KU LEUVEN

FACULTEIT WETENSCHAPPEN

Characterisation of Neuropeptide Signalling Systems in Learning

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ABSTRACT

It has been shown that the nematode *Caenorhabditis elegans* possesses a basic system of associative learning. It can be conditioned to associate several environmental stimuli – such as odorants and temperature – with positive or negative conditions. One example of this behaviour is gustatory plasticity. If *C. elegans* is temporarily pre-exposed to a low salt concentration in the absence of food, it will show an aversion to salt for a short period of time. This is contradictory to the worms' usual behaviour, as they normally migrate towards low concentrations of salt. In this project we assess the involvement of several neuropeptides and G protein-coupled receptors (GPCRs) in associative learning behaviour using the gustatory plasticity assay. We did so in the prospect of acquiring a deeper knowledge into the mechanisms underlying both invertebrate and vertebrate learning and memory processes. We tested five mutant strains. The mutated genes of the first three strains encode GPCRs, which are membrane-spanning proteins that are crucial for the relay of information from outside to inside cells. The final two strains have a mutation in genes coding for the ligands of these GPCRs, which are neuropeptides, another important class of molecules with a plethora of functions in the nervous system. Analysing our results has led us to quite confidently reject the involvement of LMR (Learning Modulating receptor)-3 and LMR-1 in salt-induced associative learning, and we also deem it unlikely that LMR-2 and LMN (Learning Modulating Neuropeptide)-2 play a role in this process. Our data does however point out a potential role for the neuropeptide LMN-1 in the regulation of this learning behaviour, but further experiments are needed to confirm this statement.

Het is aangetoond dat de rondworm *Caenorhabditis elegans* een fundamenteel systeem van associatief leren bezit. De rondworm kan worden geconditioneerd om diverse omgevingsstimuli – zoals geurstoffen en temperatuur – te associëren met positieve of negatieve omstandigheden. Een voorbeeld van dit gedrag heet smaakplasticiteit. Als *C. elegans* tijdelijk wordt blootgesteld aan een lage zoutconcentratie in afwezigheid van voedsel, zal het voor een korte tijdspanne een afkeer voor zout vertonen. Dit is in tegenspraak met het gebruikelijk gedrag van de wormen, aangezien ze gewoonlijk naar lage zoutconcentraties toe migreren. In dit project evalueren we de rol van een aantal neuropeptiden en G-proteïnegekoppelde receptoren (GPCR's) in het associatief leergedrag, met behulp van de smaakplasticiteitsassay. We deden dit in het vooruitzicht van het verwerven van een diepere kennis over de mechanismen die ten grondslag liggen van zowel ongewervelde en gewervelde leer- en geheugenprocessen, om zo een eerste inzicht te verkrijgen in deze complexe processen. We hebben vijf mutante lijnen onderzocht. De gemuteerde genen van de eerste drie lijnen coderen voor GPCR's, membraan-overspannende eiwitten die cruciaal zijn voor het doorgeven van informatie van buiten naar binnenin de cel. De laatste twee lijnen hebben een mutatie in hun genen die coderen voor de liganden van deze GPCR's, namelijk neuropeptiden, een belangrijke klasse van moleculen met een overvloed aan functies in het zenuwstelsel. Het analyseren van onze resultaten heeft geleid tot het verwerpen van de rol van LMR-3 en LMR-1 in zout-geïnduceerd associatief leren, en we achten het ook onwaarschijnlijk dat LMR-2 en LMN-2 in dit proces een rol zouden spelen. Onze gegevens wijzen echter wel op het feit dat het neuropeptide LMN-1 mogelijks dit leergedrag zou reguleren. Gezien de dubbelzinnigheid van de bekomen resultaten zal verder onderzoek nog moeten uitwijzen of LMN-1 effectief betrokken is in dit bepaald leergedrag.

INTRODUCTION

C. elegans is a free-living, soil-dwelling nematode with a length of approximately 1 mm. *C. elegans* can be male or hermaphroditic. Hermaphrodites usually outnumber the males by about a thousand to one. This means that the majority of reproduction occurs through self-fertilization rather than through mating. This nematode is eutelic, meaning the adult worm has a fixed number of cells. In the hermaphrodite's case this number is 959, while a male organism has 1031 somatic cells. Despite their smaller number of cells, the hermaphrodites are slightly thicker and longer than their male counterparts.[1]

This nematode has a body plan typical to most nematodes. It has a tubular and unsegmented body that tapers to a point at both ends. It consists of two tubes separated by a pseudocoelom. The worm's excretory, muscular and nervous systems are located in the outer layer, while the digestive and reproductive systems can be found within the inner tube. The life cycle of *C. elegans* is also akin to that of other roundworms. The embryonic stage is followed by four larval stages, L1 to L4, which precede adulthood. If the normal reproductive cycle is followed, there are approximately three days between two consecutive generations.[1]

C. elegans is used all over the world as a model organism in research of all kinds. There are several advantages to using *C. elegans* in research. It is small, relatively easy to culture and has a short life cycle. Its genome is compact and fully sequenced. Additionally, *C. elegans* has a fixed number of cells, and many of those cells' functions are known. All of these characteristics make it a well-suited organism to be used in scientific research worldwide, including in our search for molecules that play an important role in the process of learning and memory.[1]

While *C. elegans* doesn't have a brain, it does have a nervous system with a large number of neurons concentrated around its head. Several of these neurons have a sensory function, much like our own olfactory or taste receptors. Despite its nervous system's relative simplicity compared to that of vertebrates, *C. elegans* has the capacity to learn. It displays both non-associative and associative learning, and these behaviours can be short- or long-term. In this study we concentrated on short-term associative learning.[2] More specifically, we examined which ligands and receptors play an important role in the functioning of this system. To do so, we compared the behaviour of wild-type worms to mutant ones in a memory assay relying on taste perception.

