M1 teaching unit, January 2025
Neuroscience Technology Module

Olfaction, adaptation, learning and forgetting in the nematode *Caenorhabditis elegans*

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"*You have made your way from worm* to *man, and much in you is still worm*"
F. Nieztsche (Thus Spoke Zarathustra, Prologue)

Anatomy and general organisation of the nematode Caenorhabditis elegans

Nematodes, or roundworms, are filiform metazoan protostomes with bilateral symmetry. Their body is unsegmented and protected by a thick cuticle. The anatomical plan is simple: it consists of a first tube containing the cuticle, hypodermis, muscles and nerve cells, separated from a second tube by a large visceral cavity. This second tube formed the digestive tract. The gonads are contained within the general cavity. Nematodes have colonised practically all environments (terrestrial, marine and biological). Caenorhabditis elegans is a worm that lives in the soil and on rooting fruits, where it feeds on bacteria.
At the end of the 1960s, Sydney Brenner had drawn up a set of criteria for choosing a multicellular biological model that would allow him to carry out a complete study of the mechanisms of development of the nervous system and its functioning in a metazoan. He chose Caenorhabditis elegans. This choice was particularly wise because this nematode has many experimental advantages that have made it a model of choice in biology.

1.1. Some basic knowledge on *C. elegans* biology

1. 1. 1. Morphology

C. elegans appears as a small cylindrical body about 1 mm long and 70-80 micrometres in diameter (adult stage, Figure 1). The mouth is located at the anterior end. The anus is located in the subterminal posterior position. While hermaphrodites have a tail that ends in a point, the tail of males is distinguished by the presence of a complex fan-shaped structure containing the neurons and muscles involved in copulation.

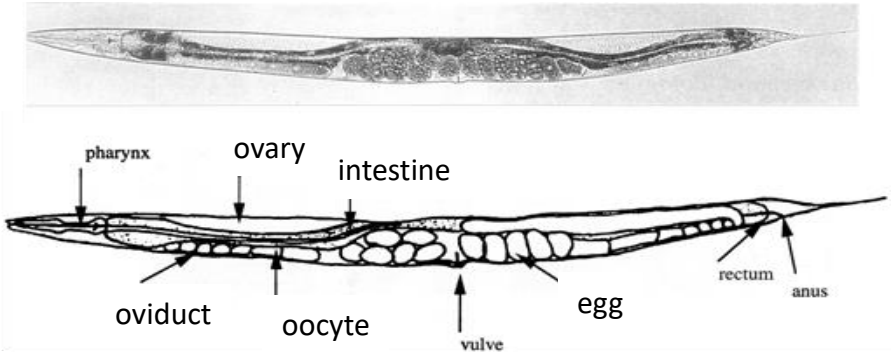

Figure 1. Anatomy of an adult hermaphrodite. Photograph and diagram of a lateral view under microscopic observation. The anterior part is on the left and the ventral side faces downwards..

1. 1. 2. Digestive system

C. elegans feeds on bacteria. They are ingested thanks to the depression caused by the rhythmic contractions of the pharynx, which acts as an autonomous muscular pump,sucking

The bacteria are picked up, crushed and passed into the intestine. The intestine is made up of multifunctional cells. They secrete digestive enzymes and absorb and store nutrients. The excretory apparatus consists of a single cell whose cytoplasm forms 2 ducts that are located in the lateral ectodermal fields along the entire length of the animal. These ducts lead to a ventral excretory pore located just below the pharynx.

1. 1. 3. The reproductive system

Hermaphrodites and males coexist in the C. elegans population. Males make up only one in about 500 animals. The reproductive system of the hermaphrodite consists of a gonad, which contains two symmetrically curved ovaries that end in two spermathecae and then in the common uterus in the centre of the animal. The vulva is an opening in the epidermis on the ventral side that allows for oviposition and copulation with males. At the distal end of each ovary is a syncytium of germ cell nuclei. The first 300 cells to commit to the proximal part of the gonad to undergo meiosis give rise to the male germ cells, which are stored in the spermathecae (located in the proximal part of the gonad). The remaining germ cells give rise to oocytes. The oocytes move towards the proximal part of the gonad to mature by passing through the spermathecae, where self-fertilisation takes place. The egg is released 450 minutes after fertilisation. The embryo develops into an L1 larva, which hatches 800 minutes after the first egg division. Development then continues through 3 further larval stages (L2, L3, L4) before reaching the young adult stage.

1. 1. 4. The nervous system

The hermaphrodite has 302 neurons of 118 different types. Most neurons are grouped in head ganglia around the pharynx (Figure 2). The nerve ring, which surrounds the central part of the pharynx, does not have a cell body but a large number of nerve bundles and connections. The ventral nerve cord contains the motor neuron cell bodies and axonal processes. The dorsal nerve cord is composed only of nerve projections.

