FUNCTIONAL REGULATION OF AIY INTERNEURONS IN OLFACTORY IMPRINTING AND ITS INFLUENCE ON LEARNING AND MEMORY IN *Caenorhabditis elegans*

A THESIS PRESENTED BY

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TO

SREE CHITRA TIRUNAL INSTITUTE FOR

MEDICAL SCIENCES AND TECHNOLOGY

Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

2021

DECLARATION BY THE STUDENT

I, Aswathy A R, hereby declare that I had personally carried out the work described in the thesis entitled **"Functional regulation of AIY interneurons in olfactory imprinting and its influence on learning and memory in *Caenorhabditis elegans*"** under the supervision of **Dr. Anoopkumar Thekkuveettil**, Scientist G, Division of Molecular Medicine, BMT Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala. No part of the thesis has been submitted for award of any other degree or diploma prior to this date.

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The thesis entitled **"Functional regulation of AIY interneurons in olfactory imprinting and its influence on learning and memory in *Caenorhabditis elegans*"** was carried out under my direct supervision. No part of the thesis has been submitted for award of any other degree or diploma prior to this date.

Date: 30-12-2021 **Dr. Anoopkumar Thekkuveettil**

The thesis entitled

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for the degree of

Doctor of philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
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ACKNOWLEDGEMENT

First and foremost, I owe my deepest gratitude to GOD ALMIGHTY without whose blessings, this Ph.D. training and its successful completion would not have been achieved and would have remained only a dream for me.

It gives me great pleasure in acknowledging the support and help of my mentor, Dr. Anoopkumar Thekkuveettil, for his outstanding mentorship and selfless guidance throughout my time in the lab. The scientific achievements I may have in the future will mostly be because of my training under his guidance. I will always appreciate his support, patience and wonderful personality.

I cannot find words to express my gratitude to my doctoral advisory committee members, Dr. A. Maya Nandkumar and T. V. Anilkumar for their valuable time and suggestions while serving on my committee.

Furthermore, I would like to extend my heartfelt thanks to former Director Dr. Asha Kishore, Head BMT Wing Dr. Harikrishna Varma, the Registrar Dr. Santhoshkumar, Deputy Registrar Radha M and the staff of Academic affairs for providing the opportunity to work in this institute and also the necessary facilities throughout my Ph.D. tenure.

My doctoral research period was amply funded by the CSIR, HRDG fellowship. Momentary support had a role in motivating, sustaining and focusing primarily on research interests.

Molecular medicine lab’s proud legacy was built by the people who worked and contributed their intellect, patience and hard work to the team. I am profoundly grateful to former and present members of the lab, Dr. Bejoy Vijayan, Ms. Agrima Nair, Mr. Vishnu Raj, Ms. Rasitha S, Ms. Swathy. S. Nair, Mr. Amal Wilson Varghese, Ms. Deity Thomas, Ms. Geethu G. S, Ms. Radhika, Mr. Vipin, Mr. Fayad, Ms. Nikhitha Mathews, Mr. Jose Jacob, Ms. Anusree, Ms. Anjana U, Ms. Ria Elza Varghese, Mr. Arumugam Selva Sharma for their commitment, integrity and friendship and allowing me to inherit, inculcate and pass on the interests and values in scientific research. I shall always remain indebted to my colleagues who supported me during my stay in the lab.

There are no words to express how grateful I am to my family for their unconditional love, advice, support and motivation. I am eternally thankful to my father, my mother, my husband and our little world for their patience, endurance and love in difficult times. I am what I am today because of them.

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LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technology
BT	Butanone
CaMK	Calcium calmodulin-depended protein kinase
cAMP	Cyclic Adenosine Monophosphate
CCD	Charge Coupled Device
CGC	Caenorhabditis Genetic Center
CI	Chemotaxis Index
CREB	cAMP Response Element Binding Protein
CS	Conditioned Stimulus
DNA	deoxy ribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethidium
GFP	Green Fluorescent Protein
GPCR	G-protein coupled receptor
IAA	Isoamyl alcohol
IPTG	Isopropyl beta D-1-thiogalactopyranoside
ITAM	Intermediate-term Associated Memory
LB	Luria Bertani
LTAM	Long-term Associated Memory
NGM	Nematode Growth Medium

NMDA	N-Methyl-D-Aspartic acid
OD	Optical Density
PCR	Polymerase Chain Reaction
PKA	Protein kinase A
PST	Parr-Smolt Transformation
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
SDS	Sodium Dodecyl Sulphate
STAM	Short-term Associated Memory
TAE	Tris Acetate EDTA
US	Unconditioned Stimulus
UV	Ultraviolet

SYNOPSIS

Introduction

Memory is a complex process that leads to a more or less permanent change in the behavior of an organism. The formation of memory involves changes in neural connections due to synaptic plasticity – and these are believed to alter behaviour. Understanding the cellular components involved in memory is critical because many neurological disorders primarily impair memory formation and recall pathways. For example, Autistic disorder is associated with connectome alterations and is significantly influenced by the environment during the early developmental stage. Alzheimer's disease impairs memory recall because of extensive neuronal damage.

The fundamental pathways involved in learning and memory are highly conserved across species. This study focused on two different types of memory: imprinted memory and learning and memory.

Imprinting is a kind of phase-sensitive learning which is rapid and independent of the consequences of behaviour. Imprinting also has a critical period - mainly during the early developmental stages. There are two categories - sensory imprinting and genomic imprinting. Sensory imprinting produces life-long attachment to environmental features experienced during a critical period of early development and is an important form of adaptive behavioural plasticity. Genomic imprinting occurs as the suppression of specific genes on chromosomes, depending on which parent they received.

Learning and memory are processes through which new information is acquired during a lifetime and alters behavioural patterns. Learning and memory are mainly of two types- associative learning and non-associative learning. Associative learning happens along with a condition, but non-associative learning place due to a stimulus.

Much of what has been learned about the neural and molecular mechanisms of learning and memory have come from using model systems that are amenable to cellular analyses. *Caenorhabditis elegans* is an excellent model system to study imprinting as well as learning and memory. In this study, I tried to understand the effect of imprinting on learning and memory. Another important aim was to elucidate the neuronal connectome involved in both the type of memory formation.

Hypothesis

Epigenetic mechanisms during early development can lead to behavioural changes in the organism. I hypothesized that does these changes lead to synaptic alterations and other behaviour like learning and memory pathways? In *C. elegans*, it is known that the AIY interneuron has a role in the imprinting pathway. I also hypothesized that the additional neuronal connectomes to AIY neurons could play a critical role in this form of memory.

Materials and Methods

Wild type Bristol N2 strain. Mutant strains include RB816 (*sra-11(ok630) II*), JC2209 (*olrn-1(ut305) X*), RB594 (*glc-3(ok321) V*), KP4 (*glr-1(n2461) III*), RB1808 (*glr-2(ok2342) II*), VM487 (*nmr-1(ak4) II*), OH910 (*otIs77 II, Kal-1*), IK705 (*njIs10 [glr-3::GFP*]), OH3976 (*otIs151; otEx2314* - Expresses GFP in ASI, RIA, PVT and

AWA), RB1690 (*ser-2(ok2103) X*), UA44 (*baIn11 [Pdat-1::A-synuclein, Pdat-1::GFP*]), ZM9078 (*hpIs587 [flp-14p::GCaMP6::wCherry + lin-15(+)*]) (all the strains from CGC) and AT9203 (*ttx-3p::GCaMP6::mCherry*, 50ng/µl and 5ng/µl, was generated by microinjection of the plasmid created by our lab), DCR744 (*cima-1(wy84) IV; wyIs45 X*), DCR775 (cima-1 rescue- cima-1 genomic region with 1.9kb promoter), DCR2188 (*ttx-3p::CD4::GFP(11) + glr-3p::CD4::GFP(1-10) + ttx-3p::mCherry::rab-3 + unc-122p::GFP*) (The DCR strains were the gift from Colon Ramos, Yale University). All nematodes were grown on NGM containing *E. coli* strain OP50 at 20^0C. siRNA worms were grown in HT115 *E. coli* culture and kept at 17 ^{0}C.

Chapter 1:

This chapter mainly includes imprinting during the critical period of development and its effect on adult life. Protocols were designed to identify how the early environmental stimulus can change the behaviour of an organism through neuronal plasticity. Mainly olfactory imprinting towards isoamyl alcohol was carried out. Synchronized eggs up to their L1 stage (24 hours) were exposed to 1/300 diluted isoamyl alcohol (IAA). The adult behavioural analysis was done using primary assays like chemotaxis and quadrant assays to determine the difference between non-imprinted and imprinted strains. siRNA knockdown of a specific gene to find out the imprinting in AIA having sra-17 receptor. The general movement pattern was recorded using a camera (SONY ILCE 7M III K with SONYFE 50MM M2.8 Lens) and was analyzed using the worm tracker software (WormLab, MBF Biosciences, USA). Actinomycin D and

Cycloheximide were used to detect the role of transcriptional and translational level changes involved in the imprinting pathway. Different neuron-specific gene mutants were used to find its involvement in olfactory imprinting, main neurons such as AWC (*str-2*), AIY (*sra-11, kal-1, glc-3, ser-2*), AIB (*glr-1, glr-2*), RIA (*cima-1*), and RID (*flp-14p::GCaMP6*). Semi-quantitative RT-PCR method was used to quantify sra-11 expression levels. Transgenic strains (*ttx-3p::GCaMP6*) with GCaMP6 specifically targeting AIY were used for calcium imaging. Non-imprinted and imprinted worm's AIY and RID neuronal calcium patterns were plotted based on the fluorescent intensity change.

Chapter 2:

In this chapter, the involvement of olfactory imprinting in associative learning and memory was studied. Associative learning and memory were created in the presence of 1/10 butanone (BT) along with OP50. Non-imprinted and imprinted worms were conditioned for short-term and long-term olfactory associative memory training in wild type and mutant strains to elucidate the pathways shared between these two processes. Calcium imaging of non-imprinted and imprinted worms undergone long-term associative memory training was used to identify the alterations in neuronal calcium flux.

Major Findings

Chapter 1

Imprinted worms showed an increased attraction towards IAA compared with the non-imprinted wild-type strain, and this attraction was persistent in its later stages. The track pattern of the wild-type imprinted worms showed a smooth pattern, but the non-imprinted worms showed many stop points. I quantified the wave parameter such as the wavelength of the non-imprinted and imprinted N2 wild-type strain. The imprinted worms reached significantly faster at the target, and the wave amplitude was higher than non-imprinted ones.

The quadrant assay of imprinted worms showed an increase in attraction towards odorants, both IAA and BT, suggesting an overall change in odorant attraction behaviour in imprinted worms compared to the control. Blocking transcription and translation with actinomycin D and cycloheximide, respectively, resulted in a significant reduction in imprinted behaviour, indicating that gene expression variations are essential in imprinting.

In the AWC sensory neuron mutation (JC2209-*str-2*), olfactory detection is defective. If the *str-2* gene is mutated, it will switch off both the AWC neurons. AWC neuron is the sensory neuron that can detect volatile odorants like isoamyl alcohol, butanone, benzaldehyde, 2,3-pentanedione, and 2,4,5 trimethylthiazole. AIY interneuron is immediately downstream of the AWC sensory neuron. There are 26 synaptic connections present between AWC sensory neuron and the AIY interneuron. The role of the AIY interneuron was tested in imprinted behavior and found that mutation of a seven-transmembrane receptor *sra-11* significantly impaired the imprinting pathway. siRNA knockdown of seven-transmembrane receptor *sra-17* uniquely present in AIA interneuron was found to have no critical role in olfactory imprinting.

It was confirmed that any defects in the connectome between the AIY interneuron and upstream or downstream neurons cause a deficiency in olfactory imprinting by studying various mutant strains. The mutant *cima-1 (cima-1(wy84) IV; wyIs45 X*), a glial protein, which helps maintain the proper connection between AIY to RIA mutation, leads to imprinting an olfactory defect. But the *cima-1* rescue strain regained the imprinting, thereby confirming the involvement of *cima-1* and the need for proper AIY to RIA connection in imprinted memory. Studying the various mutant strains -*str-2* (AWC), *sra-11, cima-1, kal-1, glc-3, ser-2* (AIY), *glr-1, glr-2* (AIB), *nmr-1* (AVA), *sra-17* (AIA)- I could elucidate that the neuronal involvement for imprinting starts from AWC sensory neuron to AIY interneuron to RIA motor neuron. Semi-quantitative RT-PCR results showed that *sra-11* expression level is maintained low in *cima-1* mutants compared to the wild-type N2 worms. Imprinted worms also showed a reduction in *sra-11* level compared to the control. During imprinting, there is an attenuation of the *sra-11* gene expression level. In *cima-1* strain, the level of *sra-11* has maintained a constant low level.

Calcium imaging data confirms that both AIY and RID neurons are involved in solvent recognition. However, calcium levels maintained a constant level both in AIY and RID neurons in imprinted worms, suggesting worms have habituation behavior to the solvent after imprinting.

Chapter 2

To find out the effect of imprinting in learning and memory, the non-imprinted and imprinted worms were conditioned for short-term associative memory (STAM) and long-term associative memory (LTAM) training. The results showed that the imprinted worms could recall the memory for a longer period than the non-imprinted worms. AWC mutant worms (JC2209) were defective in odorant detection. AIY neuron-specific mutation of *sra-11* (RB816) showed an extended STAM memory but a defective LTAM. AIY-RIA connectome alteration mutant (*cima-1* mutant strain -DCR744) was imprinting defective and also associative memory defective. But the imprinted *cima-1* mutant worms showed a short-term memory pathway intact. *cima-1* rescue worms (DCR2188) showed normal STAM, but its LTAM is defective.

KAL-1 protein is required for the normal axonal branching in the AIY interneuron. In the *kal-1* mutants (OH910), both STAM and LTAM were defective, but the imprinted kal-1 mutant worms showed an ability to acquire STAM. This result was similar to that of the *cima-1* mutant. We would like to hypothesize that during imprinting, some of the synaptic connections are consolidated and maintained even in the background of genetic mutations that generally alter the synaptic connectomes. My results suggest that for LTAM memory formation, both GLR-1 and GLR-2 receptors are required, but for STAM, GLR-1 is essential. Another important finding is that after imprinting, the *glr-2* mutant strain showed STAM similar to the N2 wild type. This study could identify that the SER-2 receptor is necessary for positive olfactory imprinting and associative memory training.

Calcium imaging of AIY neurons in long-term associative memory trained worms showed no spike in $Ca^{2+,}$ confirming our hypothesis that habituation attenuates calcium flux. The RID interneuron is involved in the functional migration of the worm, especially in the forward movement. When the RID: GCaMP6 labeled strains were imprinted and memory trained, the Ca^{2+} spike was significantly higher than non-imprinted worms. These results suggest that the solvent recognition pattern has significantly altered in imprinted worms than non-imprinted controls.

Significance of this study:

This study shows that imprinting has a critical impact on the associated learning and memory training. This study also shows the memory pathway involves both AIY and RIA neurons. This study represents a significant step forward in understanding the neuronal connectome during early development and its interaction with environmental factors.

I. INTRODUCTION

Learning and memory are the fundamental processes through which animals synchronize environmental cues into behavioural changes. Memories could last for a short term (minutes to hours) or long term (days to years). Learning process happening during the critical period of early development is termed as imprinting and can last lifelong (Remy, 2010).

Memory formation during the early stages of development is vital to the survival of any organism. The environmental conditions during the early development stage are found to leave a mark or imprint on the organism, creating behavioural alterations. Organisms such as salmon (Wisby *et al*., 1954; Scholz *et al*., 1976; Hasler *et al*., 1983; Nevitt *et al*., 2004; Hiroshi, 2010; Bett *et al*., 2016), geese (Lorenz, 1979), zebrafish (Harden *et al*., 2006; Garlach *et al*., 2019) and higher mammals (Hudson *et al*., 1995) have been found with imprinted behaviours.

Learning and memory have a stronger impact on the behaviour of an organism. Under experimental conditions, learning and memory formation occurs through non-associative or associative training. In the case of non-associative training the stimulus is given to the organism without any condition, whereas in associative training, the stimulus is given in association with another unrelated stimulus. One of the examples for such long-term memory formation is the gill withdrawal response in Aplysia (Kandel, 2001; Kandel and Tauc, 1965). Aplysia takes a reversal upon a tactile stimulus. On repetitive stimuli, the reversal amplitude and frequency, indicate habituation.

Caenorhabditis elegans is an excellent model system for studying learning and memory (Hedgecock, 1975; Sasakura, 2013). In the present work, *C. elegans* was used as the model system to understand the connectomes involved in imprinting and learning and memory. Sydney Brenner (1963) was the first person to use this free-living non-parasitic soil nematode as a model organism. In addition to its obvious advantages such as having a short life span, a fast reproductive cycle, and a transparent body, its neuronal connectome is well mapped. Techniques such as calcium imaging, laser ablation, or optogenetics may be used to study the functioning of the nervous system. siRNA knockdown experiments to specifically knock-down the gene of interest can be performed in these organisms by feeding them the specific plasmids. Transgenic strains with neuron-specific fluorescent labelling may be generated to study the connectome alterations.

C. elegans shows both attractive and aversive types of olfactory imprinting (Remy *et al.*, 2005; Jin *et al.*, 2016). Since nematodes are highly sensitive to olfactory sensation (Bargmann *et al*., 1993; Torayama *et al*., (2007), touch (Rankin *et al*., 1990; Rose *et al*., 2002), temperature (Hedgecock and Russel, 1975; Chi *et al*., 2007; Kimata *et al*., 2012), chemicals (Bargmann and Horvitz 1991), oxygen level (Fenk *et al.*, 2017) and light (Chew *et al*., 2018), it will be interesting to study the influence of imprinted behaviour upon its learned behaviour.

Olfactory imprinting in *C. elegans* has been studied with olfactory agents such as benzaldehyde, isoamyl alcohol and citronellol (Remy *et al*., 2005, Remy, 2010). These studies have shown that SRA-11, one of the seven-transmembrane receptors present on the surface of the AIY interneuron, plays a vital role in positive olfactory imprinting (Remy *et al*., 2005).

The olfactory adaptive learning and memory of *C. elegans* depend on a training paradigm. For example, combining a certain odour with food during the training session will lead to enhanced chemotaxis to that specific odor. This effect is called odor enhancement and lasts for hours (short-term memory, if a single training is given). On the other hand, a repeated training session will produce a long-term memory and enhanced chemotaxis that lasts >24 hours (Kauffman *et al*., 2011). Volatile odorants such as butanone, benzaldehyde, isoamyl alcohol, 2,3 pentanedione and 2,4,5 trimethylthiazole were detected by the AWC sensory neurons (Bargmann *et al*.,1993). For the associative learning and memory training session, the first set of neurons involved in detecting the volatile odorants are the AWC neurons (Torayama *et al*., 2007). The signals are transmitted from the AWC sensory neurons to interneurons and motor neurons.

Elucidating the connectomes and neuromodulators involved in olfactory imprinting and associative learning and memory in *C. elegans* will give better insight into the mechanisms involved in diseases such as Alzheimer’s, Autism etc. where memory functions are compromised.

I.1. Hypothesis

Imprinting as well as learning helps different types of memory formation in an organism. One of the hypotheses is that, imprinting during the critical period of development modulates neural wiring and synaptic plasticity. However, the role of imprinting in normal learning and memory pathways in our brain is not clear. We hypothesized that the early neuronal plasticity during imprinted memory formation must have a crucial role in learning memory pathways.

I.2. Objectives of the study

The major objectives of this study were designed as follows. Olfactory imprinting during early life generates somewhat a long-term memory. In this study, we observed how this early experience influences adult learning and memory. Second the connectome involved in the imprinted pathway as well as the associative learning and memory pathway. AIY neurons are known to be involved in imprinted memories, hence, one of the objectives was to identify how this modification in AIY interneuron generates a behavioural change with the help of other neurons. In this study, a set of mutants defective in specific receptors or with defective connectomes were used to assess these objectives.

II. REVIEW OF LITERATURE

The behaviour of an organism depends on the environmental influence during any stage of its life. Changes in behaviour occur due to alteration or plasticity occurring in the nervous system of the organism based on its environmental stimulus. These changes may occur either during the early developmental stages of the organism or during their lifetime. The changes happening during the critical period of development due to environmental cues are long-lasting. An example of such learning is "imprinting". Another type of learning occurs due to experience during their lifetime. If an organism undergoes repeated stimuli, it will help to reorient the neurons to create behavioural changes based on the stimulus it has been exposed to. This learned behaviour is either short-term or long-term. For remembering this information, it should be first stored. Learning followed by storing and then retrieving is termed as "memory". So, the terms learning and memory are closely related.

Imprinting is a particular form of learning in which the acquired behaviour is expressed when similar conditions repeat during the course of its lifetime. Olfactory imprinting is considered as an epigenetic phenomenon (the term epigenetics was coined by **Sir Conrad Waddington**), which occurs in a critical period during early development. Such olfactory imprinting has been observed in neonatal attachment to the mother in mammals as well as homing behaviour of salmons. This form of memory is a special type of unconditioned learning and can last during adulthood. One of the best examples for such an imprinting process is Konrad Lorenz (1935) experiments in goslings. He experiments to demonstrate environmental influence such as visual effects on imprinting in goslings. Upon hatching, the goslings follow and become attached to the first moving object they encounter and they consider them as their mother. Another example for visual imprinting is the preference towards prey of juvenile Sepia cuttlefish which they have seen early in life (Darmaillacq *et al*., 2006). Many imprinting experiments such as these have been conducted from higher to the lower model systems. Imprinting is considered to be a long-lasting memory.

Learning and memory are essential processes of the nervous system of both vertebrates and invertebrates for survival. Another type of learning and memory mechanism occurs in an organism based on their life experiences. Repetitive training to an organism with or without food will generate associative (Wen *et al.*, 1997) or non-associative memory (Timbers & Rankin, 2011) by changing the neuronal plasticity. One of the classic examples for associative memory training is reward-based salivation in dogs conducted by Pavlov. Lower organisms such as *Caenorhabditis elegans* have also been found to adapt to several learning paradigms including imprinted learning (Remy *et al.*, 2005), olfactory learning (Wen *et al.*, 1997; Stein and Murphy, 2014) and salt chemotaxis learning (Tomioka *et al*., 2006).

Non-associative learning includes habituation and sensitization. Sensitization results in an increase of response due to the increase in the number of synaptic terminals at sensory and motor neurons. Tail shock in aplysia is one of the examples for sensitization (Glanzman *et al*., 1989; Cohen *et al*., 1997). Habituation results in the decrease of response due to the decrease in the number of synaptic terminals at sensory and motor neurons. Gill withdrawal response in aplysia is due to habituation (Kandel, 2001).

II. 1. Early life experiences change the neural plasticity – effect of imprinting in memory formation.

Epigenetics is the study of heritable changes that modify genome expression profiles without altering the genetic material. Most of the epigenetic mechanisms are due to histone modifications like methylation, phosphorylation, acetylation etc. One effect of epigenetics is imprinting. Higher mammals including humans show epigenetic memory formation or imprinted memory formation. One such example for imprinting that happened in humans is the “Dutch hunger winter”. The effect of famine during this period (November 1944 to the late spring of 1945) affected the birth weight of babies who had been in the womb. Scarcity of food during the first 3 months of pregnancy gave birth to babies of normal size but more prone to obesity. Scarcity of food during the later stages of pregnancy gave birth to small-sized babies but are healthy.

Environmental conditions during the critical period of development in organisms cause changes in their behavioural pattern. Most of the unfavourable conditions will affect the neurological variations causing changes in their normal life, among which the major change occurs in the learning and memory mechanisms. These environmental conditions mainly cause changes or modifications in non-DNA regions. Such modifications include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation and other epigenetic mechanisms such as regulation by non-coding RNAs

(Sleutels *et al*., 2002; Rinn *et al*., 2012. Out of these, the most studied epigenetic mechanism include methylation in the CpG island resulting in the silencing of that gene. Inactivation of one of the X chromosomes for dosage compensation is an example for imprinting in mammals (Lyon, 1961).

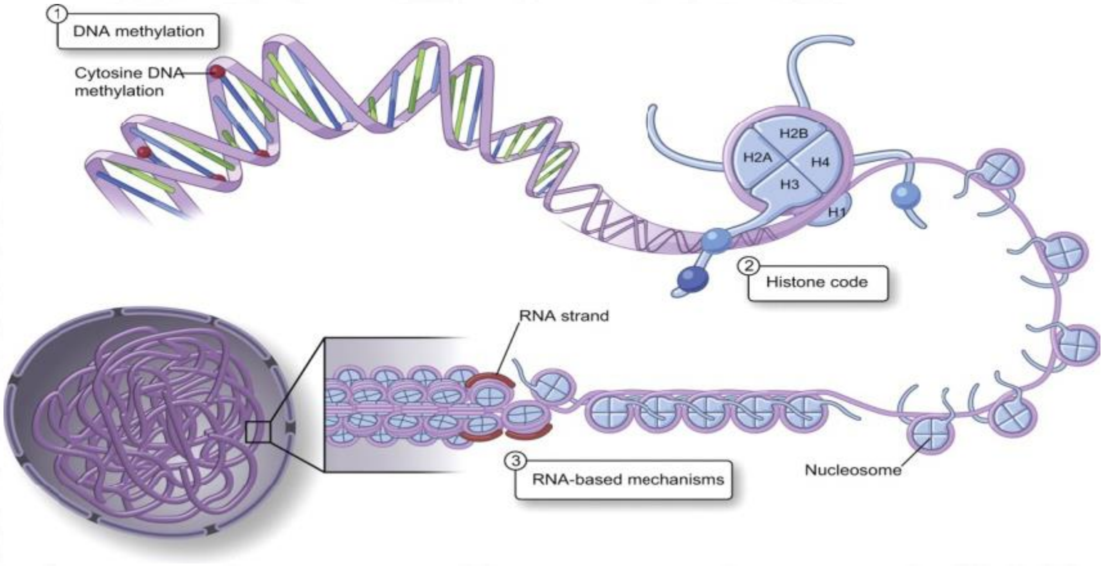


Fig. 1**: Schematic representation of 3 fundamental mechanism of epigenetic gene regulation.** DNA methylation, histone modification and RNA-based mechanisms. (Yan *et al*., 2010). With copyright permission from the journal.

II.1.1 Evidences for olfactory imprinting in model systems

Many model organisms like salmon, zebrafish and higher mammals show olfactory imprinting. Studies showed that when zebrafish were exposed to phenyl alcohol (PEA – phenylethyl alcohol) for the first 3 weeks post-fertilization, then switched to ordinary water up to the adult stage, these zebrafish showed a preference for these PEA in contrast with control fish when subjected to a preference test in Y maze (Harden *et al*.,2006). Study on another model system, Salmon fishes returned to an arbitrary stream mixed with PEA or morphine due to their early exposure to this chemical cue during their parr-smolt transformation (PST) (Bett *et al*., 2016). Coho salmon smolting coincides with surges in plasma thyroid hormone levels that are believed to be important for olfactory imprinting (Folmar *et al*.,1980, Hoar *et al*., 1988, Hasler *et al*., 1983, Scholz *et al*., 1976, Nevitt *et al*., 2004). It is hypothesized that T3 acts on the neural substrate for imprinting. Smolting leads to physiological and behavioural changes as early as hatching in the salmon which include increased gill Na+/K+ ATPase activity, silvering of the body, a shift in rheotactic orientation and the ability to tolerate salt water (Hasler *et al*., 1972, Hara *et al*., 1989). Apart from imprinting on chemical cues, zebrafish showed imprinting on chemical compounds released by kin, environmental cues and visual cues as well.

Salmon can be imprinted by 1mM Proline or Glutamate before and after PST and this will expire after PST (Hiroshi *et al*., 2010). Salmon olfactory imprinting-related genes (SOIG) encode a protein of 252 amino acids and share identity with the urokinase-type plasminogen activator receptor (Hiroshi *et al*., 2010). A similar type of protein has been isolated from *C. elegans*, *odr-2*, which has the function of regulating olfactory neuron signaling required for chemotaxis (Chou *et al*., 2001). NR1 subunit of the N-methyl, D- aspartate receptor influences memory formation in salmon and could be critical to successful imprinting during the downstream migration (Bett *et al*., 2016). But zebrafish could not make any olfactory imprinting for amino acids. Major histocompatibility complex (MHC) Class II genotype codes for olfactory imprinting and kin recognition in zebrafish larvae (Gerlach *et al*., 2019).

The activation of the odor receptor in fish due to imprinted stimulus provides an intracellular cAMP and cGMP signaling (Breer *et al*., 1990; Nakamura *et al*., 1987). The imprinted salmon can increase the cilia guanylyl cyclase activity to the chemicals that they have experienced during the juvenile stage compared to naïve adults, indicating that cGMP controls olfactory receptor neurons (ORN) sensitivity in olfactory imprinting (Dittman *et al*., 1997).

Higher mammals like rabbits also show olfactory imprinting. If a rabbit eats aromatic juniper berries during pregnancy, her pups also preferred to eat juniper (Hudson *et al*., 1995). In mice and mammals, H19/Igf2 parental expression has a role in phenotypic development. The expression of these genes regulates the size of the babies. The maternal expression of H19 and the paternal expression of Igf2 produce normal-sized mice. In such cases, Igf2 is maternally imprinted or silenced and the H19 gene is paternally silenced. The maternal Igf2 expression and paternal H19 expression results in smaller mice and their overexpression results in larger mice. The expression of these genes is controlled by the ICR (Imprinting Control Region) in humans and DMD (Differentially Methylated Region) in mice (Hark *et al*., 2000).

II.1.2 Involvement of epigenetics in neurological disorders.

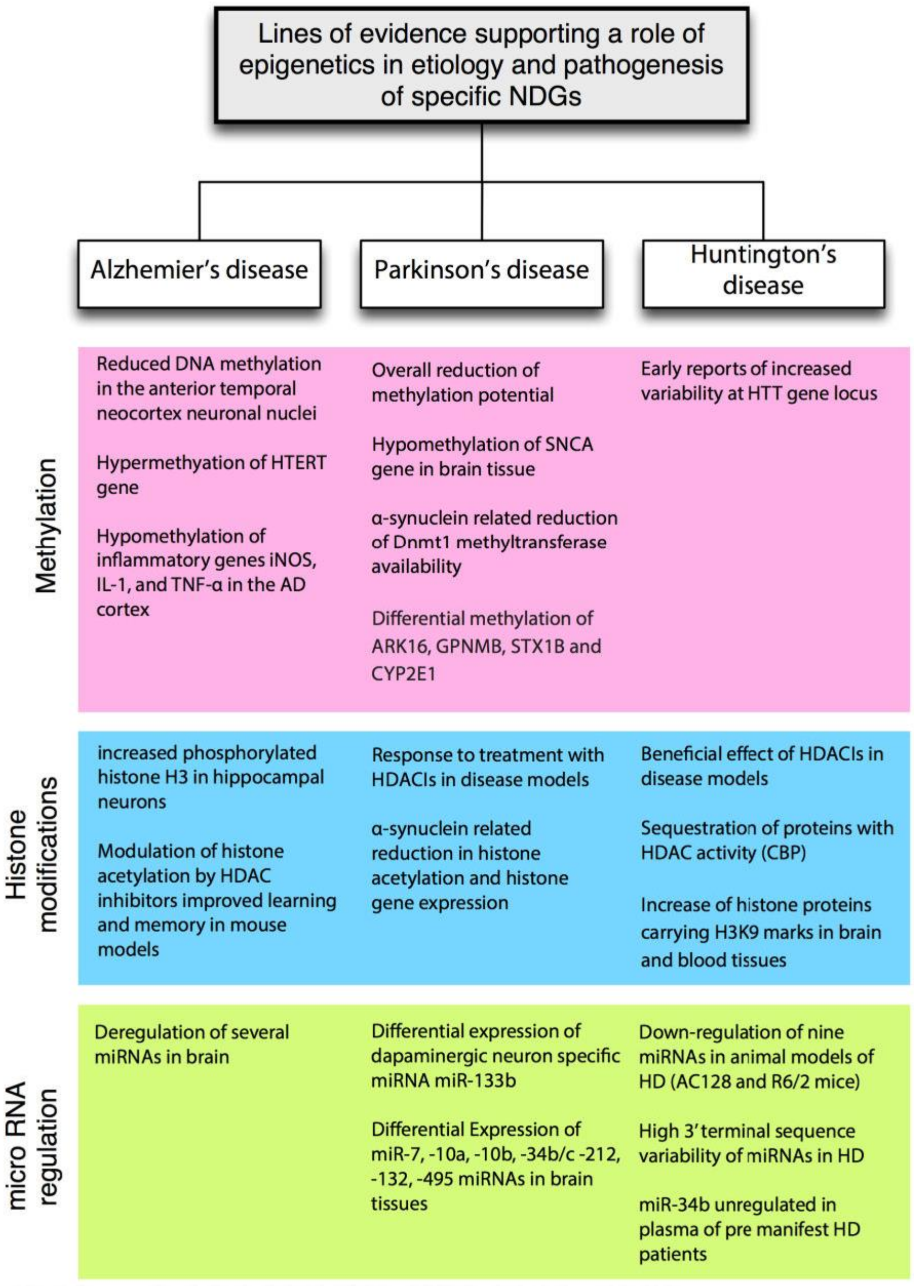
Many diseases have been associated with modifications in the neurons. Understanding the molecular pathways could help in diagnosis as well as the development of treatment modalities for diseases like Parkinsonism, Alzheimer’s, schizophrenia and autism. Histone deacetylase inhibitors (HDAC) help to increase the methylation pattern in the CpG island thereby reducing the risk for Huntington’s disease (HD) in mice. Y chromosomes are more prone to this methylation pattern and could elicit transgenerational effects. This type of methylation pattern is important in epigenetic modifications and could be transmitted through the germline (Morris *et al.*, 2015). Post-mortem brain tissue from Alzheimer’s disease (AD) patients shows specific age-related increased methylation patterns. Some regions of the brain such as the entorhinal cortex (Bollati *et al*., 2011) are hypomethylated and some regions such as the dorsolateral prefrontal cortex are hypermethylated (Chouliaras *et al*., 2013). The covalent modification in the DNA could also normalize memory formation. The methylation pattern in the memory suppressor gene PP1 leads to memory formation in the rat model (Miller *et al.*, 2007).

