wash the worms 1–2 times using M9 buffer to clean remaining bacteria out and discard the supernatant.

   Note: For step3, we suggest avoiding 'spinning down' as this will force any OP50 and small larvae down into the worm 'pellet' in the bottom of the tube.

5. Use the bleaching solution to collect eggs for the purpose of generating synchronized nematode populations.
   a. Add 1 mL of bleaching solution. Vortex the 1.5 mL tube for 30 s to 1 min (to break the worm's hard cuticle), and manually shake the tube in a gentle way.

b. Pellet the eggs by centrifuging at 1,000 × *g* for 30 s in 20°C, and carefully remove the supernatant using a pipette.
c. Wash the egg pellet with 1 mL M9 buffer and centrifuge the pellet at 1,000 × *g* for 30 s in 20°C.
d. Repeat step 5c three times to completely wash away the bleaching solution.

⚠ CRITICAL: Bleaching is a way to obtain a highly synchronized population of worms for the following experiments. The principle of this method relies on the biology of the adult worm which makes its body sensitive to the bleaching buffer while the embryos are 'relatively' protected by their hard eggshell during short periods of exposure. However, long-term exposure to the bleaching solution will reduce the hatching ratio of eggs as well as causing developmental and health issues in hatched worms.[10] Thus, it is suggested to limit the exposure of the eggs to the bleaching solution to less than 5 min. Additionally, repetitive bleaching of worm cohorts kept for colony maintenance and propagation must be avoided.

Note: Manually shake the tube to further dissolve the worms' cuticle, maximizing the numbers of eggs released from the dissolving body.

6. Re-suspend the eggs in 500 μL M9 buffer.
7. Pipette 20 μL of egg-containing M9 buffer (150–200 eggs) and spread the solution in a prepared medium plate (with OP50 lawn, with/without test drugs).
8. Wait for 5 min to enable M9 solution to be adsorbed by the NGM.
9. Seal the plates with paraffin to maintain humidity and reduce chances of contamination.
10. Place them upside down in a 20°C incubator for 3 days until the animals reach the 1-day of adulthood.

⚠ CRITICAL: Worms should never be starved. A good control of the total number of animals/plate is essential to avoid starvation; we suggest keeping 150–200 worms on a medium-size maintenance plate. Early starvation will halt the development of the worms, and late starvation will induce unexpected changes that will then cause experimental failure. Additionally, overcrowded and/or contaminated cultures should not be assayed.

Note: In step 6, adjust the volume to achieve a concentration of 75–100 eggs / 10 μL. For step 7, to attain enough adult worms for the experiments, we suggest the preparation of four plates/group. For step 10, the day after L4 is considered adult Day 1, not adult Day 0 as used in some laboratories. For worm strains with slower/faster development speed, days of incubation to reach adult Day 1 should be individually observed.

Collection of synchronized population to perform conditioning assay using isoamyl alcohol (IA)

Timing: 100–120 min

These steps show how to collect and transfer synchronized populations of worms from each treated group to conditioning plates. For each group, worms are equally divided into two plates (no food) for 90-min conditioning (naïve and IA).

11. Wash the *ad libitum*-fed adult Day 1 (or worms of other age) animals off an NGM plate using 1.5 mL M9 buffer.
12. Transfer the worms to a 1.5 mL tube using a 1 mL pipette tip.
13. After the worms have settled to the bottom via gravity, gently wash the pellet of worms with M9 buffer three more times.

⚠ CRITICAL: For step 13, a significant portion of nematodes may stick to the inner wall of plastic 1 mL pipette tips during transfer. Glass pipettes are an alternative that will reduce

loss of worms during transfer steps. Repeat the washing step three times to remove/decrease the bacterial population. Do not spin the tube down, but just let the worms settle down by gravity. To avoid overstressing the nematodes, the total washing time should be limited to 5 min.

14. Discard most of the M9 buffer and keep 200–300 μL of buffer with worms for the next step.
15. Transfer worms to a conditioning plate using a pipette and dry off excess buffer using a small piece of Kimtech™ delicate task wipe under a microscope.
    a. For naïve plates (no OP50), seal plates with parafilm, and place the plates upside down (for uniformity in the next step) before step 15b.
    b. For IA plates (no OP50), place a drop of pure IA (10 μL) on the internal surface of the IA plate's lid and spread it around evenly. Seal plates with parafilm immediately and place the plates upside down.
    c. Incubate all plates at 20°C–22°C for 90 min.

