Understanding molecular mechanism of learning and memory in *Caenorhabditis elegans*

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Certificate of Examination

This is to certify that the dissertation titled "**Understanding molecular mechanisms of learning and memory in *Caenorhabditis elegans***" submitted by Mr. Shivam Bhardwaj (Reg. No. MS08047) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: April 26, 2013

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Kavita Babu at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

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(Candidate)

Dated: April 26, 2013

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Kavita Babu

(Supervisor)

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Abstract

How do we learn and memorize has been a question in neurobiology for a long time. There have been numerous experiments to decipher the process of how information is processed, stored and retrieved by an organism. C. elegans has been used a model system to address several such questions, including our question: What is the molecular mechanism of learning and long term memory formation? What are the molecules involved and what the pathways for information processing and storage are? In this current work, we show a learning test to identify and quantify the learning in worms after training. We also show that the worms mutant in CREB show severely impaired learning. Using ChIP assays, single neuron ablation and bioinformatics analysis we plan to identify the molecules involved in long term memory formation.

Contents

Chapter 1

1.1 Introduction

Caenorhabditis elegans is a small, free-living, transparent nematode utilized as a model system to address fundamental questions in developmental biology, neurobiology and behavioral biology[1]. This project was aimed at understanding the molecules involved in the process of long term memory formation in the model system *C. elegans*.

1.1.1 Learning and Memory

Learning is the process of acquisition of information from the environmental clues, which is followed by modulations in the nervous system of an organism for retention in the form of memory.[2] There are two forms of memory based on the duration: short-term and long term, which in turn are based on the number of training trials and the time spent in consolidation.[3] Short-term memory is usually the working memory, lasting seconds to hours, whereas long-term memory last for hours to years, and in some cases, even a lifetime.[4] The short-term form requires only covalent modification of preexisting proteins, whereas the long term memory formation requires a gene expression program and de novo protein synthesis.[5,6]

Worms have been shown to have an advanced nervous system displaying various forms of learning such as associative learning, non-associative learning and imprinting.[2] They also show habituation in response to mechanical and chemical stimuli, as well as have olfactory, gustatory and thermal senses.[2] The model system is justified for the questions that we wanted to address because no other system provides simple one neuron receptors, which project onto one single interneuron, which can be ablated.[7]

1.1.2 Training Worms

A worm training protocol was standardized, which included simultaneous exposure to two volatile chemicals, an attractant Isoamyl alcohol and an aversive chemical, 2-Nonanone.[15] Isoamyl alcohol is a metabolic product of the bacteria that worms feed on and is sensed by AWC neuron, whereas the repellant 2-Nonanone is the metabolic product of pathogenic bacteria, which worms perceive through AWB neuron and selectively avoid it.[16] Worms show associative learning after spaced training of these stimuli.[18] These neurons project on to a single interneuron RIA, which in turn, produces motor response after modulating these signals.[19, 20]

1.1.3 CREB

It has been well reported in literature that novel protein synthesis is required for long-term consolidation of memory.[8,9,10] This protein synthesis can either by translation of existing mRNAs or by transcription followed by translation.[9] CREB (CyclicAMP Response Element Binding Protein) is a mammalian cellular transcription factor and its activation has been shown to have major roles in long term memory (LTM) in Drosophila, Aplysia and mice.[11] *Crh-1* (CREB homolog family member 1) is the worm homologue of CREB,[12,13]. The two major domains of crh-1, the kinase inducible domain and the basic and leucine zipper domain are highly conserved, showing 80 and 95% identity respectively.[31] Catherine Rankin and Tiffany Timbers have shown in 2011 that loss-of-function mutants of *crh-1* have memory defects. They used a mechanical tap-withdrawal assay coupled with 1-Propanol as a volatile compound, to show that wild type worms show withdrawal response to tap after training, whereas the *crh-1* mutants do not show any such learning. [33]

In order to identify the molecules involved in LTM, we tried to find out all the genes which interact with crh-1 which could purportedly be the potential molecules in the long term potentiation pathway[13]. For this, we did a pull-down of the genome after training to freeze the gene interactions, with flag antibodies for crh-1 followed by Chromatin Immunoprecipitation-Sequencing (ChIP seq) analysis. ChIP sequencing is a technique used to sequence the fragments of genomic DNA which co-precipitate with a DNA binding protein such as chromatin-modifying enzymes or transcription factors.[14]