Many model systems are available to study the learning and memory deficits associated with neurodegenerative diseases. Epigenetic changes happening in the early life stage have a pathogenic effect on Alzheimer’s disease. Alzheimer’s disease is an age-related one and epigenetic changes have been found in aging tissues (Wu *et al*., 2008). The study conducted in the mice model reveal that beta-amyloid plaque formation leading to Alzheimer’s disease development causes learning deficit (Chen *et al.*, 2000).

One of the studies in familial Alzheimer’s disease (FAD) mice found that the presence of histone methylation H3K9me2, linked to gene silencing is increased in the hippocampus and prefrontal cortex which associates with the abnormal epigenetic regulation of glutamate receptor transcription. In Alzheimer’s disease patients, the increased localization of H3K9me2 in the transcription start site of glutamate receptor affects the glutamate receptor transcription. To recover this, they inhibited the euchromatic histone methyltransferases which catalyse H3K9me2 to repress transcription. This improved the cognitive impairment by glutamate receptor recovery and synaptic function (Zheng *et al*., 2019). The use of histone deacetylase (HDAC) inhibitors in Huntington’s disease (HD) in mice helps to change DNA methylation thereby helping to cure the disease. The F1 progeny also showed a better HD phenotype. The same showed an effect on human fibroblasts (Morris *et al*., 2015).

The reason that mice showed some autistic behaviours, they could be used as a tractable model for autism spectrum disorders (ASD), but there are limitations such as finding the synaptic functions in ASD (Calahorro, 2011). Based on modern technologies like anatomical and functional imaging, it suggests that there occurs a disconnection between the higher-order association area and the frontal lobe area of the brain during development (Geschwind *et al*., 2007).

Role of epigenetics in neurological disorders



Lines of evidence supporting a role of epigenetics in etiology and pathogenesis of specific NDGs

	Alzhemier's disease	Parkinson's disease	Huntington's disease
Methylation	Reduced DNA methylation in the anterior temporal neocortex neuronal nuclei Hypermethyation of HTERT gene Hypomethylation of inflammatory genes iNOS, IL-1, and TNF-α in the AD cortex	Overall reduction of methylation potential Hypomethylation of SNCA gene in brain tissue α-synuclein related reduction of Dnmt1 methyltransferase availability Differential methylation of ARK16, GPNMB, STX1B and CYP2E1	Early reports of increased variability at HTT gene locus
Histone modifications	increased phosphorylated histone H3 in hippocampal neurons Modulation of histone acetylation by HDAC inhibitors improved learning and memory in mouse models	Response to treatment with HDACIs in disease models α-synuclein related reduction in histone acetylation and histone gene expression	Beneficial effect of HDACIs in disease models Sequestration of proteins with HDAC activity (CBP) Increase of histone proteins carrying H3K9 marks in brain and blood tissues
micro RNA regulation	Deregulation of several miRNAs in brain	Differential expression of dapaminergic neuron specific miRNA miR-133b Differential Expression of miR-7, -10a, -10b, -34b/c -212, -132, -495 miRNAs in brain tissues	Down-regulation of nine miRNAs in animal models of HD (AC128 and R6/2 mice) High 3' terminal sequence variability of miRNAs in HD miR-34b unregulated in plasma of pre manifest HD patients

***Fig. 2*: Key epigenetic changes in AD, PD and HD.**
(http://www.intechopen.com/books/neurodegenerative-diseases).

II.2. Associative Learning and Memory

Learning and memory studies were done in many model organisms including humans. Canadian physiologist Donald Hebb describes synaptic plasticity during learning and memory. According to him a weak synaptic connection becomes strong when it is repeatedly stimulated, giving rise to the phrase “cells that fire together, wire together” (Hebb, 1949). Learning is the acquisition of knowledge based on experience while memory is the expression of that learned behaviour due to alterations in the neural connections. The main mechanism of learning is synaptic plasticity.

Learning and memory studies were done in lower invertebrate model systems to the higher vertebrate model systems. One of the most studied invertebrate model systems for learning and memory is in *Drosophila*. In *Drosophila*, odorant-based behavioural studies such as learning and memory were carried out to identify the specific genes and map the region involved in olfaction (de Belle *et al.,* 1994). *Drosophila* possesses learning and memory paradigm for olfaction, visual, appetitive courtship etc. In *Drosophila* the olfactory conditioning is given by pairing the odour with footshock (Benzer *et al*., 1974).

A detailed study in *Drosophila* identified the brain areas involved in learning and memory such as mushroom bodies (Tully *et al.*, 2001) and innervating neurons (MV1 And MP1 dopaminergic neurons). The innervating neurons are required for long-term memory. Other than these, protein synthesis in the dorso-anterior-lateral neurons is also required for LTAM formation (Guérin G *et al*., 2012). Like higher model systems the learning and memory pathway in *Drosophila* also require the activation of G-protein coupled receptors to produce cAMP signaling (Quan *et al*., 1991; Bolduc *et al.*, 2015).

II. 2. 1 Molecular mechanism involved in learning and memory

Learning could be differentiated into non-associative and associative. One of the classic examples of associative learning experiment was conducted by Ivan Pavlov in dogs by pairing an unconditioned stimulus (US - food) with a conditioned stimulus (CS - bell) to produce a conditioned response (Pavlov, 1927). A classic example of non-associative learning is the gill withdrawal response (habituation) in Aplysia (Kandel *et al*., 2000 & 2001). Based on the temporal classification, memory is of two types, short-term memory (STAM) or working memory and long-term memory (LTAM).

When an organism receives a stimulus, the presynaptic neuron secretes neurotransmitters or sends signals through gap junctions to the postsynaptic neurons. The major receptors NMDA and AMPA in neurons are involved in receiving the neurotransmitters. Studies showed that for associative learning and LTAM formation, the Ca^{2+} flux through NMDA receptors is required and also the Mg^{2+} block mechanism. The *Drosophila* study showed that Mg^{2+} block mutants are defective for LTAM memory formation (Miyashita *et al*., 2012). The neurotransmitters activate the downstream neurons to release second messengers such as Ca^{2+}, cAMP (Brunelli *et al*., 1976; Abrams *et al*., 1991), etc. which in turn activate cAMP response element-binding protein (CREB) (Montminy *et al*., 1997) to activate transcription or translation.

The non-NMDA and NMDA type glutamate receptors have the main role in memory formation. One of the non-NMDA glutamate receptors GLR-1 is found to be essential for both associative and non-associative learning in *C. elegans* (Morrison *et al*., 2001). Based on this study expression pattern of *glr-1* suggests that AIB is the first neuron common to both the CS and US cellular circuitry that expresses *glr-1* in *C. elegans*. Previous studies regarding the STAM and LTAM prove that for STAM, calcium and cAMP signaling is essential while LTAM requires both transcription and translation processes and also the involvement of CREB. Transcription inhibition during STAM training does not affect STAM formation and retention. Contrary to this, translation inhibition affects both memory maintenance and forgetting in *C. elegans* (Kauffman *et al*., 2010; Stein *et al*., 2014). Like vertebrates, many genes are involved in associative learning and memory in *C. elegans* such as *asic-1, magi-1, glr-1, hen-1* and *casy-1* (Rankin *et al*., 2010).

II. 2. 2 Neurological diseases affecting learning and memory

Memory impairment generally occur at older ages. Upon aging, the cognitive functions decrease and is termed as dementia. Most of the neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and Schizophrenia have cognitive deficits. Loss or lesion of neurons or neuronal regions can cause neurodegenerative disorders. Reasons for this degeneration are varying such as epigenetic effects due to environmental variation, age, neurofibrillary tangles like tau and beta-amyloid plaques, accumulation of inclusion bodies like Lewy bodies (alpha-synuclein) etc.

Aloiz Alzheimer in 1906 found pathological events in the brain of an old woman having progressive dementia. The name “Alzheimer’s disease” was coined by Kraepelin in 1910. There are many hypotheses emerging for explaining AD such as beta amyloid hypothesis, tau hypothesis, calcium hypothesis and neurotransmitter hypothesis. Loss of synaptic terminals, synaptic dysfunction, abnormalities in spatial memory tests, and inflammation are the main problems arising due to AD (Brien *et al*., 2011).

One of the reasons for memory dysfunction mainly loss of episodic memory in AD patients occurs due to the presence of neurofibrillary tangles in the hippocampal and parahippocampal regions. The formation of beta-amyloid plaques in AD patients was generated by the activation of monoamine oxidase (MAO). The production of beta-amyloid plaques could be reduced by treatment with MAO inhibitors (Zhiyou Cai, 2014). Another remarkable identification of AD patients is the cholinergic neuronal loss and decrease in choline acetyltransferase. Early stages of AD patients could be treated with drugs such as acetylcholine esterase inhibitors (Panegyres, 2004).

The major causes and pathogenesis of PD are the degeneration of dopaminergic neurons, genetic factors, free radicals like MAO and neurotoxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT). In PD patients, the degeneration occurs in the substantia nigra resulting in dysfunction of the striatum but in AD patients’ striatum looks normal. Parkinson’s disease patients undergo degeneration of both the dopaminergic and cholinergic systems, which leads to a deficit in dopamine and acetylcholine at synapses. Studies have shown that PD patients have deficits in recall, recognition and prospective memory, and memory deficits increase with disease severity (Whittington *et al*., 2006). Dysfunction in the medial prefrontal cortex results in learning and forgetting impairment in PD patients (Faglioni *et al.*, 1997).

Huntington disease is an autosomal dominant mutation of the IT15 gene. The IT15 gene produces the Huntington gene. The cause of HD is the aggregation of polyglutamine (polyQ) due to the CAG repeats in the exon1 region of Huntington's gene (The Huntington's Disease Collaborative Research Group, 1993). Application of the drug HDAC inhibitors helps to promote histone acetylation and protect from poly-Q mediated toxicity (Chen *et al*., 2015). In HD patients, the primarily affected area is the region of the forebrain which is involved in the regulation of motor movement.

II.3. *C. elegans* as a model system

Caenorhabditis elegans is an excellent model to study changes in neuronal architecture, both cellular and molecular level, during development, in learning process as well as in neurodegeneration. Many studies regarding imprinting as well as learning and memory were carried out with this model system because of its limited number of neurons and short life span. *C. elegans* is a free-living soil nematode having a 1mm length and is a non-infectious non-parasitic non-pathogenic organism. Most of these nematodes are hermaphrodites but few are males. The main anatomical difference between these two is seen in the tail region. Hermaphrodites have a tapering tail while the male shows fan-like structures in the tail region. Also, the male is short compared to the hermaphrodite. *C. elegans* was used as a model system for many neuro and microbiology studies. In 1963 **Sydney Brenner** used *C. elegans* as a model organism first time for studying neuronal development.

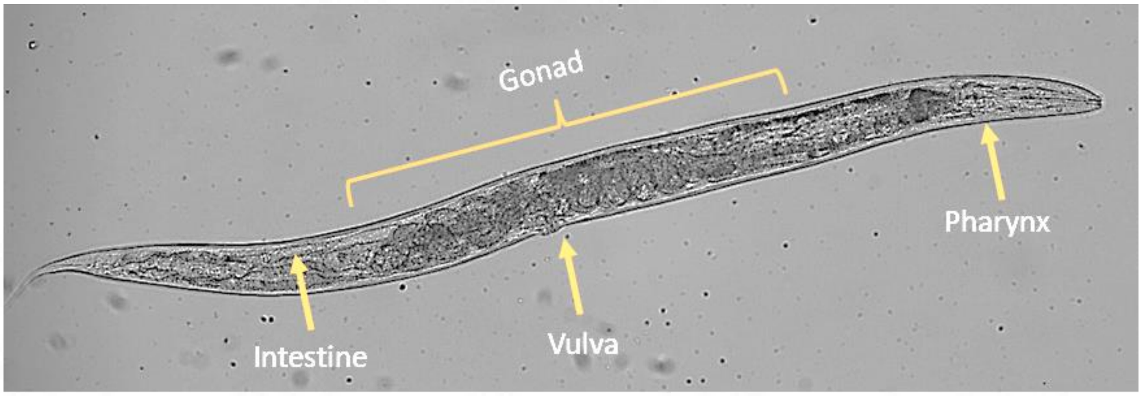


***Fig. 3*: Microscopic image of adult *C. elegans* hermaphrodite**.

The life cycle of *C. elegans* starts from egg to many larval stages to adult within 3 days under optimal conditions (20^0 C). It has a short life span of 21 days. In laboratory conditions, it grows on agar plate containing auxotrophic mutant of *E. coli* strain OP50, which is considered as a food source for the nematode. The transparent body gives the advantage of viewing internal structure under a microscope and neurons with GFP labelling could be visible under a fluorescent microscope. The ease of creating genetic manipulation in the worm is another advantage which makes the nematode, a good model system.

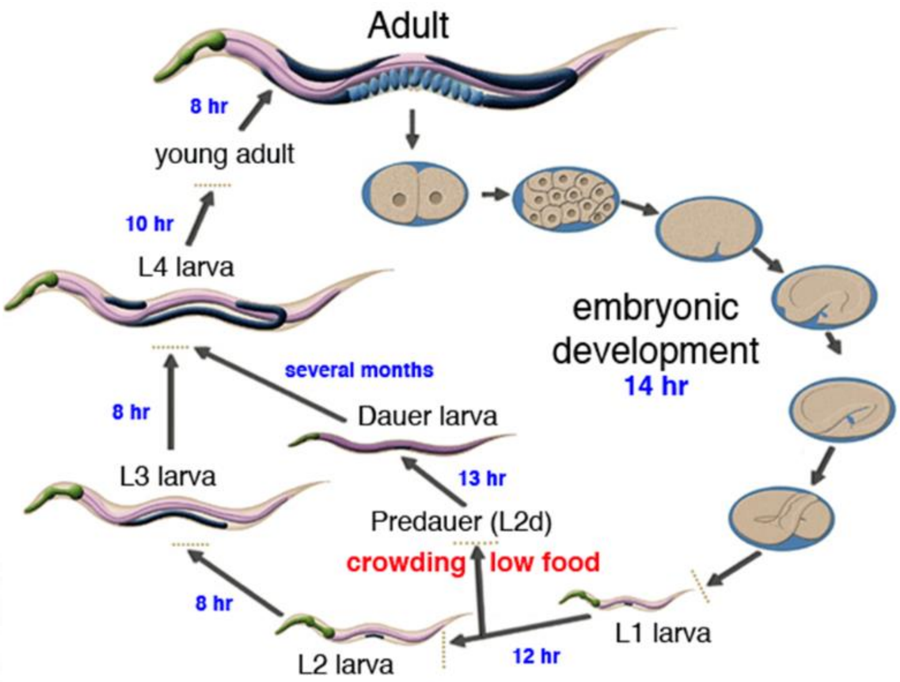


Fig. 4*: Life cycle of *C. elegans. Adapted from http://www.sfu.ca/biology/faculty/hutter/hutterlab/index.html. With copyright permission from the journal.

The entire highly stereotyped nervous system of *C. elegans* contains 302 neurons, and all synaptic connections between neurons have been defined by serial reconstruction of electron micrographs (White *et al*., 1986). It contains many sensory inter and motor neurons and the neurons are connected by chemical or electrical synapses. The neuronal circuits involved in mechano-sensation (Chalfie *et al*., 1985), olfactory sensation (Bargmann *et al*., 1993), salt chemotaxis (Bargmann and Horvitz, 1991; Iino and Yoshida, 2009), thermotaxis (Mori and Ohshima, 1995), and navigation (Gray *et al*., 2005; Tsalik and Hobert, 2003) have been well characterized. The worm could sense different odours through different sensory neurons. AWC sensory neuron detects volatile odours like butanone, isoamyl alcohol, benzaldehyde, 2,3-pentanedione, 2,4,5-trimethylthiazole (Bargmann *et al*., 1995) while diacetyl, 2,4,5-trimethylthiazole and pyrazine are detected by the AWA sensory neuron (Bargmann *et al*., 1995). The neuronal discrimination to odours makes the worm susceptible to olfactory studies. However, it is not well understood how the nervous system differentiates between different olfactory signals and thereby makes a conscious choice and if the same is based on its innate ability or through the learning process. Like higher model systems, *C. elegans* also shows neurotransmitter release for neuronal communications. Neurotransmitters such as cholinergic, glutamatergic, GABAergic and aminergic neurotransmitters are distributed in various neurons (Pereira *et al*., 2015; Serrano-Saiz *et al*., 2013; McIntire *et al*., 1993; Chase and Koelle, 2007).

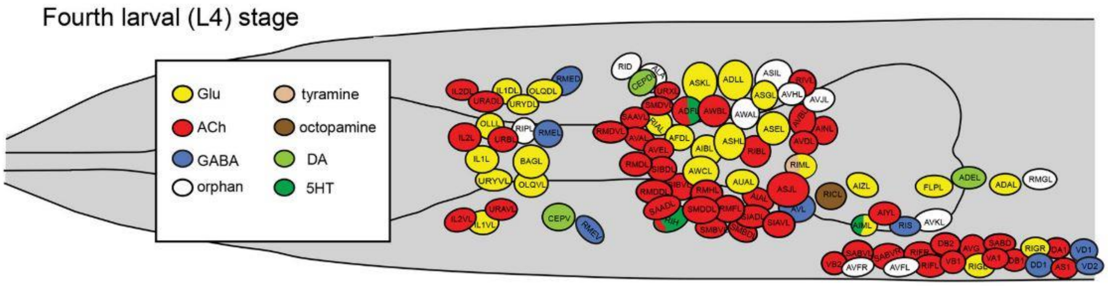


***Fig. 5*: Distribution of neurotransmitters in the nervous system**. Location of neurons with different neurotransmitter identities in the head ganglia in the hermaphrodite (Pereira *et al*., 2015). With copyright permission from the journal.

Many behavioural studies have been done in *C. elegans* based on the external environmental stimuli. The tap withdrawal response in *C. elegans* is one of the extensively studied behavioral responses (Rankin *et al*., 1990). Worms respond and chemotaxis to various salts, many attractants and repellents in the presence or absence of food (Ward *et al.,* 1973; Dusenbery *et al.,*1974). The behavioural changes were regulated by neural connections. The worm chemotaxes towards many water-soluble chemicals like NaCl, biotin and cAMP and volatile odorants such as butanone, isoamyl alcohol, benzaldehyde, acetone, etc., (Bargmann, 2006; Berger *et al*., 2007; Pereira *et al*., 2012). The activity of neurons could be analyzed by methods like calcium imaging, laser ablation of specific neurons, knocking in or knocking down the receptors or genes expressed on specific neurons and expressing light-gated channels in the neurons such as channel-rhodopsin (Shen, 2018).

Although *C. elegans* is a simple invertebrate model, it provides a highly conserved genome with humans, and this could lead to the study of the cellular and molecular mechanisms underlying neurological diseases like Alzheimer's, Parkinson's and other neurodegenerative disorders. The complete sequencing by the '*C. elegans* Sequencing Consortium' reveal that approximately 38% of human orthologs are present in *C. elegans*, among which many diseases-related genes such as APP (Amyloid Precursor Protein) and tau are present. The orthologs of genes involved in Alzheimer's disease have been identified such as *apl-1* (Daigle and Li, 1993), the APP-like gene and tau-like protein homolog such as *ptl-1* (Goedert *et al*., 1996; McDermott *et al*., 1996; Alexander *et al*., 2014). There is a transgenic worm for studying Parkinson's disease in which alpha-synuclein is incorporated in the dopaminergic neurons (Lakso *et al*., 2003). Many genes involved in autism disorder such as neurexin (*nre-1*), neuroligin (*nlg-1*) and shank (*shn-1*) also have orthologs in *C. elegans* (Bourgeron *et al*., 2009; Calahorro *et al*., 2015).

II.3.1 Regulation of AIY interneuron

C. elegans shows olfactory imprinting towards benzaldehyde, isoamyl alcohol and citronellol (Remy *et al*., 2005). For volatile olfactory detection, AWC is the main sensory neuron. Only one of the neural pair could generate a stimulus. This is how the asymmetry of AWC neurons distinguishes between different chemical cues (Chuang *et al*., 2014). According to J J Remy and O Hobert an interneuron AIY is required for olfactory imprinting. AIY receives volatile olfactory stimulus from the AWC sensory neuron. A specific G protein coupled seven trans-membrane receptors - SRA-11- present on the surface of the AIY interneuron is required for positive olfactory imprinting.

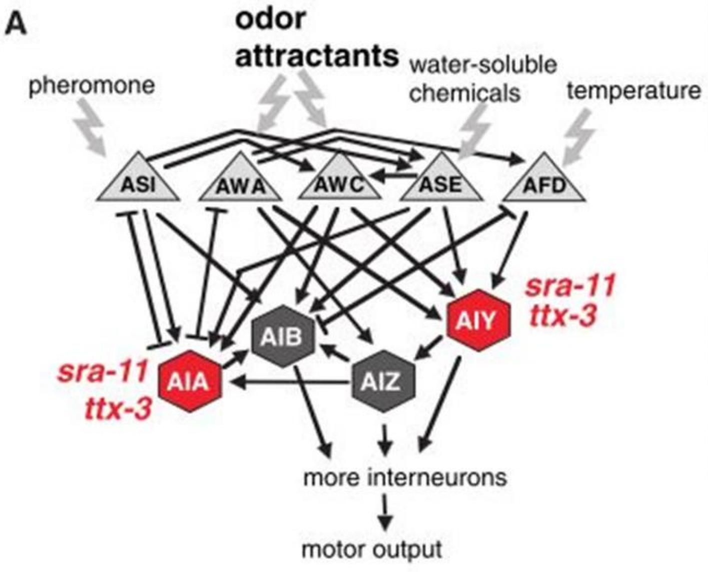


***Fig. 6*: Schematic representation of the AIY synaptic connectivity**. Connection of AIY interneuron with sensory neurons and to motor neurons to produce motor output. Involvement of sra-11 in the AIY interneuron for imprinting. (Remy *et al*., 2005). With copyright permission from the journal.

The development and function of AIY interneuron is regulated by a homeobox domain. Homeodomain box provides the regulation for the molecular mechanisms of homeodomain protein function. Mainly the collective interaction of CEH-10 and TTX-3 regulate AIY interneuron. Both are co-expressed only in AIY interneuron. Many sub batteries are activated by an AIY dependent transcription factor such as CEH-23 and are not linked through the AIY. Orthologs of CEH-10 and TTX-3 (*chx10* and *Lhx2*/Apterous) affect neuronal differentiation in other species. AIY can express at least six ion channels regulated by four different ligands such as acetylcholine, GABA, glutamate, and serotonin. Hence, it can participate in an enormous neurochemical signaling pathway (White *et al.*, 1986).

The collective interaction of *ceh-10, ttx-3* and *ceh-23* regulates the expression of many neurons. The *ceh-10* is expressed only during embryogenesis (Svendsen *et al.*, 1995). TTX-3 and CEH-23 expression started during embryogenesis and is maintained throughout adulthood. The *ttx-3* gene is the central regulator of AIY interneuron differentiation. The loss of *ttx-3* function strongly affects the expression of the *sra-11* gene. *ttx-3* dependent regulatory events were under the control of CEH-10. The major regulatory function of *ceh-23* is on the expression of *sra-11* gene but it does not have any impact on the expression of KAL-1, UNC-17 and SER-2 (other receptors present on AIY interneuron) (Gultekin *et al*., 2001).

The receptor glutamate-gated chloride channel (GLC-3) is solely present on the AIY interneuron (Horoszok *et al*., 2001; Cully *et al.*, 1996). The *glc-3* gene has a similarity to the glycine receptor in mammals. GLC-3 in *C. elegans* helps for turning behaviour to find the food source. When the AWC sensory neuron releases glutamate neurotransmitter, it activates the glutamate-gated chloride channel thereby inhibiting the activation of AIY inter neuron leading to turning in *C. elegans* (Wolstenholme *et al.*, 2012). Dropping the glutamate input via odour application leads to a Ca^{2+} spike (Oka *et al.*, 2019).

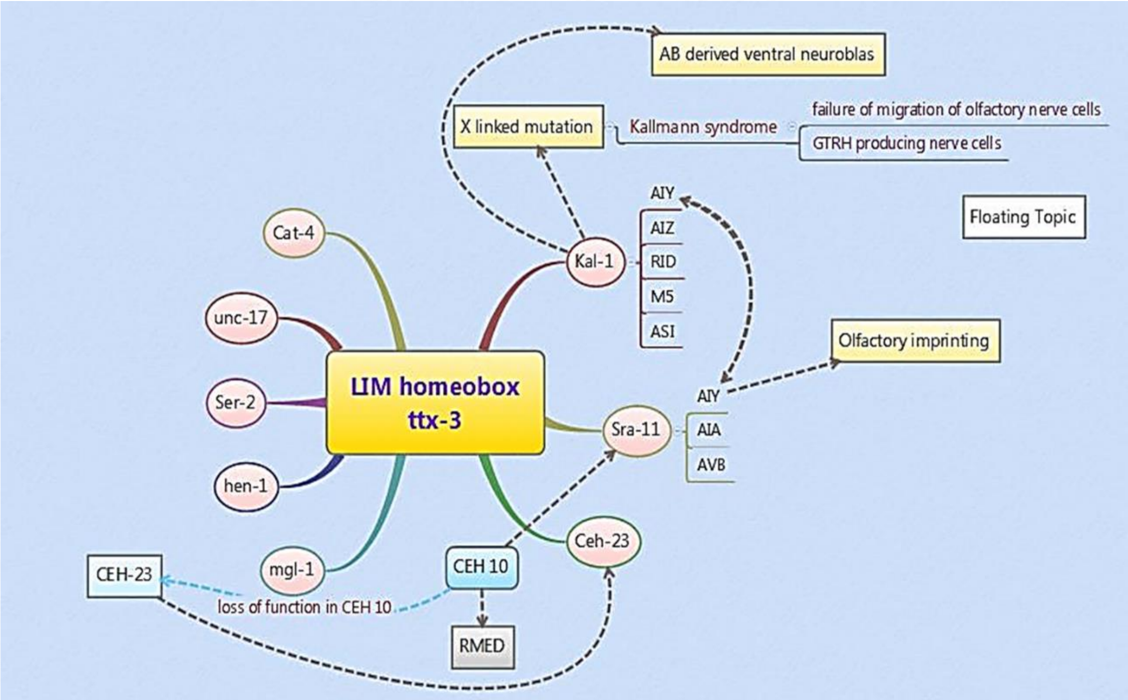


***Fig. 7*: Regulation of LIM homeobox ttx-3:** LIM homeobox domain regulation on different neurons specifically on AIY inter-neuron.

II.3.2 Olfactory imprinting and neuronal involvement

It has recently been found that positive olfactory imprinting behaviour such as acquired behaviour is stably inherited in *C. elegans* (Remy, 2010). Like positive olfactory imprinting, the worms showed aversive olfactory memory formation and retrieval to pathogenic bacteria (Jin *et al.*, 2016). According to this the tyramine neurotransmitter released from the RIM interneuron interacts with the SER-2 receptor present on the AIY interneuron. Here the AIY interneuron helps for memory retrieval function while for adaptive learning, involvement of AIB is noted.

The AIY interneuron play a very critical role in many behavioural changes. As a candidate for olfactory imprinting, the AIY inter-neuronal analysis is very critical. One such behaviour performed by *C. elegans* is its reversal frequency to control the movement towards or away from the smell and is found by mutant analysis and microsurgical mapping. Also, this AIY controls other command inter-neurons like AVA, AVB, AVE etc. (Tsalik, E. L *et al*., 2003) indicating that loss of function of AIY inter neuron can affect many behavioural changes in these nematodes.

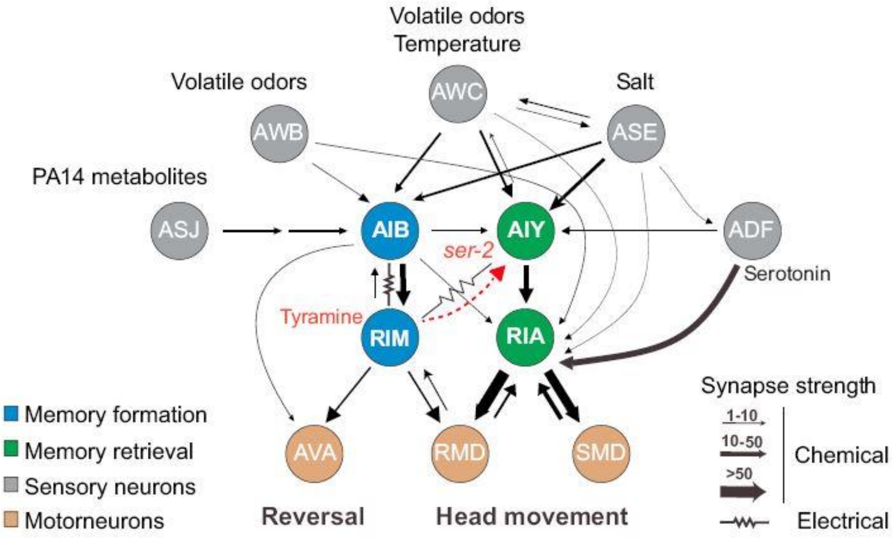


***Fig. 8*: synaptic partners in aversive olfactory imprinting**. The involvement of AIB, RIM, AIY and RIA in aversive olfactory imprinting. Adult learning requires either AIB or AIY but for aversive imprinting both AIB and AIY required (Jin *et al*., 2016). With copyright permission from the journal.

II.3.3 Learning and Memory in *C. elegans*

Learning and memory training in *C. elegans* creates neuronal plasticity in the nematode. The worms chemotax to various chemicals and volatile odours. One of the examples for this is chemotaxis to NaCl and is sensed by the ASE sensory neuron. When the worms were exposed to NaCl for a prolonged period in the absence of food, the worms show negative chemotaxis towards NaCl. But the same done in the presence of food does not make any difference in chemotaxis (Saeki *et al*., 2001). The worms have a negative learning to NaCl exposure without food. G-protein, Ca^{2+}, cAMP is responsible for this learning paradigm.

Olfactory learning in *C. elegans* depends on the odour given during the training. But one of the studies in *C. elegans* showed cross-adaptation to benzaldehyde and isoamyl alcohol (Colbert and Bargmann, 1995). Worms have aversive learning towards pathogenic bacteria PA14. Upon training the worm chemotaxis index decreases towards PA14. This pathogenic aversion pathway starts from the AWC sensory neuron to the downstream neurons such as AIY, RIM, RIA, etc., (Jin *et al*., 2016). The neuronal plasticity for short-term and long-term memory of *C. elegans* depends on NMDA (N-methyl-D-aspartate receptor) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor) type receptors. Mutations in the NMDA receptor NMR-1 subunit reveals that it is required for both the memory formation (Takashi *et al*., 2008). AMPA-type glutamate receptor is also required for olfactory associative and non-associative learning (Morrison *et al*., 2001). *crh-1* which encodes the CREB transcription factor in *C. elegans* is required for long-term memory (Sasakura *et al*., 2013; Amano *et al*., 2011).

The volatile odours are recognized by the AWC sensory neurons. Learning and memory training of butanone conditioning paired with food results in short-term and long-term memory in the worm. Single conditioning results in short-term memory and repeated training results long-term memory (Kauffman *et al*., 2011). The odour detection by the sensory neuron AWC is similar to that of vertebrate cone and rod cells signaling. When the odour stimulus is received, AWC become OFF and it will activate the AIB interneuron and inhibit the GLC-3 chloride channel in the AIY interneuron. In vertebrates, when light is received by the photoreceptors it becomes inactivated and will in turn activate ionotropic glutamate receptor and inhibit metabotropic glutamate receptors (Zhang *et al*., 2005; Wassle *et al*., 2004; Chalasani *et al*., 2007).

The similarities in neural connections and neural mechanisms bring *C. elegans* to become an excellent model system for studying learning and memory as well as cognitive deficits happening in humans.