⚠ CRITICAL: As worms are very sensitive to temperature shifts and smells, which could lead to changes in olfactory sensitivity and behavioral scores, it is important to keep a stable temperature and a clean environment during all experimental stages. It is necessary to avoid direct exposure of the conditioning plates to sunlight or airflow from an air-conditioner; other than the IA smell, the experimental environment should have no other noticeable smells, such as the smells of shampoo, perfume, or any other pungent chemicals. To enable even and quick IA dispersal within the conditioning plate, it may help to release the 10 μL IA slowly in a spiral pattern rather than releasing it as a single drop.

Prepare chemotaxis experimental plates

Timing: 10–30 min

These steps detail preparation of chemotaxis experimental plates including marking of plates with 'gradient' (IA), 'trap' (T), and 'start' (S) points, labeling of the designated information (strain name, drug information, naïve/IA condition), and adding of sodium azide (20 mM, 15 μL) at both point 'IA' and point 'T' at 15 min before next step.

16. Mark chemotaxis assay plates to define the points for IA, T, and S.

Note: To do this, draw an isosceles triangle with two upper corners (to be labeled 'IA' and 'T') equidistant (4.5 cm) from the start corner, while the distance between 'IA' and 'T' should be 6.5 cm (Figure 1).

17. Mark the designated name on each assay plate (Figure 1).
18. 15 min before step 19, add sodium azide (20 mM, 15 μL) at both point 'IA' and point 'T'.

Chemotaxis assay

Timing: 150–180 min

These steps show how to collect and place the worms on the assaying plates after the conditioning period. Worms are then allowed to explore the area and the number of worms in each zone is counted after 2 h.

19. Collect worms into 1.5 mL tubes from each conditioning plate via washing each plate with 1.5 mL M9 buffer.

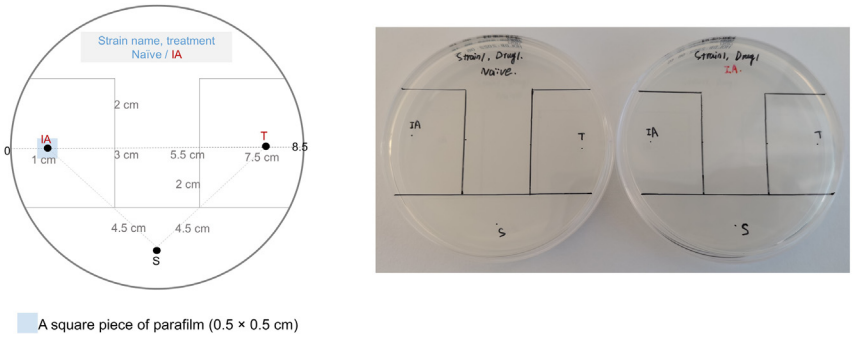

Figure 1. Demonstration image of assaying plate

A chemotaxis assay plate (large-size petri dish with NGM, 8.5 cm internal diameter, no OP50) with the three designated 'dots'. Left is a schematic model while the right two plates are demonstrations of an experiment. 'IA', isoamyl alcohol; 'T', trap; 'S', start point (location where all the worms being tested are loaded).

⚠ CRITICAL: A glass pipette is recommended for transferring of the worms since the animals may stick to the inside of plastic pipette tips. Make sure to use a new pipette/pipette tip for each group to avoid cross-contamination. To avoid unexpected stress and harm, the total washing time should be limited to 5 min. To save time and avoid mistakes (such as mixing the different groups), it is recommended that you pre-label the 1.5 mL tubes and plates carefully up to one day ahead of time.

20. Transfer the worms with an individual pipette from the 1.5 mL tubes to point 'S' on the matched assay plates.
21. Dry the excess buffer using a small piece of Kimtech™ delicate task wipe.
22. Place a square piece of parafilm (size of 0.5 × 0.5 cm) at the 'IA' spot on the agar (Figure 1).
23. Add 4 μL of diluted IA (ratio 1:50 in water) on top of the parafilm.
24. Immediately seal the plates using parafilm and keep the plates at 20°C–22°C for 2 h.
25. After 2 h, count the number of worms at each point under a microscope and record the numbers accordingly.

⚠ CRITICAL: For step 22, it is important to add the diluted IA on the parafilm as it will be quickly absorbed by the NGM agar if plated directly on the 'IA' spot. One may wonder why a similar square of parafilm is not placed to the 'T' spot. This is because we aim to 'trap' the worms with poor memory by sticking them to the 'T' spot once they arrive there. The addition of the parafilm square would prevent this from happening, and therefore we avoid this step. For step 24, in any labs with a big temperature shifts during the experimental period (normally 4–6 h), it is suggested that the experiments be performed inside a temperature-controlled incubator set to between 20°C - 22°C. For step 25, it is not uncommon for a small portion of worms to remain at 'S' even at the end of the experiment. In our experience, most of them are injured worms, likely due to physical damage during the experimental procedures; if this is the case, these 'injured' worms can be excluded for statistics. If the number of injured worms reaches to over 20% of the total worms in a group, and/or this is happening in many groups, it is recommended to repeat the experiment.