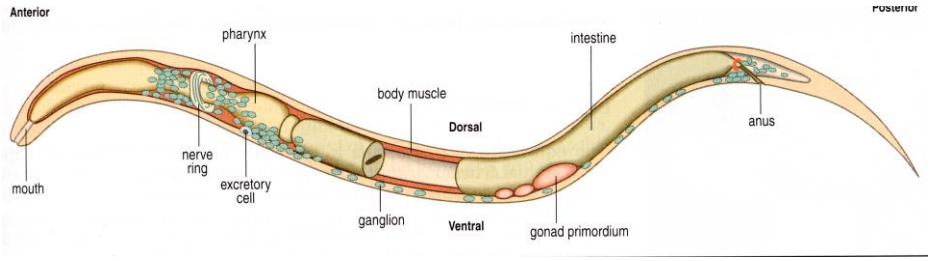

Figure 2. Schematic representation of the nervous system in C. elegans. General view of the nervous system of an L1 larva. The nerve ganglia of the head are located on either side of the nerve ring (consisting of nerve extensions without a cell body). The retrovesicular and preanal ganglia limit the ventral nerve cord made up of numerous neurons and nerve extensions. The dorsal chord has no neurons.

1. 1. 5. The displacement is carried out by a sinusoidal movement

The musculature of the animal's body is responsible for movement. It is composed of 95 mononuclear cells organized in four rows: 2 dorsal and 2 ventral. Each row consists of 23 or 24 muscle cells that partially overlap. These muscle cells contain contraction units (sarcomeres) made up of oblique striae. The nematode is laid on the flank. It moves forward, initiating a flexion at the level of the head which spreads all along the body towards the back. The sinusoidal propagation movement is maintained by the alternating, coordinated contraction of the dorsal and ventral muscles. These alternate contractions are caused by interactions between inhibitory and activating motor neurons in the ventral nerve cord.

1. 2. Experimental advantages.

The transparent body of C. elegans makes it possible to follow all the stages of its development by direct observation. The use of Nomarski optics (differential interference contrast) adds relief to this observation by transparency. It is an animal that grows very easily in the laboratory at 20°C on an agar medium in Petri dishes inoculated with a strain of E. coli. It is characterized by great prolificacy since a hermaphrodite can give birth by self-fertilization to 300 individuals which will become adults in 3 days (Brenner, 1974). Its simple organization (959 somatic cells in the hermaphrodite including 302 neurons) allowed the establishment of the cell lineage (Sulston, 1988) and the exact topography of all the nerve connections, since it is invariant from one individual to another (White et al., 1986). Isolation of mutants is facilitated by rapid segregation of homozygotes due to self-fertilization of hermaphrodites. The males are then used for crosses directed by the experimenter. Obtaining numerous mutants made it possible to establish an extremely detailed and precise genetic map. The first works of S. Brenner (1974) allowed the selection of more than 700 mutants and the establishment of linkage groups, confirming the presence of 6 chromosomes. C. elegans has a relatively compact genome of 98 Mb (20 times the genome of Escherichia coli, 1/30 of the human genome) divided into five autosomes and one sex chromosome (hermaphrodites are XX, and males X0). In 1998 the sequencing of its genome was completed. The transgenesis of nematodes is relatively easy. It allows the cloning by transformation and the in vivo study of regulatory sequences controlling a reporter gene.

Major biological discoveries have been obtained through the use of the C. elegans model. Some go well beyond the scope of nematode biology, such as: the elucidation of the genetic pathway for programmed cell death (Brenner, Horvitz, Sulston, **Nobel Prize 2002**), the identification of molecules such as netrin involved in axonal guidance (Ishii et al ., 1992) the discovery of the epigenetic mechanism of gene inactivation by double-stranded RNA (Andrew Fire and Craig Mello **Nobel Prize 2006**); the first in vivo use of GFP (Martin Chalfie, **Nobel Prize 2009**) and the discovery of microRNA (Victor Ambros and Gary Ruvkun **Nobel Prize 2024**).

In addition, the nematode C. elegans makes it possible to model the study of certain human pathologies. For example, the salt-12 gene of C. elegans has 50% homology with the two presenilin genes ps1 and ps2, the mutations of which are linked to familial cases of Alzheimer's disease (Rogaev et al., 1995). The authors have shown that the expression of the human ps1 and ps2 genes in the C. elegans sel-12 mutant enables the nematode to revert to a wild-type phenotype (Levitan et al., 1996). This homology of sequence and function suggests that the sel-12 and ps1/ps2 genes act in a similar way. Genetics experiments on C. elegans will find the genes whose products interact with sel-12, for example by looking for suppressor mutants, and then trace them back to the human counterparts.

