Probing the role of CREB homolog, CRH-1, in innate and learned behaviours in *C. elegans*

Saloni Rose

A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

Supervised by

Dr. Kavita Babu

April 2018

Certificate of Examination

This is to certify that the dissertation titled "Probing the role of CREB homolog, CRH-1, in innate and learned behaviours in *C. elegans*" submitted by Ms. Saloni Rose (Reg.No. MS13019) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Sudip Mandal Dr. Shravan K. Mishra Dr. Kavita Babu
(Superviser)

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Kavita Babu at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Saloni Rose

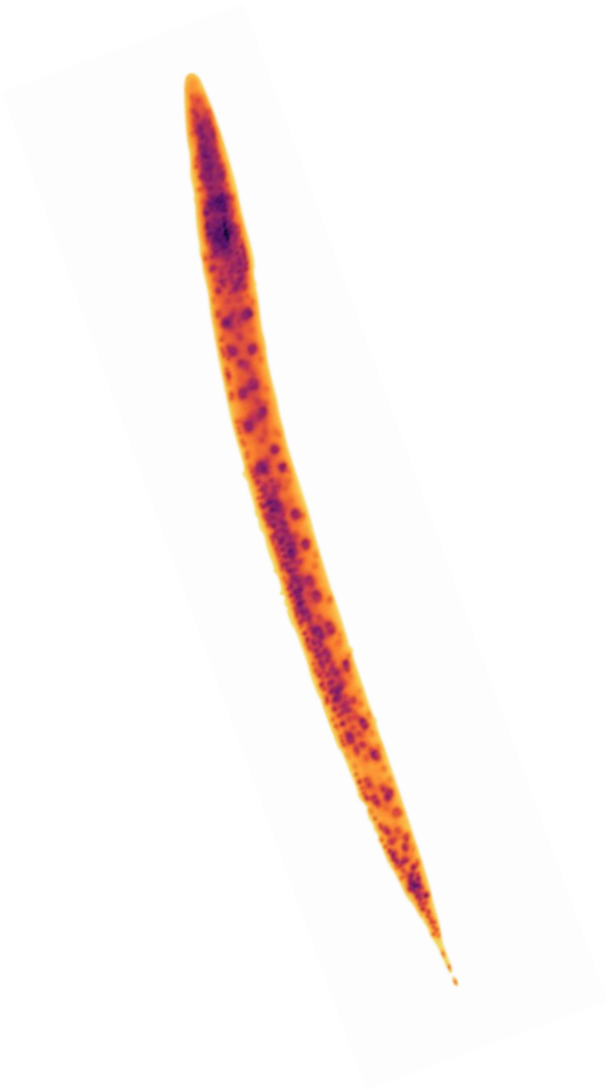
(Candidate)

Dated: April 20, 2018

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

Dr. Kavita Babu

(Supervisor)



"You have made your way from worm to man, and much within you is still worm."

- Friedrich Nietzsche, *Thus Spoke Zarathustra*

Acknowledgements

I would like to thank Dr. Kavita Babu for giving me the opportunity to work in her lab, providing me with space and resources. Her calm and composed personality is very encouraging. I express my deepest gratitude to Dr. Yogesh Dahiya for letting me work under his mentorship. He is one of the few polymaths I have met in real life and his knowledge on any subject is extensive and elaborate. Endless discussions on science with him inspired me to further my career in Academia. I would like to acknowledge Shruti for introducing me to basic *C. elegans* handling techniques and molecular biology tools. I thank Ankit Negi for routine help and Pallavi Sharma for her joyous company in the lab. I also wish to thank the rest of the lab for their support. I am most indebted to my parents and my friends Simran and Thomas for their unconditional love and encouragement. Many thanks to my father for introducing me to badminton, without which I wouldn't have developed discipline and perseverance. Finally, I would like to thank Brenner and his intellectual descendants for introducing the *C. elegans* to the scientific community.