1.1.4 Single neuron ablation

Alternatively, experiments were designed to ablate the interneuron, which is the potential site of the de novo protein synthesis to check the effects on LTM.[21] For this, two approaches were taken. A QF-QS system, which is analogous to the Gal4-UAS system, was used to selectively induce apoptosis in the interneuron of the learning circuit.[22] In another approach, a recent finding on mitochondrial mini Singlet Oxygen Generator (miniSOG) will be used to ablate the interneuron using blue light illumination.[23]

1.2 Materials and Methods

1.2.1 : Worm Handling and Maintenance

C. elegans is grown in the laboratory on Nematode Growth Medium (NGM) agar petri plates with *E. coli* strain OP50 as a food source, which is a uracil auxotroph with limited growth on NGM plates.[24] The worms were maintained at 20^0C throughout the experiments.

Bleaching is done to prepare eggs and maintain synchronization in the life stage of worms. For learning and memory assays, all the worms should preferably be in same stage at the time of training. [24, 25] Bleaching is particularly useful in removing the contaminations from worm plates[24].

Worm stocks can be frozen for several years. This is achieved by putting freshly starved L1 or L2 worms in a freezing mixture of S. Basal and glycerol and storing at -80^0C.[26]

1.2.2 Acquisition of Strains

The strains that we used for our experiments included **N2** (wild type worms) and **YT17**. N2 was acquired in the lab from Dr. Sandhya P. Koushika from NCBS. YT17 strain was acquired from CGC. YT17 is a 6x outcrossed mutant for *crh-1(tz2) III*.[28] *crh-1* encodes a *C. elegans* homolog of the cyclic AMP-response element binding protein (CREB) and is involved in the transcription of CRE-sequence dependent genes activated by a CaM kinase cascade.[27] A mutant for *crh-1* was used as a negative control to assess the efficiency of memory formation in absence of homolog of CREB protein.

1.2.3 Outcrossing

The worms obtained from sources were outcrossed at least 2x to confirm the phenotype and remove background mutations. Genotyping was done by PCR amplifying the DNA using gene specific primers from single worms to find out the correct strain.

1.2.4 Experimental Design

In order to address the quest of the molecules involved in long term memory formation, two approaches were undertaken. One included the CREB mediated learning followed by ChipSeq, whereas in the other case we tried to look at the single neuron (RIA interneuron) ablation to assess the effects.

1.2.5 Worm Training

The worms were synchronized to a stage by egg preparation before training. The fresh eggs were then kept at 20^0C for 3 days. These worms were then given a spaced training of repeated exposure to a mixture of two volatile organic compounds, Isoamyl alcohol and 2-Nonanone. Isoamyl alcohol is an attractant and is perceived by the AWC neuron. 2-Nonanone is a repellant and is perceived by the AWB neuron. An inter-stimulus interval (ISI) of 0 minutes (i.e. exposing worms to the two chemicals simultaneously) and an inter-training interval of 7 minutes with 5 times conditioning were standardized and repeated throughout the experiments. After training, the worms were kept at 20^0C for 18-24 hours before the chemotaxis experiments were performed.