We decided to investigate the involvement of several G protein-coupled receptors (GPCRs): LMR-1, LMR-2 and LMR-3, as well as their respective neuropeptide ligands. LMR-1 and LMR-2 are activated by both LMN-2a and LMN-2b, whereas LMR-3 is activated by peptides of the LMN-1 precursor protein. The LMR-1 and -2/LMN-2 signalling system is homologous to an important and well-known signalling system in vertebrates which has been shown to be involved in distinct learning and memory pathways in several animals. The LMR-3/LMN-1 system has homologous counterparts in *Drosophila* and in vertebrates. There are strong indications that these signalling systems are involved in learning and memory as well, which is why we decided to also examine LMR-3 and LMN-1.[3,4]

GPCRs have been shown to play prominent and diverse roles in *C. elegans*, which is why they were chosen as potential regulators of associative learning in this study. GPCRs are present in all eukaryotes, acting as intracellular signal transducers. They function as a messenger that can relay a signal from outside the cell to the cell's interior. Their ligands are

diverse, and can vary from ions to proteins, or neuropeptides. Of the one thousand or so GPCRs found in *C. elegans*, it is estimated that over 150 of them are neuropeptide receptors.[5]

Neuropeptides are short polypeptides that play a crucial role in the modulation of neuronal activity. They are important signalling molecules in the animal kingdom, acting as neurotransmitters and hormones. Their functions vary, and there are at least 250 different neuropeptides predicted in the *C. elegans* genome. In *C. elegans*, these peptides can be classified into three categories: insulin-like peptides (INS), FMRFamide-like peptides (FLP), and neuropeptide-like proteins (NLP). This last group is rather diverse, as it contains all neuropeptides not belonging to the other two families. The two neuropeptides we investigated are both NLPs. As neuropeptides have so many different functions, especially in the nervous system, they were definitely potential candidates for the regulation of associative learning.[5,6]

As mentioned previously, *C. elegans* exhibits several forms of learning. In associative learning paradigms, this nematode can be conditioned to link certain environmental stimuli (conditioned stimuli) with beneficial or unfavourable conditions (unconditioned stimuli). Examples of such conditioned stimuli include odorants, temperature and oxygen concentration, while unconditioned stimuli could be the presence or absence of food or the presence of an aversive chemical. This kind of learning behaviour can be compared to the famous experiment done by Ivan Pavlov at the turn of the twentieth century, in which he conditioned dogs to associate the sound of a bell with receiving food. We did not use sound as the conditioned stimulus, but taste, the unconditioned stimulus being the absence of food.[2]

In the gustatory plasticity assay, we used salt as the conditioned stimulus. Under normal circumstances, *C. elegans* shows an attraction to a low sodium chloride (NaCl) concentration (0.1-200 mM) and migrates towards it. But if subjected to the absence of food and a low concentration of NaCl, it temporarily avoids salt when brought onto a plate containing a salt gradient. This shift in response to a salt cue is termed gustatory plasticity. Several different neurons have been shown to play a role in gustatory plasticity towards NaCl, but one of the important cells is the ASE neuron. ASE is a gustatory neuron responsible for the recognition of, among other substances, sodium and chloride ions. ASE neurons always occur in pairs, labelled ASEL (Left) and ASER (Right). Although they are structurally each other's mirror image, they show discrepancies in their function: ASEL is primarily sensitive to Na^+, while ASER detects Cl^-.[7,8] ASE is an amphid neuron, meaning it is situated in *C. elegans*' amphids. These are nematodes' main sensory organs, located on their head and open to the outside world. Each of the two amphids consists of twelve sensory neurons, of which ASE is the most relevant to our research.[9]

Several different proteins and pathways have already been shown to be essential in associative learning behaviour, such as nematocin and ODR-3. The former is a vasopressin/oxytocin-related peptide, while the latter is a Gα protein. But nematocin and ODR-3 are only two of the many vital components of *C. elegans*' memory process, and in this study we wish to identify more of those key elements. That way we will come one step closer to understanding how not only a nematode's, but also a human's memory functions, in all its complexity.[10,11]

OBJECTIVES

The ultimate goal of our study is to identify neuropeptidergic signalling systems that are important to the functioning of memory. More specifically, we wish to determine if the genes *lmr-1*, *lmr-2*, *lmr-3*, *lmn-1*, *lmn-2* and the GPCRs and neuropeptides they encode have a part in *C. elegans*' associative learning process. If we discover that one or more of these GPCRs and neuropeptides are pivotal to *C. elegans*' ability to learn, this could mean that their homologous counterparts might play an essential role in the memory process of higher vertebrates and maybe even humans. The human brain is one of the least understood organs of the human body, and any small advance in our knowledge of its inner workings is a contribution towards a better understanding of its intricate method of functioning.