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Abstract

Animals display innate behaviours that are genetically hardwired and can be performed in response to a cue. Although they are stereotypic, some innate behaviours can be modified through experience. One such behaviour is chemotaxis towards an attractant isoamyl alcohol (IAA) in *Caenorhabditis elegans*. *C. elegans* is a soil dwelling nematode that lives on microbes for its food source. They move forward in a sinusoidal wave pattern and their forward movement is punctuated by frequent stops and events of backward movement called reversals. The main strategy is to reduce the frequency of reversals when the environment becomes more favourable. *crh-1* (homolog of mammalian CREB1) null mutants have severely compromised ability to change the reversal frequency in response to the gradient of attractant IAA. This defect is also manifested as a learning defect in *crh-1* null worms. Our experiments employ a learning paradigm where the IAA was paired to heat and show that CRH-1c and CRH-1e (2 out of 6 CRH-1 isoforms) are required for innate behaviours as well as learned. Consistent with the behavioural data, the spatial localisation of ionotropic glutamate receptor subunit GLR-1 was found to be defective in CRH-1c and CRH-1e deletion lines. These experiments provide important insight into mechanistic understanding of CREB1/CRH-1 transcription factor in mediating innate and learned behaviours.

Contents

1 Introduction

"CREB's opposing regulatory actions provide a threshold for memory storage, presumably to ensure that only important, life-serving experiences are learned. Repeated shocks to the tail are a significant learning experience for an Aplysia, just as, say, practicing the piano or conjugating French verbs are to us: practice makes perfect, repetition is necessary for long-term memory."

-Eric R. Kandel, *In Search of Memory* (2006)

1.1 Learning and memory formation

The Holy Grail of neuroscience has always been to understand the biological basis of learning and memory formation. Learning can be defined as an individual's ability to modify its behavior in response to past experience and current environment, and memory as the ability to store the learned information and thus maintain the modified behavior for variable time periods (Davis et al. 2005). An alternate definition of learning is "the acquisition of neuronal representations of new information" (Dukas et al. 2008).

Learning is an ubiquitous phenomenon in the animal kingdom and comes in various forms. The simplest forms include non-associative paradigms, like habituation where there is decrease of the behavioral response to exposure to a repetitive neutral stimulus (Rankin et al. 2009). Animals also display more complex forms of learning like classical conditioning that involves association of biologically relevant stimulus with another neutral stimulus or operant conditioning, where strength of a behavior is modified by reinforcement or punishment (Levitan and Kaczmarek, 1991). Learning results in at least short term memory that involves chemical changes in synaptic properties and often long term memory which requires gene transcription and translation into proteins (Dukaas et al. 2004).

1.2 *C. elegans* as a model system

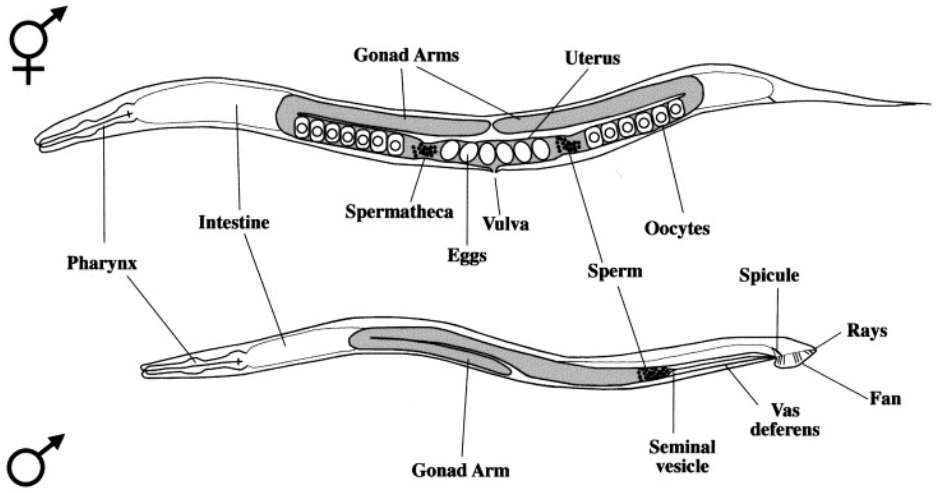

Figure 1.1: Anatomy of *C. elegans*: A. Hermaphrodite, B. Male. Adapted from Hansen et al. 1999.