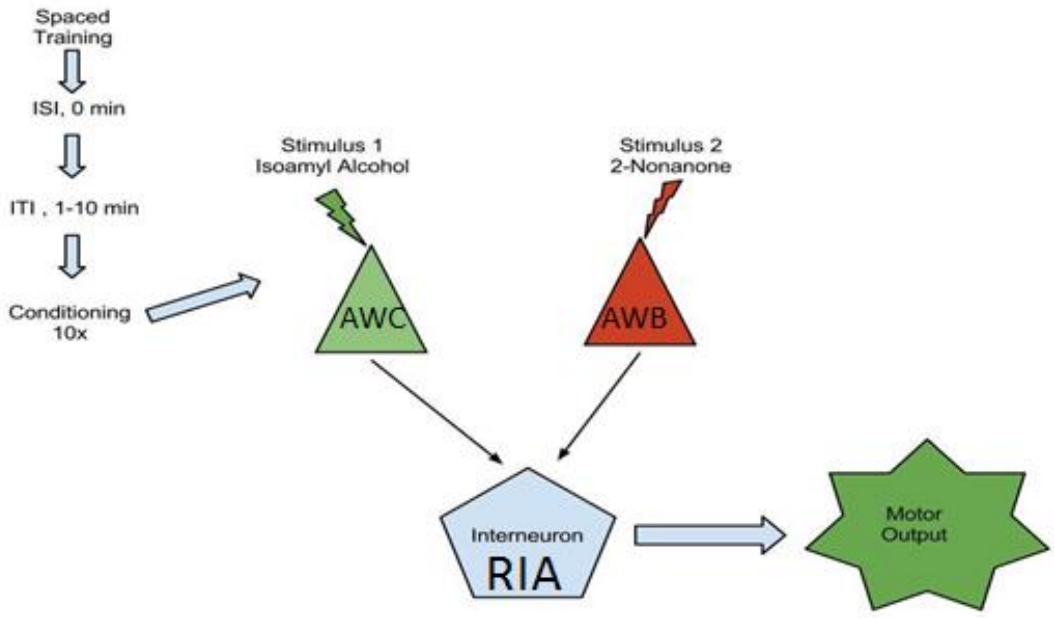


Fig. 1 : Worm training Protocol. Worms were given spaced training with Isoamyl alcohol and 2-Nonanone, which had an inter-stimulus interval (ISI) of 0 min and Inter-training

interval of 1-10 minutes. The conditioning was done for up to 10 times initially. Later, a training of 5 times with an ITI of 7 minute was standardized.

1.2.6 Chemotaxis

The well-fed worms on Day 4 after hatching were put on a standard 90mm petri plate using wide bore low retention tips. The food was washed away before putting up the worms on chemotaxis plate. This was done to minimize the effects of age, locomotion and olfactory sensitivity on chemotaxis assay.

The chemotaxis plate was marked into 7 different grids, and worms were put in central grid of the plate. A drop of attractant or repellant chemical is kept at one end of the plate. On both the ends of the plate a drop of Sodium azide was put. Sodium azide is a paralyzing agent and inhibits the activity of catalase, peroxidases and cytochrome oxidase. This ensures that the worms will not show random movements after being attracted towards an attractant or away from a repellant.

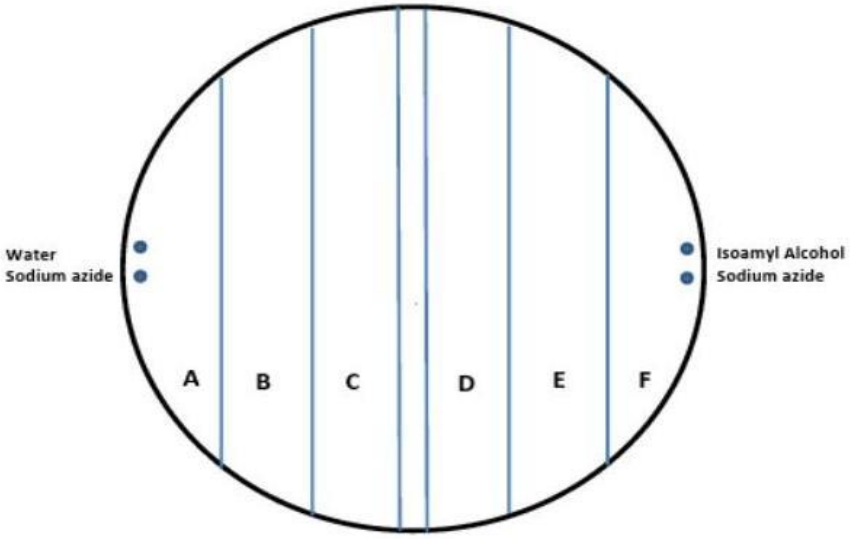


Figure 2 : Standard Chemotaxis Plate

Based on the movement of worms on the plate, a chemotaxis index (CI) is calculated using the following formula:[29]

$$CI_{volatile} = \frac{(\text{No. worms at attractant}) - (\text{No. worms at control})}{\text{Total no. worms}}.$$

1.2.7 Learning Index

The simultaneous conditioning of both the attractant and repellant chemicals induce learning in the worms. This learning can be characterized using the following formula:[30]

Learning Index = CI of **Control Untrained** worms - CI of **Trained** Worms

For all the chemotaxis and learning experiments, we used 100 worms on each petri-plate. All the trainings were made in standardized conditions and the assays were done in triplicates. The data was statistically analyzed with students' t-test and the graphs were plotted in Prism software.