Our first subsidiary goal will be to outcross the mutant strains of *C. elegans* so as to obtain a population of individuals only containing the desired mutation, with limited background mutations. Next, we will have to synchronise the nematodes, allowing us to obtain a population of worms that are all in the same developmental stage. Then, the importance of the GPCRs and neuropeptides in associative memory with salt as the conditioned stimulus will be assessed. We will achieve this objective by comparing each of the mutant worms' behaviour to that of a control population. Doing this, we should be able to deduce which mutants' learning behaviour has been adversely affected by the absence of a specific GPCR or neuropeptide, thus concluding that that GPCR or neuropeptide plays a crucial role in *C. elegans*' associative memory.

MATERIALS AND METHODS

As *C. elegans* is the organism central to this project, a thorough knowledge of their culturing is imperative. *C. elegans* is grown on plates containing Nematode Growth Medium (NGM). Their source of food is usually the OP50 *E. coli* strain. This strain has a limited growth on NGM plates, which is favourable as it enhances visibility when counting worms and doesn't impede *C. elegans* mating. First, the NGM solution is prepared and aseptically poured into a plate. Once the agar has solidified a drop of *E. coli* liquid culture is added, which is subsequently spread out across the plate. Here, attention must be paid to not streaking it out too far. This could result in loss of worms due to dehydration, caused by worms crawling up the wall of the plate.[12]

It is often necessary to transfer single or multiple worms to a new plate. It must be done every two to three days to prevent starvation, as the plate's bacterial population will be exhausted. A first method to do this is called 'chunking'. A portion of the old plate is removed using a sterilized spatula, and placed onto a new plate. This way a large number of worms will be transferred. Another method, used for transferring individuals or small groups, uses a flattened platinum wire to pick them off the plate and place them on the fresh plate. A microscope can be useful to help select individual worms.[12]

C. elegans can be frozen for storage purposes, and then thawed when they are needed for an experiment. This was done to some of the mutants in our experiment once they had been crossed out six times, as a backup for if something went wrong with the worms in culture. It is best to use L1 and L2 worms from an exhausted plate, as they have the largest chance of survival. The worms are washed from their plates using basal buffer, and Soft Agar Freezing Solution is added to the receptacle. This solution is then distributed over four vials for freezing. It is important that the nematodes are chilled slowly. This can be achieved by putting them in a Styrofoam holder so that the temperature decreases gradually. Once at -80 °C they can be taken out of the holder for long-term storage. To see if the worms have survived, one of the four vials can be removed after they have reached -80 °C, thawed and inspected under the microscope. [12]

1. Crossing-out of the acquired mutants

After acquiring *lmr-1*, *lmr-2*, *lmr-3*, *lmn-1* and *lmn-2* mutants from the *Caenorhabditis* Genetics Center (CGC), it is necessary to outcross the nematodes to reduce background mutations. The EMS and UV/TMP treatment that is used in mutagenesis also results in unwanted mutations, which could alter the organisms' phenotype, thus influencing the experiment's result. To avoid this, the mutants are crossed with wild-type worms in a controlled way to reduce the occurrence of such background mutations. All mutants used in our experiments were crossed out six times. We did this ourselves, with the exception of *lmr-2*, which had already been crossed out eight times by a colleague. However, we did wish to design and optimise primers for this mutation as well, to be used in possible future assays.

Before the actual crossing-out could be started, the appropriate primers had to be designed and optimised. NetPrimer (http://www.premierbiosoft.com/netprimer/) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) were used to find primers with the right specifications. Next, the optimal annealing temperature had to be determined.

This was done by running the PCR at different annealing temperatures, and seeing which one had the highest yield.

Materials

- 35 mm NGM plates
- Proteinase K (10 mg/mL) (*Tritirachium album* (Sigma-Aldrich))
- Advantage PCR template buffer (1x) (Clontech)
- PCR Thermal Cycler (TProfessional Thermocycler (Biometra))
- Forward and reverse primers (Sigma-Aldrich) (see Table 1)
- REDTaq® ReadyMix™ (Sigma-Aldrich)
- Tris-Acetate-Ethylenediaminetetraacetic acid (EDTA) (TAE) buffer (50x) (242.5 Trizma®base (Sigma-Aldrich); 34 g sodium acetate trihydrate (Sigma-Aldrich); 18.5 g Tritriplex III (Sigma-Aldrich); Milli-Q until 1 L, pH 8)
- Agarose (1%) (to 1 g of agarose (Sigma-Aldrich) add 100 mL of TAE buffer (1x) and dissolve by heating in the microwave oven)
- GelRed™ (10000x in DMSO) (Biotium)
- 1 kb DNA ladder standard (Fermentas)
- ProXima 2500-T gel imaging system (Isogen Life Sciences)

Methods

- Cross about eight L4 homozygous wild-type males with one or two L4 homozygous mutant hermaphrodites on a NGM plate (see (1) in Figure 1)
- Approximately three to four days later all offspring should be heterozygous. Place eight of the F1 males on a new plate, alongside one or two L4 homozygous wild-type hermaphrodites (see (2) in Figure 1)
- Around the ninth day after starting the process, half of the second filial population will be heterozygous. Single out ten F2 hermaphrodites, of which on average five will be heterozygous, and allow them to self-fertilize (see (3) in Figure 1)
- Once these worms have laid eggs, which will be about two to three days later, test their genotype using PCR
    - First the worms will be digested and their DNA will be released using proteinase K. Thaw the proteinase K and the template buffer on ice
    - Add 100 µL template buffer to 5 µL proteinase K, and then distribute this solution over the sample vials so that each vial contains 6 µL of solution
    - Pick the adult worms to be digested from their plate and add each one to a separate vial. Do the same for a confirmed mutant and a wild type, as controls
    - Cool the vials to a temperature of -80 °C for 15 to 20 minutes
    - Heat for 60 minutes at 60 °C to activate proteinase K and then 15 minutes at 95 °C to inactivate it