Data analysis and figure preparation

Timing: 10–60 min

These steps describe calculation of the chemotaxis index using a simple equation.

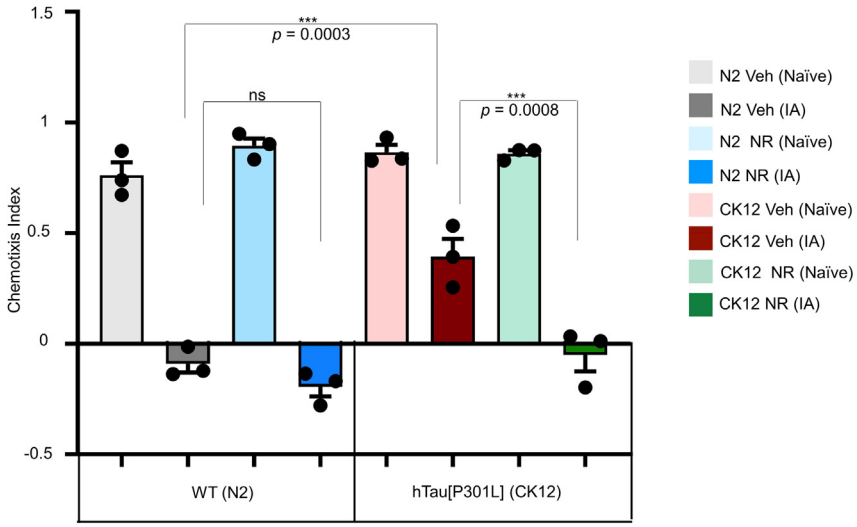

Figure 2. The NAD$^+$ precursor NR mitigates memory loss in the AD-like hTau[P301L] (CK12) *C. elegans* model

N2 and CK12 worms were treated with vehicle (Veh) or NR (2 mM) from egg hatching to adult Day 1, followed by IA-based chemotaxis on the same day. Compared with the N2 worms (column 2 from the left), the CK12 worms had reduced memory (column 6 from the left). Although NR did not improve memory in the N2 worms (column 4 from the left), it dramatically improved memory in the CK12 worms (column 8 from the left) compatible to that of the N2 worms (Veh) (column 2 from the left). The data are shown as mean ± S.E.M. from three biological repeats with one dot denoting one biological repeat. Two-way ANOVA followed by Tukey's multiple comparisons test was used for data analysis. ns, no significance; *p < 0.05; **p < 0.01; and ***p < 0.001. Data were generated specifically for this protocol paper and were not published elsewhere.

26. Calculate chemotaxis index according to the equation = (IA - T)/ (IA + T + S).
27. Analyze and graph the chemotaxis index data utilizing software such as GraphPad Prism.

⚠ CRITICAL: For data validation and statistics, at least three biological replicates are needed.

EXPECTED OUTCOMES

C. elegans utilizes olfactory and chemosensitivity to search for food, determine directions, and find mates, and shows many chemotaxis-related behaviors.[11,12] In this assay, naïve (IA-free) and IA conditions are applied for each group. In the conditioning step (step 15), both conditions are food-free, but IA is used to emulate food resources and to test associative learning, memory, and olfactory adaptation in examining *C. elegans strains*; after conditioning, worms with learning and memory capacity will associate IA with 'no food' and will crawl in the direction opposite to the 'IA' spot, that is, towards the 'T' spot and then become trapped there. Therefore, a lower score (for N2 wild type worms, the score could be –0.4 to 0) correlates with better learning/memory. Conversely, animals from naïve conditioned plates preferentially reside in the 'IA' spot as this is their first time being exposed to IA, giving a positive score (for N2 wild type worms, the score could be between 0.5-0.9).[1–3]