The nematode C. elegans is therefore one of the main organisms used in developmental biology. It is more than likely that the experimental advantages of this animal will be at the origin of significant advances in the knowledge of the physiopathology of certain diseases; this type of knowledge is currently one of the missing keys to implementing therapeutic solutions.

Nomenclature used for mutants, genes and proteins in C. elegans:
By convention, the genes of C. elegans are designated by three lowercase letters in italics followed by a number. The three letters refer either to the gene (ace: acetylcholineterase; myo: myosin; cha: choline acetyltransferase) or to the phenotype observed when the gene is mutated (unc: "uncoordinated"; smg: "small morphogenetic defects in genitalia"). For the same product or the same phenotype, the various responsible genes are numbered in the order of their identification (ace-1, ace-2, ace-3). The code consisting of a letter and a number in parentheses following the name of a gene designates the allele of the gene in question (e.g.: lin-3 (e134) characterizes the e134 allele of the lin-3 gene). Gene products (proteins) are designated by the same three capital letters (ACE-1, the ace-1 gene product, acetylcholinesterase class A)

Olfaction: attraction, repulsion, in the nematode Caenorhabditis elegans

I. foreword

One of the problems of modern neurobiology is to understand how organisms can discriminate a particular odor among hundreds of others, how this recognition occurs at the level of an olfactory neuron and the nature of the molecular processes involved. The mechanism of chemical sensation, chemosensation, is one of the neuronal functions whose understanding requires an approach at the molecular and cellular levels. This is a real problem of integrative neurobiology since it requires multidirectional approaches. The nematode C. elegans is a very good animal model to try to answer this question because of the ease of manipulating it at the genetic level and the possibility of carrying out very targeted cell ablations.
The nematode, like most multicellular organisms, must be able to integrate information from its environment and modify its behavior accordingly. Caenorhabditis elegans, lives in soil interstices, at the air-water/soil interface or on the surface of rotting fruits. It is permanently exposed to chemical information, which is transported both by water and by air. In fact, its perception of the external environment is essentially based on this information, which allows it to manage competition for food with other species, to avoid predators, parasites and toxic compounds. The nematode is particularly very sensitive to the molecules emitted by its prey, the bacteria, which produce secondary metabolites such as alcohols (with a small carbon chain), esters, ketones.
The nematode is therefore capable of transforming the presence of a chemical molecule into a molecular message. This detection is then integrated by his nervous system which then causes an ad hoc modification of his behavior.

II. Aims

This practical session aims to show how, practically, an experimenter can approach the integrated study of an animal's behavior. The study of behavior is complex to envisage because it must integrate the study of a series of elementary phenotypes. It is necessary to carry out an exhaustive description of the behavior before undertaking a study of it. The first step is to define the strategy used to quantify the behavior to be studied. This approach should make it possible to establish a simple, reproducible measure of behavior so as to be able to highlight any changes. This requires controlling the stage of development of the animals used to carry out the measurements and precisely controlling the environment in which these

measurements are carried out. You will choose and implement a rigorous experimental protocol allowing you to show that (i) the nematode can detect a large number of different volatile compounds, (ii) these compounds can be either attractive molecules or repellents or neutral molecules, (iii) that the nematode exercises a preference for certain attractive compounds, (VI) that it is possible to determine the nature of the genes involved in these different aspects of olfaction by working on different C. elegans mutants of olfaction.

III. Protocols

1. Strains

Animals at the young adult stage of the wild N2 strain (Bristol variety) and mutants obtained from the N2 strain by random mutagenesis. The animals are washed twice in buffer S and once in distilled water before being used in the Bargmann olfactory test.

2. Chemotaxis assay

To test chemotaxis, we used fresh bacteria-free NGM 85 mm test plates. We then spotted in A 2 µL of diacetyl at a dilution of 1:1,000 and in B 2 µL of ethanol. 10 min prior to the start of the chemotaxis experiment, 1 µL of 1M sodium azide was added next to the diacetyl and ethanol spots for worm immobilization. Approximately 100 worms were transferred to the center of the plate using a pipette. The worms were gently dried with a Kimwipe, the lids of the plates were closed, and the worms were allowed to move freely for 30 min. Subsequently, the number of worms on the odor side (A) of the plate and an equivalent region (B) on the other side of the plate were counted (Figure S7). Worms within the start point region were excluded from the count. The chemotaxis index was calculated using the formula:
Chemotaxis Index = (#Side A - #Side B)/(#Side A + #Side B).
This index can vary between +1 (perfect attraction) and –1 (perfect repulsion).