- Then a PCR is run to amplify the released DNA. Move half of the contents of each vial to a new vial. Of each pair of vials, the contents of the one will be amplified using external primers and that of the other will be amplified using an external and an internal primer. These primers can be found Table 1 (see *Results*)
- Add 9.5 µL Milli-Q water, 1 µL of each of the appropriate forward and reverse primers and 12.5 µL of REDTaq® ReadyMix™ to each sample
- Run the PCR according to the primer-specific PCR program documented in Table 2 (see *Results*)
- Load the samples onto a 1% Agarose gel containing GelRed™
- Start the agarose gel electrophoresis at 150 V and 200 mA
- Visualise the result using the ProXima 2500-T gel imaging system

- Single out fifteen of the F3 hermaphrodites whose F2 parents are confirmed heterozygotes, and allow self-fertilization (see (4) in Figure 1)
- Two to three days later, these individuals will have laid their eggs. Their genotype is then tested using PCR as described above
- The F4 offspring of confirmed F3 homozygous mutants have been crossed out twice and can be used for further crossing-out cycles or for the gustatory plasticity test. These individuals are marked by a red box in Figure 1

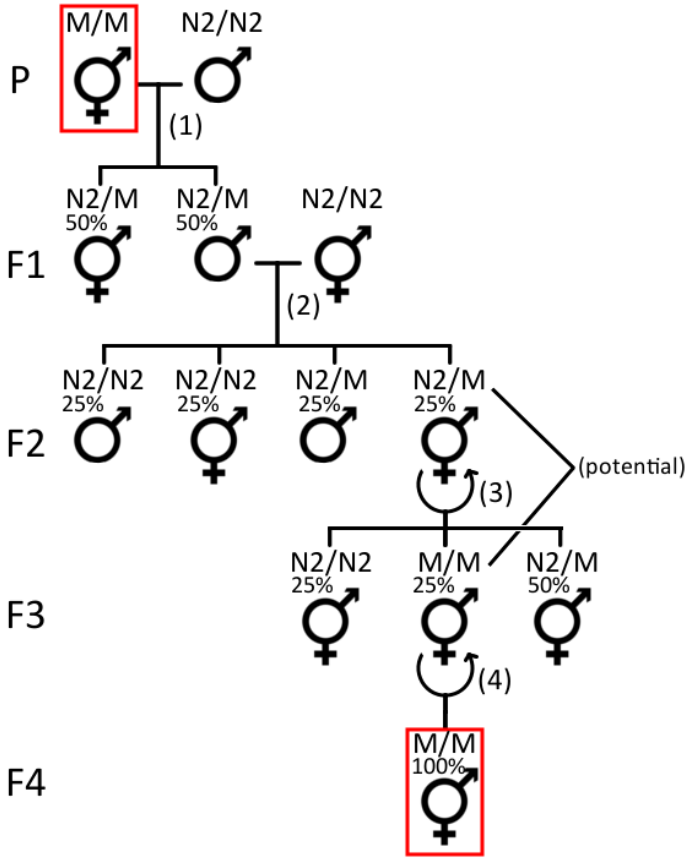

Figure 1 Outcrossing of C. elegans. In steps (3) and (4), all hermaphrodites are crossed, but only the offspring of F2 N2/M and F3 M/M worms are used in the subsequent steps.

2. Gustatory plasticity assay

Once the mutants have been crossed out six times, we are ready to move on to the experiment that is central to our study – the gustatory plasticity assay. In this test, *C. elegans* is pre-exposed to a low salt concentration in the absence of food. If the worms' memory is working properly, they should show an aversion towards salt for a short period of time afterwards. This is contrary to their usual behaviour, as naive worms are attracted to low salt concentrations. We can test their response to conditioning by placing naive and conditioned animals on plates divided into quadrants, two containing salt and two not. In wild-type worms – which are used as a negative control – the majority of the conditioned organisms should move towards the salt-free quadrants, whereas naive wild-type worms will chemotax towards the salt containing quadrants. If a mutation has altered *C. elegans*' ability to learn, this shall become apparent due to indifference or preference to salt of conditioned animals. CX10 worms (*osm-9*(*ky10*)) are used as a positive control, as they are known to have an impaired learning ability. The assay is performed in the climate room at 40% relative humidity and 20 °C.

An important step in this nine-day process is the synchronization of the worm population. The nematodes are synchronized so that all of the worms in the population are in the same developmental stage throughout the experiment. Doing so eliminates the risk of making faulty conclusions due to age differences influencing the results. To synchronize the worms, gravid hermaphrodites are treated with a lethal bleach solution. This will destroy the worms, while their eggs – which are encased in a protective cuticle – manage to survive for a limited time span. Next, the eggs are brought onto seeded plates. This will result in them all evolving into the next larval stage, and all subsequent stages will be passed through more or less synchronously.