III. MATERIALS AND METHODS

III.1. Materials:

III.1.1. Strains:

Wild type Bristol N2 strain. Mutant strains include RB816 (*sra-11(ok630) II*), JC2209 (*olrn-1(ut305) X*), RB594 (*glc-3(ok321)V*), KP4 (*glr-1(n2461) III*), RB1808 (*glr-2(ok2342) III*), VM487 (*nmr-1(ak4) II*), OH910 (*ttx3p::kal1 + usc122p::GFP*), RB1690 (*ser-2(ok2103) X*), GR1373 (*eri-1(mg366*), ZM9078 (*hpIs587 - GCaMP6*) (all the strains from CGC) and AT9203 (*ttx-3p::GCaMP6::mCherry*, 50ng/µl and 5ng/µl, was generated by microinjection of the plasmid created by our lab), DCR744 (*cima-1(wy84) IV; wyIs45 X*), DCR775 (*cima-1(wy84) IV; wyls45 X; olaEx459*; *cima-1* rescue is the *cima-1* genomic region with 1.9kb promoter), DCR2188 (*ttx-3p::CD4::GFP(11)+glr-3p::CD4::GFP(1-10)+ttx-3p::mCherry::rab-3 + unc-122p::GFP*) (The DCR strains were the gift from Colon Ramos, Yale University). All nematodes were grown on NGM containing *E. coli* strain OP50 at 20^{0}C. siRNA worms were grown in HT115 *E. coli* culture and kept at 17 ^{0}C.

III.1.2. Chemicals:

Volatile odorants such as isoamyl alcohol (IAA - central drug house (p) Ltd, Bombay-New Delhi) and butanone (BT - HIMEDIA), ethanol, Drugs such as Actinomycin D and Cycloheximide (SIGMA Aldrich), Sodium alginate powder (SDFCL- S D Fine-Chem Ltd, Mumbai), Sodium hydroxide (NaOH), Agar-agar, Dipotassium hydrogen phosphate (K_2HPO_4), sodium azide ($NaNO_3$) from Merck, Calcium chloride ($CaCl_2$), potassium dihydrogen phosphate (KH_2PO_4), peptone, Magnesium sulphate ($MgSO_4$), Luria Bertani agar, Luria Bertani broth, Ethylenediaminetetraacetic acid (EDTA), sodium carbonate (Na_2CO_3) from HIMEDIA, Whatman filter paper (Whatman 1001-917, 11µ), ethidium bromide, cholesterol from SRL, Disodium hydrogen phosphate (Na_2HPO_4), sodium hypochlorite (NaClO) from SIGMA Aldrich, agarose HE (Ultrapure) from Usb, glycerol, Trizol (Ambion), Tris HCl, sodium dodecyl sulphate (SDS), proteinase K, RNase A, Phenol, chloroform, isopropanol, sodium acetate.

III.2. Equipment:

Class II biosafety cabinet (Esca, PA, USA), Stereo microscope (Magnus Analytics, India), Leica DMi8 automated fluorescent microscope (S/N 455551), Olympus IX51 inverted microscope (Olympus Imaging, Center Valley, PA, USA), Inject-Man 4, centrifuge 5418R, thermomixer and Master cycler X50s (Eppendorf AG, Hamburg, Germany), Dino camera, Sony camera, Mille Bifocal water purification system (Merck Millipore, MA, USA), Autoclave (Sanyo, Japan), Gel apparatus (Amersham Biosciences, NJ, USA), Uviol gel documentation system (Vitec, Cambridge, UK), weighing balance (Sartorius, Germany), water bath (Boston, India), Cooling water bath (Amersham Biosciences, NJ, USA), shaker incubator (IA, Germany), QuantStudio 5 and HERAtherm microbiological incubator (Thermo Scientific, USA), ECHO-therm chilling incubator (Torrey Pines Scientific, California, USA), IKA incubator shaker KS 3000 IC control (IKA, ProfiLab24 GmbH, Berlin), B. O. D incubator (Labline instruments, India), SFA stereo microscope fluorescence adapter and light base (NIGHTSEA, USA).

III.3. Software

Statistical analysis and graphical representations were done using GraphPad Prism (Version 6.01), Images were captured using NIS Elements AR (Nikon, USA) and LAS X imaging software (Version 3.3.16799.0, Leica Microsystems), worm tracking software (WormLab, MBF Biosciences, USA) and ImageJ (Version 1.52p, NIH, USA).

III.4. Methods:

III.4.1. Nematode growth medium (NGM):

For 1L NGM media 17g of Agar, 3g of NaCl and 2.5g of peptone were dissolved in 1L distilled water. After autoclaving, the media was cooled to 55^0C and then added 1ml of 1M $CaCl_2$, 1ml of 5mg/ml cholesterol (cholesterol dissolved in 99% ethanol), 1ml of 1M $MgSO_4$ and 25ml of 1M KPO_4 buffer (pH 6.0). 20 ml of the media were transferred to petri-plates and allowed to dry. *E. coli* strain OP50 (food source) was seeded to the plate and kept at 37^0C overnight.

III.4.2. Maintenance of *C. elegans*

All strains were maintained at 20^0 C B.O.D incubator except some mutants, maintained at 17^0 and 15^0C on nematode growth medium (NGM) containing OP50 (auxotrophic mutant strain of *E. coli*) bacteria as a food source (Brenner, 1974).

III.4.3. Chemotaxis plate:

For 1L chemotaxis media added 20% agar-agar and made up to 1L using distilled water. After autoclaving cooled the media to ~55^{0}C and added 5ml of KPO_4 buffer, 1ml of 1M $MgSO_4$ and 1ml of 1M $CaCl_2$. 20ml of agar was poured into 10cm petri-plates and allowed to dry overnight (Bargmann and Horvitz, 1991). Used for chemotaxis assay.

III.4.4. Worm synchronization:

The purpose of worm synchronization is to obtain the worms at the first larval stage, L1 (Stiernagle, 2006). For this the well-fed egg-laying gravid adult *C. elegans* were collected in an Eppendorf tube by washing the plate with M9 buffer (3g KH_2PO_4, 6g Na_2HPO_4 and 5g NaCl for 1L). Centrifuged the liquid at 1300g (4000 rpm) for 1 minute. Removed the supernatant by keeping a few microliters of solution in the tube. 1 ml of freshly prepared bleach solution (for 1ml bleach solution mix 100µl of 5N NaOH, 5% sodium hypochlorite and sterile water) was added into the tube. The solution was vortexed to mix. The vial was kept for 7 to 10 minutes for the worm to digest in the bleach solution, with intermittent vortex mixing. The tube was centrifuged at 1300g for 1 minute at 20^{0}C to pellet the released eggs. Removed the supernatant by aspiration. Washed the pellet using M9 buffer to remove all the traces of the bleach solution. Resuspended the eggs in 100µl M9 buffer and plated in the petri-plate containing OP50.

III.4.5. Chemotaxis assay:

Chemotaxis assays were used to calculate the chemotaxis index (Ward, 1973; Bargmann and Horvitz, 1991). Two kinds of chemotaxis assays were used to explore the involvement of various neurons and receptors in the *C. elegans* when various chemicals were exposed to them. Chemotaxis assays were performed on petri-dishes having 8.7 cm diameters. The chemotaxis plates were prepared as previously mentioned in Bargmann *et. al.,* 1993 (instead of 1.7% agar concentration, 2% agar was used). Worms were placed on the center of the plate and odorants were spotted on two opposite ends as control and odorant along with sodium azide. Sodium azide was used to anesthetize the worms when it reached the spot. Approximately 50 worms were placed in the center of the plate. The plates were closed and kept undisturbed. After 20 minutes the chemotaxis index was determined by how many worms moved to the attractant or to the control (*Fig. 9a*). This value ranges between -1.0 to +1.0.

$$CI = \frac{\text{No. of worms on the test spot} - \text{ No. of worms on the control spot}}{\text{Total number of worms}}$$

For quadrant chemotaxis assay the plate was divided into four quadrants (*Fig. 9b*). This helped to determine the comparative attraction of worms towards two different attractants or repellents. Here the control solvent was spotted in opposite quadrants and the test in the next opposite quadrants. Calculated the choice index of the worm using the following:

$$Choice\ index\ of\ odorant\ A = \frac{\text{No. of worms in A} - \text{ No. of worms in B}}{\text{Total no. of worms}}$$

$$Choice\ index\ of\ odorant\ B = \frac{\text{No. of worms in B} - \text{No. of worms in A}}{\text{Total no. of worms}}$$

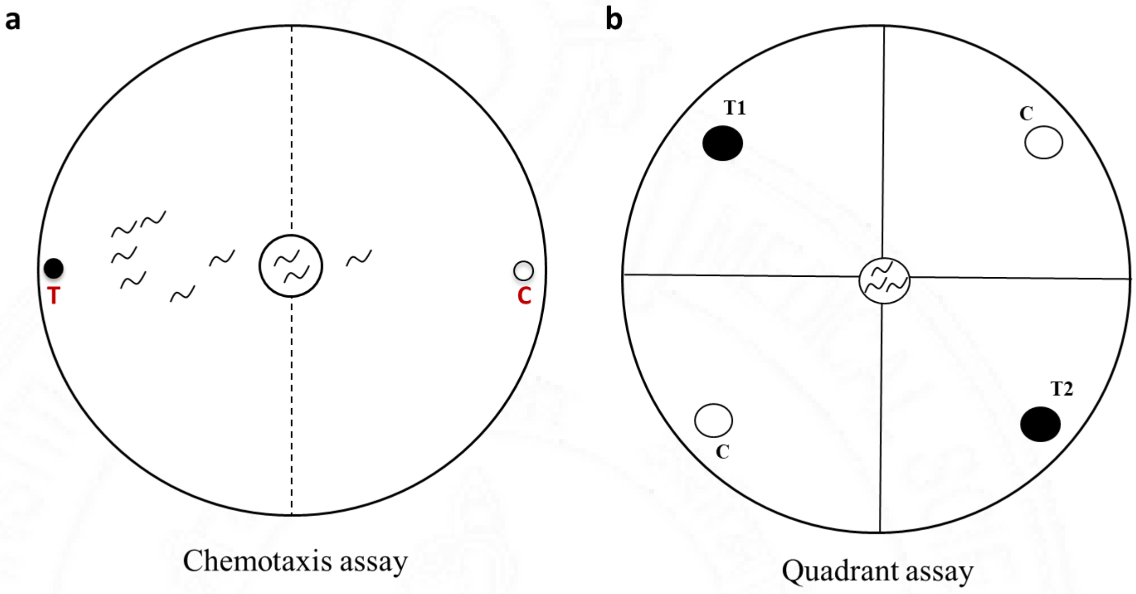


***Fig. 9*: Schematic diagram of chemotaxis plate.** a) chemotaxis plate in which worms migrate or repel to a single cue. b) Quadrant assay plate in which worms migrate or repel to two different cues.

III.4.6. Imprinting procedure:

Healthy adult hermaphrodite worms were synchronized to get eggs and transferred to a new NGM plate containing OP50. Streaked 4 µl of 1/300th dilution of IAA (1 µl IAA diluted in 299 µl of water) on the lid of the plate and sealed with parafilm (*Fig. 10*). Control experiments were carried out by placing 4 µl of water on the lid. Kept these plates at 20^{0}C for 24 hours. After 24 hours (during this time eggs develop to L1 larvae), the lid was replaced with a new one to prevent further exposure of the odorant and allowed the worms to grow at 20^0 C (Remy *et al*., 2005). On the 3rd day, (i.e., the first day of adult worms), collected the worms by washing with M9 buffer (1ml) and allowed them to settle in an Eppendorf tube (this takes around 2 minutes). Discarded the supernatant and worms were washed thrice with M9 buffer till the supernatant became clear. These worms were used immediately for chemotaxis assay.

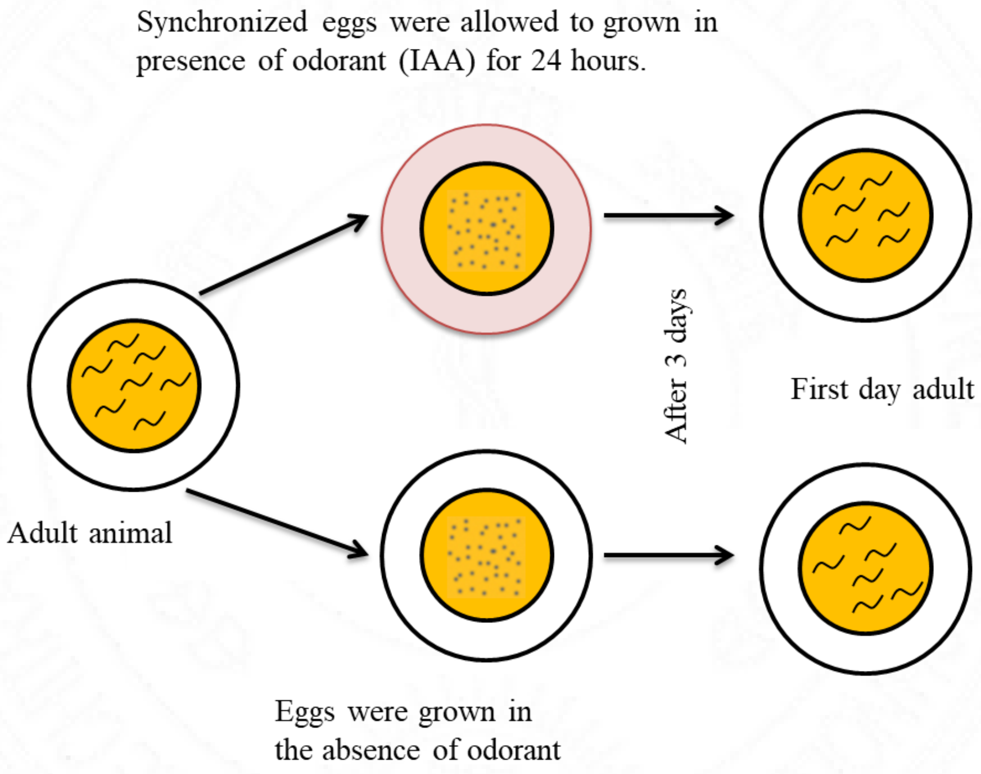


***Fig. 10*: Schematic diagram of imprinting procedure**

III.4.7. Short-term associated memory training (STAM):

The procedure was adapted from Kauffman *et al*., 2011 with some modifications (*Fig. 11*).

For short-term associated memory training, synchronized worms were allowed to grow till their day 1 adult stage. Washed the worms thrice in M9 buffer (till the supernatant become clear). The worms were starved for one hour in 1ml M9 buffer in an Eppendorf tube, after which they were transferred to a 60mm NGM plate containing liquid OP50 and streaked 4 µl of 10/100th dilution of BT (10 µl BT+90 µl 95% ethanol) on the lid (Conditioning plate). Plates were immediately sealed using parafilm and kept for one hour at 20⁰C. Worms were then washed off from the conditioned plate using M9 buffer 3 times till the supernatant was clear. Chemotaxis assays were conducted at 0th hour, 1st hour, 2nd hour and 3rd hour. During the period in between the assays, the worms were kept on the food plate.

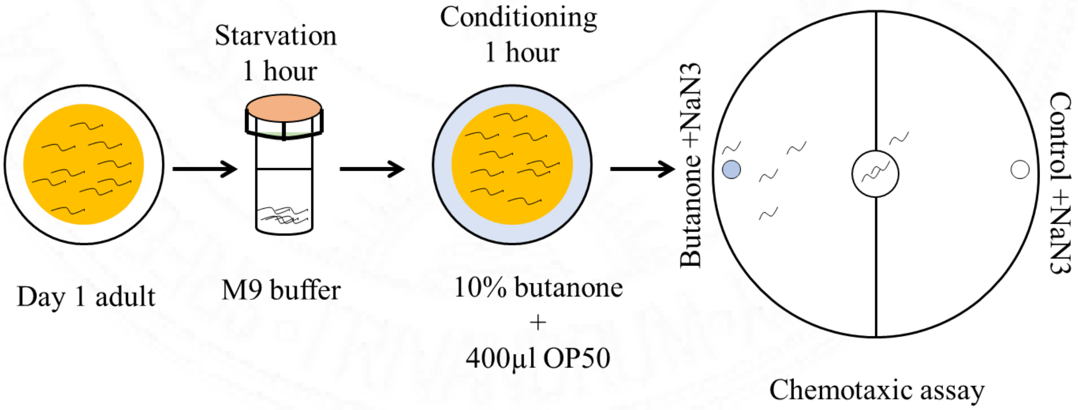


***Fig. 11*: Schematic diagram of short-term associated memory training**

III.4.8. Long-term associated memory training (LTAM):

For long-term associated memory training, synchronized worms were allowed to grow till they became day 1 adults. The worms were washed thrice in M9 buffer (till the supernatant became clear). Worms were starved for 1 hour in 1ml M9 buffer in an Eppendorf tube. After this the worms were transferred to NGM plate and conditioned with 400 µl liquid OP50 and 4 µl of 10% BT (in 95% ethanol) for 30 minutes. Plates were sealed with parafilm for every conditioning time and kept at 20^0C. This process of starvation and conditioning (for 30 minutes intervals each) was repeated 7 times (Kauffman *et al*., 2011). To transfer the worms from one plate to another, they were washed with M9 buffer. Before transferring worms from the conditioning plate to the starvation plate, they were washed with M9 buffer to avoid food contamination. After repeated conditioning, the worms were kept in food plate before transferring for chemotaxis assay (*Fig. 12*). Chemotaxis assays were conducted at 0th, 16th, 24th and 48th hours.

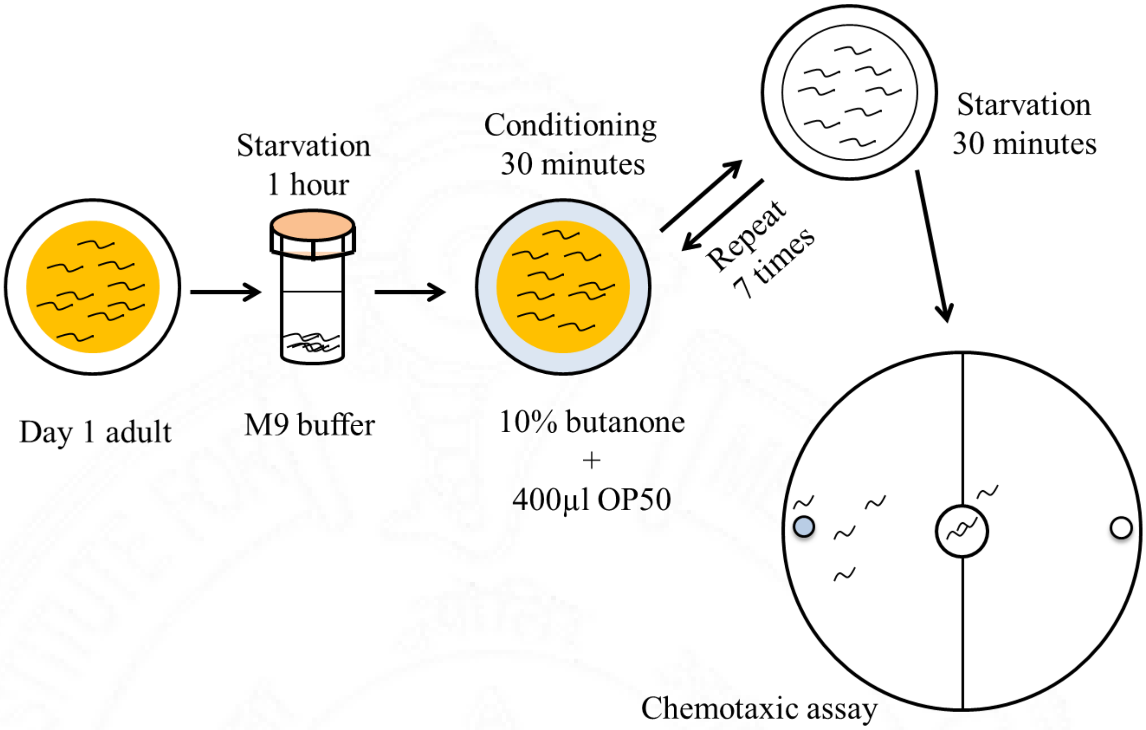


***Fig. 12*: Schematic diagram of long-term associated memory training**

III.4.9. Combining imprinting and associative memory training:

First, the worms were imprinted with 1/300th dilution of IAA. These imprinted worms were conditioned for short-term and long-term associated memory towards BT as independent experiments.

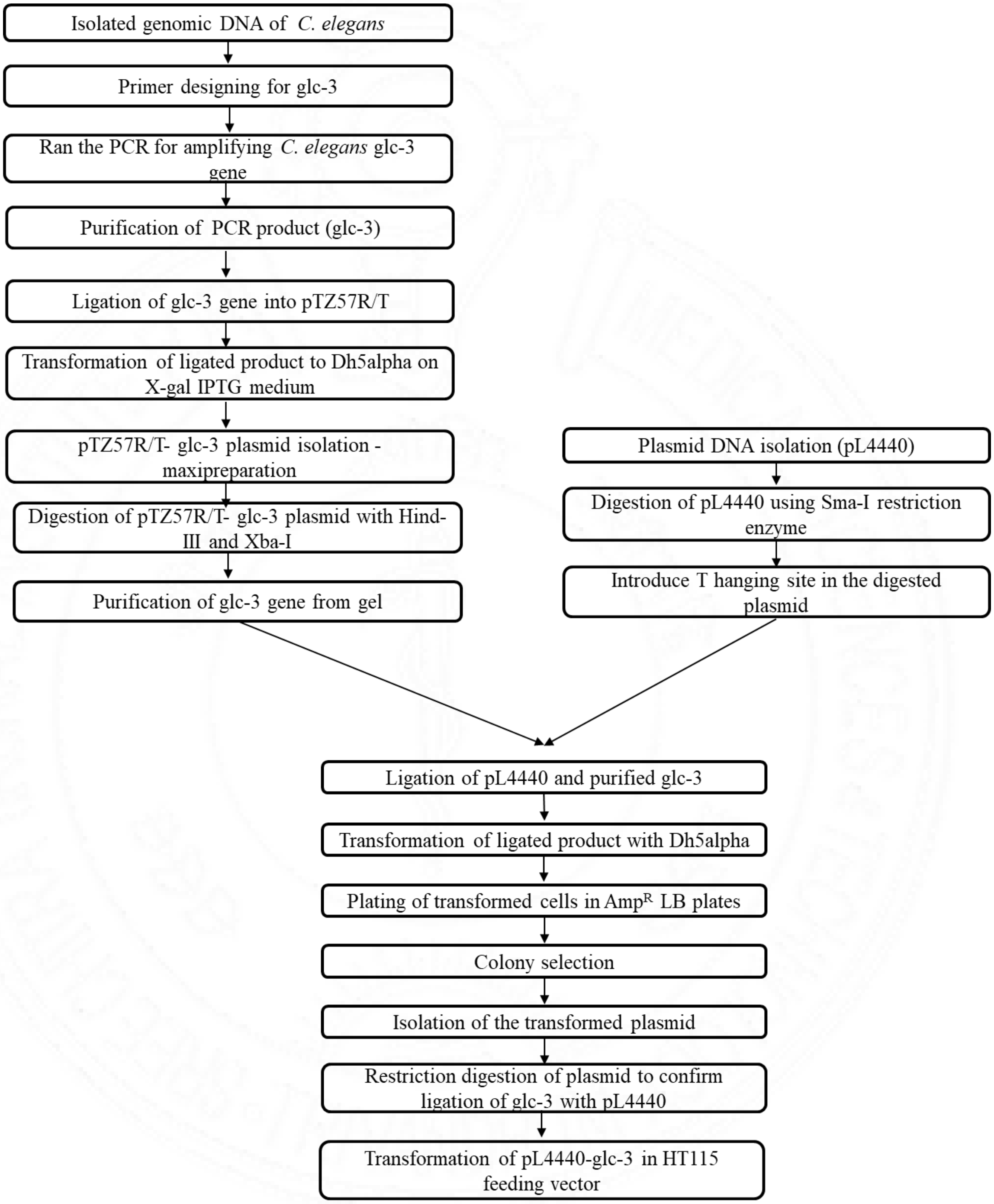
III.4.10. Transcription-translation inhibition assay:

Transcription blocking was done by the drug Actinomycin D (200µg/ml) and translation blocking was done using cycloheximide (0.8mg/ml) (Stein *et al.*, 2014). Worms were treated with the respective chemicals from their egg stage to day 1 adult stage. The chemicals were added along with OP50 to grow the worms. The day 1 adult worms were washed with M9 buffer to completely remove the traces of the chemicals and OP50. These worms were used immediately for the chemotaxis assay towards IAA or BT.

III.4.11. Worm tracking:

For the tracking assay, 3-4 worms were placed on an agar plate. Odorant was spotted 3.5 cm away from the worm. Video recording was carried out using a Sony camera (SONY ILCE 7M III K with SONYFE 50MM M2.8 Lens) till the worms reached the odorant spot. The recorded video was analysed using worm tracking software (WormLab, MBF Biosciences, USA) for the speed, reversals, omega turn, wavelength, time taken to reach the odorant spot etc.

III.4.12. Flow chart for creating RNAi feeding vector for *C. elegans*



siRNA feeding vector:

a) glc-3

172 bp region of *glc-3* gene was amplified from N2 genomic DNA and cloned into pTZ57R/T vector before subcloning to pL4440 using gene-specific primers: MOM_GLC3FP1- forward (5'-AATGGGCATATCAATCACAACA-3') and MOM_GLC3RP1- reverse (5'-ATATTAAATCCGCCCTTTTCG-3'). The PCR product was cloned to PTZ57R/T vector using the T/A cloning protocol. Glc3-pTZ57R/T vector was digested using Hind-III and Xba-I restriction enzymes and the 218 bp product containing *glc-3* gene was gel purified and eluted using AxyPrep DNA gel extraction kit (Product No. AP-GX-250). The purified *glc-3* gene fragment was cloned to the pL4440 plasmid. This plasmid has an IPTG inducible gene for two opposable T7 polymerases to create the siRNA feeding vector. The siRNA feeding vector pL4440-glc-3 was expressed in the *E. coli* strain HT115. N2 worms were fed with siRNA plasmid (from egg stage to L1 larval stage) in the siRNA feeding plate (4mM IPTG + 0.5µg/ml Ampicillin) seeded with the pL4440 plasmid vector (control) or pL4440-glc3 and siRNA expressing HT115 bacteria (test). Day 1 adult worms were used for chemotaxis assay to confirm the involvement of the *glc-3* gene in naïve and imprinted animals (See annexure for more details).

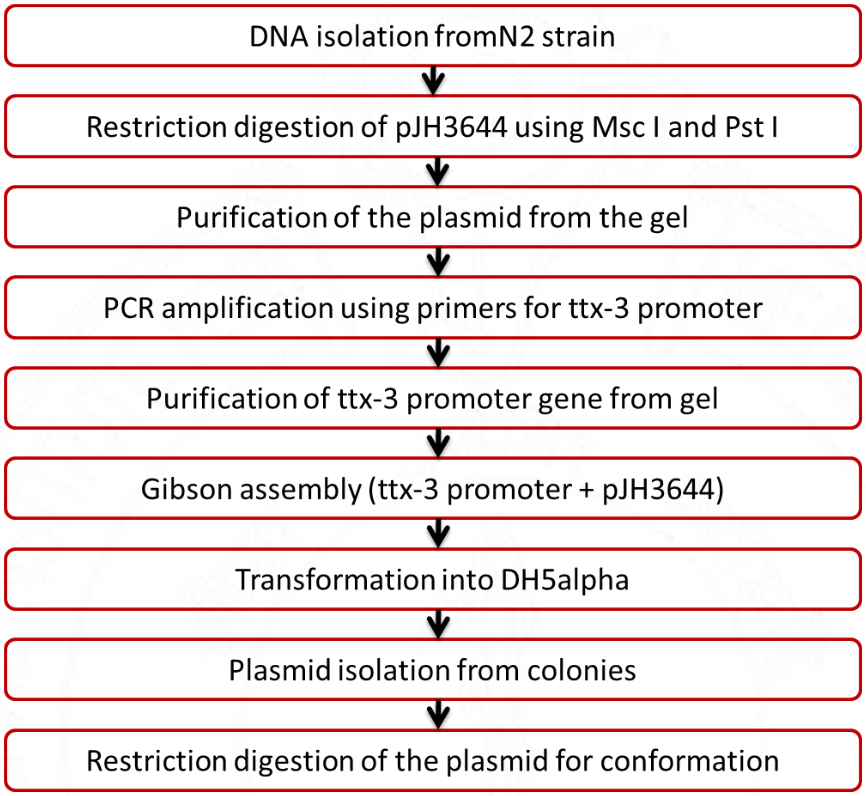
b) *sra-17*

Gibson assembly primer designing to construct *sra-17* siRNA vector. *sra-17* primer designing was done using the ERNAi online software. *sra-17* gene fragment having an amplicon size of 158 bp was amplified using the primers, forward (5’-cgaggtcgacggtatcgataagcttTATGGAGTTCCACTTGTTG-3’) and reverse (5’-ccaccgcggtggcggccgctctagaTGCTTTTCTGATTTTACGC-3’). This 208 bp *sra-17* fragment (158 bp PCR amplicons with 50 bp pL4440 flanking sequence) was inserted into the Hind-III and Xba-I restriction digested pL4440 plasmid using the Gibson assembly master mix (See annexure for preparation of Gibson assembly master mix and the vector map). Add 15µl of the Enzyme reagent Master mix. Flick the tube several times and centrifuge to collect the sample at the bottom of the tube. Incubate at 50˚C for 1 hour. Transform DH5α bacteria using a 10µl reaction product.

GR1373 (*eri-1(mg366*) worms were used to feed the *sra-17* siRNA feeding vector to knock-down the *sra-17* gene present in AWA neurons. Chemotaxis assays on day 1 adult worms were used to find the participation of AWA neurons in imprinted memory formation.

Creating AIY neuron-specific GCaMP6 expressing plasmid for calcium imaging

Steps to Gibson assembly for creating ttx-3p::GCaMP6 plasmid:



III.4.13. Construction of transgenic strain:

To study the involvement of AIY interneuron in imprinting and learning and memory, an AIY specific GCaMP6 strain was created using the standard protocol (Mello *et al*., 1991). 3163 bp genomic coding sequence for *ttx-3* promoter was amplified from the genomic DNA of N2 strain was amplified using the primers MOM19_TTX3p forward (5’-AATAAGCTTGCATGCCTGCAGGATCCTAGTGATAAAGTTTAG-3’) and MOM19_TTX3p reverse (5’-CATACCTTTGGGTCCTTTGGTTGAAAAGTAGGAAGCATTG-3’). From the pJH3644 vector [Pflp-14::GCaMP6::Cherry] (gift from Dr. Mei Zhen, University of Toronto), the 6080 bp of the *pflp-14* fragment was removed by double digestion with Msc-I and Pst-I restriction enzymes (see the details of the promoter sequence in the annexure). The PCR product was cloned to the purified vector pJH3644 without *pflp14* using Gibson cloning protocol and named the plasmid as *ttx-3p::GCaMP6.*

For constructing the transgenic strain, *ttx-3p::GCaMP6*plasmid (concentration – 50ng/µl) and a co-injection marker pCFJ104 (concentration – 5ng/µl) were microinjected in the N2 (See annexure for more details). The transgenic strains were screened for F2 generation carrying GFP on the AIY neuron and RFP on the body wall muscle.

III.4.14. Calcium imaging:

For calcium imaging, live worms were entrapped in 5% sodium alginate – calcium chloride gel. The worms were briefly placed in a 5% sodium alginate-coated coverslip and kept for two minutes to rest the worms and added 100 µl of 100mM $CaCl_2$ to accelerate an immediate gel formation, thereby entrapping the worms and allowing very less movement (*Fig. 13*).

For odour delivery during recording, a thin strip of Whatmann filter paper was fixed to a capillary tube and connected to InjectMan (Eppendorf). Timelapse images of worms without odour exposure was taken for 30 sec and then the worm was exposed to 5 µl of the desired odour dropped to the filter paper for 20 seconds. The filter paper was retracted after 20 sec. Timelapse images were recorded up to 2 minutes at a rate of 2 frames/ second. Leica microscopic system was used for capturing the images at 20X magnification. Calcium imaging was done in ZM9078 (*hpIs587::GCaMP6*) and AT9203 (*ttx-3p::GCaMP6::mCherry*) strains.