1.2.8 ChIP Sequencing

Young adult worms were trained using the protocol described above. After training, the worms were lysed and the DNA-protein complex was cross-linked using Formaldehyde. DNA was then digested using nuclease into smaller fragments (~500bp). A flag-tag Antibody was generated against CREB and the protein DNA complex was kept overnight for antibody incubation. It was followed by washing to remove non-specific binding and Proteinase K treatment. The reverse crosslinking was done overnight and DNA was recovered subsequently, which then was detected by PCR.[14]Untrained N2, Trained YT17 and Untrained YT17 were used as the controls for the ChIP experiments. The samples were sent to C-CAMP, Bangalore for sequencing. However, due to poor sample concentration and possible loss of sample in transportation, the reaction could not be completed.

1.2.9 Neuron Ablation Strategies

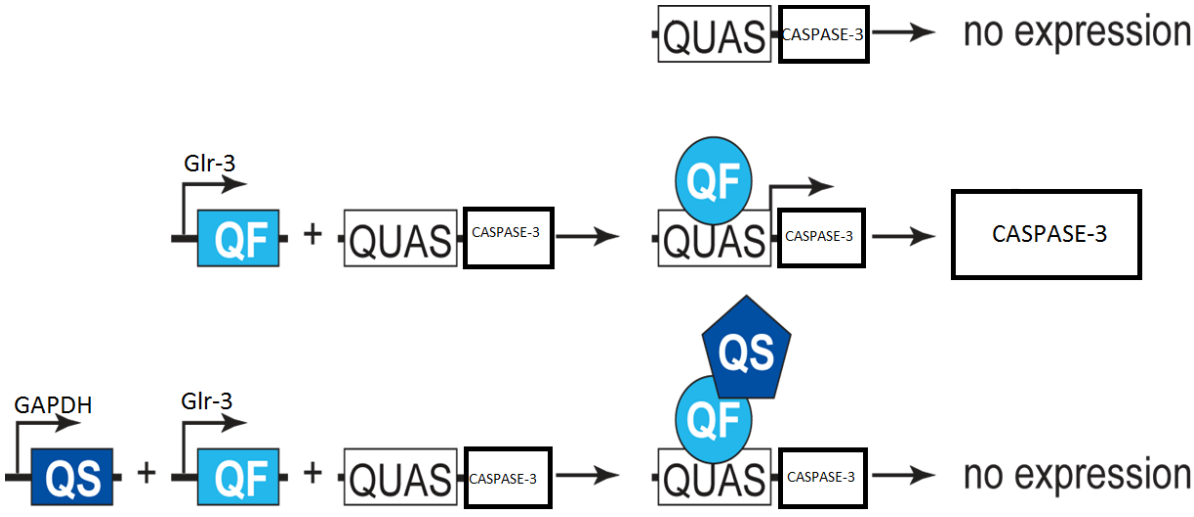
We tried using two alternative strategies to ablate the interneuron RIA to understand the possible role in mediating memory formation. In first method, we designed constructs to

induce a caspase mediated apoptosis. While we were working towards it, a novel method was reported in literature, in which a mitochondrial targeted mini SOG was used to selective ablate a cell without causing detectable damage to surrounding cells.

1.2.10 Constructs

In order to selectively ablate the interneuron, we induced caspase-mediated apoptosis in the RIA interneuron of the learning circuit. For this, a QF-QS system was used. QF-QS system is a gene expression system similar to the yeast GAL4/UAS system but based on genes from the *Neurospora crassa qa* . It consists of two regulatory proteins (QF and QS) and a binding site for QF called QUAS (QF upstream activating sequences). QF is a transcriptional regulator which after binding to QUAS causes downstream gene activation. QS is a repressor for QF and is an effective blocker for downstream gene activation of QF. Quinic acid can be used for suppression of repression by QS. This system offers an advantage of low basal expression in the absence of the transcriptional activator QF, high level of expression in presence of QF, and QF repression by QS.