Materials

- KH_2PO_4/K_2HPO_4 (1 M) (to 108.3 g of KH_2PO_4 (Sigma-Aldrich), and 35.6 g of K_2HPO_4 (Sigma-Aldrich) add 50 mL of DM water and filter through a vacuum filter system)
- $CaCl_2$ (1 M) (to 7.35 g of $CaCl_2$ (Sigma-Aldrich) add 50 mL of demineralized (DM) water and filter through a 0.22 µm filter)
- $MgSO_4$ (1 M) (to 12.32 g of $MgSO_4$ (Sigma-Aldrich) add 50 mL of DM water and filter through a 0.22 µm filter)
- Cholesterol (5 mg/mL) (to 0.250 g of cholesterol (Sigma-Aldrich) add 50 mL of ethanol (95%) (VWR Prolabo Chemicals) and filter through a 0.22 µm filter)
- 1 L NGM in Milli-Q (17 g agar (Sigma-Aldrich); 3 g NaCl (Sigma-Aldrich); 7.5 g BactoPeptone (BD); 5 mL KH_2PO_4/K_2HPO_4 (1 M); 1 mL $CaCl_2$ (1 M); $MgSO_4$ (1 M); 1 mL Cholesterol in Ethanol (5 mg/ml); Milli-Q until 1 L)
- Liquid OP50 culture (one OP50 colony in 50 mL Luria-Bettani medium (to 25 g of LB Broth (Sigma-Aldrich) add DM water up to 1 L and autoclave)
- Agar (Sigma-Aldrich)
- M9 buffer (6 g Na_2HPO_4; 3 g KH_2PO_4; 5 g NaCl; 1 mL $MgSO_4$ (1M); Milli-Q until 1 L)
- CTX buffer (5 mL KH_2PO_4/K_2HPO_4 (1 M); 1 mL $CaCl_2$ (1 M); $MgSO_4$ (1 M); Milli-Q until 1 L)

- CTX buffer supplemented with NaCl (5 mL KH_2PO_4/K_2HPO_4 (1 M); 1 mL $CaCl_2$ (1 M); $MgSO_4$ (1 M); 20 mL NaCl (5 M); Milli-Q until 1 L)
- Bleach solution (2 mL NaOH (4 M); 3 mL 13% NaOCl (Acros Organics)
- NaCl (5 M) (to 14.61 g NaCl add 50 mL Milli-Q)
- Buffered agar (1 mL $CaCl_2$ (1 M); 1 mL $MgSO_4$ (1 M); 5 mL phosphate buffer (1 M); 900 mL 1.7% agar solution; Milli-Q until 1 L)
- Buffered agar supplemented with NaCl (1 mL $CaCl_2$ (1 M); 1 mL $MgSO_4$ (1 M); 5 mL phosphate buffer (1 M); 5 mL NaCl (5 M); 900 mL 1.7% agar solution; Milli-Q until 1 L)
- Four-quadrant Petri Dishes (Phoenix Biomedical)
- 90 mm Petri Dishes
- Table top centrifuge (Centrifuge 5415D (Eppendorf))
- 0.22 µm millipore filter (Millex^{R}GP)
- Vacuum filter system with 0.2 µm Nylon membrane (Corning)

Methods

Day 1 – Preparing the plates

- Prepare NGM in Milli-Q
- Pour the NGM into 90 mm petri plates by adding 28 mL per plate
- Allow to dry overnight at room temperature
- Inoculate *E. coli* OP50 into 50 mL LB medium

Day 2 – Seeding the plates

- Seed the plates with 200 µL of liquid OP50 culture and spread it out evenly using a sterilised curved metal rod
- Allow overnight growth on the bench then store at 4 °C if necessary

Day 3 – Chunking the plates

- If stored at 4 °C, allow the plates to acclimate to room temperature
- Chunk two or three plates for every stain. Do this for all five mutants, wild-type worms (negative control) and the CX10 strain (positive control)
- Grow the worms at 20 °C for three days

Day 6 – Synchronizing the worms

- Wash the worms of their plates using sterile M9 buffer in Milli-Q
- Collect in a 14 mL falcon tube and allow to settle for 5 minutes
- Discard the supernatant until 2 mL remains
- Resuspend and transfer to a 2 mL microcentrifuge tube
- Allow worms to settle for 5 minutes
- Discard the supernatant until 0.5 mL remains

- Add 250 µL bleach solution, invert and incubate for no longer than 3,5-4 minutes
- Add 800 µL M9 buffer and invert
- Centrifuge for 1 minute at 2000 rpm
- Remove the supernatant until 0.5 mL remains
- Add 1.5 mL M9 buffer
- Resuspend and repeat the entire washing step four additional times
- Remove the supernatant until 0.5 mL remains
- Resuspend the pellet and distribute on an OP50 seeded NGM plate
- Allow eggs to hatch and grow at 25 °C

Day 7 – Chunking the plates

- Allow the plates to acclimatise to room temperature
- Transfer the worms onto new plates
- Grow at 25 °C

Day 8 – Preparing the agar

- Dissolve 17 g agar in 900 mL Milli-Q
- Autoclave and store overnight at 50-60 °C