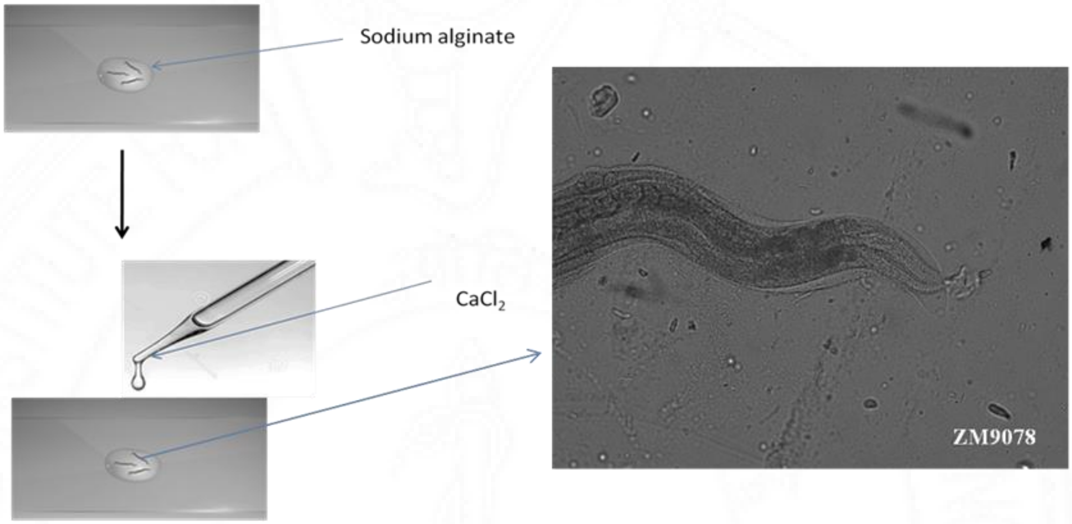


Fig. 13**: Entrapping of the worm using sodium alginate**

III.4.15. RNA isolation:

The worms were washed using M9 buffer in a nuclease-free 1.5ml Eppendorf tube. The worms were pelleted and stored at $^{-}80^{0}$C for 3 hours and thawed immediately to 4^{0}C by resuspending the worms with 1ml of TriZol (Ambion). The tube was kept in a shaking bath at 4^{0} C until the worms were dissolved (~40 minutes). 200 µl of chloroform was added to the tube and mixed for 15 seconds. The tubes were centrifuged at 12000 rpm for 15 minutes at 4^{0}C and the clear top layer was transferred to a nuclease-free 1.5

ml Eppendorf tube. 500 µl of isopropanol was added to the tubes and gently mixed by inverting the tube several times. The solution was kept at room temperature for 10 minutes. The tubes were then centrifuged at 12000 rpm for 10 minutes at 4^{0}C. The supernatant was decanted; the pellet was washed using 70% ethanol and dissolved in 10 µl of nuclease-free water (Ketting *et al.*, 2006) and stored at $^{-}80^{0}$C in small aliquots till further use.

III.4.16. cDNA synthesis:

Isolated RNA was used for cDNA synthesis using Eurogentec Reverse Transcriptase core kit using gene-specific reverse primers. Eppendorf PCR machine (Eppendorf Master cycler X50s; Catalog No. 6311000010) was used for the reaction.

The experimental conditions were:

Initial step – 10 min 25^{0}C

Reverse Transcription Step – 30 min 48^{0}C

Inactivation of the RT enzyme – 5 min 95^{0}C

III.4.17. Semi-Quantitative real-time PCR:

Quantitative real-time RT-PCR was carried out using the cDNA. Cdc-42 was used as the control gene. Quantstudio 5 (Thermo) qPCR machine was used for the reaction.

Primer details are given below.

cdc-42 primer details:

Forward primer: 5’-CTGCTGGACAGGAAGATTACG-3’

Reverse primer: 5’-CTCGGACATTCTCGAATGAAG-3’

sra-11 primer details:

Forward primer: 5’-TGCTCATTGCACCAAACTGC-3’

Reverse primer: 5’-GACAAAACTACTCCCGGCCA-3’

Experimental conditions for RT-PCR:

Conditions	***cdc-42***	***sra-11***
Initiation	95^0C /10min	95^0C /10min
Denaturation	95^0C/30sec	95^0C/30sec
Annealing	60^0C/ 30sec	64^0C/ 30sec
Synthesis	72^0C/30sec	72^0C/30sec
Hold	72^0C/10min	72^0C/10min

35 cycles

***Table. 1*: RT-PCR conditions for *cdc-42* and *sra-11* gene**

The amplified products were resolved by agarose gel electrophoresis. The fluorescence intensities of the bands were measured to quantify the gene expression. Expression levels of the *cdc-42* gene were used to normalize the values.

III.4.18. Statistical analysis:

All experiments were performed in a minimum of three independent replicates denoted by 'N'. For the chemotaxis assays, each independent experiment contains approximately 50 worms (n~50). For calcium imaging 'n' represents an independent worm experiment. The statistical analysis of the data was done using Graph pad Prism 6.01 software. Paired 't'-test, one-way and two-way ANOVA analysis was used to determine the statistical significance. Calcium imaging analysis were done using Fiji (Version 1.52p, NIH, USA).

IV. RESULTS

IV. IA. Chapter 1: Olfactory imprinting in *C. elegans*

IV.1.1 Olfactory imprinting during early development results in behavioural responses:

Olfactory imprinting known to cause behavioural alterations in the model system *C. elegans*. ((Remy & Hobert, 2005; Remy, 2010). In wild-type N2 worms imprinted with 1/300 diluted isoamyl alcohol (IAA) showed significant enhancement of attraction towards the odorant on adult stages and was persistent on later stages of adult life (*Fig: 14*). These results suggest that imprinting behaviour is highly robust in these nematodes and it is also persistent throughout their lifetime.

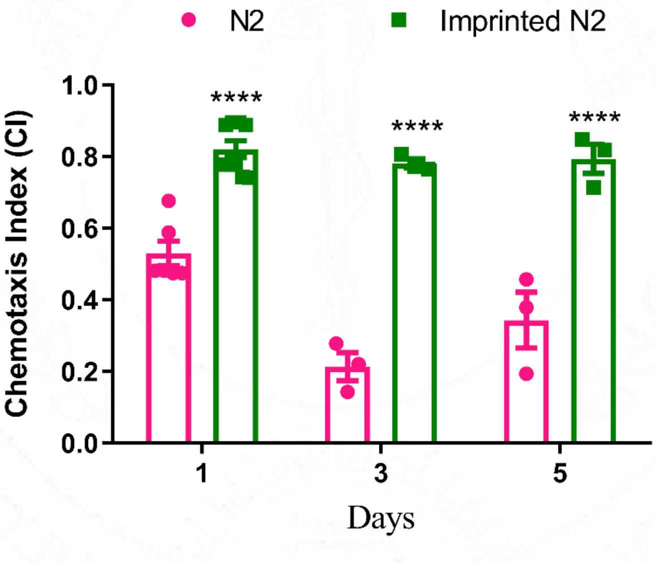


***Fig. 14*: Olfactory imprinting in N2 wild type strain on different days.** Chemotaxic index of naïve and imprinted N2 worms to IAA were calculated on 1st, 3rd and 5th day of the adult worm and the values are expressed as mean ±SEM. N= 3 or more (n~50), 2way ANOVA, $p<0.0001$ (****).

Migratory patterns in the imprinted vs naïve worms were tracked to understand the differences. The imprinted worms have a smooth tracking curve with limited halts compared to the naïve ones, which showed frequent change of directions in IAA (*Fig. 15a & 15c*). To the less attractive 1/10 BT both non-imprinted naïve N2 and imprinted N2 showed frequent changes in direction (*Fig. 15b & 15d*).

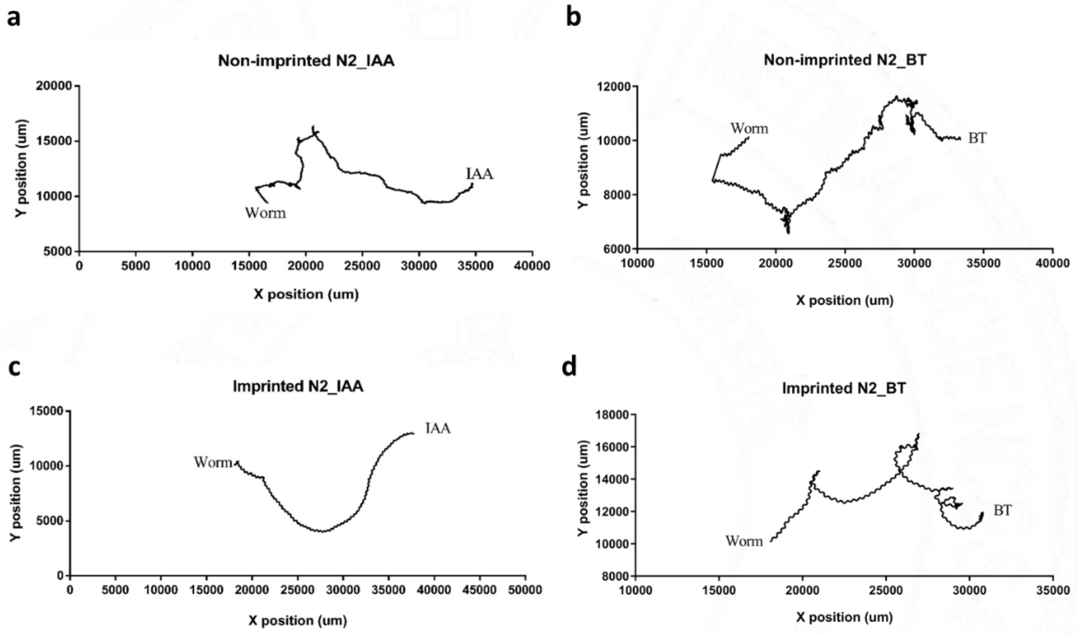
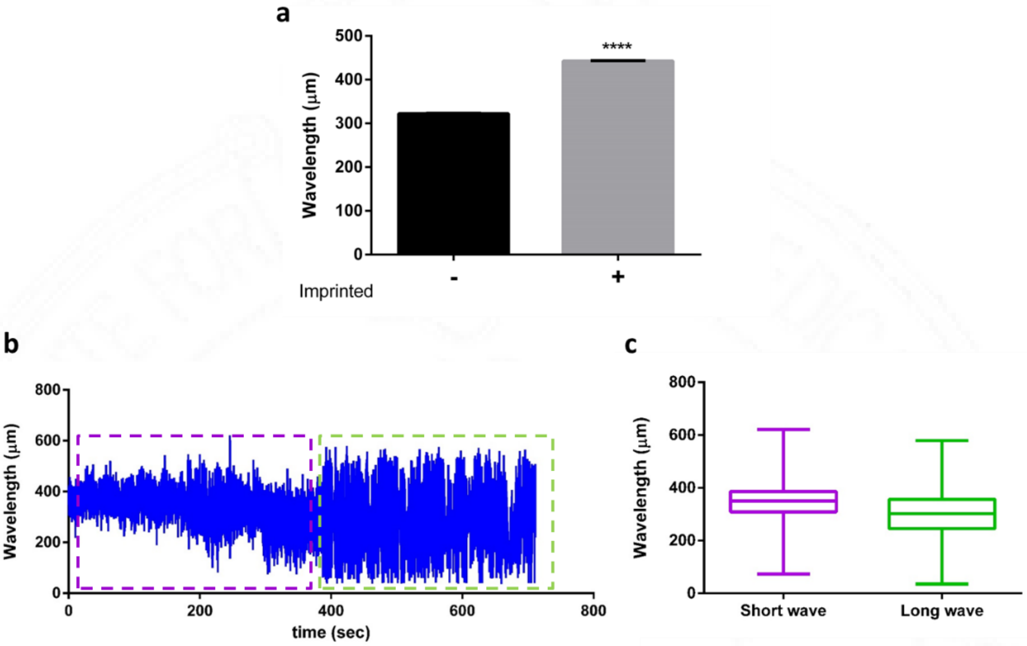
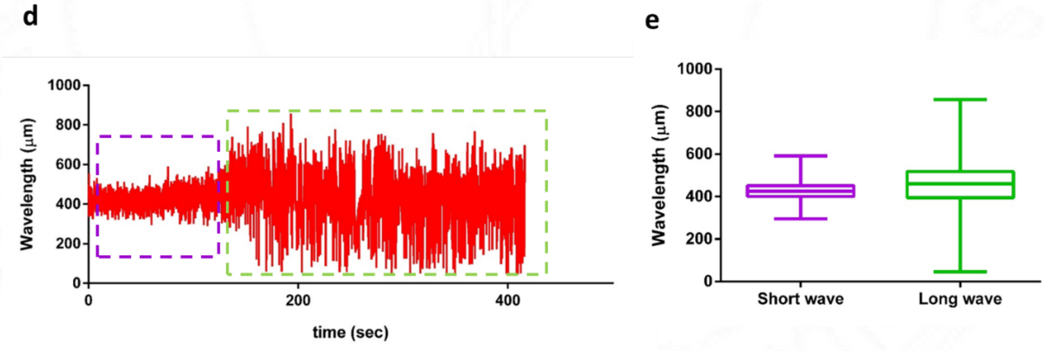


***Fig. 15*: Track pattern of non-imprinted and imprinted N2 strain towards 1/300 diluted IAA and 1/10 diluted BT.** a) track pattern of wild type N2 worm towards IAA; b) track pattern of wild type N2 towards BT; c) track pattern of imprinted N2 to IAA; d) track pattern of imprinted N2 to BT.

Analysis of the wavelength of the migratory pattern towards IAA, showed that there is an increased frequency in the imprinted worms than the non-imprinted worms at an early time point (*Fig. 16a*) and reaching the target faster (*Fig. 16b & 16d).* The initial shorter wavelength showed a range of ~50-600 um and ~300-600 in control and imprinted worms respectively (*Fig. 16c and 16e*). When the worms reached closer to the solvent the wavelength pattern showed a longer wave pattern (*Fig. 16c and 16e*).



Wavelength of N2 towards 1/300 diluted IAA



Wavelength of imprinted N2 towards 1/300 diluted IAA

***Fig. 16*: Wavelength of non-imprinted and imprinted N2 strain towards 1/300 diluted IAA.** Wavelength of the adult worms were drawn using the WormLab software. a) difference in the wave frequency of non-imprinted and imprinted N2 worms to 1/300 diluted IAA. b) wavelength of N2 worms to 1/300 diluted IAA. c) Mean wavelength of N2 worms to 1/300 IAA before recognizing the odorant (short wave) and reaching near to the odorant (long wave). d) wavelength of imprinted N2 worms 1/300 diluted IAA. e) Mean wavelength of imprinted N2 worms to 1/300 IAA before recognizing the odorant (short wave) and reaching near to the odorant (long wave). The values are expressed as mean ± SEM. n=8.

IV.1.2. Transcription-translation blockers reveal the mRNA and protein level modification in olfactory imprinting:

To understand the need for transcriptional and translational modifications for imprinted behaviour, actinomycin D (transcription blocker) and cycloheximide (translation blocker) were used. Blocking transcription, as well as translation, resulting in a significant reduction in imprinted behaviour (*Fig. 17a and 17b*), indicating that there are alterations in both mRNA and protein expression levels during the imprinting process.

Previous studies (Stein *et al.*, 2014) have shown that long-term adaptive memory (LTAM) needs both translational and transcriptional modifications, drawing parallels to the imprinted ones.

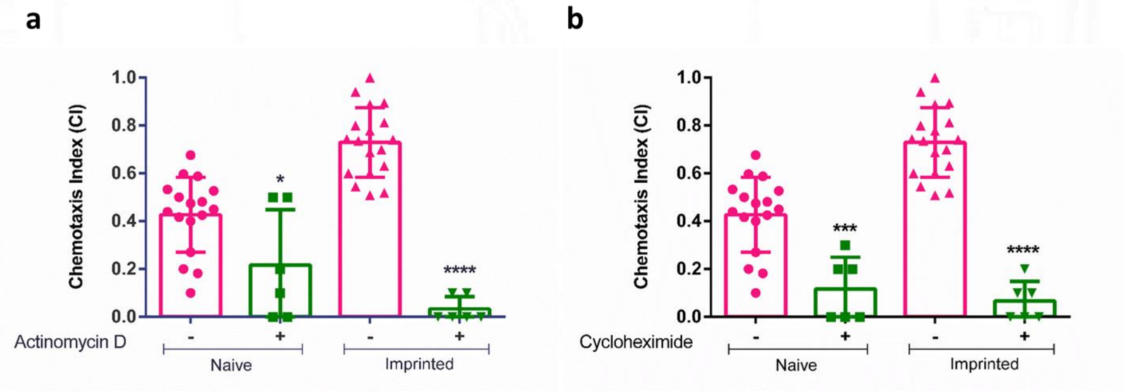


***Fig. 17*: Transcription and translational level blocking in non-imprinted and imprinted N2 strain.** a) Transcription blocking of naïve and imprinted N2 was done with 200µg/ml Actinomycin D; b) translation blocking of naïve and imprinted N2 with 0.8mg/ml cycloheximide. The values are expressed as mean ± SEM, N=6 or more (n~50), one-way ANOVA, $p<0.05$ (*), $p<0.001$ (***), $p<0.0001$ (****).

IV.1.3. Imprinting requires sensory neuronal communication to AIY interneurons:

In *C. elegans*, AWC is the major sensory neuron which detects volatile odours like benzaldehyde, butanone, isoamyl alcohol, 2,3-pentanedione, and 2,4,5-trimethylthiazole (Bargmann *et al*., 1993). The expression of receptor STR-2 in AWC neurons determines the olfactory sensation (Sasakura *et al*., 2013). Deletion mutation of *str-2* results in AWC-OFF (Sasakura *et al*., 2013). To test the role of AWC neurons in imprinting, a mutant strain with *str-2* gene knockout was used (JC2209: *olrn-1(ut305) X*). The results showed that, these mutants were less attractive towards volatile odours and showed very low imprinting behaviour towards IAA (*Fig: 18*).

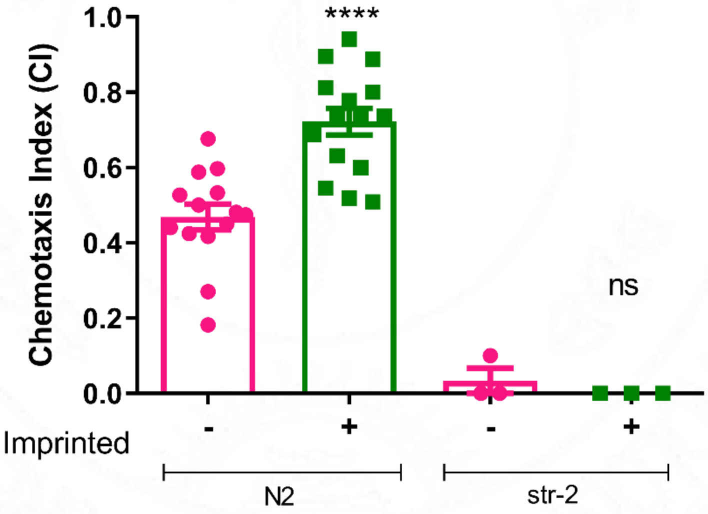


***Fig. 18*: AWC-OFF mutant (**JC2209: *olrn-1(ut305) X*) **lacks imprinting behaviour.** AWC-OFF mutant (*str-2*) were used for the imprinting study. It showed that the mutant could not detect the odour in the inactivation of AWC neuron. The values are expressed as mean ± SEM, N=3 (n~50), Student's t test, not significant (ns).

IV.1.4. Imprinting deficient worms do not show attraction to odour stimulus:

SRA-11, a transmembrane receptor present on the surface of AIY inter-neuron, has a critical role in olfactory imprinting in *C. elegans* (Remy and Hobert, 2005). To confirm, AIY specific *sra-11* mutants (RB816: *sra-11(ok630) II*) were tested for imprinted behaviour. The results showed a significant defect in olfactory imprinting compared to the wild-type N2 (*Fig. 19a*). These results reiterate the role of AIY neurons in the memory-recalling pathway (Jin, X. *et al.*, 2016). To test whether the G-protein coupled receptor SRA-17 present on AWA sensory neurons (Taniguchi *et al.*, 2014), could have a similar impact, the gene was knocked out using siRNA in GR1373 (*eri-1(mg366*) worms. The results showed that the olfactory imprinting was intact in these worms (*Fig. 19b*) suggesting SRA-17 in the AWA neurons does not have a role in positive olfactory imprinting.

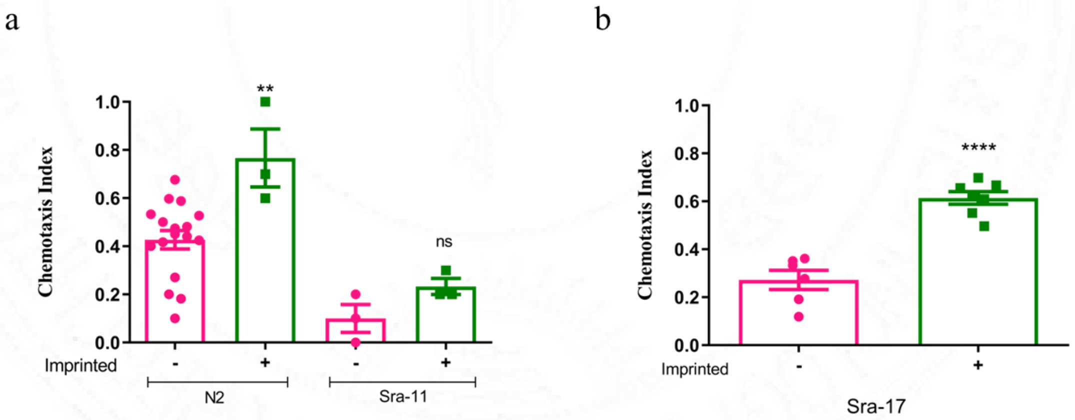


***Fig. 19*: Imprinting is defective by SRA-11 receptor mutation on the AIY; but SRA-17 receptor is not involved.** a) the graph indicates that the *sra-11* mutation (RB816: *sra-11(ok630) II*) affect the olfactory imprinting. b) siRNA mediated *sra-17* mutation in the AWA neuron not affected the olfactory imprinting. The values are expressed as mean ± SEM, N=3 or more (n~50), one-way ANOVA, $p<0.01$ (**), $p<0.0001$ (****), not significant (ns).

IV.1.5. Connectome alteration makes imprinting defect:

To further investigate the downstream connectomes from AIY interneurons, worms defective in AIY-RIA synapse formation were used in the study. *cima-1*(DCR744: *(wy84) IV; wyIs45 X*), mutant strain is defective in synapse formation between AIY and RIA neurons (Shao, Z *et al*., 2013). Deletion of *cima-1* gene leads to an ectopic branching of AIY neurons and results in disruption in the proper AIY-RIA connection. *cima-1* strain showed significant impairment in imprinting behaviour (*Fig. 20a*). To confirm this further, *cima-1* rescue worms (DCR2188: *ttx-3p::CD4::GFP(11)+glr-3p::CD4::GFP(1-10)+ttx-3p::mCherry::rab-3 + unc-122p::GFP*) were tested and found to regain imprinting behaviour (*Fig. 20b*). The result suggests that there is imprinting information, which is coded to multiple neurons, especially in AIY and RIA interneurons.

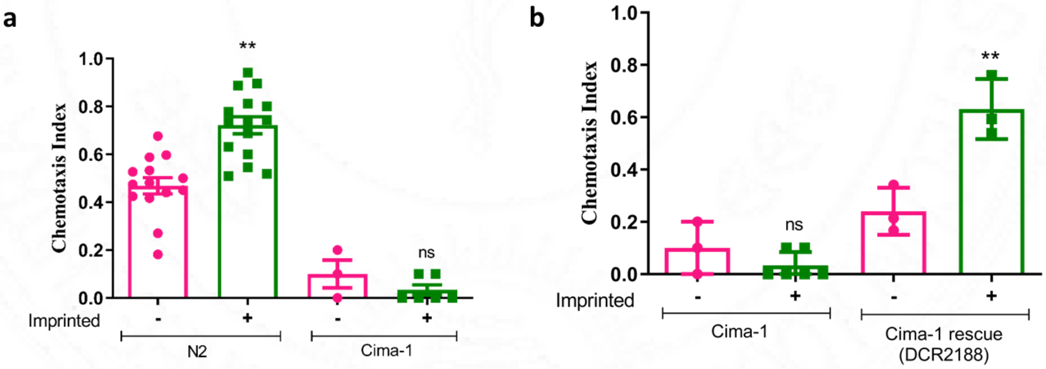


***Fig. 20*: IAA attraction is defective in *cima-1* mutant; *cima-1* partial rescuing restored the IAA attraction.** a) *cima-1* mutant (*wy84) IV; wyIs45 X*) showed imprinting defect. b) *cima-1* rescue strain (*ttx-3p::CD4::GFP(11)+glr-3p::CD4::GFP(1-10)+ttx-3p::mCherry::rab-3+unc-122p::GFP*) showed reintroduction of imprinting behaviour. The values are expressed as mean ± SEM, N=4 or more (n~50), one-way ANOVA, p<0.01 (**), not significant (ns).

IV.1.6. Semi-quantitative PCR reveals the imprinting effect:

Since *sra-11* gene has a critical role in imprinting, the gene expression levels of *sra-11* were quantified in non-imprinted and imprinted worms. The molecular mechanism of imprinting in *C. elegans* was studied using semi-quantitative PCR. For this, expression levels of *sra-11* gene were quantified both during normal and imprinted conditions. The mRNA of cell division control protein – 42 (CDC-42) was used as the internal control. RT-PCR experiments were carried using RNA samples from N2 (wild type), DCR744 (*cima-1* mutant) (*cima-1(wy84) IV; wyIs45 X*), DCR775 (*cima-1(wy84) IV; wyls45 X; olaEx459*; *cima-1* rescue - *cima-1* genomic region with 1.9kb promoter) and DCR2188 (*ttx-3p::CD4::GFP(11)+glr-3p::CD4::GFP(1-10)+ttx-3p::mCherry::rab-3 + unc-122p::GFP; olaEx765* rescues AIY presynaptic defects in *cima-1(wy84)* mutants).

The mRNA expression levels of *sra-11* in different strains during non-imprinted and imprinted conditions were quantified after resolving bands in agarose gel (*Fig. 21A*).

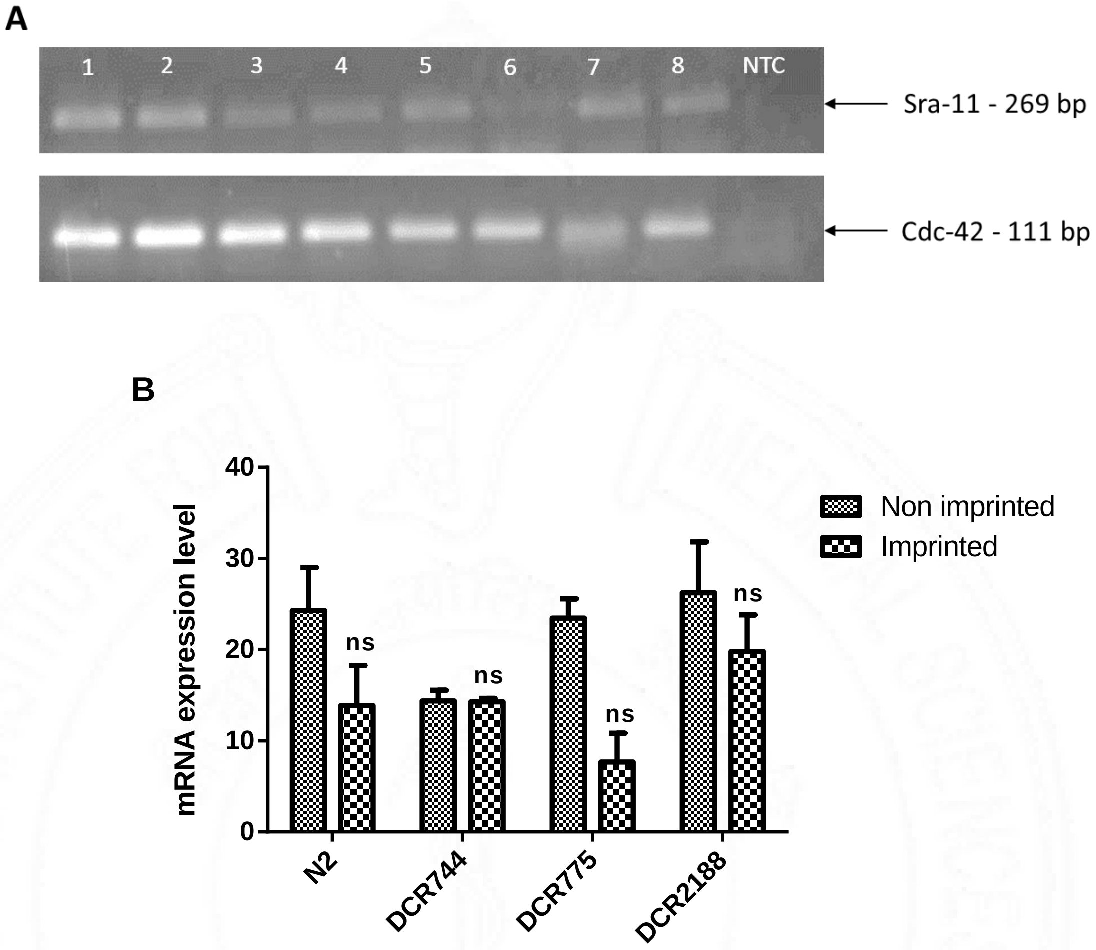


***Fig. 21*: A. Representative gel images of RT-PCR amplicons demonstrating the expression of *cdc-42* gene (product size 111bp - internal control) and *sra-11* gene (269bp) in non-imprinted and imprinted conditions of different strains.** PCR amplicons of *sra-11* and *cdc-42* after separation by 1% agarose gel electrophoresis. 111bp amplicon represent the *cdc-42* internal control and 263bp amplicon represent the *sra-11* gene. Lane 1 - N2, 2- imprinted N2, 3 - DCR744 (*cima-1(wy84) IV; wyIs45 X*), 4 - imprinted DCR744, 5 - DCR775 (*cima-1(wy84) IV; wyls45 X; olaEx459*), 6 - imprinted DCR775, 7 - DCR2188 (*ttx-3p::CD4::GFP(11)+glr-3p::CD4::GFP(1-10)+ttx-3p::mCherry::rab-3+unc-122p::GFP*), 8 - imprinted DCR2188 and NTC.

B. Quantification of mRNA expression level of *sra-11* gene in non-imprinted and imprinted conditions of different strains. Different mutants were used for profiling *sra-11* gene expression. The quantification of *sra-11* expression was normalization with *cdc-42* gene expression. The values are expressed as mean ± SEM, n=3, one-way ANOVA.

The results showed that during imprinting *sra-11* gene expression level was maintained at a lower rate as compared to the non-imprinted control in N2, *cima-1* rescue worms such as DCR775 and DCR2188 (*Fig. 21B*), suggesting that alterations in RNA profile is critical in maintaining an acquired behaviour.

IV.1.7. Genes and neurons involved in positive olfactory imprinting:

To elucidate additional genes and the connectomes involved in imprinting the following approaches were taken.

IV.1.7.1. Olfactory imprinting is normal in *kal-1* mutant, defective for AIY axonal branching.

kal-1 mutant strain (OH910 - *ttx3p:kal1+usc122p::GFP*), defective in AIY neurite branching was used to study the olfactory imprinted memory. *kal-1* overexpressing mutants showed unusual neuronal branching of AIY interneurons (Rugarli *et al*., 2002). During the early stages of this mutant strain, AIY interneurons are normal. As a result of the *kal-1* over expression during the adult stage, unusual axonal branching develops in the AIY interneuron resulting in defects in the olfactory system. The *kal-1* mutant showed a defect in the detection of 1/300 diluted IAA compared with the IAA attraction of N2 strain ($p<0.0001$). The imprinted *kal-1* mutant showed normal imprinting behaviour similar to the wild-type (*Fig. 22*). The imprinting results, strongly suggest that imprinting is involved in maintaining certain synapses which formed during imprinting conditioning at early developmental stages and these synapses survive even under a genetic mutation which induces synaptic alterations and neuronal branching during their adult stage.

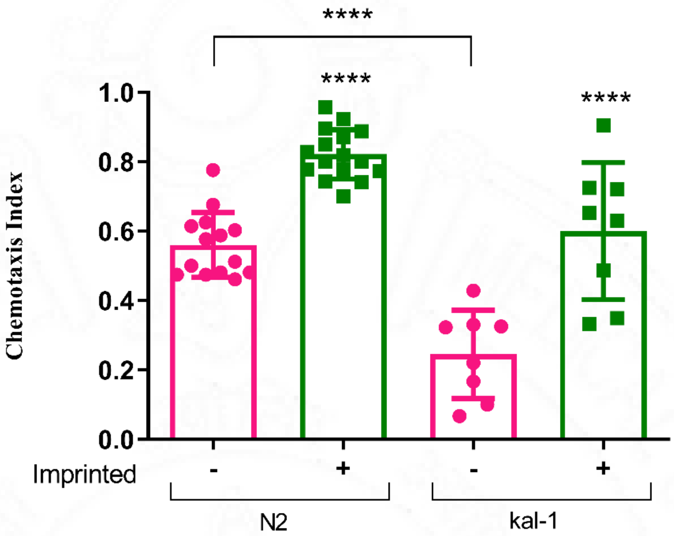


***Fig. 22*: AIY connectome alteration during adult stage could not make any change in imprinted memory.** Behavioural analysis of the imprinted kal-1 strains (OH910 - *ttx3p::kal1* + *usc122p*::GFP) showed intact imprinted memory. The values are expressed as mean ± SEM, N=6 (n~50), one-way ANOVA, p<0.0001 (****).

IV.1.7.2. Imprinting is independent of ionotropic glutamate receptors but dependent on glutamate-gated chloride channels.