In our demonstration experiment (Figure 2), we investigated whether the NAD$^+$ precursor NR (2 mM) could improve memory in a *C. elegans* model of AD, hTau[P301L] (CK12) worms. N2 worms were used as healthy controls. We treated N2 and CK12 worms with 2 mM NR from egg hatching stage to adult Day 1, followed by completion of the memory assay on adult Day 1. The N2 animals (Veh) of the naïve group showed a positive score of 0.76 ± 0.06, suggesting normal chemotaxis in the N2 worms (Figure 2); after IA-conditioning, the N2 animals (Veh) showed a negative score, indicating these worms had punishment-induced aversion towards starvation-paired chemosensitivity. A lower

numerical value in the 'IA' condition was found in comparison to those in the naïve condition, indicating a high chemotaxis-associated memory in the N2 worms. In comparison, memory in the CK12 worms was damaged as the score was 0.39 ± 0.08 in the IA conditioned group, which was higher than that of N2 (IA conditioning, -0.09 ± 0.04) ($p = 0.0003$). NR reduced the memory score from 0.39 ± 0.08 (Veh) to -0.05 ± 0.07 ($p = 0.0008$) in the IA-conditioned CK12 worms, indicating that NR increased memory in the CK12 worms. These data are comparable to previously published data,[4] indicating the high stability of this assay. Importantly, NR-induced memory preservation in AD worms is conserved in AD mice[13]; this cross-species conservation is also true for other small molecules,[5] highlighting the translational value of the AD worm-based chemotaxis assay for the screening of anti-AD drug candidates.

Collectively, this IA-based chemotaxis assay in worms is simple, fast, and highly cost-effective for mechanistic studies of learning and memory, and for initial screening of drug candidates against AD and potentially other neurodegenerative diseases. It is likely that memory data from the worms may, to some extent, apply to mammals, highlighting its value in translational studies.

LIMITATIONS

This worm-based chemotaxis assay is a sensitive and challenging assay, with a potential for large variations in results due to changes in worm conditions (e.g., food availability, population density, temperature fluctuation during maintenance), worm handling methods during the experiments (variation such as harsh or gentle washing could change the results), and environmental factors (e.g., substantial temperature shifts, exposure to sunlight, excessive airflow or exogenous smells during the experiments). Therefore, good quality control is required. We suggest the below tips to help reduce data variation. First, the amount of OP50 seeded per plate should be optimized to ensure an *ad libitum* food condition. Second, it is necessary to maintain worms in temperature/humidity-controlled incubators as well as keeping the lab at a constant temperature of around 20°C–22°C. Third, to avoid the influence of exogenous smells other than IA during the assay, do not use perfume, fragrant body lotion, or wear smelly clothes during the entire experimental period. If working in an open laboratory, make sure no other pungent chemicals are hanging in the air. Additionally, it is recommended that a fresh IA solution is prepared on the day of the experiment to ensure the most stable IA chemotaxis effect.

The aforementioned experiments are designed for the evaluation of memory in adult Day 1 worms. However, this protocol may also be applied for the evaluation of memory of worms at older ages, such as adult Day 2–5. If worms at older age are used for experiments, make sure there is enough OP50 to avoid starvation, or change the plates periodically (about every 2 days). If memory assays are to be performed in older worms (e.g., post Day 1), daily transfer of worms to a new food plate (with/without drug based on the settings) or the adding of 5-fluro-2'-deoxyuridine (FUdR) (from late L4 stage at a dose of 75 μM, to stop the hatching of eggs laid by the gravid worms) is required. These extra steps could avoid the mixing of the synchronized parental worms with their progeny. In addition, since this chemotaxis assay is dependent on the movement of the worms to the designated regions, it is suggested that researchers avoid using worms older than adult Day 5 or worms with impaired movement; as this chemotaxis assay is dependent on locomotion (to move from 'S' to 'IA' or 'T', Figure 1), worms with locomotion defects (due to aging or genetic reasons) are not suitable for this assay. Similarly, this method should not be used for any worm strains with severe movement restrictions, even at a young age (e.g., adult Day 1). For worms with these issues, an alternative, less movement-intense method for monitoring the 'learning index', should be considered.[14]

As with most behavior experiments, the IA-based chemotaxis assay is an approach that provides valuable information for mechanistic studies in a broad array of fields, such as biology, neuroscience, medicine, gerontology, and drug discovery. For translational studies, experimental discoveries in worms should be validated in higher model systems, such as *Drosophila*, mice, and human cells.

For the purpose of drug development against AD and aging, data from the worms should also be further verified using complementary experiments in different experimental models, such as the Y-maze or Morris water maze in murine AD models.[5]

TROUBLESHOOTING

Problem 1

Worms are starved on any steps before the 'conditioning' step.

Potential solution

An *ad libitum*-fed synchronized worm population is a prerequisite for the experimental success and accuracy of data collection. To avoid worm starvation during any step, sufficient OP50 should be added to each plate; the appropriate feeding level should be determined through the use of pilot experiments as each disease/genetically modified animal may have distinct eating capacity. Furthermore, it is necessary to add a 'reasonable' amount of eggs/worms per plate; no more than 150–200 worms should be added to a medium-size test drug plate (step 7).