Caenorhabditis elegans (*Caeno* meaning recent; *rhabditis* meaning rod; *elegans* meaning nice), a free living nematode, is widely used as a model system in developmental biology and neuroscience (Brenner et al. 1974). *C. elegans* has five pairs of autosomes and one pair of sex chromosome and their populations are mainly hermaphroditic in nature, only 0.01% of them are males (Wormbook). Hermaphrodites have two X Chromosomes while males have only one X Chromosome (Nikon et al. 1949). Being a very simple organism, all the cell lineages have been extensively mapped and studied (Sulston et al. 1983). An adult wild type worm consists of 959 cells, 302 of which are neurons (Fig 1.1, WormAtlas). Simple neural and genetic architecture of invertebrate model systems like *C. elegans*, *Drosophila* and *Aplysia* have enabled us to study the neural pathways and cellular mechanisms involved in memory formation (Honjo and Furukubo-Tokunaga et al. 2009).

1.2.1 *C. elegans* connectome

The entire connectome of *C. elegans* has been reconstructed using electron micrographs by J. G. White, E. Southgate, J. N. Thomson and S. Brenner. *C. elegans*

nervous system consists of 302 neurons that are organised in several ganglia in the head, tail and the ventral nerve cord (Fig 1.2). The neurons are divided into 118 classes and wired into 5000 chemical synapses, 2000 neuromuscular junctions and 600 gap junctions (White et al. 1986).

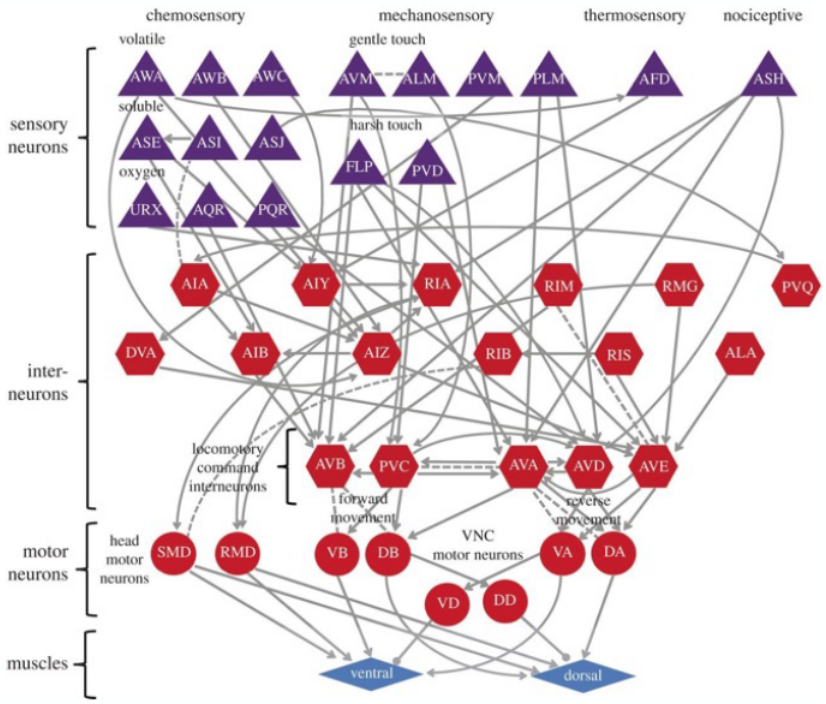

Figure 1.2: Partial circuit diagram of *C. elegans* nervous system. Arrows represent excitatory or inhibitory chemical synapses and dashed lines represent electrical synapses. Adapted from Fang-Yen et al. 2015.

The majority of the neurons of *C. elegans* are situated in the head and arranged in groups of sense organs, known as sensilla (Ward et al. 1975). The sensory neurons receive input from different modalities like olfaction, mechanosensation, thermosensation and nociception. The sensory information is processed and integrated by a set of interneurons which translate the output to muscles via motor neurons (Fang-Yen et al. 2015). The neurons contain many of the neurotransmitters known in other animals, including serotonin, dopamine, glutamate, acetylcholine, and gamma-aminobutyric acid (GABA) (de Bono and Maricq, 2005).