Day 9 – Performing the gustatory plasticity assay

- Prepare the buffered agar solution and the buffered agar solution supplemented with NaCl
- Prepare four-quadrant Petri plates
    - Fill opposite quadrants of the four-quadrant plates with 13.5 mL of the NaCl-supplemented agar
    - Fill the two remaining quadrants with 13.5 mL of the agar that doesn't contain NaCl
    - Allow to solidify at room temperature for approximately one hour. Meanwhile prepare the CTX buffers
    - Fill the splits between quadrants with NaCl-free agar using a Pasteur pipette just before performing the assay
- Wash the worms off their plates into a 14 mL falcon tube using CTX buffer (to test naive worms) and CTX buffer supplemented with NaCl (to test conditioned worms)
- Allow worms to settle for 5 minutes
- Discard the supernatant, keeping 1 mL of the solution
- Add 10 mL of CTX buffer (supplemented with NaCl), and repeat these washing steps twice
- Place 100-200 animals at the intersection of the four-quadrant assay plate and remove excess fluid

- Allow the worms to migrate for 10 minutes. Place both plates at different angles to compensate for potential environmental influences
- Count the number of worms in each quadrant, excluding the ones present in the agar added onto the splits
- Calculate the chemotaxis index using the formula *(A-C)/(A+C)*, with *A* the number of worms in the two quadrants containing NaCl and *C* the number of worms in the two remaining quadrants

Figure 2 shows the results that can be expected from conditioned wild types and mutants whose learning capacities are affected, such as the positive control, CX10.

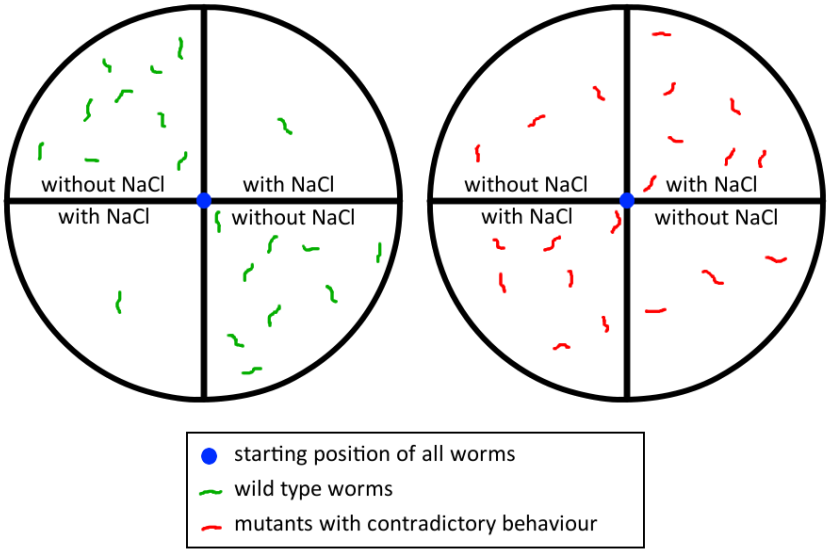

Figure 2 Gustatory plasticity assay. The behaviour of conditioned worms.

RESULTS

1. Crossing-out of the acquired mutants

During the first several weeks of the study, mutants were crossed out six times. This was done successfully by us for all mutants save *lmr-2*, which had already been crossed out. As mentioned in *Materials and Methods*, for each mutant a specific set of external and internal primers was used to identify heterozygotes and homozygous mutants.

When using external primers, the wild-type allele will result in a long fragment while the mutant gene will form a shorter one, due to the deletion in its DNA. This means a homozygous wild type (N2/N2) can be recognised by a single longer fragment, a heterozygote (M/N2) by two distinct bands and a homozygous mutant (M/M) by a single shorter strand. Theoretically this is sufficient to distinguish between all three genotypes, but in practice this is not always the case. In our experiment we realised that *lmr-1* and *lmn-2* heterozygotes sometimes showed up as homozygous mutants, hence the use of internal primers was required.

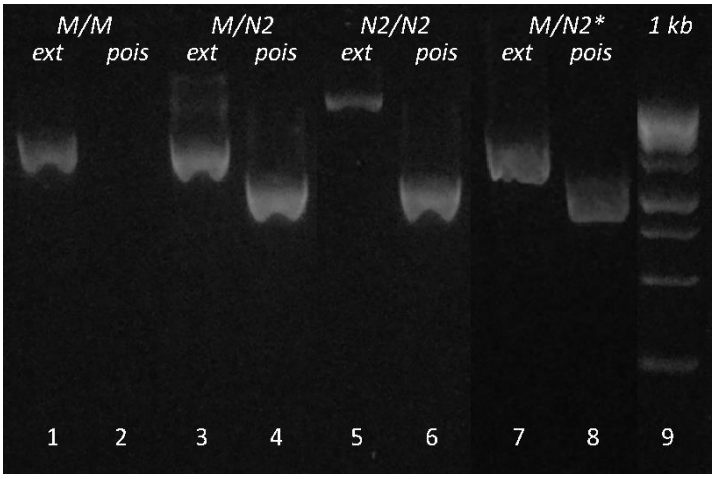

Figure 3 Agarose gel electrophoresis performed for outcrossing of *lmr-1*. Lanes 1, 3 and 5: expected outcome when using external primers for M/M, M/N2 and N2/N2 worms. Lane 7: would be interpreted as an M/M individual when using only external primers. Lane 8: use of an internal primer clearly shows that it also contains a wild-type allele, so its genotype must be M/N2.