Since glutamate receptors have an important role in learning and memory, further experiments were conducted to decipher the role of ionotropic or metabotropic glutamate receptors in the imprinting process. The following glutamate receptor mutants were used in the study: *glr-1* (KP4 - *glr-1(n2461) III*), *glr-2* (RB1808 - *glr-2(ok2342) III*), *nmr-1* (VM487 - *nmr-1 (ak4) II*), *ser-2* (RB1690 - *ser-2(ok2103) X*) and *glc-3* (RB594 - *glc-3(ok321) V*) for behavioural study. *C. elegans* have all four types of glutamate receptors such as AMPA (GLR-1, GLR-2), NMDA (NMR-1), G-protein coupled receptors (SER-2), and glutamate-gated chloride channel (GLC-3) (Chalasani *et al.*, 2007). These glutamate receptors are present on specific neurons such as GLR-1 in AIB, RIM and RIA neuron, GLR-2 present on AIB, AIY, AIZ, AIA and RIA neuron, NMR-1 present on RIM neuron, SER-2 present on AIY, AIZ and RIA neuron and GLC-3 genes present on AIY interneuron. The mutants of metabotropic glutamate receptors such as *ser-2* and *glc-3* showed a lack of imprinted memory indicating that these receptors are involved in olfactory imprinting (*Fig. 23*). The naïve result of *ser-2* and *glc-3* were showing higher chemotaxis to IAA, and hence no statistically significance was observed between naïve and imprinted strains. However, imprinted memory is independent of NMDA (NMR-1) and non-NMDA (GLR-1 and GLR-2) type receptor. All the strains with a mutation in ionotropic glutamate receptors showed positive olfactory imprinting similar to the N2 strain (see *Fig. 14*).

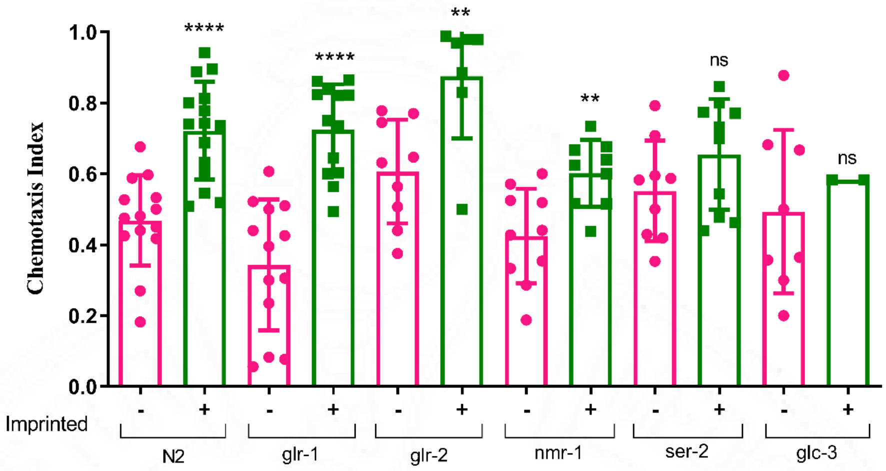


Fig. 23**: The non-NMDA and NMDA type receptor does not involve in olfactory imprinting but the glc-3 chloride channel is involved.** The non-NMDA receptors such as glr-1 (KP4 - glr-1(n2461) III), glr-2 (RB1808 - glr-2(ok2342) III) and the NMDA receptor such as nmr-1 (VM487 - nmr-1 (ak4) II) showed normal imprinted memory but the metabotropic glutamate receptor such as ser-2 (RB1690 - ser-2(ok2103) X) and glc-3 (RB594 - glc-3(ok321) V) were defective for imprinted memory. The values are expressed as mean ± SEM, N=3 or more (n~50), one-way ANOVA, $p<0.01$ (**), $p<0.0001$ (****), not significant (ns).

IV.1.8. Calcium imaging of AIY interneuron after positive olfactory imprinting:

To understand the calcium transients in the AIY interneuron during the exposure of 1/300 diluted IAA in non-imprinted and imprinted worms, a transgenic strain was generated by microinjecting 50 ng/µl concentration of *pttx-3::GCaMP6* vector (*ttx-3* promoter sequence was amplified from the wild-type N2 worm DNA and Gibson cloned to the pJH3644 by removing the *pflp-14* region) along with a co-injection marker pCFJ104 with a concentration of 5 ng/µl and named the strain as AT9203 (see the materials and methods for the details regarding transgenic strain generation). Calcium imaging of AT9203 (*pttx-3::GCaMP6::mCherry*) showed that fluorescence change in the imprinted strain is significantly less compared with the non-imprinted strain (*Fig. 24*). This indicates a strong habituation behaviour of imprinted worms towards IAA recognition, resulting in lowered Ca^{2+} spike.

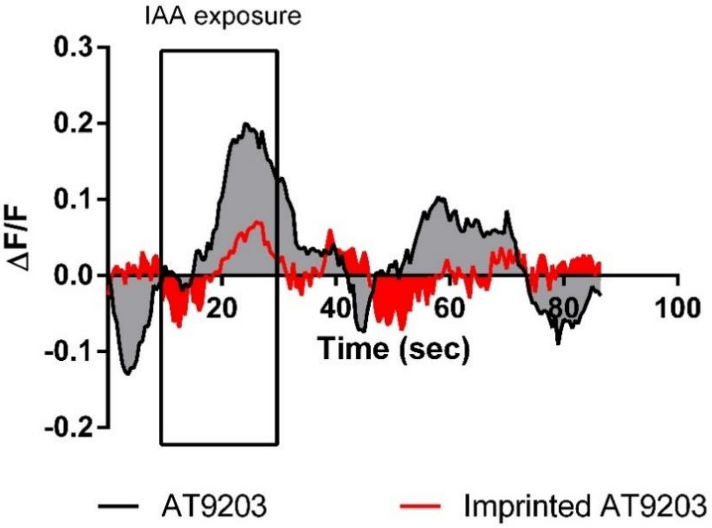


***Fig. 24*: Calcium responses of AIY interneuron after imprinting.** AIY calcium responses during 1/300 diluted IAA exposure for 20 seconds in naïve (black) and imprinted (red) AT9203 (ttx-3p::GCaMP6::mCherry) showed an increased calcium fluctuation in the naïve and a baseline calcium firing in the imprinted animals. The values are expressed as mean ± SEM, n=6 or more, one-way ANOVA, p<0.0001 (****).

IV.1.9. Calcium imaging of RID interneuron after positive olfactory imprinting:

To further confirm the habituation behaviour of the neurons, a strain expressing GCaMP-6 in the RID interneuron (ZM9078 - hpIs587 - GCaMP6) was used for calcium imaging. The RID interneuron is involved in the functional migration of the worm especially during forward movement. There is no direct synaptic connection between AIY and RID interneuron. For positive olfactory sensation the worm has to move towards the odorants so that RID neuron will also help for its movement indirectly.

RID is a single neuron, the sister undergoes apoptosis during embryonic development (Wang *et al.*, 2015). The functional calcium imaging data showed that the fluorescent fluctuations in the imprinted animals were significantly less compared with non-imprinted animals (*Fig. 25*). IAA is a strong olfactory attractant for the worms, hence inducing Ca^{2+} spike in the non-imprinted worms on odour exposure. Imprinting results in strong habituation to the IAA odour and shows a baseline Ca^{2+} spike during 1/300 diluted IAA exposure but the speed towards the odorant increased (*Fig. 16*).

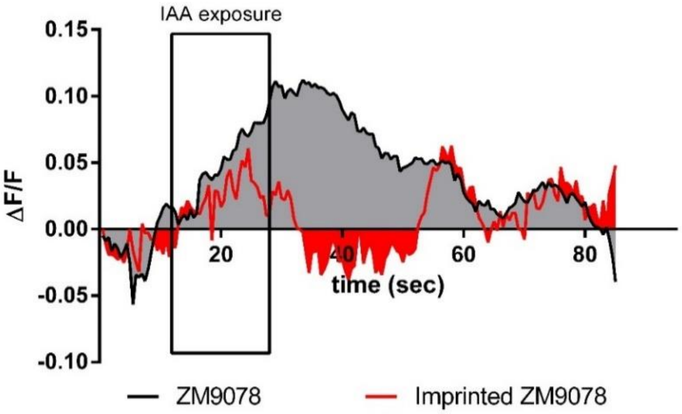


***Fig. 25*: Calcium responses of RID interneuron for 1/300 diluted IAA.** RID calcium responses for 1/300 diluted IAA for 20 seconds in naïve (black) and imprinted (red) ZM9078 (hpIs587 - GCaMP6) showed increased calcium firing in naïve animals compared with baseline calcium firing in imprinted animals. The values are expressed as mean + SEM, n=5 or more, one-way ANOVA, p<0.0001 (****).

IV.1B. Discussion

This study focused on the detection of neuronal connectome participating in positive olfactory imprinting in *C. elegans*.

In *C. elegans* olfactory imprinting is induced during their critical period of development. For positive olfactory imprinting in *C. elegans,* SRA-11 receptors present on the AIY interneuron play a critical role (Remy *et al*., 2005 and this study). However, for aversive olfactory imprinting SRA-11 receptors do not play a critical role (Jin *et al*., 2016). Hence, imprinted memory formation involves complex cellular and molecular mechanisms.

The chemotaxis assay of imprinted N2 wild-type worms towards IAA showed a long-lasting attraction even on the 5th day, supporting the observation that, early imprinting has a significant behavioural alteration in worms (Remy *et al*., 2005). The tracking pattern showed the imprinted worms have a significant change in the undulatory gait and take a faster route towards the attractant compared to the control. Such changes in gait patterns in worms have been documented under various conditions like food (Angstman *et al*., 2016), temperature (Parida *et al*., 2014), environment (Park *et al*., 2008) etc. Imprinting also has an impact in modified locomotory behaviours in worms. Worms were taking short wavelengths on the first set but were migrating more closer to the solvent of attraction. Once the worms reach closer to the solvent, they alter the gait pattern to a longer wavelength. The controls also showed a similar longer wavelength pattern as they reach closer to the solvent of attraction.

C. elegans showed transcriptional and translational level changes for the LTAM to function but not for the STAM (Kauffman *et al*., 2010; Stein *et al*., 2014; Li *et al*., 2013). Transcription mediated CREB activity in the AIM interneuron has a major role in modifying LTAM including synaptic plasticity (Lakhina *et al*., 2015; Suo *et al.,* 2006). The transcription and translation blocking with actinomycin D and cycloheximide during imprinting, a long-lasting memory, also explains the importance of mRNA and protein level requirement for imprinted memory. Blocking of transcription and translation resulted in the significant reduction of imprinted memory.

AWC sensory neuron is the primary neuron which detect IAA odorant in *C. elegans* (Bargmann *et al*., 1993). When the worms were exposed to IAA, AWC become OFF which results in the activation of the AIY interneuron initiating the local search behaviour of the worm (Bargmann *et al*., 2001). AWC-OFF happens by lowering the glutamate neurotransmitter release by AWC neurons and this in turn inhibits the glutamate-gated chloride channel (GLC-3) present on the AIY neuron (Horoszok *et al*., 2001) resulting in the inhibition of turns and allowing the worms to move towards the odorant (Chalasani *et al*., 2007). A similar kind of neuronal and molecular mechanisms happened in the vertebrate rod and con photoreceptors (Zhang *et al.*, 2005).

Even though the AWC neuron is OFF during IAA detection, the absence or mutation of AWC could not make any odour detection in *C. elegans*. The AWC (specific to *str-2* gene -2AWC-OFF) mutant which has BT enhancement abnormal property (Troemel *et al*., 1999; Wes *et al*., 2001) showed defects in IAA detection and it is also unable to create olfactory imprinting to IAA. These results underscore that the functioning of AWC sensory neurons is essential in the detection of volatile odour as well as in imprinted memory.

SRA-11 G-protein coupled receptor in AIY interneuron has an important role in positive olfactory imprinting (Remy *et al*.,2005), but is not sufficient for generating long-lasting imprinted memory. CEH-23, one of the LIM homeobox domains also has an effect on *sra-11* (Gultekin *et al*., 2001). *sra-11* mutant was defective for positive olfactory imprinting but the seven transmembrane receptors on the AWA neuron *sra-17* have no significant role in imprinted memory showing intact imprinted memory in *sra-17* mutants.

Negative olfactory imprinted memory in *C. elegans* showed the involvement of AIY and RIA neurons along with the effect of RIM releasing neurotransmitter tyramine (Jin *et al*., 2016). Based on this information, I assumed that positive olfactory imprinting is not made by the AIY interneuron alone. The results also support this working hypothesis.

This study could identify the participation of CIMA-1 in the olfactory imprinted memory. *cima-1* mutation produces an ectopic connection in the zone 1 region of AIY during the adult stage, thereby disrupting the AIY-RIA connection (Shao *et al*., 2013). However, the worms have a normal connectome between AIY-RIA during the larval stage (Shao *et al*., 2013), the stage at which the imprinting process is initiated. CIMA-1 is not required for distributing the presynaptic connection but is essential for maintaining it (Shao *et al*., 2013).

CIMA-1 is homologous to vertebrate lysosomal transporter sialin linked to neurodegenerative disease (Reimer, 2013; Sreedharan *et al*., 2010). Most of the neurodegenerative diseases have the common symptom of memory loss. Olfactory imprinting showed a significant defect in *cima-1* mutants and could regain the behaviour by rescuing the *cima-1* gene. These results suggest AIY-RIA synaptic integrity is critical for imprinted memory recall. The impact of synaptic loss in memory formation has been well documented (Tonegawa *et al*., 2015; Takeuchi *et al*., 2014). This observation was further confirmed using KAL-1 overexpressing strain (*ttx3p::kal1 + usc122p::GFP*) which results in abnormal branching of AIY neurites (Bulow *et al.*, 2002), and shown that imprinting results in consolidation of synapse even under strong genetic mutation and rescue the attractive behaviour to IAA. In human KAL-1 helps for the olfactory axon guidance and the homolog in *C. elegans* influence the neural outgrowth and helps the neurons for migratory mechanisms (Rugarli *et al*., 2002).

The semi-quantitative RT-PCR analysis displayed the expression level of *sra-11* gene showed a decrease during the imprinted state. mRNA profile shows significant variations in the memory pathway (Alberini *et al.*, 2015). SRA-11 is a GPCR (Troemel *et al*., 1995). GPCRs can regulate synaptic plasticity both at pre and post-synapse. At pre-synapse various GPCRs can influence the neurotransmitter release either positively or negatively (Tedford *et al.*, 2006; Betke *et al*.,2012). At post-synapse GPCRs can regulate long-term potentiation and long-term depression, alter synaptic connections, and the memory process (Rojas *et al*., 2013; Leung *et al.,* 2017). SRA-11, similar to other GPCRs, must have a significant role in regulating the AIY neuronal function and its plasticity (Jong *et al.*, 2018)

Since synaptic integrity is essential for imprinted memory formation, as shown in *cima-1* mutant, this observation was reconfirmed in *kal-1* mutant.

The analysis on the major glutamate receptors such as AMPA and NMDA receptors involved in the LTAM (Rose *et al*., 2003; Zamanillo *et al.*, 1999) confirms the non-involvement of these receptors in positive olfactory imprinted memory. AMPA receptor mutants for the genes *glr-1* and *glr-2*, mainly expresses in AIB interneuron (Maricq *et al*., 1995), showed intact positive olfactory imprinting and the *nmr-1* mutant (NMDA type glutamate receptor) mainly expresses in RIM neurons (Kano *et al*., 2009), showed significantly increased attraction after imprinted conditioning.

SER-2 receptor present on the AIY interneuron binds to the neurotransmitter, tyramine released from the RIM interneuron (Jin *et al.*, 2016). SER-2 has been identified as one of the critical molecules for aversive olfactory imprinting (Jin *et al*., 2016). My study shows SER-2 is also essential for positive olfactory imprinting. These results point out that alteration in the pre or postsynaptic terminals of AIY neurons could impact olfactory imprinted behaviour.

The calcium release pattern of AWC, AIB and AIY neurons reveals the participation of these neurons during odour presentation and removal (Chalasani *et al*., 2007). During odour presentation, AWC became inactivated resulted in the activation of the AIY interneuron (Chalasani *et al*., 2007). The calcium measurement in AIY and RID neurons indicates that these neurons are involved in the detection of IAA. RID neuron helps in the forward movement of the worm (Lim *et al*., 2016). The data suggest that there is a decrease in calcium flux in the AIY and RID interneuron in imprinted worms during odour exposure. These results suggest imprinting results in modification in neuronal firing patterns similar to habituation behaviour.

Worms are known for fast adaptation to repetitive stimuli such as light (Ardiel *et al*., 2016), taping (Wicks *et al*., 1997; Bozorgmehr *et al.*, 2013) etc. Imprinting is a long-lasting memory resulting in significant neuronal modifications – the firing pattern similar to habituation is a strong indication of the involvement of multiple cellular processes. For example, habitation alters the gill withdrawal reflex in Aplysia (Kandel *et al*., 2004) and olfactory inhibition in *Drosophila* (Twick *et al*., 2014) are due to depression of excitatory synapses.

The results from this study shows that the complex connectome and receptor interactions with the AIY interneuron is critical for coding olfactory imprinting in *C. elegans*.

IV. 2A. Chapter 2: Imprinting alters learning and memory

IV.2.1. Imprinting enhances learning and memory.

It is essential to have transcriptional and translational changes to establish long-term memory (Kauffman *et al*., 2011; Murphy *et al*., 2014). Since imprinting lasts for a very long time, it is assumed that a series of synaptic changes must be occurring due to transcription/translation variations during the memory coding process. If so, I hypothesized that the learning process in olfactory imprinted worms would be more robust when trained for the olfactory adaptive learning paradigm.

Short-term olfactory adaptive learning (STAM) towards 1/10 BT (Kauffman *et al.,* 2011) was significantly long-lasting (>2 hours; $p<0.05$) in imprinted N2 wild type worms compared to the control worms (*Fig. 26a*). Long-term olfactory adaptive learning (LTAM) also showed a significantly extended period of memory recalling in imprinted worms: >48 hours compared to 24 hours in control ($p<0.0001$) (*Fig. 26b*).

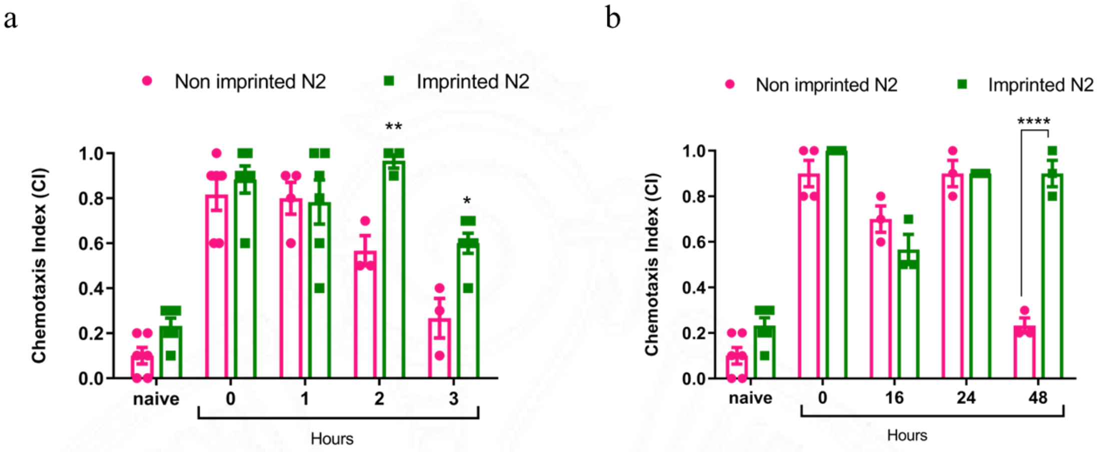


Fig 26**: a) STAM of non-imprinted and imprinted N2**. Wild type N2 after STAM training showed memory towards 1/10 diluted BT up to 2 hours after that CI value reached to the naïve level. But the STAM of imprinted N2 showing a significantly increased memory extension capacity towards 1/10 diluted BT till 3rd hour.; **b) LTAM of non-imprinted and imprinted N2.** LTAM training of wild-type N2 resulted the memory holding capacity to 1/10 diluted BT till 24th hour after that the value dropped to the naïve level. From the graph, LTAM of imprinted N2 showed an increased attraction towards BT up to 48th hour. The values are expressed as mean ±SEM. N= 3 or more (n~50), 2way ANOVA, $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****).

IV.2.2. Connectomes involved in the learning paradigm.

Neuronal connectomes associated with imprinting as well as learning and memory are shown in *Fig. 27*.

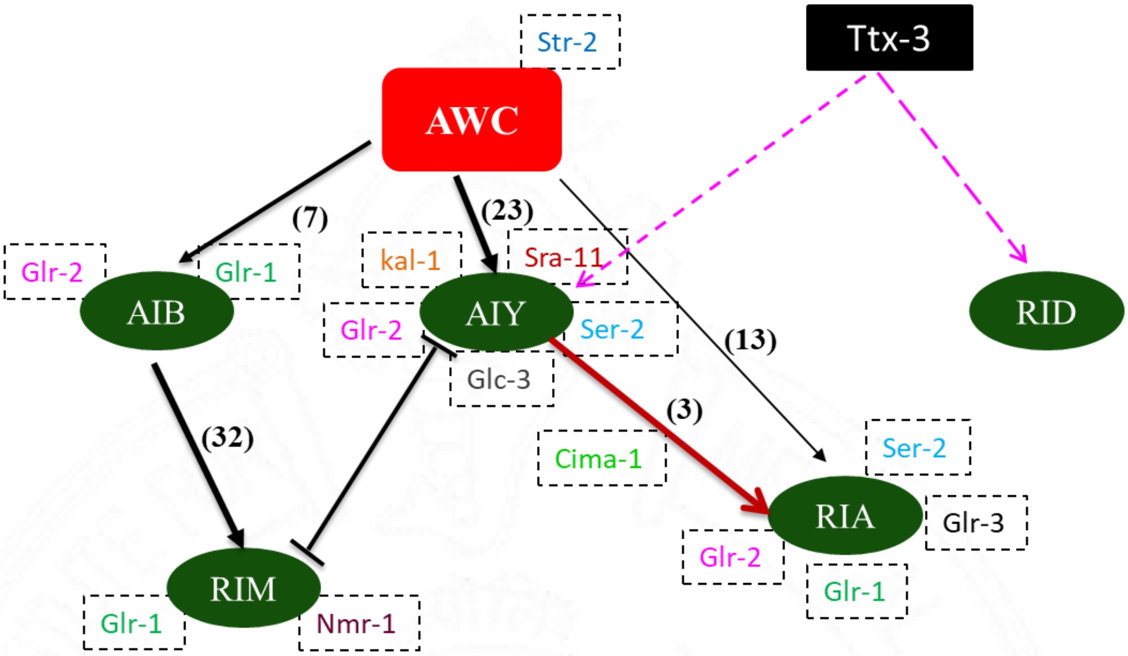


***Fig 27:* Neurons involved in associated learning and memory.** AWC, AIY, AIB, RIA, RIM and RID neurons, and receptors or genes associated with these neurons are selected as the candidates for this study. Arrows represents synaptic connections. Thickness of the arrows indicates the synaptic strength. Blunt line represent the gap junction. Number of synapses formed between neurons were noted in brackets. *ttx-3* represents the gene (part of homeobox domain) which regulate the AIY and RID neuronal development.

IV.2.3. Role of volatile sensory neuron, AWC, in learning and memory.

In *C. elegans*, AWC neurons are the first set of neurons involved in volatile olfactory sensation (Bargmann *et al*., 1995). AWC mutant (JC2209 - *olrn-1(ut305) X*) showed defective short-term and long-term adaptive learning (*Fig. 28a and 28b*). Without sensory recognition of the odorant, the worms cannot establish learning and memory.

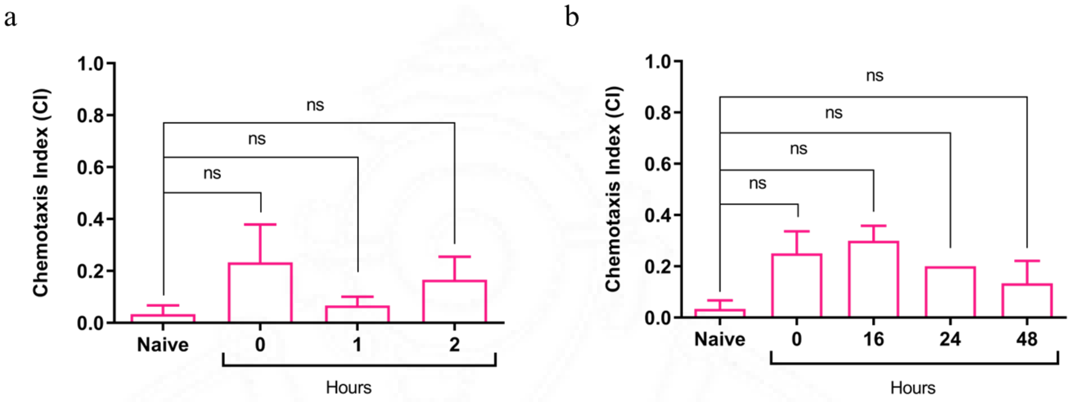


Fig 28**: a) STAM of AWC mutant** (JC2209 - *olrn-1(ut305) X*). The *str-2* mutation causes loss of sensation in AWC neuron resulting in STAM defect.; **b) LTAM of AWC mutant**. The *str-2* mutation also creates LTAM defect. The values are expressed as mean + SEM, N=3 or more (n~50), one-way ANOVA, not significant (ns).

IV.2.4. SRA-11 receptors are vital to imprinting and for long-term memory.

AWC neurons have 23 synaptic connections to AIY interneurons (Wormweb.org). There is evidence to support SRA-11, the G protein-coupled seven-transmembrane receptor (GPCR) presents in the AIY interneuron, is essential to establish imprinted behaviour (Remy *et al*., 2005). Surprisingly, *sra-11* mutant worms (RB816 - *sra-11(ok630) II*) showed extended short-term adaptive memory (*Fig. 29a*) while the worms were unable to form long-term memory (*Fig. 29b*). Both imprinted memory and long-term adaptive memory formation lasts a longer period and require translation and transcription of a series of genes. The results suggest that *sra-11* expression in AIY neuron is essential to establish these memories. It is not immediately obvious to us the reasons behind extended short-term memory in *sra-11* mutants which showed a similar pattern of memory formation to that of imprinted N2 worms (*Fig. 26a*). One possibility is that short-term memory recalling is AIY interneuron independent pathway or *sra-11* has antagonistic action in AIY neurons under both short-term and long-term memory formation.

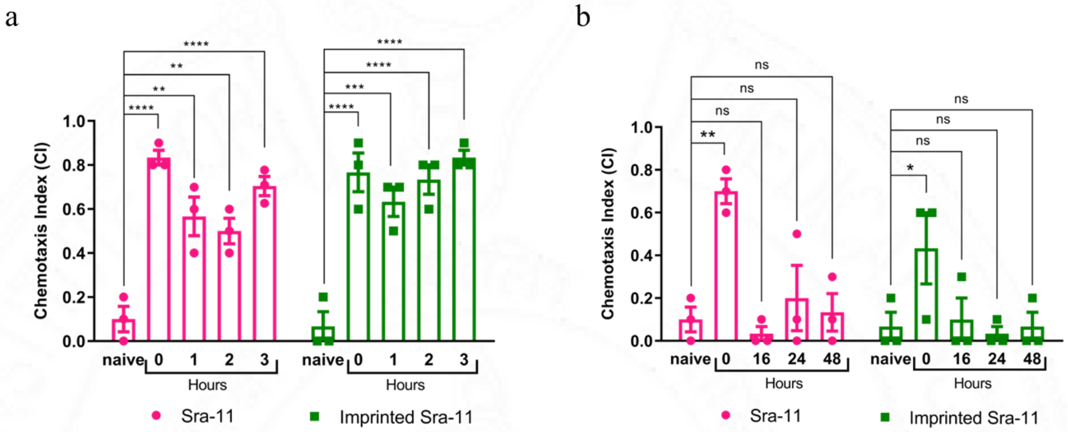


***Fig 29*: a) STAM of non-imprinted and imprinted *sra-11* mutant** (RB816 (*sra-11(ok630) II*)**.** *sra-11* mutation in the AIY interneuron did not affect the STAM training. This mutant showed an increased attraction towards 1/10 BT after short-term memory training.**; b) LTAM of non-imprinted and imprinted *sra-11* mutant**. The LTAM memory formation is defective for *sra-11* mutant and showing the 0^{th} hour adaptation in naïve and imprinted animals. The values are expressed as mean ± SEM, N=3 (n~50), 2way ANOVA, p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****), p not significant (ns).

IV.2.5. Role of RIA motor neuron in learning and memory.

AIY interneurons forms a strong synaptic connection (13 synapses) with RIA motor neuron (Wormweb.org). The strain DCR744 (*cima-1(wy84) IV; wyIs45 X*) is defective in proper synapse formation between AIY-RIA neurons as it develops into adults. However, synaptic connections between the AIY-RIA are normal during the early larval stage (Shao *et al*., 2013).

It may be noted that our protocol on imprinting was carried during the early stages of development (from egg to L1 larvae) and the learning paradigm was carried out in day 1 adult worms (*Fig. 30a*).

The DCR744 (*cima-1* mutant) worms showed significantly low memory formation after short-term and long-term learning and memory training (*Fig. 30b and 30c*). This result suggest that AIY-RIA synaptic connections are critical for memory formation. 0th hour memory formation after long-term olfactory adaptive training showed a significant chemotaxis index ($p<0.001$) compared to the naïve control (*Fig. 30c*) suggesting immediate memory recall and long-term memory storage could involve various other connectomes.

cima-1 mutant worms were imprinted and tested for both short-term and long-term adaptive learning and memory. Imprinted worms showed a rescue of short-term memory pathway (*Fig. 30b*), while unable to rescue the long-term memory (*Fig. 30c*). These results suggest that imprinting did rescue some of the early synapse formations between AIY-RIA neurons to reestablish the short-term memory recall pathway.

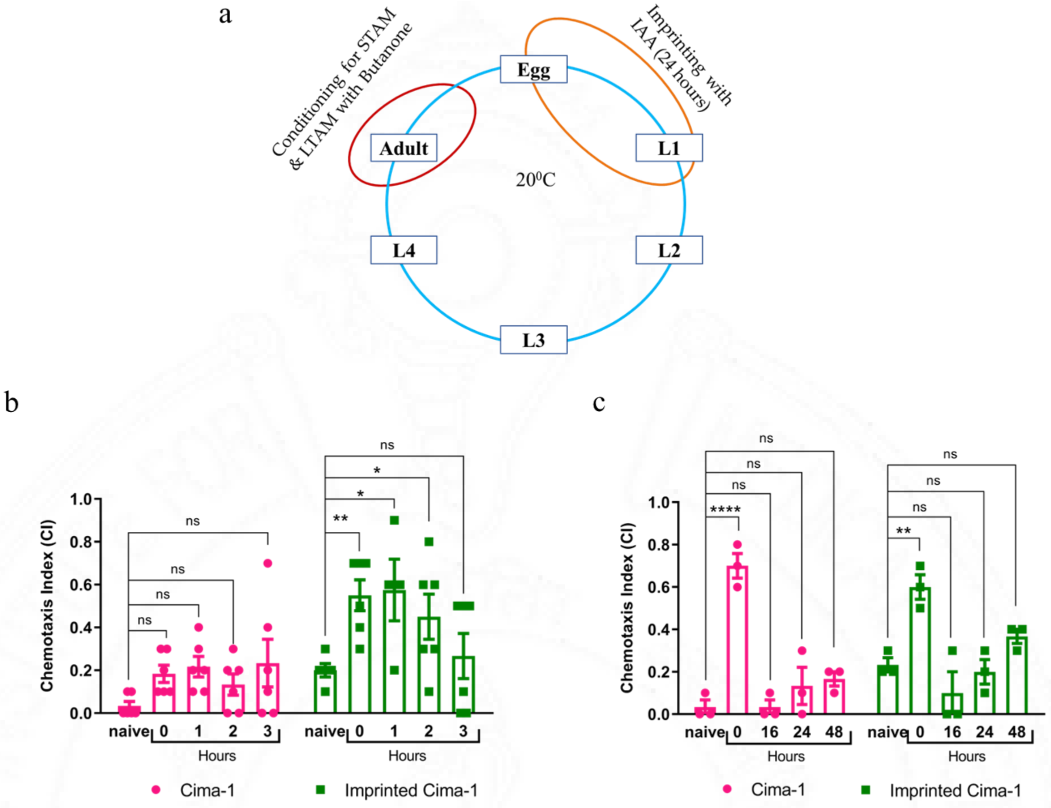


Fig 30**: a) Diagrammatic representation of the combined conditioning of imprinting and learning and memory training.** Imprinting was given during the egg to L1 stage and the memory training was given in the 1st day adult worms.**; b) STAM of non-imprinted and imprinted *cima-1*** (DCR744 (*cima-1(wy84) IV; wyIs45 X*). The imprinted *cima-1* mutant regained the short-term memory compared with its naïve**; c) LTAM of non-imprinted and imprinted *cima-1*.** The long-term memory of naïve and imprinted *cima-1* mutant shows defect but 0th hour adaptation occurs. The values are expressed as mean ± SEM, N=6 (n~50), 2way ANOVA, $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****), p not significant (ns).

To confirm the role of *cima-1*, worms which were rescued with *cima-1* (DCR775 (*cima-1(wy84) IV; wyls45 X; olaEx459*) were tested with learning and memory paradigm. The rescue worms showed extended short-term memory compared to the *cima-1* mutant (*Fig. 31a*), confirming AIY-RIA synaptic connections are critical for the memory pathway. Introduction of the 1.6kb promoter region of *cima-1* to the *cima-1* mutant enhances the 0th hour adaptation of the rescue worm after long-term memory (*Fig. 31b*). *Fig. 31c* represent the remarkable variation in short-term memory of *cima-1* mutant and *cima-1* rescue worms ($p<0.01$).