Problem 2

There are few worms (e.g., less than 200 worms/group) for the 'conditioning' assay (step 15).

Potential solution

One could prepare 3 - 4 medium-size plates of worms for each group. If there are 150–200 worms on a medium-size test drug plate (step 7), there are a total of 450–800 worms/group before the chemotaxis experiment. Therefore, even a potential large loss of worms (e.g., 50%) during any designated step (e.g., steps 11–15, steps 19–20) would leave one with around 125 worms/group for the final scoring step. Based on our in-house experience, it is recommended that the number of worms is kept within the range of 200–250 worms/plate in step 20.

Problem 3

Overcrowded population during worm maintenance and/or assay process.

Potential solution

A common and easy neglected reason for false positive/negative data and data variation is the use of experimental worms from an overcrowded population (step 1) or the application of too many worms in the scoring stage (step 20). Recent studies show that larval crowding accelerates development but shortens worm lifespan[8]; by extrapolation, worms grown in an environment of dense population could change their molecular, cellular, and behavioral characteristics. Thus, we suggest following our suggestions for the number of worms in different conditions (e.g., at steps 1 and 7). It is also necessary to keep the numbers of worms within the range of 200–250 worms/plate in step 20, as too many worms may interrupt/block the chemotaxis-directed movement of worms to 'IA' or 'T'.

Problem 4

Increase in paralyzed nematodes and death events during and/or after conditioning step (step 15).

Potential solution

During the worm washing and transferring steps, it is important to treat them gently. The use of either a P1000 pipette with wide orifice tip or glass pipettes during washing and transferring steps could reduce stress and injury to the worms. Pay attention to avoid pipetting the nematodes up and down during their transfer. In steps 15 and 21, please gently remove the excess M9 buffer using a Kimtech™ delicate task wipe to avoid any damage to the worms.

Problem 5

Trouble shooting 'weird' results, e.g., the N2 worms do not learn.

Potential solution

In standard conditions, the wild worms (N2) with IA-conditioning show a negative score (in our example, the scores range from around – 0.4 to 0 in step 26; Figure 2). Thus, it is important to always have the N2 (Veh) worms included as an internal control for the chemotaxis assay.

However, periodically, the N2 worms with AI-conditioning may show a score similar to their naïve counterparts, indicating that the N2 worms are not learning. Several conditions may cause such unanticipated results, including interference from unrelated experimental factors or changes in the genetics of the worms. For example, the existence of other exogenous smells (in addition to IA), such as perfume, scented body lotion, or any unrelated smelly chemicals in the experimental environment, may cause changes in the results. If after 2–3 repetitions of the memory assay, the N2 worms still show negative results, one could consider the possibility that an endogenous mutation has occurred in the original population, or that the strain has been contaminated by worms from other strains. There are a few possible solutions: If you suspect a strain cross-contamination, you can perform phenotyping, genotyping, or sequencing of the strains to validate the identity of the strain. If you suspect a naturally occurring endogenous mutation(s), you can back-cross the strains or thaw a new vial of the original batch of the designated worm(s). As it is not uncommon to see naturally occurring endogenous mutations, we suggest discarding the worms once they have been propagated for 4–6 months, and thawing a new vial. For each fresh thawed strain, we strictly require 4 generations of propagation (20°C, *ad libitum* feeding) to normalize any potential epigenetic changes during the 'freeze', 'storage', and 'thaw' steps, before a formal experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Evandro F. Fang (e.f.fang@medisin.uio.no).

Materials availability

All materials, unless otherwise specified, are commercially available with vendors' information provided. Materials unique to the Fang laboratory are available upon reasonable request.

Data and code availability

Original data shown in Figure 2 are available upon reasonable request. This study did not generate or analyze any additional datasets.

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AUTHOR CONTRIBUTIONS

E.F.F. supervised the protocol and provided resources of research experiments. S.Q.C. performed the experiments. E.F.F., K.P., and N.T. validated the experimental results. S.Q.C., E.F.F., and H.L.W. wrote the protocol. E.F.F., K.P., and N.T. revised the protocol, and all approved the final protocol.

DECLARATION OF INTERESTS

E.F.F. has a CRADA arrangement with ChromaDex (USA) and is a consultant to Aladdin Healthcare Technologies (UK and Germany), the Vancouver Dementia Prevention Centre (Canada), Intellectual Labs (Norway), and MindRank AI (China). E.F.F. and S.Q.C have a commercialization agreement with Molecule AG (https://www.molecule.to/).

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