We used a Glr3 promoter tagged to QF and a GAPDH ubiquitous promoter linked to QS. Our gene of interest for caspase-mediated apoptosis was tagged downstream of QUAS.



(Adapted from Christopher J. Potter, Johns Hopkins University)

Fig. 3 : Neuron Ablation Strategy using QF-QS system

The specific constructs made are the following:

1. QS with ubiquitous promoter in pPD49.26 vector
2. QF with specific promoter (eg. Glr-3) in pPD49.26 vector
3. 5x QUAS in pPD49.26 vector

1.2.11 miniSOG constructs

miniSOG is an engineered mini singlet oxygen generator protein. It is a 106 amino acid fluorescent protein which generates singlet oxygen in quantum yield upon blue light illumination**.** Transgenically expressed mitochondrial targeted miniSOG (mito-miniSOG), on blue light illumination, causes rapid and effective death of neurons in a cell autonomous manner without detectable damages to the surrounding tissues.

For this we are in the process of designing a **sl2-glr3-miniSOG-mCherry** construct. SL2, or the spliced leader 2, is a non-coding RNA sequence which trans-splices in lower eukaryotes, to give distinct gene products. Glr-3 is a promoter expressed in RIA interneuron.

1.2.12 Worm Microinjections

The constructs were injected in the worm gonads using an eppendorf FemtoJet and Injectman worm injector. Worms were fixed on a 2% agarose gel pad and a drop of halocarbon oil was put on the pad to avoid dehydration of worms. Nikon Eclipse Ti microscope was used to visualize the worms while injecting. The needles for injections were pulled using a Sutter P1000 needle puller. The injections were made exactly at the gonads of the worms, and post injection, worms were suspended in M9 buffer for revival. After revival, each injected worm was put on a separate plate with food and progeny were tracked for few days to identify the transgene.

2.1 Results

2.1.1 Chemotaxis in untrained wild type worms

Control untrained worms show a clear response to the attractant and repellant during chemotaxis. Worms show a CI of -0.9 for 2-Nonanone, suggesting that worms are repelled from it. On the other hand, worms show a CI of 0.8 in response to exposure to Isoamyl alcohol, representing that worms are attracted towards it. Worms do not show any directional movement in response to water, which is represented as a chemotaxis index of 0 for it.

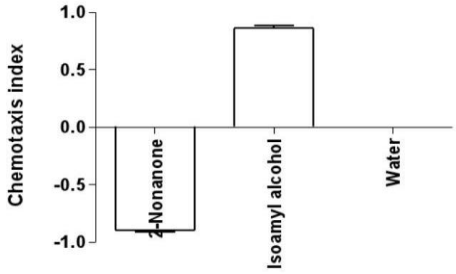
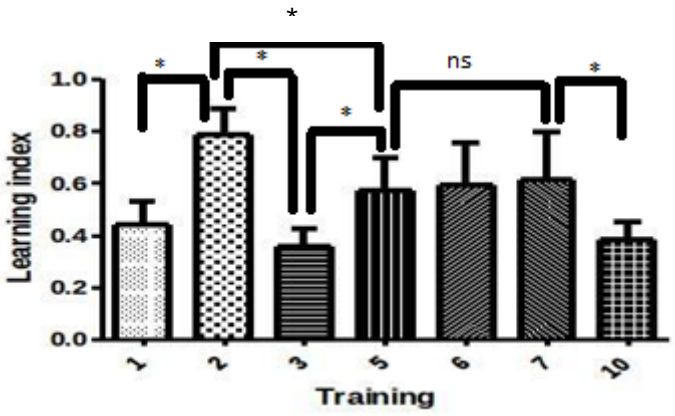


Fig. 4 : Chemotaxis index of N2 wildtype worms without training. Worms are repelled from 2-Nonanone, are attracted towards Isoamyl alcohol and show neutral response when exposed to water.