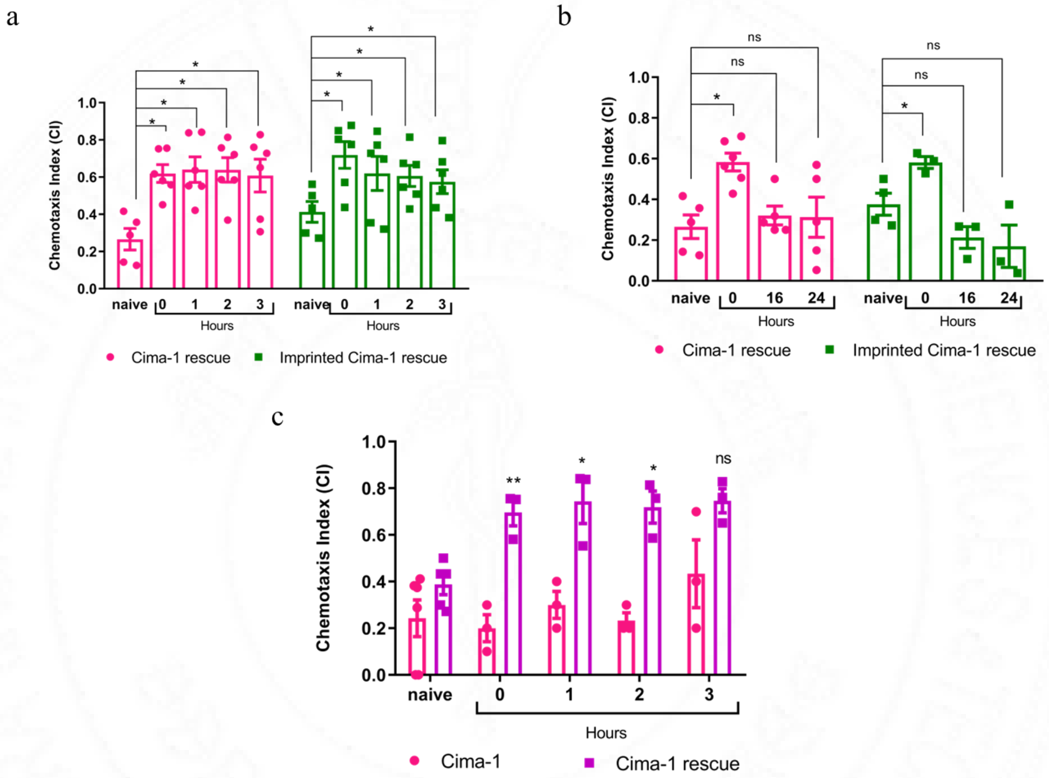


***Fig 31*: a) STAM of non-imprinted and imprinted *cima-1* rescue (**DCR775 (*cima-1(wy84) IV; wyls45 X; olaEx459*)**.** *cima-1* rescue showed extended STAM in both naïve and imprinted animals.**; b) LTAM of non-imprinted and imprinted *cima-1* rescue.** LTAM memory formation is defective for *cima-1* rescue but 0th hour adaptation is maintaining.**; c) Difference of STAM training in *cima-1* mutant (**DCR744 (*cima-1(wy84) IV; wyIs45 X*) **and *cima-1* rescue strains.** The values are expressed as mean ± SEM, N=5 or more (n~50), 2way ANOVA, $p<0.05$ (*), $p<0.01$ (**), p not significant (ns).

IV.2.6. Significance of AIY interneuron in learning and memory.

kal-1 overexpressing mutants (OH910 - *ttx3p::kal1* + *usc122p::GFP*) show non-specific sprouting of AIY neurons (Hobert *et al*., 2002) resulting in erratic synapse formations. Hence, we used this strain to test whether imprinting stabilizes synapses critical for memory formation pathways, as seen in *cima-1* mutants. OH910 worms were defective in olfactory adaptive learning and memory both short-term and long-term (*Fig. 32a* and 32*b*). However, on imprinting the worms showed rescue in short-term memory, while long-term memory continued to be defective (*Fig. 32a* and 32*b*).

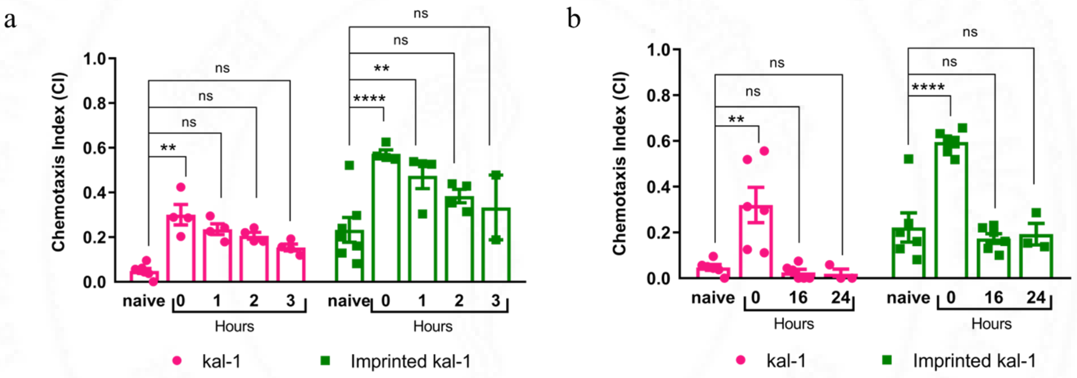


Fig 32*: a) STAM of non-imprinted and imprinted *kal-1(OH910 - *otIs77 [ttx-3p::kal-1 + unc-122p::GFP]) II*). *kal-1* mutant showed short-term memory defect but the short-term memory of imprinted animals recovered the memory**; b) LTAM of non-imprinted and imprinted *kal-1*.** Both naïve and imprinted long-term memory trained animals showed defect in memory formation but shows the 0^{th} hour adaptation**.** The values are expressed as mean ± SEM, N=4 or more (n~50), 2way ANOVA, p<0.01 (**), p<0.0001 (****), p not significant (ns).

IV.2.7. Role of glutamate receptors specific to AIY neurons in imprinted associated modifications in learning and memory.

RB594 strain (*glc-3(ok321) V*) lacks AIY specific *glc-3* expression (Chalasani *et al.*, 2007). The worms showed short-term olfactory adaptive learning pattern similar to that of N2 (*Fig. 33*). On imprinting these worms followed the extended memory similar to that of N2 (*Fig. 33* and see *Fig. 26a*), suggesting that lack of *glc-3* does not impact the imprinted associated modifications in the learning pathway.

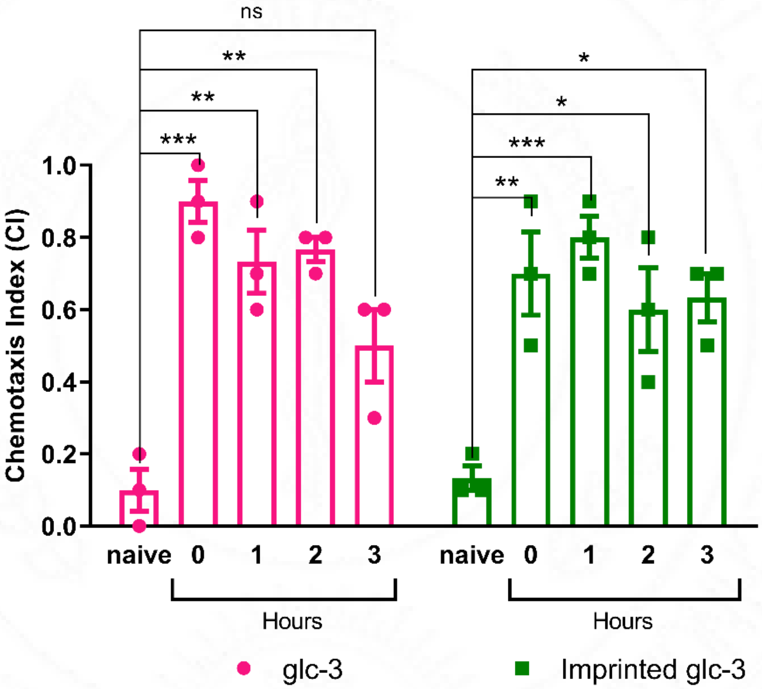


***Fig 33*: STAM of non-imprinted and imprinted *glc-3* mutant** (RB594 - (*glc-3(ok321) V*). *glc-3* mutation resulted in an extended short-term memory in naïve and imprinted animals. The values are expressed as mean ± SEM, N=3 (n~50), 2way ANOVA, p<0.05 (*), p<0.01 (**), p<0.001 (***), p not significant (ns).

IV.2.8. Role of the SER-2 receptor, present in RIA and AIY neurons.

To test the possible role of SER-2 receptor present in RIA and AIY neurons (Tsalik *et al.*, 2003), a mutant strain (RB1690 - *ser-2(ok2103) X*) was used to study the short-term associated memory and imprint associated modifications. The results showed the worms were defective in learning and memory pathways and imprinting did not rescue these defects (*Fig. 34*), suggesting *ser-2* receptor expression in AIY-RIA neurons is critical for learning and memory.

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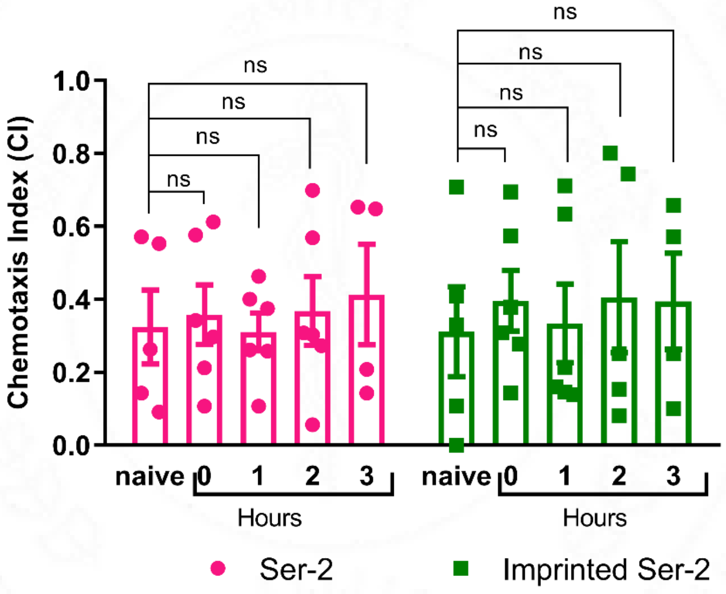


***Fig 34*: a) STAM of non-imprinted and imprinted *ser-2* mutant** (RB1690 - *ser-2(ok2103) X*). *ser-2* mutation causes short-term memory defect. Imprinting in *ser-2* mutation does not overcome the defect in short-term memory. The values are expressed as mean ± SEM, N=4 or more (n~50), 2way ANOVA, p not significant (ns).

IV.2.9. Ionotropic glutamate receptors in learning and memory.

glr-1 **(KP4 -** ***glr-1(n2461) III*****) mutant strain.**

The *glr-1* mutant (KP4 - *glr-1(n2461) III*) was defective for short-term associated memory and imprinting did not show an impact in rescuing the memory (*Fig. 35a*). A similar trend was also observed in long-term associate memory in this mutant strain. (*Fig. 35b*). These results suggest that GLR-1 receptor is critical for the formation of memory in the worms.

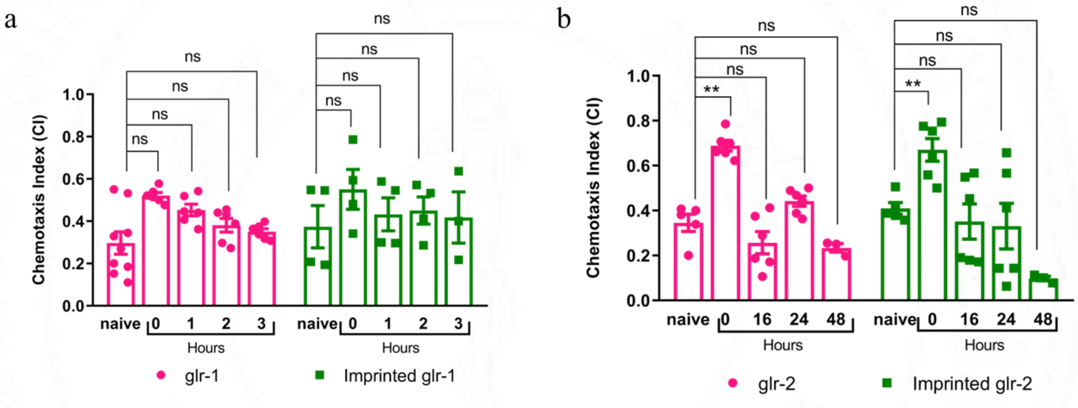


Fig 35**: a) STAM of non-imprinted and imprinted** ***glr-1*** (KP4 - *glr-1(n2461) III*). *glr-1* mutation showed short-term memory.**; b) LTAM of non-imprinted and imprinted** ***glr-1*****.** The long-term memory result also showed memory defects. The values are expressed as mean ± SEM, N=6 (n~50), 2way ANOVA, p<0.01 (**), p not significant (ns).

glr-2 **(RB1808 -** ***glr-2(ok2342) III*****) mutant strain.**

glr-2 receptor mutant (RB1808 - *glr-2(ok2342) III*) result showed the short-term memory similar to that of the N2 wild type worms (*Fig.36a* and see *Fig. 26a*). Imprinting showed an extended short-term associated memory in these mutant worms similar to that of wild-type N2 worms (*Fig. 36a and see Fig 26a*). However, the *glr-2* mutants were defective for long-term memory and imprinting did not rescue this defect (*Fig. 36b*). These results suggest GLR-2 receptor has a critical role in long-term adaptive memory formation in *C. elegans*.

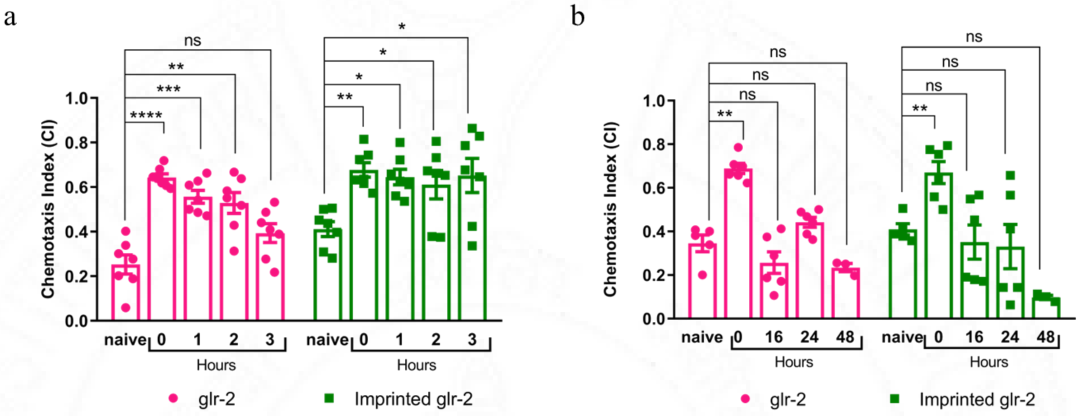


Fig 36*: a) STAM of non-imprinted and imprinted *glr-2 (RB1808 - *glr-2(ok2342) III*). *glr-2* mutation showed normal short-term memory and the imprinted *glr-2* mutants showed extended short-term memory similar to imprinted N2 worms.**; b) LTAM of non-imprinted and imprinted *glr-2*.** *glr-2* mutation affected the long-term memory in naïve and imprinted animals. Long-term training showed only 0th hour adaptation in both naïve and imprinted worms. The values are expressed as mean ± SEM, N=6 or more (n~50), 2way ANOVA, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****), p not significant (ns).

IV.2.10. Calcium imaging RID interneurons.

RID interneurons involved in the forward movement of the worms and has synaptic links between AIY and RIA interneurons (*Fig. 37*).

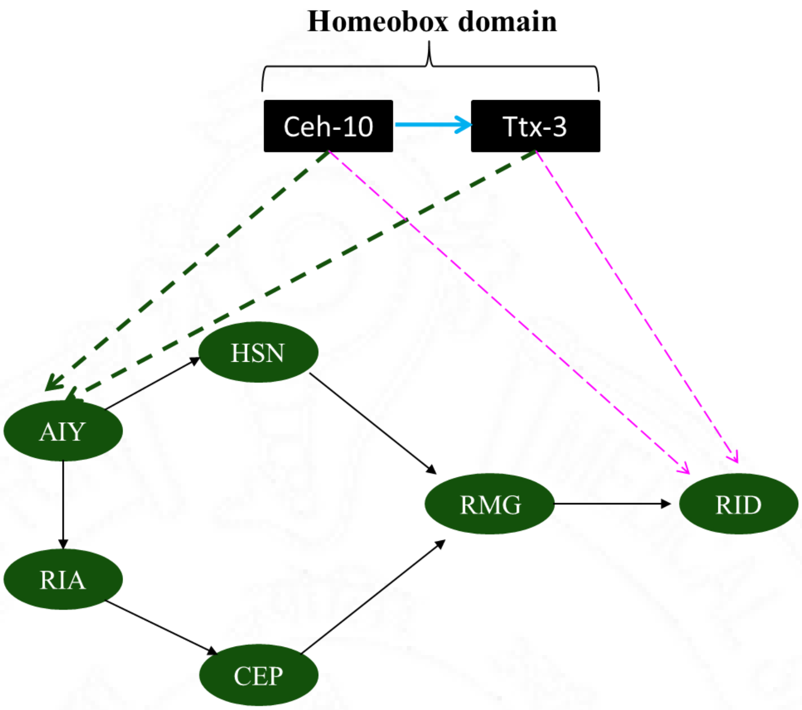


***Fig. 37*: Schematic representation of the synaptic connection between AIY, RIA and RID neurons.** The homeobox domain ceh-10 and ttx-3 involved in the AIY and RID neuronal differentiation. There is no direct synaptic connection between AIY and RIA to RID neuron.

To test the firing pattern of RID neurons after long-term olfactory adaptive training as well as after imprinting, calcium intensity signals were quantified using the ZM9078 strain (*hpIs587 - GCaMP6*). These worms are GCaMP6 labelled specifically to RID neurons which on calcium influx will exhibit enhanced GFP fluorescence (*Fig. 38*).

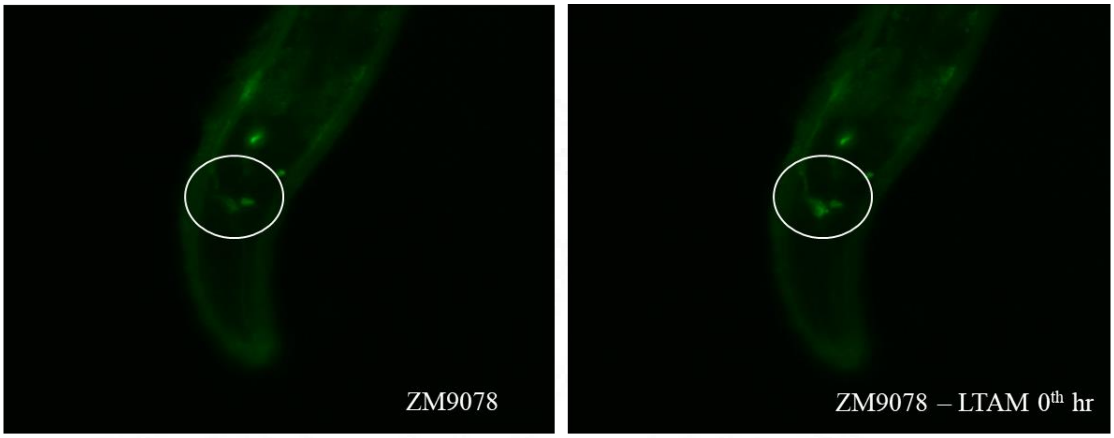


***Fig 38*: Representative images of fluorescent intensity change in 0th hour LTAM trained ZM9078** (*hpIs587 - GCaMP6*) **strain before and after BT exposure.** GCaMP6::GFP marker was expressed in RID neurons in this strain.

The worms trained for long-term adaptive olfactory memory showed an increase in calcium influx compared to the non-trained worms (*Fig. 39a*) Imprinted worms also showed an enhanced calcium influx in RID neurons similar to the long-term memory trained worms (*Fig. 39b*) suggesting that both long-term memory and imprinting share same neuronal connectome.

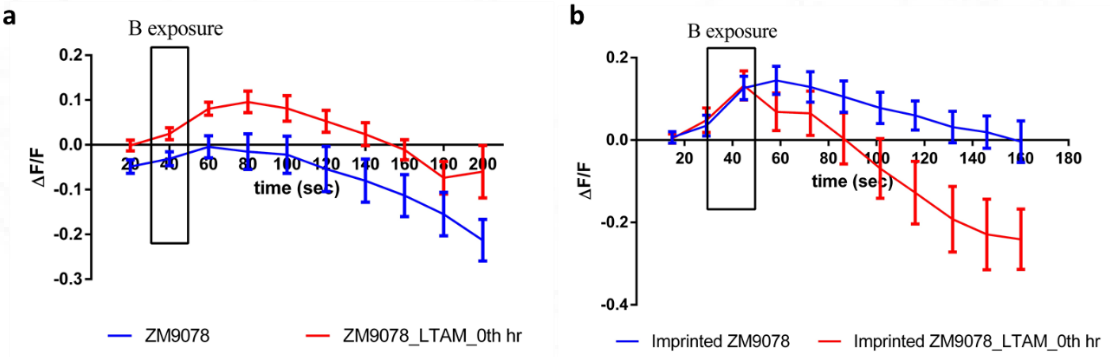


***Fig 39*: a) Average RID calcium responses to 20 seconds during BT.** 0th hour LTAM trained animals showed an increased fluorescent intensity in the RID neuron compared with the naïve animals when exposed to 1/10 diluted BT**; b) Average RID calcium responses to 20 seconds during BT exposure in imprinted and 0th hour LTAM trained imprinted animals.** The fluorescent intensity increases in the imprinted and 0th hour of LTAM trained imprinted animals in the RID neuron. The values are expressed as mean, n=7 or more.

IV.2.11. Calcium imaging AIY interneurons.

To analyze the role of AIY interneuron in learning and memory the firing pattern of AIY interneuron was examined after long-term memory and the involvement of imprinting on long-term memory using calcium imaging technique. Using AIY::GCaMP6 labelled worms (AT9203 - GCaMP6 and *mCherry -ttx-3_GCaMP6* in AIY) for calcium imaging to capture the firing of AIY interneuron during BT exposure. The result showed the AIY interneuron express baseline firing in naïve and long-term trained AT9203 animals (*Fig. 40a*) suggesting that the AIY interneuron has no role in 0th hour adaptation. Imprinted AT9203 strain also showed no calcium firing in long-term trained animals during BT exposure (*Fig. 40b)* again confirming AIY is not involved in immediate recalling memory. *Fig. 40c* indicates the combined result of 0th hour long-term trained non-imprinted and imprinted animals showing no difference in the fluorescent intensity while sensing the odorant, BT.

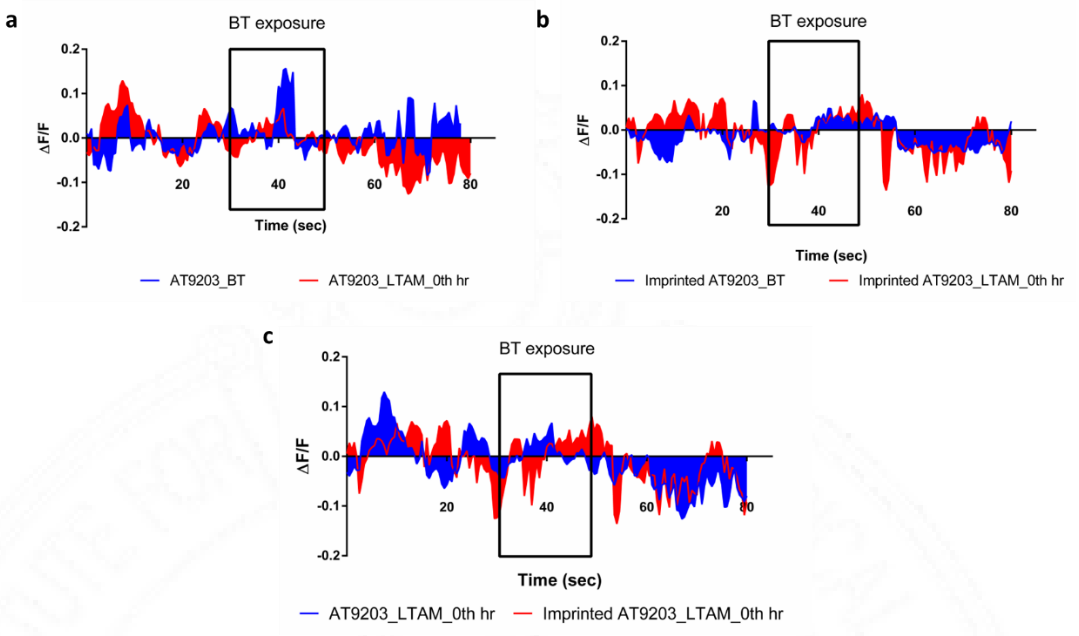


***Fig. 40*: a) Average calcium responses to 20 seconds during BT exposure in naïve and 0th hour of LTAM trained animals.** The results showed baseline fluorescence in the naïve and 0th hour long-term trained animals in the AIY interneuron. **b) Average AIY calcium responses to 20 seconds during BT exposure in imprinted worms and 0th hour of LTAM trained imprinted animals.** The results showed there is no calcium spike in the imprinted and 0th hour of LTAM trained imprinted animals in the AIY interneuron during 1/10 diluted BT exposure. **c) Calcium response to 20 seconds during BT exposure in 0th hour of LTAM trained non-imprinted and imprinted animals.** The average values are expressed as mean, n=7 or more.

IV.2B. Discussion

Learning and memory are essential processes of the nervous system of vertebrates and invertebrates for survival. *C. elegans* has been found to adapt to a number of learning paradigms including associative learning and memory towards olfaction (Kauffman *et al*., 2011), pH (Murayama *et al*., 2013), temperature (Hedgecock *et al*., 1975; Kimata *et al*., 2012 and salt (Bargmann and Horvitz, 1991; Iino and Yoshida, 2009).

Here I have shown that larval imprinting has a significant impact on the adult learning paradigm. Linking of two independent kinds of learning paradigm together, olfactory imprinted memory (IAA imprinting) and associative learning and memory (learn to enhance the BT attraction associated with food) have given new insights to the connectomes and molecules that are shared between them.

It is unknown whether imprinting has a long-lasting effect on normal learning and memory processes or not. I addressed this question by inducing imprinted olfactory memory in *C. elegans* and assayed for fluctuations in associative olfactory learning and memory. Previous studies showed that imprinted memory could be preserved after cryopreservation and revival (Vita-More *et al*., 2014).

The results have shown that positive olfactory imprinting has a constructive involvement in learning and memory. N2 worms showed extended chemotaxis imprinted animals - the memory holding capacity for both STAM and LTAM increased by >2 hours and >24 hours respectively. These results suggest a significant overlap between imprinting and associative learning pathways, probably sharing the common neuronal circuits. A study on chicks showed that exposure to an object during imprinting results in the boosting of IMHV neurons specific to that object (Horn, 1998).

Most of the conditioned and unconditioned stimulus in associative and non-associative learning initiates through AWC sensory neurons in *C. elegans* (Colbert *et al.*, 1997; Chalasani *et al.*, 2010; Lin *et al.*, 2010; Pereira *et al*., 2012). My study also confirms that defects in AWC sensory neurons disrupt the associative learning and memory towards BT. For the aversive imprinting and adult learned aversion AWC-AIB neural activity has the main role but the memory retrieval requires AIY- RIA neuronal involvement (Jin *et al*., 2016).

SRA-11, a GPCR receptor, in the AIY interneuron plays a major role in olfactory imprinting (Remy *et al*., 2005). The imprinted *sra-11* mutant (RB816 (*sra-11(ok630) II*) worms showed improved STAM - extending the memory holding capacity >2nd hour. However, the LTAM showed defect confirming that SRA-11 have a role in long-lasting memory formation (Arey *et al*., 2018). These data confirm that SRA-11 is a common receptor involved in imprinting and LTAM. The data also indicate that STAM and LTAM information processing differs at the molecular level.

The presynaptic distribution during *C. elegans* post-development required the involvement of a glial protein, CIMA-1 which mainly modifies the AIY presynaptic distribution with RIA. During the L-1 larval stage, the AIY synaptic distribution is normal in *cima-1* mutants and alters as the worm grows to adult (Shao *et al*., 2013). In vertebrates also interaction of astrocytes found to modulate synapse formation (Allen *et al*., 2012). In *cima-1* mutants (DCR744 (*cima-1(wy84) IV; wyIs45 X*) were defective in both STAM and LTAM. Imprinted worms were found to recover the STAM to a large extend. One of the possibilities for such a recovery memory could be the due strengthening of synapses during the imprinting process and are maintained to a larger extend even under *cima-1* gene mutation. Rescuing of CIMA-1 also rescued STAM behaviour strongly suggesting proper synapse formations between AIY-RIA are essential in learning and memory.

This observation was reiterated using *kal-1* mutant strain (OH910 (*ttx3p::kal1 + usc122p::GFP*). KAL-1 is essential for olfactory axonal guidance and is mainly involved in the proper axonal branching of AIY neurons and target recognition (Rugarli *et al*., 2001; Bulow *et al*., 2002). Mutation in the *kal-1* gene results in abnormal axonal branching of AIY neurons. The short-term associative learning and memory experiments showed *kal-1* mutant is defective in STAM. Imprinting during the early larval stages rescued this memory defect in these mutants – reiterating the hypothesis that imprinting could result in the strengthening of synapses. These studies also strengthen the observation that AIY and RIA neurons participate in learning and memory. From these studies, it is clear that both for imprinting and associative learning and memory AWC - AIY - RIA connectomes are critical.

AIY neuron activation and inactivation depend on the inhibitory chloride channel GLC-3 (Horoszok *et al*., 2001; Chalasani *et al*., 2007). STAM for the non-imprinted and imprinted *glc-3* mutant (RB594 (*glc-3(ok321)V*) showed extended memory. Since in these mutants AIY has maintained the ON stage because of the absence of GLC-3, it is evident that AIY -ON stage has been one of the essential requirements for the memory pathway.

The SER-2 tyramine receptor (Zhang *et al*., 2005) is mainly expressed in the AIY interneuron (Gultekin *et al*., 2001) and has been found to have a role in imprinted aversive memory (Jin *et al*., 2016). *ser-2* mutants (RB1690 (*ser-2(ok2103) X*) showed defective STAM and could not rescue this defect by imprinting. Functional defects in AIY neurons have a significant impact on both imprinting as well as olfactory adaptive learning and memory.

Studies on the glutamate receptors such as AMPA and NMDA have an important role in associative learning and memory (Rose *et al*., 2003; Zamanillo *et al*., 1999; Kauffman *et al*., 2010). In *C. elegans* also these receptors play a critical role. The absence of GLR-1 glutamate receptors had a significant impact on STAM and LTAM. On the other-hand GLR-2 receptor is essential for LTAM and not for STAM. Imprinting did not rescue the defects in memory in the mutant strains, suggesting mutations in these receptors have a long-term impact on the learning pathway.

Functional imaging studies on neurons are critical to assess their involvement in recognizing a particular stimulus (Ha *et al*., 2010; Chalasani *et al*., 2007). Calcium imaging studies on the RID interneuron showed an enhanced fluorescence in those worms trained for LTAM, suggesting signals for forward movements are enhanced in these worms after the training paradigm. It may be noted that imprinted worms also showed a similar behaviour where activation of RID interneuron were higher than the naïve worms – suggesting a strong shared interconnection between imprinting and olfactory adaptive learning and memory.

On the other hand, calcium imaging of the AIY interneuron, being the central essential interneuron for imprinting and learning and memory, maintains a baseline fluorescence after olfactory associative training. It may kindly be noted that similar behaviour was observed in imprinted worms as well. The naïve worms when exposed to BT showed calcium influx in the AIY interneuron suggesting, this neuron participates in the solvent recognition pathway. On conditioning either by learning paradigm or by imprinting the neuronal firing pattern in AIY interneuron plateaus confirmed that there is a habituation behaviour in AIY interneuron. This has been discussed in detail in the chapter *IV.1B*

In conclusion, the results suggest there is a common connectome and molecular pathways between olfactory imprinting as well as learning and memory-associated behaviour. The major neuronal circuits involved are AWC, AIY, RIA, RID and RIM. Among them, AIY interneuron is the most critical in both conditional learning as well as imprinted learning. This study also suggests that early exposure to a favourable environmental condition could have a positive impact on the learning process of an organism by strengthening or modifying synaptic connections.