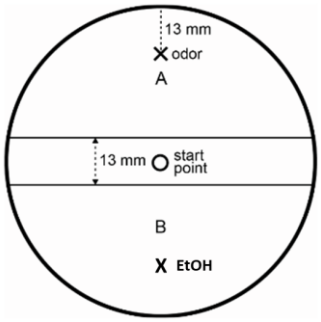

Diagram illustrating the annotation of the plates for the chemotaxis experiment

3. Odorants used

Volatile compounds used and their concentrations (between brackets) : 2,3 butanedion =diacetyl (1:100,10^{-1}), benzaldehyde (1:200, 5.10^{-3}), l'isoamylalcool, ethanol (pur, 10^{0}).

Adaptation, learning and forgetting in the nematode *Caenorhabditis elegans*

I. Foreword

One of the most amazing properties of the human brain is its ability to store vast amounts of information drawn from experience and retrieve most of it at will. We give the name of learning to the process by which our brain acquires new information and that of memory to the mechanism of storing and retrieving this information. But the ability to forget is also important for the functioning of the brain and the control of organism behavior.

Organisms generally face a complex environment whose physico-chemical parameters can change in space and time. This situation constantly challenges an organism, forcing it to adapt in real time, as conditions become drastic and/or change rapidly. To do this, anticipation mechanisms exist which make it possible to predict a future scenario based on past experiences on the memory of past events. This is why associated memory plays a central role since conditioning clues encountered in the past evoke a memory and by association predict an imminent changing situation.

You learn a lot through experience. When we repeatedly encounter a signal that is coupled with either a reward or a punishment, we end up remembering that the two occur together. This phenomenon is called learning by association. In the brain different groups of neurons are responsible for the signal which is associated on the one hand with the information signal and on the other hand with the reward or punishment. In addition to storing each individual information (non-associative memory), neurons communicate. Learning and memory are probably universal capacities in animals and it is interesting to study these mechanisms in an animal like *C. elegans*.

During this practical work we will learn how to test experimentally in C. elegans its ability to learn to associate an odor with a noxious stimuli, and that it is also able to forget this memory and that there are genes that control these different mechanisms in C. elegans.

The nematode is probably also able to adapt. Adaptation, or diminished perception of a stimulus from the environment, is an important modulator of nerve activity and actually allows animals to focus on changes in perception of a stimulus rather than static stimulation. in time. The nematode must also be able to forget that it has been confronted with an attractive stimulus, i.e. forget its olfactory adaptation.
Active forgetting, i.e. the mechanism that allows memories to be lost through neural regulation, is important for animals to avoid overstoring capacity and to prevent interference between memories.

II. Aims

The objective of this session is to use a conditioning protocol to test the ability of a nematode to learn by association between two sensory and physiological experiences: presence of an odor and a specific noxious environment: acidic condition.
Then, to show that the nematode is capable of forgetting and that there are genes that control this forgetting mechanism.

III. Protocol

Aversive conditioning:
Synchronized adult worms were gently rinsed off their growth plates using CTX buffer and transferred into standard Eppendorf tubes with a pipette. After a 1 min settling period, the

supernatant was carefully removed, and replaced by 700 µL CTX. This washing process was repeated 5 times to ensure thorough removal of any residual bacteria. In the final wash M9 buffer was used instead of CTX. Subsequently, the worms were subjected to a 2 h starvation period by placing them on fresh bacteria-free NGMplates. Following starvation, the worms were collected into new Eppendorf tubes, and excess supernatant was gently removed.
In each tube, 250 mL of the conditioning or control solution was added. The conditioning solutions comprised Diacetyl in a 1:10,000 dilution, 1 mM (pH = 3) HCl, or a combination of both. The worms were then incubated with the conditioning solution for a total of 15 min. **Throughout this period, the tubes were positioned on their side on an isolated shelf to minimize environmental disturbances and facilitate worm dispersion within the tube.** Approximately 30 s before the conclusion of the 15 min training period, the tubes were returned to their upright position, allowing the worms approximately 25 s to settle. Following the 15 min conditioning period, the conditioning solution was carefully aspirated, and the worms were rinsed once with 700 µL of CTX. After the rinse, the tubes where briefly centrifuge for 1 min in mini centrifuge at 1,000 RPM, and the supernatant was removed. Chemotaxis testing was performed immediately after rinsing the worms. However, when testing memory at later time points, testing for post conditioning memory retention, worms were transferred to fresh NGM plates pre-seeded with OP50 bacteria, and incubated at 15°C, after which worms were collected from the plates and washed three times with 700 µL CTX before proceeding to the chemotaxis assay.

Security rules:
-Wear a lab coat in the lab room
-Eating or drinking in the lab room is strictly forbidden
-Sort and dispose of the various waste generated by the experiments in the appropriate containers (autoclave bags, glass container, etc.)
-In case of injury or discomfort, notify the teacher supervising the practical work session as soon as possible.