2.1.2 Standardization of worm training

In order to standardize the training procedure, a set of combinations were tried using different exposure times and inter-training interval as well as the number of trainings (1-10). The learning index was calculated for each number of training. The worms show maximum learning when trained twice, however this significantly reduced when the conditioning was repeated thrice. We used a standard training of 5 times for our experiments as it was the minimum number of trainings which was completely replicable and learning index was conserved for it.

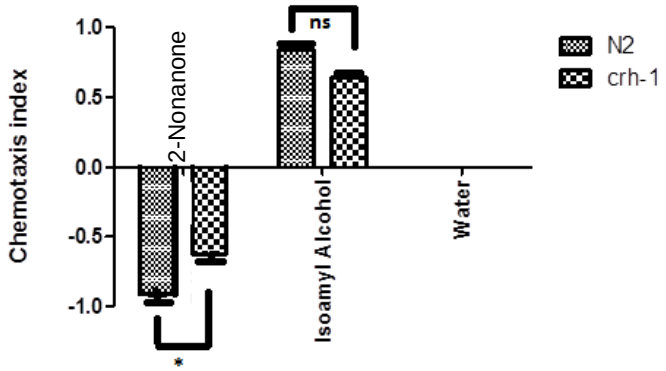


Key: ns : non-significant ; * : Significant, 0.01< p <0.05 (Students t-test)

Fig. 5: Standardization of the training protocol. Worms display higher learning when given a 5 to 7 times spaced training. A high learning index at 2x training is not consistent when we go for 3x training.

2.1.3 Chemotaxis in wild type vs mutant worms

We also checked for the levels of chemotaxis in the crh-1 mutants (CREB homologue) to verify the response to the two chemicals. The mutant worms show a similar response to the two chemicals with respect to the wild type, N2 worms. This shows that the mutant worms do not have any problem with the odor receptors for Isoamyl alcohol, and hence, can be used as control for the learning experiments.

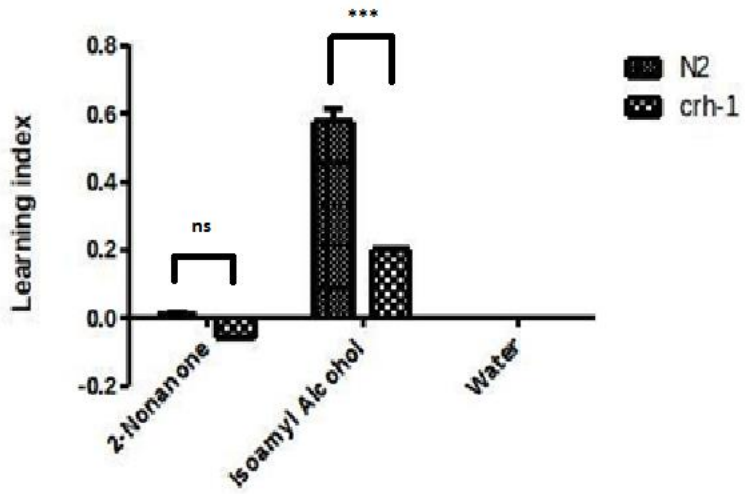


Key: ns : non-significant, p>0.05; * : Significant, 0.01< p <0.05 (Students t-test)

Fig. 6: Chemotaxis index for N2 wild-type worms compared to the crh-1 mutant worms. These worms show a decreased response to the two chemicals, 2-Nonanone and Isoamyl alcohol. Both type of worms show neutrality towards water.

2.1.4 Learning in wild type worms vs mutants

We now wanted to check the learning index in mutant worms to understand the defects in learning. In case of chemotaxis with 2-Nonanone, the two different strains do not show a major difference. With Isoamyl alcohol, mutants show severely impaired learning (3.5-fold, $p<0.001$, Students t-test).



Key: ns: non-significant, p>0.05; *** : Highly Significant, p <0.001 (Students t-test)

Fig. 7: Learning Index Comparison. The learning is severely reduced in case of mutants of *crh-1* when compared to the wild type worms.