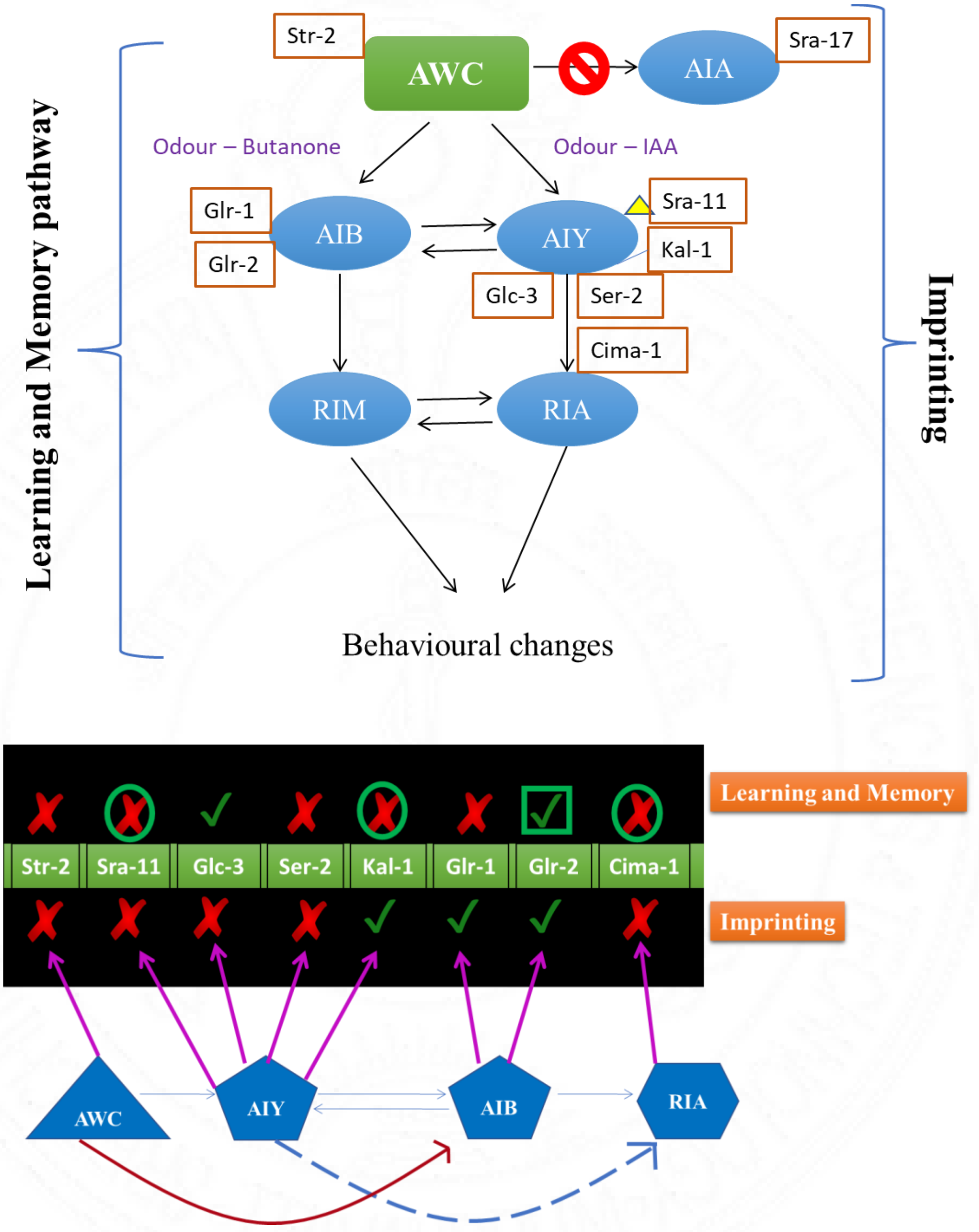
V. SUMMARY AND CONCLUSION

This study brings evidence that imprinting at the early developmental stage alters neuronal plasticity and behaviour in *C. elegans*. A series of interneurons and receptors play a critical role in these behavioural changes. The major neurons involved in the connectome for imprinting are AWC, AIY, AIB, RIM and RIA, among them AIY neuron plays a significant role. *Fig.41* summarizes the major interactive molecules identified in this study.

This study also brings supporting evidence for imprinting associated alterations in learning and memory pathways. The data suggest both imprinting and adaptive olfactory learning pathway are majority of the molecules in establishing long-term memory. These results, in fact, reiterate how the environment during early development influences the behaviour, including the learning and memory pathway; and its profound effect on the physiological function of the nervous system.

AIY neurons expresses or interacts with a series of molecules like CIMA-1, KAL-1, SER-2 and GLC-3 which all have a supporting role in olfactory imprinting and associative learning. Further understanding on how AIY interneuron regulates the complex process of memory will give new insights into the biological process involved in information coding.

Overall schematic representation of the study



	Str-2	Sra-11	Glc-3	Ser-2	Kal-1	Glr-1	Glr-2	Cima-1
Learning and Memory	✗	⊗	✓	✗	⊗	✗	☑	⊗
Imprinting	✗	✗	✗	✗	✓	✓	✓	✗

Fig. 41: **Pathways share common neurons.** Imprinting and learning and memory pathways share common neurons and receptors or genes. The major neurons involved in imprinting and learning and memory pathways are AWC, AIY, AIB, RIM and RIA. The involvement of receptors or genes present on these neurons is represented in this figure.

VI. BIBLIOGRAPHY

Abrams T. W, Karl K. A, Kandel E. R. (1991). Biochemical studies of a stimulus convergence during classical conditioning in Aplysia: dual regulation of adenylate cyclase by Ca2+/calmodulin and transmitter. *J. Neurosci*., 11:2655–2665.

Alberini, C. M. & Kandel, E. R. (2015). The Regulation of Transcription in Memory Consolidation. *Csh. Perspect. Biol.* 7, a021741.

Alexander, A. G., Marfil, V., & Li, C. (2014). Use of *C. elegans* as a model to study Alzheimer's disease and other neurodegenerative diseases. *Front. Gene.*, 5(JUL), 1–21. https://doi.org/10.3389/fgene.2014.00279.

Allen, N.J., Bennett, M.L., Foo, L.C., Wang, G.X., Chakraborty, C., Smith, S.J., and Barres, B.A. (2012). Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors. *Nature* 486, 410–414.

Altun-Gultekin, Z., Andachi, Y., Tsalik, E. L., Pilgrim, D., Kohara, Y., & Hobert, O. (2001). A regulatory cascade of three homeobox genes, ceh-10ttx-3 and ceh-23, controls cell fate specification of a defined interneuron class in *C. elegans*. *Development*, 128(11), 1951–1969.

Amano. H, Maruyama. I. N. (2011). Aversive olfactory learning and associative long-term memory in *Caenorhabditis elegans*. *Learn. Mem.*, 18:654-665.

Angstman, N. B, Frank, H. G, Schmitz, C. (2016). Advanced Behavioural Analyses Show that the Presence of Food Causes Subtle Changes in *C. elegans* Movement. *Front. Behav. Neurosci*.;10:60. Published 2016 Mar 31. doi:10.3389/fnbeh.2016.00060.

Ardiel, E. L. et al. (2016). Dopamine receptor DOP-4 modulates habituation to repetitive photoactivation of a *C. elegans* polymodal nociceptor. *Learn. Mem.*, 23, 495–503.

Ardiel, E. L., & Rankin, C. H. (2010). An elegant mind: learning and memory in *Caenorhabditis elegans*. *Learn. & Mem.* (Cold Spring Harbor, N.Y.), 17(4), 191–201. https://doi.org/10.1101/lm.960510.

Arey, R. N., Stein, G. M., Kaletsky, R., Kauffman, A., & Murphy, C. T. (2018). Activation of GαqSignaling Enhances Memory Consolidation and Slows Cognitive Decline. *Neuron*. https://doi.org/10.1016/j.neuron.2018.03.039.

Ashida, K., Hotta, K., & Oka, K. (2019). The Input-Output Relationship of AIY Interneurons in *Caenorhabditis elegans* in Noisy Environment. *iScience*, 19, 191–203. https://doi.org/10.1016/j.isci.2019.07.028.

Bargmann, C. I. in Wormbook (2006). The C. elegans Research Community) WormBook doi/10.1895/wormbook.1.123. 1. http://www.wormbook.org.

Bargmann, C. I., & Horvitz, H. R. (1991). Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*. *Neuron*, 7(5), 729–742. https://doi.org/10.1016/0896-6273(91)90276-6.

Bargmann, C. I., & Horvitz, H. R. (1991). Control of larval development by chemosensory neurons in *Caenorhabditis elegans*. *Sci.*, 251(1972), 1243–1246.

Bargmann, C. I., Hartwieg, E., & Horvitz, H. R. (1993). Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell*, 74(3), 515–527. https://doi.org/10.1016/0092-8674(93)80053-H.

Belle, J. S. De. Heisenberg, M. (1994). Associative Odor Learning in Drosophila Abolished by Chemical Ablation of Mushroom Bodies, *Science*. https://doi.org/10.1126/science.8303280.

Berger, M., Hamid, K., Koh, A., Leslie, M., & Park, S. (2007). Attractant preference between NaCl and KCl in wild-type and mutant *Caenorhabditis elegans*.

Betke K. M and Wells C. A. Hamm H. E. (2012). GPCR mediated regulation of synaptic transmission. *Prog Neurobiol.* 96, 304–321. Review. [PubMed: 22307060]

Bett, N. N., Hinch, S. G., Dittman, A. H., & Yun, S.-S. (2016). Evidence of Olfactory Imprinting at an Early Life Stage in Pink Salmon (*Oncorhynchus gorbuscha*). *Scientific Reports*, 6(1), 36393. https://doi.org/10.1038/srep36393.

Boekhoff, I., Tareilus, E., Strotmann, J., and Breer, H. (1990). Rapid activation of alternative second messenger pathways in olfactory cilia from rats by different odorants. *EMBO J.* 9, 2453-2458.

Bolduc, F. V. (2015). From learning to memory: what flies can tell us about intellectual disability, *Journal Frontiers in Psychiatry*. https://doi.org/10.3389/fpsyt.2015.00085.

Bollati, V, Galimberti, D, Pergoli, L, *et al*. (2011). DNA methylation in repetitive elements and Alzheimer disease. *Brain Behav Immun*.;25:1078-1083).

Bourgeron, T. (2009) A synaptic trek to autism. *Curr. Opin. Neurobiol.* 19(2):231–234.

Bozorgmehr, T., Ardiel, E. L., Mcewan, A. H., & Rankin, C. H. (2013). Mechanisms of plasticity in a *Caenorhabditis elegans* mechanosensory circuit, 4(August), 1–11. https://doi.org/10.3389/fphys.2013.00088.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77(1), 71–94. https://doi.org/10.1002/cbic.200300625.

Brunelli, M, Castellucci, V, Kandel, E. R. (1976). Synaptic facilitation and behavioral sensitization in Aplysia: Possible role of serotonin and cyclic AMP. *Science*, 194:1178–1181.

Bulow, H. E., Berry, K. L., Topper, L. H., Peles, E., & Hobert, O. (2002). Heparan sulfate proteoglycan-dependent induction of axon branching and axon misrouting by the Kallmann syndrome gene kal-1. *Proc. Natl. Acad. Sci*. USA, 99(9), 6346–6351. https://doi.org/10.1073/pnas.092128099.

Byers, D, Davis, R. L, Kiger, J. A. (1981). Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in *Drosophila melanogaster*. *Nature*, 289:79–81.

Calahorro, F., Holden-dye, L., & Connor, V. O. (2015). Gene Expression Patterns Analysis of splice variants for the *C. elegans* orthologue of human neuroligin reveals a developmentally regulated transcript. *Gene Expression Patterns*, 17(2), 69–78. https://doi.org/10.1016/j.gep.2015.02.002.

Castellucci, V. F, Kandel, E. R, Schwartz, J. H, Wilson, F. D, Nairn, A. C, Greengard, P. (1980). Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioral sensitization in Aplysia. *Proc. Natl. Acad. Sci. USA*, 77:7492–7496.

Chalasani SH, Kato S, Albrecht DR, Nakagawa T, Abbott LF, Bargmann CI (2010) Neuropeptide feedback modifies odor-evoked dynamics in *Caenorhabditis elegans* olfactory neurons. *Nat. Neurosci.* 13:615–621.

Chalasani, S. H., Chronis, N., Tsunozaki, M., Gray, J. M., Ramot, D., Goodman, M. B., & Bargmann, C. I. (2007). Dissecting a circuit for olfactory behaviour in *Caenorhabditis elegans*, *Nature*. 450. https://doi.org/10.1038/nature06292.

Chalfie M, *et al*. (1985) The neural circuit for touch sensitivity in *Caenorhabditis elegans*. *J Neurosci* 5(4):956–964.

Chase, D. L., & Koelle, M. R. (2007). Biogenic amine neurotransmitters in *C. elegans*. WormBook: The Online Review of *C. elegans* Biology, 1–15. https://doi.org/10.1895/wormbook.1.132.1.

Chen, G., Chen, K. S., Knox, J. H., Inglis, J., Bernard, A., Martin, S. J., Morris, R. G. M. (2000). A learning deficit related to age and b-amyloid plaques in a mouse model of Alzheimer’s disease. *Nature,* 408(1998), 975–979. https://doi.org/10.1038/35050103.

Chen, X., Barclay, J. W., Burgoyne, R. D., & Morgan, A. (2015). therapeutic compounds for ageing - associated neurodegenerative diseases. *Chemistry Central Journal*, 1–20. https://doi.org/10.1186/s13065-015-0143-y.

Chew, Y. L., Tanizawa, Y., Cho, Y., Lu, H., Beets, I., Rabinowitch, I. (2018). An Afferent Neuropeptide System Transmits Mechanosensory Signals Triggering Sensitization and Arousal in *C. elegans*. *Neuron*, 99(6), 1233-1246.e6. https://doi.org/10.1016/j.neuron.2018.08.003.

Chi, C. A., Clark, D. A., Lee, S., Biron, D., Luo, L., Gabel, C. V, Samuel, A. D. T. (2007). Temperature and food mediate long-term thermotactic behavioral plasticity by association-independent mechanisms in *C. elegans*. *J. Exp. Biol.* 210(22), 4043–4052. https://doi.org/10.1242/jeb.006551.

Chou, J. H, Bargmann, C. I, Sengupta, P. (2001). The *Caenorhabditis elegans* odr-2 gene encodes a novel Ly-6-related protein required for olfaction. *Genetics* 157: 211–224.

Chouliaras, L, Mastroeni, D, Delvaux, E. (2013). Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer’s disease patients. *Neurobiol. Aging*;34:2091-2099.

Cohen RE, Kaplan SW, Kandel ER, Hawkins RD. (1997). A simplified preparation for relating cellular events to behavior: Mechanisms contributing to habituation, dishabituation, and sensitization of the Aplysia gill- withdrawal reflex. *J Neurosci*, 17:2886–2899.

Colbert HA, Smith TL, Bargmann CI (1997) OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation and olfactory adaptation in *Caenorhabditis elegans*. *J. Neurosci*. 17:8259–8269.

Colbert, H. A., & Bargmann, C. I. (1995). Odorant-specific adaptation pathways generate olfactory plasticity in *C. elegans*. *Neuron*, 14(4), 803–812. https://doi.org/10.1016/0896-6273(95)90224-4.

Cooper, J. C., Hasler, A. D. (1974). Electroencephalographic evidence for retention of olfactory cues in homing Coho salmon. *Science* 183:336–338.

Cully, D. F., Wilkinson, H., and Vassilatis, D. K. (1996) Molecular biology and electrophysiology of glutamate-gated chloride channels of invertebrates. *Parasitology* 113, S191–S200.

Daigle I, Li C. (1993). apl-1, a *Caenorhabditis elegans* gene encoding a protein related to the human beta-amyloid protein precursor. *Proc Natl Acad Sci* U S A 90: 12045–12049.

Darmaillacq, A.S., Chichery, R., and Dickel, L. (2006). Food imprinting, new evidence from the cuttlefish Sepia officinalis. *Biol Lett* 2, 345-347.

Davis RL, Cherry J, Dauwalder B, Han PL, Skoulakis E. The cyclic AMP system and Drosophila learning. *Mol. Cell. Biochem*. (1995) 14(9–150):271–8. doi:10.1007/BF01076588.

Dittman, A. H., Quinn, T. P., Nevitt, G. A., Hacker, B., & Storm, D. R. (1997). Sensitization of olfactory guanylyl cyclase to a specific imprinted odorant in coho salmon. *Neuron*, 19(2), 381–389. https://doi.org/10.1016/S0896-6273(00)80947-2

Dubnau, J., Grady, L., Kitamoto, T., Tully, T. (2001). Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. *Nature*. 411:476–80. doi:10.1038/35078077.

Dusenbery, D. B. (1974). Analysis of chemotaxis in the nematode *Caenorhabditis elegans* by countercurrent separation. *J. Exp. Zool* 788,41-48.

Faglioni, P, Botti, C, Scarpa, M, Ferrari, V, Saetti, M. C. (1997). Learning and forgetting processes in Parkinson's disease: a model based approach to disentangling storage, retention and retrieval contributions. *Neuropsychologia;* 35:767–79.

Fenk, L. A., & Bono, M. De. (2017). Memory of recent oxygen experience switches pheromone valence in *Caenorhabditis elegans*. *PNAS.* https://doi.org/10.1073/pnas.1618934114.

Fernando Calahorro • Manuel Ruiz-Rubio. (2011). *Caenorhabditis elegans* as an experimental tool for the study of complex neurological diseases: Parkinson's disease, Alzheimer's disease and autism spectrum disorder, *Invert Neurosci*. 73–83. https://doi.org/10.1007/s10158-011-0126-1.

Folmar, L. C, Dickhoff, W. W. (1980). The parr-smolt transformation (smoltification) and seawater ad- aptation. *Aquaculture* 21:1–37.

Gabriel Horn. (1998). Visual imprinting and the neural mechanisms of recognition memory, *Cell press*.

Gerlach, G., Tietje, K., Biechl, D., Namekawa, I., Schalm, G., & Sulmann, A. (2019). Behavioural and neuronal basis of olfactory imprinting and kin recognition in larval fish. *J. Exp. Biol*. https://doi.org/10.1242/jeb.189746.

Geschwind, D. H., & Levitt, P. (2007). Autism spectrum disorders: developmental disconnection syndromes. *Curr. Opin. Neurobiol.*, 17(1), 103–111. https://doi.org/10.1016/j.conb.2007.01.009.

Glanzman, D. L, Mackey, S. L, Hawkins, R. D, Dyke, A. M, Lloyd, P. E, Kandel, E. R. (1989). Depletion of serotonin in the nervous system of Aplysia reduces the behavioral enhancement of gill withdrawal as well as the heterosynaptic facilitation produced by tail shock. *J Neurosci*, 9:4200–4213.

Goedert, M., Baur, C. P., Ahringer, J., Jakes, R., Hasegawa, M., Spillan- tini, M. G., et al. (1996). PTL-1, a microtubule-associated protein with tau-like repeats from the nematode *Caenorhabditis elegans*. *J. Cell Sci*. 109, 2661–2672.

Gray, J. M., Hill, J. J. & Bargmann, C. I. (2005). A circuit for navigation in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci*. USA 102, 3184–3191.

H., Morris, C. D., Williams, R. M., Loring, J. F., & Thomas, E. (2015). HDAC inhibition imparts beneficial transgenerational effects in Huntington's disease mice via altered DNA and histone methylation. *Proc. Natl. Acad. Sci*., https://doi.org/10.1073/pnas.1415195112.

Ha, H. ick, Hendricks, M., Shen, Y., Gabel, C. V., Fang-Yen, C., Qin, Y., Zhang, Y. (2010). Functional Organization of a Neural Network for Aversive Olfactory Learning in *Caenorhabditis elegans*. *Neuron*, 68(6), 1173–1186. https://doi.org/10.1016/j.neuron.2010.11.025.

Hara, T. J., Zielinski, B. (1989). Structural and functional development of the olfactory organ in teleosts. *Trans. Am. Fish. Soc.* 118:183–194.

Harden, M. V., Newton, L. A., Lloyd, R. C. & Whitlock, K. E. (2006). Olfactory imprinting is correlated with changes in gene expression in the olfactory epithelia of the zebrafish. *J. Neurobiol*. 66, 1452–1466.

Hark, A. T., Schoenherr, C. J., Katz, D. J., Ingram, R. S., Levorse, J. M., & Tilghman, S. M. (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19 / Igf2 locus, 405(May), 486–489.

Hasler, A. D, Scholz, A. T. (1983). "Olfactory Imprinting and Homing in Salmon. "New York: Springer- Verlag.

Hedgecock, E.M., and Russell, R.L. (1975). Normal and mutant thermotaxis in the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci*. USA 72, 4061–4065.

Hensch, T.K. (2005). Critical period plasticity in local cortical circuits. *Nat. rev. Neurosci.*

Hoar, W. S. (1988). The physiology of smolting salmonids. In WS Hoar and DJ Randall (eds): "Fish Physiology. "New York: Academic Press, 275–343

Horn, G. (1998). Visual imprinting and the neural mechanisms of recognition memory. *Trends in Neurosci*., 21(7), 300–305. https://doi.org/10.1016/S0166-2236(97)01219-8.

Horoszok, L., Raymond, V., Sattelle, D. B., and Wolstenholme, A. J. (2001) GLC-3: a novel fipronil- and BIDN-sensitive, but picrotoxinin-insensitive, L-glutamate-gated chloride channel subunit from *Caenorhabditis elegans*. *Br. J. Pharmacol*. 132, 1247–1254.

Hsieh, Y.-W., Alqadah, A., & Chuang, C.-F. (2014). Asymmetric neural development in the *Caenorhabditis elegans* olfactory system. *Genesis*, 52(6), 544–554. https://doi.org/10.1002/dvg.22744.

Iino, Y., & Yoshida, K. (2009). Parallel Use of Two Behavioral Mechanisms for Chemotaxis in *Caenorhabditis elegans*. *J Neurosci.*, 29(17), 5370–5380. https://doi.org/10.1523/JNEUROSCI.3633-08.2009.

Jean-Jacques Remy1 and Oliver Hobert. (2005). An Interneuronal Chemoreceptor Required for Olfactory Imprinting in *C. elegans*, *Science*. 309(July), 787–790.

Jia, H., Morris, C. D., Williams, R. M., Loring, J. F., & Thomas, E. (2015). HDAC inhibition imparts beneficial transgenerational effects in Huntington's disease mice via altered DNA and histone methylation. *Proc. Natl. Acad. Sci*., 112, E56–E64. https://doi.org/10.1073/pnas.1415195112.

Jin, X., Pokala, N., Bargmann, C. I., Jin, X., Pokala, N., & Bargmann, C. I. (2016). Distinct Circuits for the Formation and Retrieval of an Imprinted Olfactory Memory. *Cell*, 164(4), 632–643. https://doi.org/10.1016/j.cell.2016.01.007.

Jong, Y.-J. I., Harmon, S. K. & O'Malley, K. L. (2018). Intracellular GPCRs Play Key Roles in Synaptic Plasticity. *Acs. Chem. Neurosci.* 9, 2162–2172.

Kandel, E. R. (2001). The molecular biology of memory storage: A dialogue between gene and synapses. *Sci*., 294(5544), 1030–1038. https://doi.org/10.1126/science.1067020.

Kandel, E.R., and Tauc, L. (1965). Heterosynaptic facilitation in neurons of the abdominal ganglion of Aplysia depilans. *J. Physiol*. 181: 1–27.

Kandel, ER, JH Schwartz and TM Jessell. (2000). *Principles of Neural Science*. New York: McGraw-Hill.

Kano, T., Brockie, P. J., Sassa, T., Fujimoto, H., Iino, Y., Mellem, David M. Madsen., Ryuji Hosono1, and Andres V. Maricq. (2009). Memory in *Caenorhabditis elegans* is Mediated By NMDA-Type Ionotropic Glutamate Receptors. *Curr. Biol.* July 8; 18(13). https://doi.org/10.1016/j.cub.2008.05.051.

Kauffman, A., Ashraf, J. M., Corces-Zimmerman, M. R., Landis, J. N., & Murphy, C. T. (2010). Insulin signaling and dietary restriction differentially influence the decline of learning and memory with age. *PLoS Biology*, 8(5), e1000372. http:// dx.doi.org/10.1371/journal.pbio.1000372.

Kauffman, A., Parsons, L., Stein, G., Wills, A., Kaletsky, R., & Murphy, C. (2011). *C. elegans* positive butanone learning, short-term, and long-term associative memory assays. *JoVE*, (49), e2490. https://doi.org/10.3791/2490.

Kimata, T., Sasakura, H., Ohnishi, N., Nishio, N., & Mori, I. (2012). Thermotaxis of *C. elegans* as a model for temperature perception, neural information processing and neural plasticity. *Landes Biosci*.

Lakhina, V., Arey, R. N., Kaletsky, R., Kauffman, A., Stein, G., Keyes, W., Murphy, C. T. (2015). Genome-wide functional analysis of CREB/Long-term memory-dependent transcription reveals distinct basal and memory gene expression programs. *Neuron*, 85(2), 330–345. https://doi.org/10.1016/j.neuron.2014.12.029.

Lakso, M., Vartiainen, S., Moilanen, A. M., Sirviö, J., Thomas, J. H., Nass, R., et al. (2003). Dopaminergic neuronal loss and motor deficits in *Caenorhabditis elegans* overexpressing human alpha-synuclein. *J. Neurochem*. 86, 165–172. doi: 10.1046/j.1471-4159.2003.01809.x.

Leung C. C. Y and Wong Y. H. (2017). Role of G Protein-Coupled Receptors in the Regulation of Structural Plasticity and Cognitive Function. *Molecules* 24;22(7) doi: 10.3390/molecules22071239.

Li, C., Timbers, T. A., Rose, J. K., Bozorgmehr, T., McEwan, A., & Rankin, C. H. (2013). The FMRFamide-related neuropeptide FLP-20 is required in the mechanosensory neurons during memory for massed training in *C. elegans*. *Learn. Mem.*, 20(2), 103–108. http://dx.doi.org/10.1101/lm.028993.112.

Lim, M. A., Chitturi, J, Laskova, V, Meng, J, Findeis, D, Wiekenberg, A. (2016). Neuroendocrine modulation sustains the *C. elegans* forward motor state. *Elife*. 5: 1–33. https://doi.org/10.7554/eLife.19887.

Lin CH, Tomioka M, Pereira S, Sellings L, Iino Y, van der Kooy D (2010) Insulin signaling plays a dual role in *Caenorhabditis elegans* memory acquisition and memory retrieval. *J. Neurosci.* 30:8001–8011.

lkue Mori and Yasumi Ohshima. (1997). Molecular neurogenetics of chemotaxis and thermotaxis in the nematode *Caenorhabditis elegans*. *BioEssays*. Vol 19. no. 12.

Lorenz, K. (1979). The year of the greylag goose, 1st American edn (New York: Harcourt Brace Jovanovich).

Lyon, M. F. (1961). Gene action in the X-chromosome of the mouse (Mus musculus L.). *Nature*;190:372– 373. [PubMed: 13764598]

Maricq, A. V., Peckol, E., Driscoll, M.&Bargmann, C. I. (1995). Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. *Nature* 378, 78–81.

McDermott, J. B., Aamodt, S., andAamodt, E. (1996). ptl-1, a *Caenorhabditis elegans* gene whose products are homologous to the tau microtubule-associated proteins. *Biochemistry* 35, 9415–9423. doi: 10.1021/bi952646n.

Mclntire, S. L., Jorgensen, E., Kaplan, J., & Horvitz, H. R. (1993). The GABAergic nervous system of *Caenorhabditis elegans*. *Nature*, 364(6435), 337–341. https://doi.org/10.1038/364337a0.

Mello, C. C., J. M. Kramer, D. Stinchcomb and V. Ambros. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* 10:3959–3970.

Miyashita, T., Oda, Y., Horiuchi, J., Yin, J. C. P., Morimoto, T., & Saitoe, M. (2012). Mg2+ Block of Drosophila NMDA Receptors Is Required for Long-Term Memory Formation and CREB-Dependent Gene Expression. *Neuron*, 74(5), 887–898. https://doi.org/10.1016/j.neuron.2012.03.039.

Montminy M. (1997). Transcriptional regulation by cyclic AMP. *Annu. Rev. Biochem*., 66:807–822.

Morrison, G. E., & Kooy, D. Van Der. (2001). A Mutation in the AMPA-Type Glutamate Receptor, glr-1, Blocks Olfactory Associative and Nonassociative Learning in *Caenorhabditis elegans*, *Behav. neurosci.* 115(3), 640. https://doi.org/10.1037//0735-7044.115.3.640.

Murayama, T., Takayama, J., Fujiwara, M., and Maruyama, I. N. (2013). Environmental alkalinity sensing mediated by the transmembrane guanylyl cyclase GCY-14 in *C. elegans*. *Curr. Biol.* 23, 1007–1012. doi: 10.1016/j.cub.2013. 04.052.

Nakamura, T., and Gold, G. H. (1987). A cyclic-nucleotide gated conductance in olfactory receptor cilia. *Nature* 325, 442-444.

Nevitt, G., & Dittman, A. (2004). A New Model for Olfactory Imprinting in Salmon. *Integr. Biol.* 215–223.

O'Brien *et al*. (2011). Amyloid Precursor Protein Processing and Alzheimer's Disease.

Ohnishi, N., Kuhara, A., Nakamura, F., Okochi, Y., & Mori, I. (2011). Bidirectional regulation of thermotaxis by glutamate transmissions in *Caenorhabditis elegans*. *EMBO J*. https://doi.org/10.1038/emboj.2011.13.

Panegyres, P. K. (2004). Review The contribution of the study of neurodegenerative disorders to the understanding of human memory, *Q. J. Med* .97(9), 555–567. https://doi.org/10.1093/qjmed/hch096.

Parida, Lipika & Neogi, Sudarsan & Padmanabhan, Venkat. (2014). Effect of Temperature Pre-Exposure on the Locomotion and Chemotaxis of *C. elegans*. *PloS one*. 9. e111342. 10.1371/journal.pone.0111342.

Park, S. et al. (2008). Enhanced *Caenorhabditis elegans* Locomotion in a Structured Microfluidic Environment. *Plos One* 3, e2550.

Pavlov IP. Conditioned reflexes. London: Oxford University Press 1927:430.

Pereira, L., Kratsios, P., Serrano-Saiz, E., Sheftel, H., Mayo, A. E., Hall, D. H., Hobert, O. (2015). A cellular and regulatory map of the cholinergic nervous system of *C. elegans*. *E Life*, 1–46. https://doi.org/10.7554/eLife.12432.

Pereira, S., & van der Kooy, D. (2012). Two Forms of Learning following Training to a Single Odorant in *Caenorhabditis elegans* AWC Neurons. *J. Neurosci*., 32(26), 9035–9044. https://doi.org/10.1523/JNEUROSCI.4221-11.2012.

Plaçais, P. Y, Trannoy ,S, Isabel, G, Aso, Y, Siwanowicz, I, Belliart-Guérin, G, (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in Drosophila. *Nat Neurosci*. 15:592–9. doi:10.1038/ nn.3055.

Quan, F, Thomas, L, Forte, M. (1991). Drosophila stimulatory G protein alpha subunit activates mammalian adenylyl cyclase but interacts poorly with mammalian receptors: implications for receptor-G protein interaction. *Proc Natl Acad Sci* US A. 88:1898–902. doi:10.1073/pnas.88.5.1898.

Quinn, W. G, Harris, W. A, Benzer, S. (1974). Conditioned behavior in Drosophila melanogaster. *Proc. Natl. Acad. Sci. U S A*. 71:708–12. doi:10.1073/pnas. 71.3.708.

R. M. (2007). Article Covalent Modification of DNA Regulates Memory Formation, 857–869. https://doi.org/10.1016/j.neuron.2007.02.022.

R.F. Ketting, M. Tijsterman, R.H.A. Plasterk, Isolation of RNA from *C. elegans*.

Rankin, C.H., Beck, C.D., and Chiba, C.M. (1990). *Caenorhabditis elegans*: a new model system for the study of learning and memory. *Behav. Brain Res*. 37, 89–92.

Reimer, R.J. (2013). SLC17: A functionally diverse family of organic anion transporters. *Mol. Aspects Med.* 34, 350–359.

Remy J.J., and Hobert O. (2005). An Interneuronal Chemoreceptor Required for Olfactory Imprinting in *C. elegans. Sci*.

Remy, J. J. (2010). Stable inheritance of an acquired behavior in *Caenorhabditis elegans*. *Curr. Biol*.. https://doi.org/10.1016/j.cub.2010.08.013

Rinn, J. L., & Chang, H. Y. (2012). Genome regulation by long noncoding RNAs. *Annual Review of Biochemistry*, 81, 145–166. https://doi.org/10.1146/annurev-biochem-051410-092902.

Rojas A and Dingledine R. (2013). Ionotropic glutamate receptors: regulation by G protein-coupled receptors. *Mol Pharmacol.* 83, 746–752. Review [PubMed: 23348498].

Rose J. K., Kaun K.R., Rankin C.H. (2002). A new group training procedure for habituation demonstrates that presynaptic glutamate release contributes to long-term memory in *C. elegans*. *Learn. Mem. 9, 130-137.*

Rugarli, E. I., Di Schiavi, E., Hilliard, M. A., Arbucci, S., Ghezzi, C., Facciolli, A., Bazzicalupo, P. (2002). The Kallmann syndrome gene homolog in *C. elegans* is involved in epidermal morphogenesis and neurite branching. *Development*, 129(5), 1283–1294.