1.3 Chemotaxis in *C. elegans*

Chemotaxis in *C. elegans* has been extensively studied, with a special emphasis on behavioral and neuronal mechanisms. Worms use chemotaxis to identify their food source, find mates and avoid toxic environments (Wormbook). Worms can detect a wide variety of volatile odors by sensory neurons AWA (attractive odors like benzaldehyde, butanone, isoamyl alcohol, 2,3-pentanedione, and 2,4,5-trimethylthiazole), AWB and AWC (diacetyl, pyrazine, and 2,4,5-trimethylthiazole) (Wormbook). At lower concentrations, worms showed attraction to the odorants but showed aversion at higher concentrations (Yoshida et al. 2013).

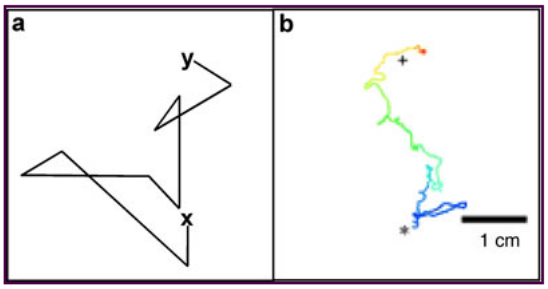

Figure 1.3: The pirouette model for chemotaxis: a. Computer simulated model of chemotaxis of a worm(x) towards an attractant(y), b. Representative trace of a wild-type animal during chemotaxis from origin (*) to attractant (+). Adapted from Wormbook

Worms move forward in a sinusoidal wave pattern and their forward movement is punctuated by frequent stops and events of backward movement called reversals or pirouettes (Croll et al. 1975). Worms adopt two strategies in moving towards attractants: klinokinesis and klinotaxis. Klinokinesis model is based on biased random walk and worms modulate the frequency of turning by sensing the concentration gradient of odour (dC/dt) over time (Pierce-Shimomura et al. 1999). When the environment becomes more favourable (dC/dt >0 for a chemoattractant), the probability of reversals is decreased (Fig 1.3, Pierce-Shimomura et al. 1999). While in klinotaxis, worms regulate curving rate bias and move by continuously adjusting toward the line of steepest ascent (Iino and Yoshida, 2009).

1.4 cAMP-response element-binding (CREB) protein

The CREB protein is an important evolutionally conserved transcription factor, with pleiotropic functions involving long-term memory, apoptosis, differentiation, metabolism, hematopoiesis, and immune activity (Fig 1.4, Mayr and Montminy, 2001).

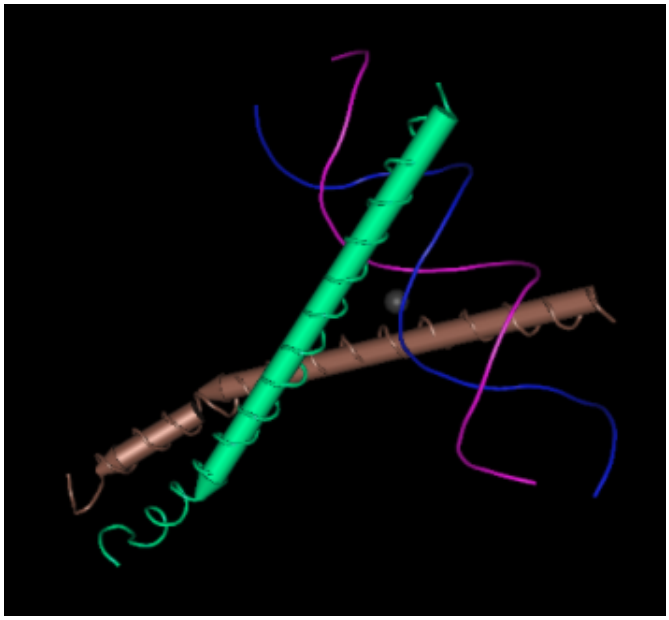

Figure 1.4: Tertiary structure of CREB1 (*Mus musculus*) heterodimer binding DNA represented in green and brown while the DNA strands are represented in pink and blue. Adapted from RSCB.