We wanted to check the effects of training on worms in both the wild-type N2 and the mutant crh-1 strain YT17. Before training, the normal and mutant worms show similar response to Isoamyl alcohol and are attracted towards it. On the other hand, after training with a mixture of two chemicals, the N2 worms moving towards Isoamyl alcohol are greatly reduced, whereas the mutants show no significant change in the movement pattern of

worms. This also suggests crh-1 (CREB homologue in worms) indeed plays a role in the learning and long-term memory formation pathway in worms.

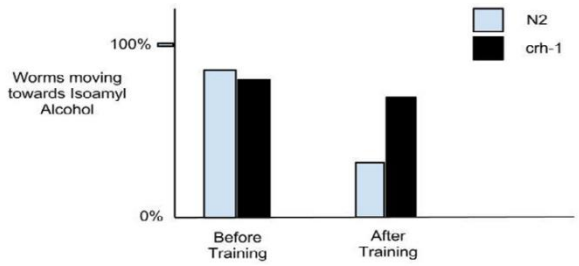


Fig. 8: Schematic of Movement of worms towards isoamyl alcohol pre- and post-training. N2 worms learn to avoid isoamyl alcohol, whereas crh-1 mutant worms do not show any significant learning.

2.1.5 ChIP Sequencing

After we knew that the learning is impaired in *crh-1* mutant worms, we wanted to find out the difference in the genes that bind to CREB or its homologue crh-1 in wild type worms and mutants. For this, a chromatin immuno-precipitation assay was done with 4 samples. To check the genes that are activated after training by CREB, we trained wild type N2 worms and 3 other controls, untrained wild type worms, trained *crh-1* mutants and untrained *crh-1* mutants to get rid of all the background interactions of CREB and find out only the genes activated during the learning and memory consolidation process. The samples were then sent to C-CAMP, Bangalore for sequencing. We were reported that only one sample (Trained wild type worms) out of the four sent had sufficient sample concentration and volume. There was a possible loss in sample quality and quantity either during preparation or transport, and hence we could not proceed with the ChIP assay. Currently Dr. Yogesh is finding out genes regulated by crh-1 by a bioinformatics approach of finding out the genes having a crh-1 binding sequence. Once these genes are shortlisted, we plan to do gene knockdowns by RNAi to assess the effects on learning in these worms.

2.1.6 Glr-3 promoter GFP microinjections

RIA is an interneuron, central to the learning circuit of this experiment. It processes the incoming signals from two neurons, AWB and AWC, and modulates the information to store it in memory or provide the directions to motor neurons. We wanted to know what happens to the learning process in case this interneuron is removed. We searched for this in literature but this kind of experiment was never done before. We tried to selectively ablate this RIA interneuron. There is a Glr-3 promoter specific to RIA interneuron. Before ablation we wanted to visualize it, and hence we tagged GFP to the promoter and then microinjected it in the worms. The F1 progeny expressed Glr-3 promoter GFP, which was found under dissecting microscope, to be expressed in a single neuron. The neuron position was found with the help of wormbook, and it exactly matched the RIA interneuron location. However, we were not able to make stable extrachromosomal arrays of Glr-3 promoter GFP.

2.1.7 Single Neuron Ablation

We now had the preliminary data that Glr-3 is a promoter specific to RIA interneuron which can be used with a marker florescent protein to identify the correct neuron, we design the constructs in two different approaches to selectively induce apoptosis or cell death of RIA.

2.1.7 (a) : QF-QS system

As discussed in the materials and methods section, I made the following constructs:

1. QS with ubiquitous promoter (GAPDH) in pPD49.26 vector
2. QF with specific promoter (Glr-3) in pPD49.26 vector
3. 5x QUAS with Caspase-3 in pPD49.26 vector

This system, similar to the UAS-Gal4 system, can cause selective gene expression. These constructs were microinjected in worms to induce caspase mediated apoptosis of RIA. However, this method had some limitations. Apoptosis generates some cell debris after

cellular degradation, which is released in extra cellular matrix (ECM) and can cause considerable damage to the surrounding cells. After we made these constructs, we found out a recent report on mitochondrial miniSOG, which is highly specific and no considerable damage is detected for surrounding cells.