Sasakura, H., & Mori, I. (2013). Behavioral plasticity, learning, and memory in *C. elegans*. *Curr. Opin. Neurobiol.*, 23(1), 92–99. https://doi.org/10.1016/j.conb.2012.09.005.

Scholz, A. T, Horrall, R. M, Cooper, J. C, Hasler, A. D. (1976). Imprinting to chemical cues: the basis for home stream selection in salmon. *Science* 1972: 1247–1249.

Semke, E., Distel, H., and Hudson, R. (1995). Specific enhancement of olfactory receptor sensitivity associated with foetal learning of food odors in the rabbit. *Die Naturwissenschaften* 82, 148-149.

Serrano-Saiz, E., Poole, R. J., Felton, T., Zhang, F., De La Cruz, E. D., Hobert, O. (2013). Modular control of glutamatergic neuronal identity in *C. elegans* by distinct homeodomain proteins. *Cell* 155:659–673. DOI: 10.1016/j.cell.2013.09.052 .

Shao, Z., Watanabe, S., Christensen, R., Jorgensen, E. M., & Colón-Ramos, D. A. (2013). Synapse location during growth depends on glia location. *Cell*, 154(2), 337–350. https://doi.org/10.1016/j.cell.2013.06.028.

Shen, C.-H. (2018). Dynamics of neural activity during chemotaxis in *Caenorhabditis elegans*. Dissertation Abstracts International: Section B: The Sciences and Engineering, 78(9-B(E)), No-Specified. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=psyc13a&NEWS=N&AN=2017-29366-032.

Sleutels, F., Zwart, R., & Barlow, D. P. (2002). The non-coding Air RNA is required for silencing autosomal imprinted genes. *Nature*, 415(6873), 810–813. https://doi.org/10.1038/415810a.

Sreedharan, S., Shaik, J.H., Olszewski, P.K., Levine, A.S., Schioth, H.B., and Fredriksson, R. (2010). Glutamate, aspartate and nucleotide transporters in the SLC17 family form four main phylogenetic clusters: evolution and tissue expression. *BMC Genomics* 11, 17.

Stein, G. M., & Murphy, C. T. (2014). *C. elegans* positive olfactory associative memory is a molecularly conserved behavioral paradigm. Neurobiology of *Learn. Mem.*, 115, 86–94. https://doi.org/10.1016/j.nlm.2014.07.011.

Stiernagle, T. (2006). Maintenance of *C. elegans*. WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.101.1, http://www.wormbook.org.

Suo, S., Kimura, Y., & Van Tol, H. H. M. (2006). Starvation induces cAMP response element-binding protein-dependent gene expression through octopamine-Gq signaling in *Caenorhabditis elegans*. *J. Neurosci*., 26(40), 10082–10090. https://doi.org/10.1523/JNEUROSCI.0819-06.2006.

Svendsen, P. C. and McGhee, J. D. (1995). The *C. elegans* neuronally expressed homeobox gene ceh-10 is closely related to genes expressed in the vertebrate eye. *Development* 121, 1253-1262.

Takeuchi, T., Duszkiewicz, A. J. & Morris, R. G. M. (2014). The synaptic plasticity and memory hypothesis: encoding, storage and persistence. *Philosophical Transactions Royal Soc B Biological Sci* 369, 20130288.

Taniguchi, G., Uozumi, T., Kiriyama, K., Kamizaki, T., & Hirotsu, T. (2014). Screening of odor-receptor pairs in *Caenorhabditis elegans* reveals different receptors for high and low odor concentrations. *Science Signaling*, 7(323), 1–10. https://doi.org/10.1126/scisignal.2005136.

Tedford H. W and Zamponi G. W. (2006). Direct G protein modulation of Cav2 calcium channels. *Pharmacol Rev*. 58, 837–862. Review. [PubMed: 17132857] 71.

The Huntington's Disease Collaborative Research Group. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72, 971–983. doi: 10.1016/0092-8674(93)90585-E.

Timbers, T. A., & Rankin, C. H. (2011). Tap withdrawal circuit interneurons require CREB for long-term habituation in *Caenorhabditis elegans*. *Behavioral Neuroscience*, 125(4), 560–566.

Tomioka, M., Adachi, T., Suzuki, H., Kunitomo, H., Schafer, W. R., & Iino, Y. (2006). The Insulin/PI 3-Kinase Pathway Regulates Salt Chemotaxis Learning in *Caenorhabditis elegans*. *Neuron*, 51, 613–625.

Tonegawa, S., Pignatelli, M., Roy, D. S. & Ryan, T. J. (2015). Memory engram storage and retrieval. *Curr. Opin. Neurobiol*. 35, 101–109.

Torayama, I., Ishihara, T., & Katsura, I. (2007). *Caenorhabditis elegans* integrates the signals of butanone and food to enhance chemotaxis to butanone. *J. Neurosci*., 27(4), 741–750. https://doi.org/10.1523/JNEUROSCI.4312-06.2007.

Troemel, E. R., Chou, J. H., Dwyer, N. D., Colbert, H. A. and Bargmann, C. I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell* 83, 207-218.

Troemel, E. R., Sagasti, A., and Bargmann, C. I. (1999). Lateral signaling mediated by axon contact and calcium entry regulates asymmetric odorant receptor expression in *C. elegans*. *Cell* 99, 387–398. doi: 10.1016/S0092-8674(00)81525-1.

Tsalik, E. L., & Hobert, O. (2003). Functional mapping of neurons that control locomotory behavior in *Caenorhabditis elegans*. *J. Neurobiol*., 56(2), 178–197. https://doi.org/10.1002/neu.10245.

Twick I, Lee JA, Ramaswami M. (2014). Olfactory habituation in Drosophila odor encoding and its plasticity in the antennal lobe. *Prog. Brain. Res*.208:3 – 38.

Vita-More, N., Barranco, D., & Vita-more, N. (2014). Persistence of Long-Term Memory in Vitrified and Revived *C. elegans*, 1–38.

Wang, J., Chitturi, J., Ge, Q., Laskova, V., Wang, W., Li, X., Huang, X. (2015). The *C. elegans* COE transcription factor UNC-3 activates lineage specific apoptosis and affects neurite growth in the RID lineage. *Development* (Cambridge), 142(8), 1447–1457. https://doi.org/10.1242/dev.119479.

Ward, S. (1973). Chemotaxis by the nematode *Caenorhabditis elegans*: identification of attractants and analysis of the response by use of mutants. *Proc. Natl. Acad. Sci.* USA, 70(3), 817–821. https://doi.org/10.1073/pnas.70.3.817.

Wassle, H. Parallel processing in the mammalian retina. (2004). *Nature Rev. Neurosci*. 5, 747–757.

Wen, J. Y., Kumar, N., Morrison, G., Rambaldini, G., Runciman, S., Rousseau, J., & van der Kooy, D. (1997). Mutations that prevent associative learning in *C. elegans. Behavioral Neuroscience*, 111, 354–368.

Wes, P. D., & Bargmann, C. I. (2001). *C. elegans* odour discrimination requires asymmetric diversity in olfactory neurons. *Nature*, 410(6829), 698–701. https://doi.org/10.1038/35070581.

Whalley, K. (2007). Learning and memory - Dynamic DNA methylation. *Nature Reviews Neuroscience*, 8(5), 323. https://doi.org/10.1038/nrn2133.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B. Biol*. Sci. 314, 1-340.

Whittington, C. J., Podd, J., & Stewart-williams, S. (2006). Memory Deficits in Parkinson' s Disease, *Journal of Clinical and Experimental Neuropsychology*, 738–754. https://doi.org/10.1080/13803390590954236.

Wicks, S. R., & Rankin, C. H. (1997). Effects of Tap Withdrawal Response Habituation on Other Withdrawal Behaviors: The Localization of Habituation in the Nematode *Caenorhabditis elegans*, I(2), 342–353.

Wisby, W. J. & Hasler, A. D. (1954). Effect of olfactory occlusion on migrating silver salmon (O. kisutch). *J. Fish. Res. Bo. Can*. 11, 472–478.

Wolstenholme, A. J. (2012). Glutamate-gated Chloride, *The Journal of Biological Chemistry*. 287(48), 40232–40238. https://doi.org/10.1074/jbc.R112.406280.

Wu, J., Basha, M. R., Brock, B., Cox, D. P., Cardozo-Pelaez, F., McPherson, C. A., et al. (2008). Alzheimer’s disease (AD)-like pathology in aged monkeys after infantile exposure to environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD. *J. Neurosci*. 28, 3–9. doi: 10.1523/JNEUROSCI.4405-07.2008.

Yamamoto Y., Hino H., Ueda H. (2010). Olfactory Imprinting of Amino Acids in Lacustrine Sockeye Salmon. *PLoS ONE*. doi: https://doi.org/10.1371/journal.pone.0008633.

Yan M. S, Matouk C. C, Marsden P. A. (2010). Epigenetics of the vascular endothelium. *J. Appl. Physiol. (1985)*. Sep;109(3):916-26. doi: 10.1152/japplphysiol.00131.2010.

Zamanillo, D., Sprengel, R., Hvalby, O., Jensen, V., Burnashev, N., Rozov, A. (1999). Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science*, 284(5421), 1805–1811.

Zhang, X. & Cote, R. H. (2005). cGMP signaling in vertebrate retinal photoreceptor cells. *Front. Biosci.* 10, 1191–1204.

Zhang, Y., Lu, H., and Bargmann, C.I. (2005). Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature* 438, 179–184.

Zheng, Y., Liu, A., Wang, Z., Cao, Q., Wang, W., Lin, L., Wang, X. (2019). Inhibition of EHMT1 / 2 rescues synaptic and cognitive functions for Alzheimer’ s disease, Brain*: A Journal of Neurology*. https://doi.org/10.1093/brain/awy354.

Zhiyou Cai. (2014). Monoamine oxidase inhibitors: Promising therapeutic agents for Alzheimer's disease (Review).

VII. ANNEXURE

Appendices

I. Buffers and its composition:

1. M9 buffer

- KH_2PO_4-3g, Na_2HPO_4-6g, NaCl-5g, $MgSO_4$(1M)-1ml, H_2O-1L. Autoclave the solution after adding all the chemicals except 1M $MgSO_4$ to the 1L water. After autoclaving add 1ml of 1M $MgSO_4$.

2. Genomic DNA lysis buffer.

- 200mM NaCl, 100mM Tris HCl (pH 8.5), 50mM EDTA (pH 8.0), 0.5% SDS.

3. KPO_4 buffer (pH 6.0).

- For 1L add 868ml of 1M KH_2PO_4 and 132ml of 1M K_2HPO_4.

4. TAE buffer

- For 1L add 242g Tris-base, 57.1mL glacial acetic acid and 100ml of 500mM EDTA (pH 8.0).

5. STE buffer (store at 4^0C)

- 10mM Tris-Cl (pH 8.0)
- 0.1M NaCl
- 1mM EDTA (pH 8.0)

6. GTE solution (store at 4^0C)

- 50mM Glucose
- 25mM Tris Cl (pH 8.0)
- 10mM EDTA (pH 8.0)

7. Alkaline lysis solution II (freshly prepared)

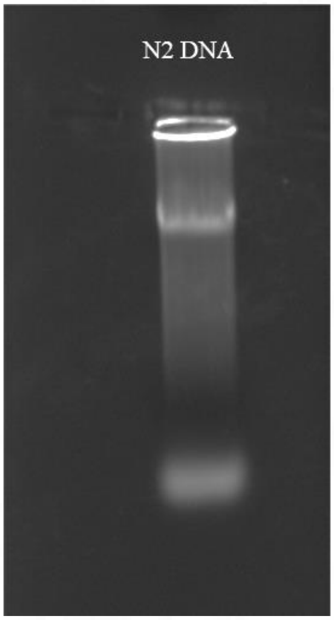
- 0.2N NaOH
- 1% SDS

8. Alkaline lysis solution III (store at 4^0C)

- 60ml 5M potassium acetate
- 11.5ml glacial acetic acid
- 28.5ml dist. H_2O

II. Construction of RNAi feeding vector for glc-3 silencing

1. Worm genomic DNA isolated from N2 strain



2. mRNA sequence for silencing glc-3 primer generation

GenBank Reference sequence: NM_072040.7

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GCTCGATTCAACGACTCCTAATGGCTCATTAGCCTTGTTATGACTTTCTTTC
GTACTACTCGACACACTGTGCGCATCAAATTAATACTTCTTGCCGCTTCTGC
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CTGCAGGACAGCAGATTTGGATGCCAGATAGTTTTTTCCAGAACGAAAAAC
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GATTCGACAGTCTGGAAATGGAAAAACTGTAAGTAGAGTAAATGGGCATA
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ACAATTAATTTCAAAATTTTAACTCTCATAATCAGACCTCTGACACCCGTGA
TTGAATTTCACTTTTTTGAATTTTTCAACAAGTTGTTGGCCAATTGTTTACGT
TGTATGTTTTTTGTGTTCAAATAAAAATACATTCCTGATATTA

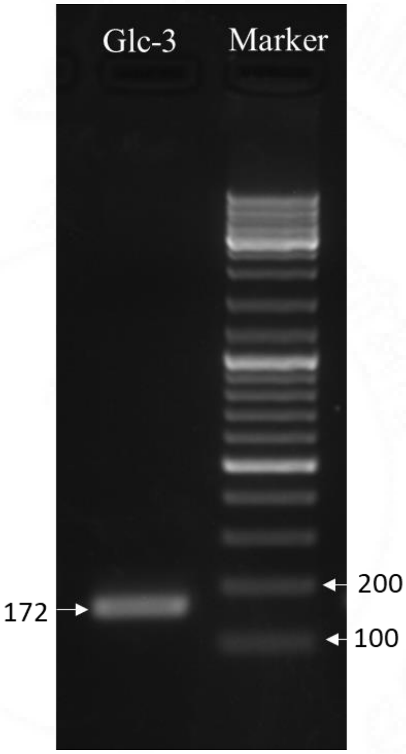
3. PCR amplification of glc-3 gene fragment from N2 DNA

The PCR conditions were;

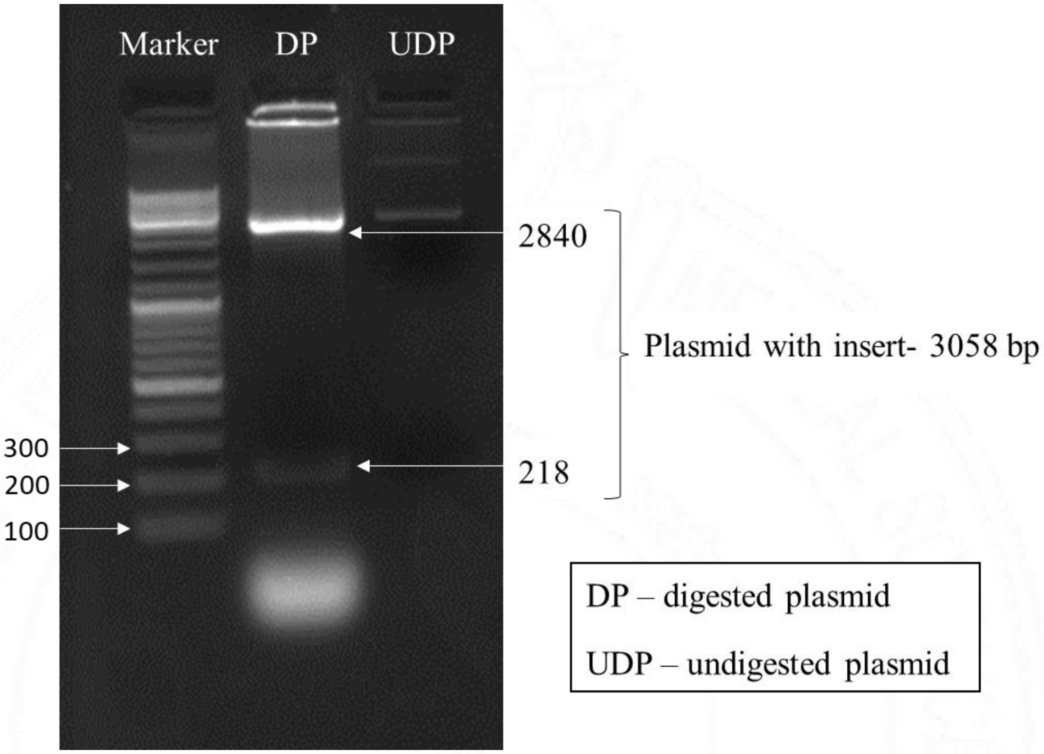
94^{0}C for 10 minutes	1 cycle
94^{0}C for 30 seconds	35 cycles
58^{0}C for 30 seconds	
72^{0}C for 30 seconds	
72^{0}C for 10 minutes	1 cycle
4^{0}C for infinity.	

4. Purification of PCR product using QIAquick PCR purification kit (Cat. No. / ID: 28104)

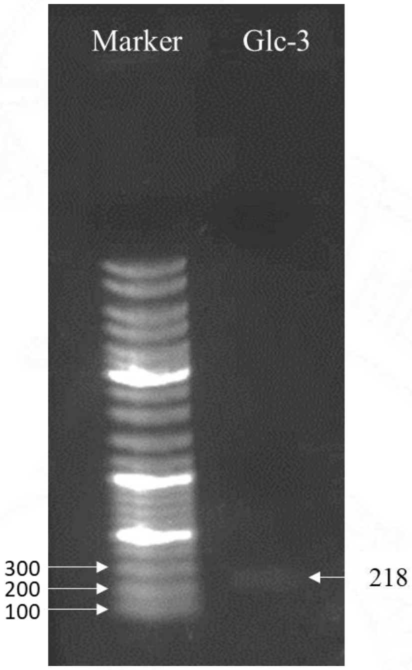
172 bp PCR amplified product. The QIAquick purified product was run on 1% agarose gel. The purified PCR product was cloned with pTZ57R/T by T/A InsTAclone PCR cloning kit (Fermentas; Product code - 10181080).



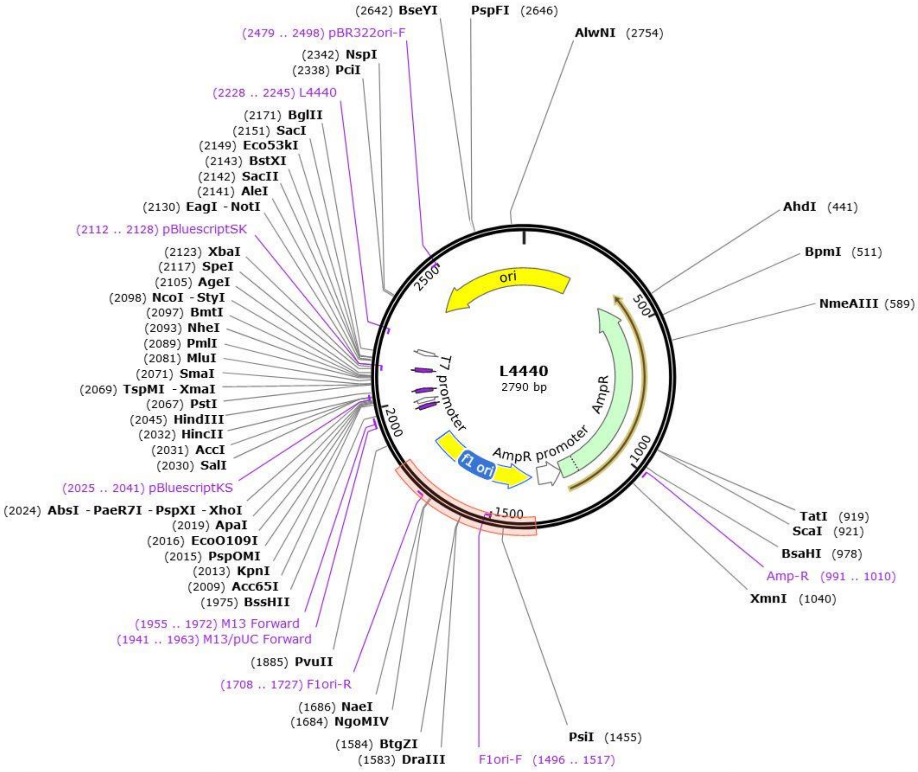
5. Restriction digestion of pTZ57R/T-glc-3 using Hind-III and Xba-I to excise the glc-3 gene fragment from the pTZ57R/T-glc-3 plasmid



6. Gel purification of glc-3 gene fragment using AxyPrep DNA gel extraction kit (Product No. AP-GX-250)



7. Subcloning of glc-3 gene fragment to pL4440



III. Construction of RNAi feeding vector for sra-17 silencing

1. Exon 3 mRNA sequence for silencing sra-17 primer generation

Reference ID: WBGene00005043.3

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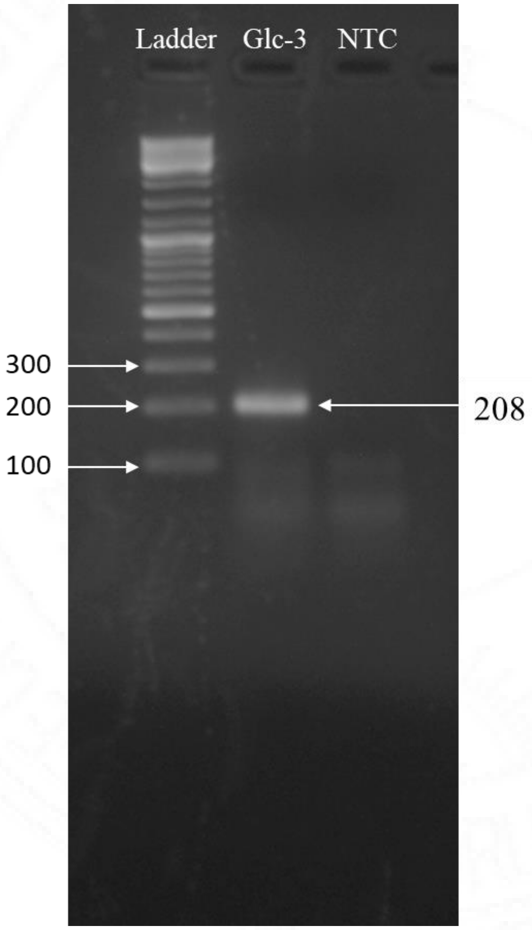
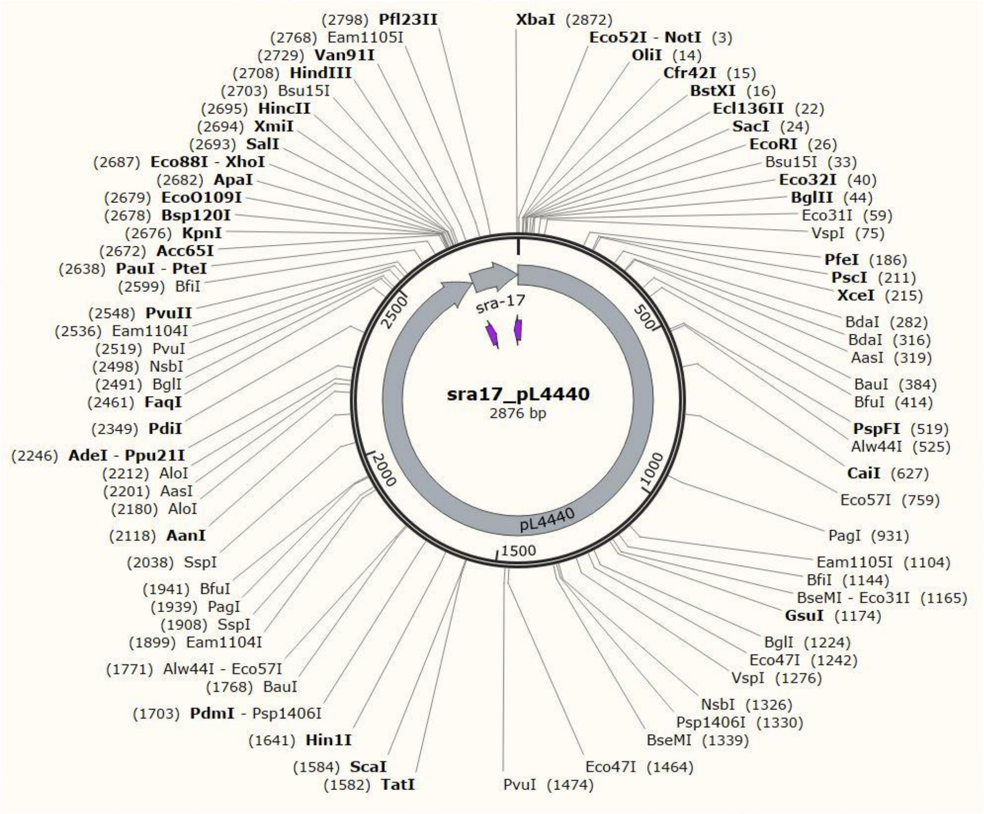
AGCGTAAAATCAGAAAAGCA

2. PCR amplified sra-17 gene fragment

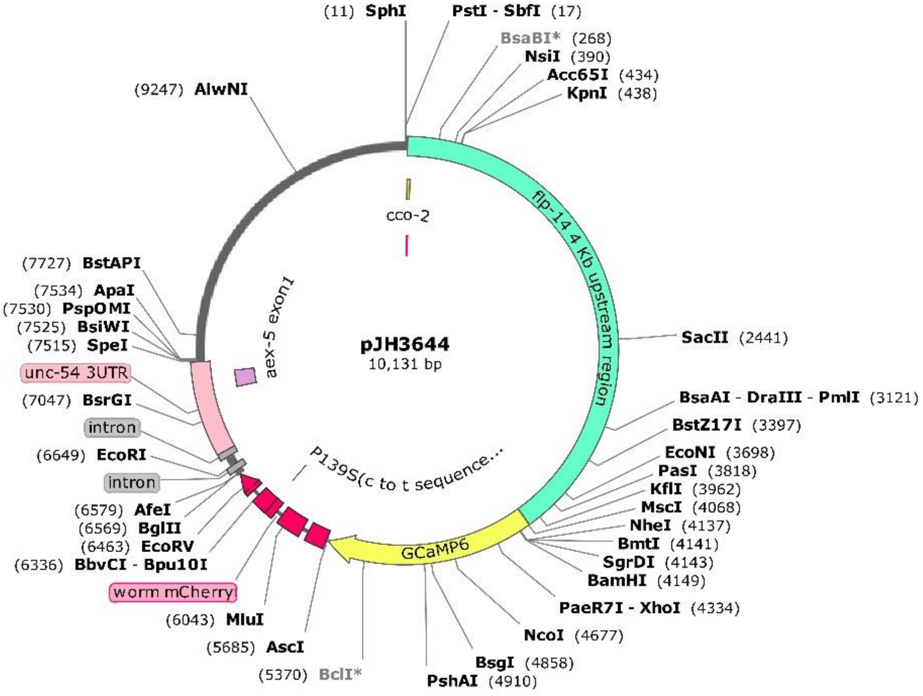


3. Subcloning of sra-17 gene fragment to pL4440



IV. Construction of plasmid for AIY calcium imaging

1. Structure of the plasmid pJH3644 containing GCaMP-6 gene.



2. Ttx-3 promoter gene sequence (3124bp)

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TCTGTCCCATTATCAGATTATCTGATCTCTCGGACATGAGCAATGTTTATGTTATTTGTCAA
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AATTTTTCAGATATCAATCAGTCAAAAATAATTGGACACAAGTAAAAAGCAGACAACATAAT
GCGGTTCAAAAAAAACATTCTGAAATTTTTTCAATCTCTATTATTTGCCTACTATCTGCGTG
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CCTCCAATTTCACTAACACTTTTACATATTGATGGGCGAGTCGAAAAAGTACTACGTTTACT
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```

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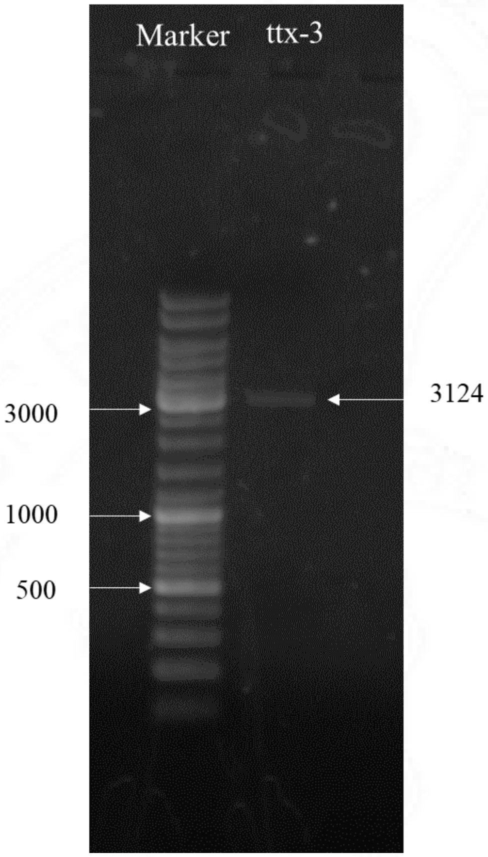
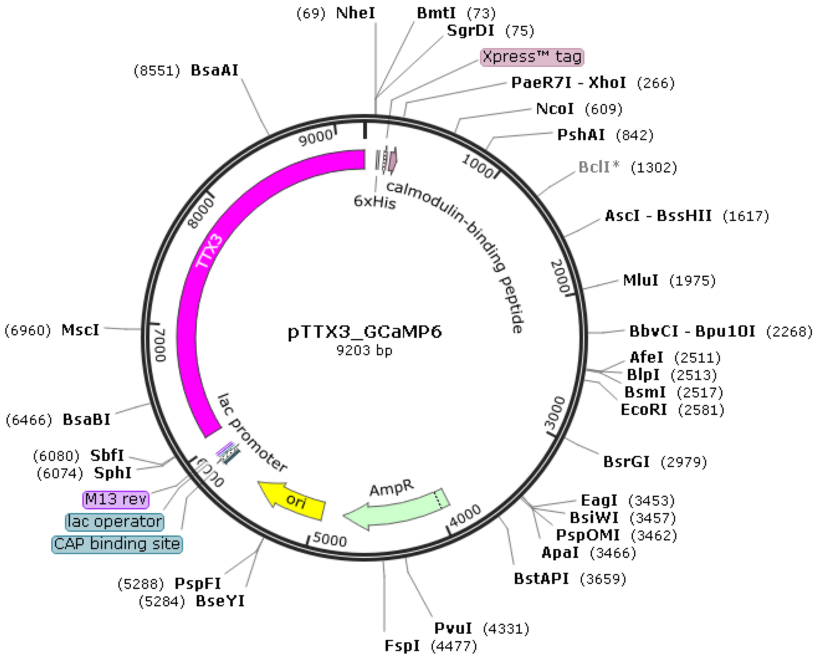
3. **Purification of ttx-3 promoter gene from the gel using Qiagen gel purification kit (Cat No./ID: 28115).**



4. Map for Ttx-3::GCaMP6 plasmid construct



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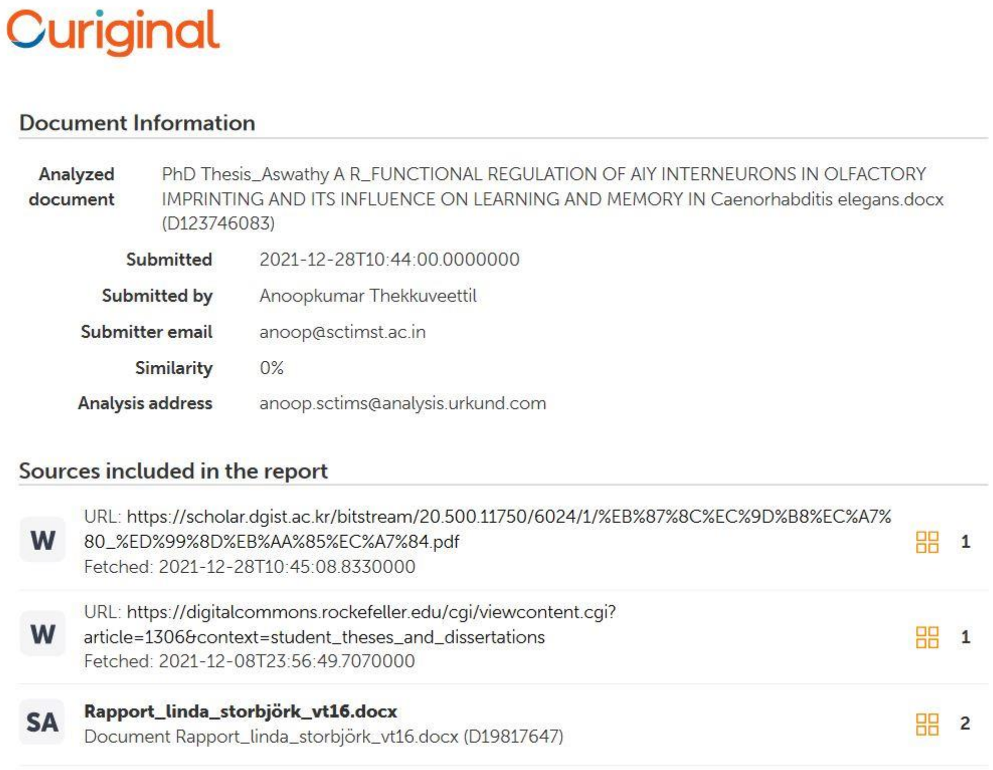

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