2. *Gustatory plasticity assay*

Once the *C. elegans* mutants had been crossed out six times, the gustatory plasticity assay was executed several times. Prior to the actual testing some optimizing assays were performed. The resulting data have not been included in the results. This leaves us with the results of in total four successfully executed assays. However, not all our mutants were assessed on four independent days. The data shown arises from two or more independent assays, done in at least duplicate.

An essential step in performing the gustatory plasticity assay was the synchronization of the worms. Also here, an optimising phase was required as primary attempts resulted in the death of the obtained eggs. Several differential parameters were tested, finally resulting in a successful bleaching.

Figure 4 a. displays the massed data of all successful experiments. Given the variability of the obtained results, it was decided to also include the data given in Figure 4 b. Here, the results of one single experiment are depicted. However, as this data arises from an assay in which a high number of repetitions was performed, it helps to draw conclusions from results that actually require additional data prior to coming to a definitive conclusion. The average chemotaxis index is displayed of naive (blue) and conditioned (yellow) worms, along with the standard error of the mean. Optimal chemotaxis indices would be 0.8 and -0.6 for respectively naive and conditioned wild-type worms, and 0.2 for both naive and conditioned CX10 worms.

In order to statistically analyse the obtained results and to determine whether the results of mutant worms differ significantly from those of wild-type worms, a one-way ANOVA followed by the Tukey post hoc test was performed. The outcome of this test is shown in Table 1, in which N2 represents wild-type worms.

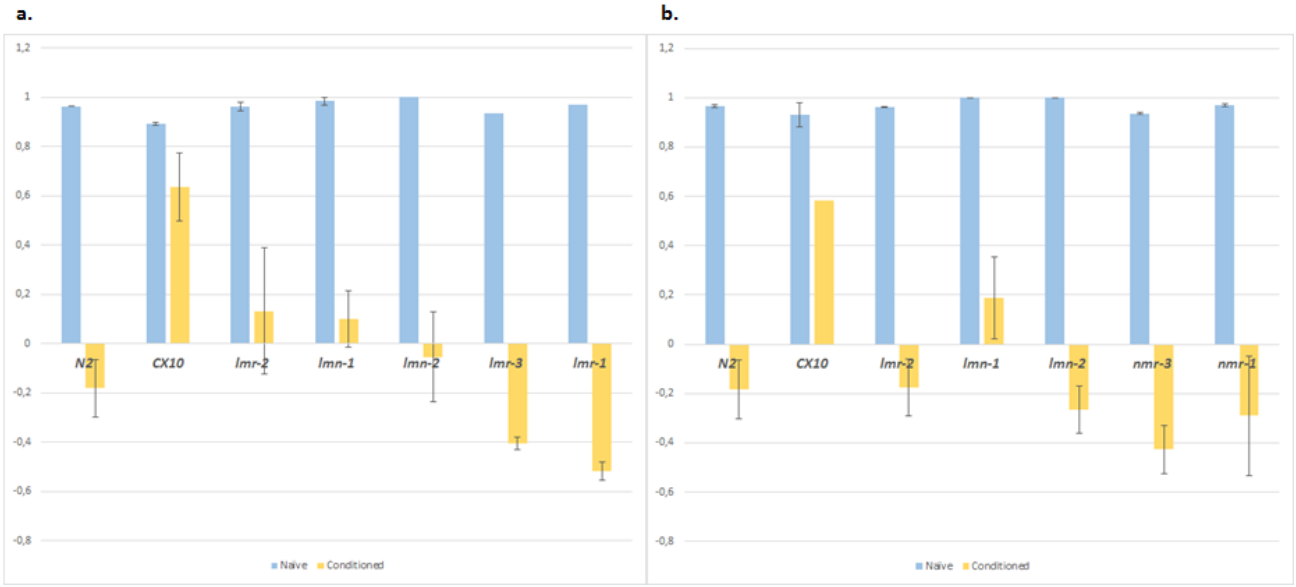

Figure 4 Chemotaxis indices of naive and conditioned worms, with the standard error of the mean (SEM) represented as error bars. **a.** Grouped data of all experiments. **b.** Data of single experiment.

Table 1 Tukey's multiple comparisons test	
Comparison	Significant?
N2 Naive vs. CX10 Naive	No
N2 Naive vs. *lmr-1* Naive	No
N2 Naive vs. *lmr-2* Naive	No
N2 Naive vs. *lmn-2* Naive	No
N2 Naive vs. *lmr-3* Naive	No
N2 Naive vs. *lmn-1* Naive	No
N2 Conditioned vs. CX10 Conditioned	Yes
N2 Conditioned vs. *lmr-1* Conditioned	No
N2 Conditioned vs. *lmr-2* Conditioned	No
N2 Conditioned vs. *lmn-2* Conditioned	No
N2 Conditioned vs. *lmr-3* Conditioned	No
N2 Conditioned vs. *lmn-1* Conditioned	No

DISCUSSION

It has been demonstrated in multiple studies that *C. elegans* possesses the ability to learn associations between different environmental stimuli, such as a sensory stimulus and hunger. Several polypeptides have already been proven to play a role in that associative learning behaviour, but hundreds of other potential regulators remain to be tested. Likely candidates are GPCRs and neuropeptides, as both play an important part in many neuronal activities. For our study we selected five such GPCRs and neuropeptides, with the aim of testing whether or not they are of any importance in associative learning. There are of course multiple ways of testing this, and as a starting point we chose one of several learning assays that are well established in the scientific community: the gustatory plasticity assay. Salt is the conditioned stimulus in this assay, absence of food the unconditioned one. Under normal circumstances *C. elegans* shows a preference for low NaCl concentrations, while after being exposed to the salt in the absence of food this behaviour is reversed. By analysing the behaviour displayed by mutants *lmr-1*, *lmr-2*, *lmr-3*, *lmn-1* and *lmn-2* and comparing it to that of wild-type worms, conclusions can be drawn about the effect of the mutation on associative learning.