2.1.7 (b) : mitochondrial miniSOG system

We currently have a construct with sl2 and we are in the process of making a sl2-glr3-miniSOG-mCherry construct. Once prepared, we will microinject this construct into worms. With blue-light illumination, the RIA interneuron, with Glr-3 promoter can be selectively targeted to rapid death. SL2 will selectively splice the RNA to give two products, glr3-miniSOG and glr3-mCherry. That will make detection of neuron to be ablated easier since we can visualize mCherry being expressed in the neuron. After the neuron is ablated, mCherry would no longer be seen, which would be a confirmation of the death of RIA neuron.

2.2 Discussion and Future Directions

C. elegans is used as a simple yet wonderful model system for learning and memory behavioral assays, with evolved forms of responses towards heat, odors, chemicals, touch and other mechanical inputs. It has been shown that the worms learn when presented to a massed or spaced training, and it can possess short term memory or long term memory respectively. Short term memory is protein synthesis independent whereas long term memory requires de novo protein synthesis. The difference between two training protocols is a time interval between two subsequent trainings (Inter training interval, ITI) in case of spaced training, which is absent in massed training. The time duration, or ITI was similar to other organisms such as drosophila, honey bees and crickets, however it was standardized for present study. It has also been reported that the more closer the stimuli are together in time, higher is the efficiency of learning. It led us to simultaneous exposure of these stimuli, attractant Isoamyl alcohol and repellant 2-Nonanone, to the worms. These two stimuli are specifically receipted by one neuron each, which then project on an

interneuron which is then responsible for motor response, or de novo protein synthesis for long term memory formation. The chosen simple circuit makes it easier to tweak the circuit by neuron ablation to identify the effects on learning mechanism.

It has been well reported in literature that de novo protein synthesis is required for long term memory formation, however the exact cellular and molecular machinery is yet far from being completely understood. The present study was aimed at finding novel molecular components of the learning and memory mechanism. CREB is an important transcription factor, whose *C. elegans* homologue is *crh-1*, central to the activation of a series of genes which are involved in long term memory formation. In this study, it was proposed to look for the genes that interact with CREB during or immediately after the spaced training, where the training would be consolidated in the form of memory by de novo protein synthesis. CREB is supposed to be activating these genes for transcription and translation. Chromatin immunoprecipitation (ChIP) is a technique used to investigate the gene-protein interactions in a cell. The cell-lysate of *C. elegans* was cross-linked with protein, which was then sheared and immunoprecipitated with a flag-tag to identify all chromatin fragments interacting with CREB. These fragments were then purified and sent for sequencing. ChIP assays in whole worms are complex because of interference by several factors such as requirement of a large population of worms and contaminating DNA from bacterial food.

Glr-3 is a RIA interneuron specific promoter and we were able to tag it to GFP and microinject in worms. The expression pattern was visualized under dissecting microscope and confirmed with the wormbook reference. It also opened up a possibility of single neuron ablation as identification of the correct RIA interneuron was possible.

RIA interneuron is the center of the neural circuit that we are using. It receives inputs from the two neurons, AWB and AWC, which then are modulated and the processed signals are sent for motor output. This is also the probable site for de novo protein synthesis for long term memory formation. We want to look at the effects on the learning and memory abilities of the worms when this central RIA interneuron is missing. For this, we selectively wanted to induce caspase mediated apoptosis in this cell using a binary Q-system (QF-QS).

We tagged Caspase-3 with QUAS, Glr3 promoter with QF and a ubiquitous promoter GAPDH with QS. The constructs were being prepared, which could then be microinjected into worms to get transgenic worms with ablated RIA interneuron.

To conclude, we have successfully developed and standardized a protocol for spaced training of worms using Isoamyl alcohol and 2-Nonanone. Worms show the behavioral response of learning in chemotaxis assays that follow training. Mutants for crh-1 have impaired learning, as previously reported in literature. The ChIP sequencing for a protein for *C. elegans* is novel, however, we could not get the desired DNA quantity and purity. The QUAS mediated apoptosis experiments were being performed when miniSOG was reported to selectively ablate cell using blue light illumination. Since there is unreported damage to adjoining cells, we have decided to proceed with miniSOG mediated neuron ablation.

We now plan to do a bioinformatics analysis of CREB binding sites in *C. elegans* genome. After a shortlisting of candidates, we will knockdown candidate gene by RNAi and see their roles in memory formation.

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