Prior to the actual experiment, several optimisation steps were required. One such stage was the crossing-out of the mutants. First of all primers were designed and optimised. This was done successfully for all mutants, including *lmr*-2, which we ourselves did not cross out. As mentioned in *Results*, the mutants *lmr-1* and *lmn-2* sometimes gave faulty results in an agarose gel electrophoresis. In these cases, heterozygotes resembled homozygous mutants after visualisation. This is probably because there is a large difference in length between the shorter and longer fragments, so that the short fragment is amplified more rapidly and will have a higher concentration than the long fragment, masking its presence and leading to a false assumption of homozygosity and the detrimental effects this could have on the continuation of the experiment. Therefore we decided to use internal primers in addition to external ones, to avoid unnecessary mistakes. As a precaution we did so not only for *lmr-1* and *lmn-2*, but for the other mutants as well.

After the several optimisation steps in the experimental process we managed to obtain data for each mutant during at least four separate experiments. The controls – both negative (wild type) and positive (CX10) – showed normal behaviour, that is to say that wild type displayed an aversion to salt after conditioning while CX10 did not. For naive wild-type worms, we obtained an even higher chemotaxis index than the usual 0.8. Conditioned wild-type worms may not be as negative as desired, but they are still below zero, and thus show normal learning behaviour. CX10's values are higher than expected, but it is clear that they do not learn as wild-type worms do. All in all, the control values are acceptable, meaning the experiments were executed correctly.

When looking at the chemotaxis indices of the mutants, *lmr-3* and *lmr-1* immediately catch the eye as the most unambiguous results. They both have a relatively negative conditioned chemotaxis index and a small SEM. This leads us to the conclusion that these two GPCRs are most likely not involved in associative learning when salt is the sensory cue. For the other three mutants, however, we cannot confidently draw such definitive conclusions. All three have chemotaxis values close to 0, with a SEM of between 0.1 and 0.3. Any conclusions made based on these results would be of little value. Tukey's multiple comparisons test shows that there is a significant difference between conditioned wild-type

worms and the positive control CX10, which is what we expected. Furthermore, Table 4 also indicates that the values found for each mutant do not differ significantly from wild-type worms. However, not enough data is available to make definitive statements. Therefore we have decided to base preliminary conclusions about *lmr-2*, *lmn-1* and *lmn-2* on data from one single experiment. In this experiment many more individual assays were performed than in the others, each one being repeated in at least triplicate, making it more reliable than any other single experiment. The results of this experiment can be found in Figure 4 b. Going by these values, it seems that *lmr-2* and *lmn-2* probably also act like wild-type worms. However, *lmn-1* does tend more towards a positive conditioned index. It is therefore possible that this mutant's associative learning behaviour is affected by its lack of the neuropeptidergic ligand LMN-1. Figure 4 a. does seem to suggest the same thing, only less obviously. Although the data does point towards an adversely affected learning ability, it would be premature to make any definitive conclusions that that is the case, as follow-up experiments are necessary to be able to do so with more certainty. The same can be said for *lmr-2* and *lmn-2*, and they should not yet be discarded as potential regulators of salt-mediated associative learning behaviour until further research has been performed.

As for the question why the assays performed over several weeks show such great variation, a likely answer is that environmental influences were the largest contributing factor. Small differences in the concentration of the buffers could have similar effects. Another element of variability is the aspect of time. While counting the first of two duplicate plates, the worms on the second plate are still migrating, which means they will be on the plate for more than ten minutes. In short, there are a lot of small factors that may together be responsible for great variation. Further optimisation could help improve this test and reduce the relatively high variability witnessed in our study.

Although it is clear that *lmr-3* and *lmr-1* do not display abnormal learning capacities in this experiment, this does not necessarily mean LMR-3 and LMR-1 are not involved in associative learning behaviour at all. There are many forms of such learning, and the one examined here is but a single one of them. It is entirely possible that a pathway activated by another sensory cue causing associative learning will be deregulated in these mutants. A possible follow-up experiment would be to examine these mutants' behaviour using another cue such as butanon by means of the butanon enhancement assay. In that test butanon is used as the conditioned stimulus and food as the unconditioned one, instead of salt and hunger, but the underlying principle is the same.

If it would turn out through additional investigation that *lmn-1* is indeed a mutation that affects *C. elegans*' ability to learn, as is suggested by our results, it would be interesting to investigate if any of its homologs in other organisms play a similar role. The function of *Drosophila*'s homologue could be examined in more detail, as well as the homologous system in vertebrates. Research into those systems could bring us closer to learning more about the vertebrate brain, and how it enables us to learn and remember, among the hundreds of other things the human brain allows us to do.

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