The DNA binding domain consists of a basic region that binds to negatively charged DNA molecules and a bZIP domain that consists of a series of amino acids (leucine zipper) which helps in dimerisation (Fig 1.5, Meyer et al. 1992). The transactivation domain facilitates interactions with coactivators and components of the transcriptional machinery and consists of P-box that has a conserved serine residue that is phosphorylated (Meyer et al. 1992). A variety of stimuli induce CREB dependent transcription of target genes including peptide hormones, growth factors, and neuronal activity. They activate a variety of protein kinases including protein kinase A (PKA), mitogen-activated protein kinases (MAPKs), and Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) that further phosphorylate CREB thus activating it (Hunter and Karin, 1992).

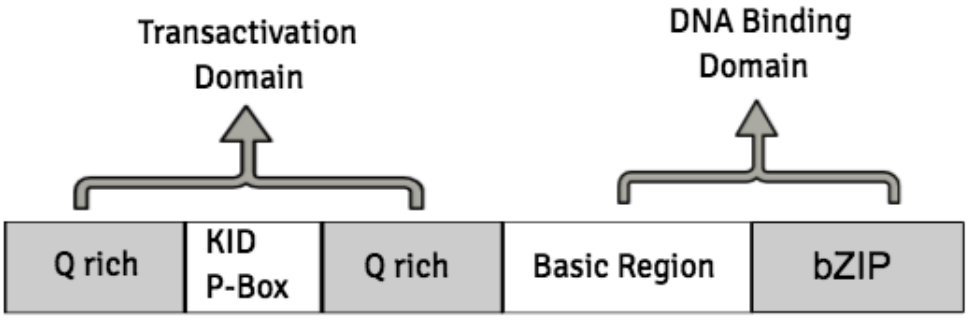

Figure 1.5: Topographical Structure of CREB Protein. Adapted from Meyer et al. 1992

The first mechanistic insights into the role of cAMP-CREB-dependent gene expression in learning and long term memory formation was shown in Aplysia (Dash et al. 1990). Using an olfactory memory paradigm in Drosophila, geneticists identified mutant genes "Rutabaga" and "Dunce" that were key regulators of the same pathway, modulating intracellular cAMP levels (reviewed in Waddell and Quinn, 2001). Yin et al. showed that dominant negative mutation of CREB itself blocked long term memory formation (Yin et al. 1994). Studies have shown that overexpression of CREB in Mammalian brain leads to enhancement of long term memory formation (Josselyn et al. 2001).

1.5 *C.elegans* CREB homolog, CRH-1

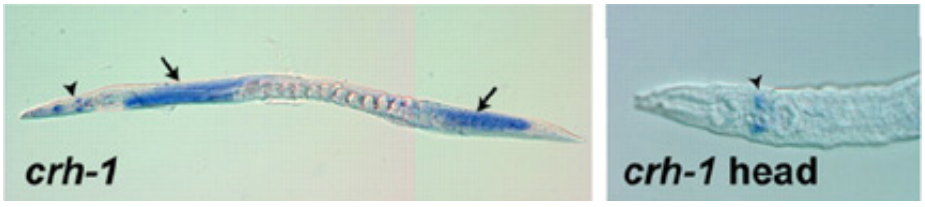

Figure 1.6: Expression pattern of *crh-1* using Insitu Hybridisation (Kimura et al. 2002)

CRH-1 is a CREB (Cyclic AMP-response element binding protein) homolog found in *C. elegans* and is involved in the transcription of CRE-sequence dependant genes activated by a CaM kinase cascade (Wormbase). The Kinase Inducible

Domain and bZIP region are 80% and 95% similar to the Mammalian CREB (Kimura et al. 2002). Kimura et al. identified a strain from a screen of a library of mutagenized nematodes, *crh-1(tz2)*, carrying a deletion of 979 nucleotides that eliminates 38 amino acids at the C-terminal of the bZIP region (Kimura et al. 2002). They used insitu-hybridisation to look at *crh-1* mRNA expression, which is limited to head neurons including AFD, the gustatory neurons ASE, and the olfactory neurons AWC and the intestine and gonads (Fig 1.6, Kimura et al. 2002). Previous biochemical studies had shown that CMK-1 phosphorylated CRH-1 at S48 (Kauffman et al. 2010; Kimura et al. 2002). CRH-1 has shown to be important for long term memory formation in several contexts, including aversive olfactory learning, tap withdrawal pathogen avoidance and thermotaxis (Prithika et al. 2016, Chen YC et al. 2016, Nishijima et al. 2017).

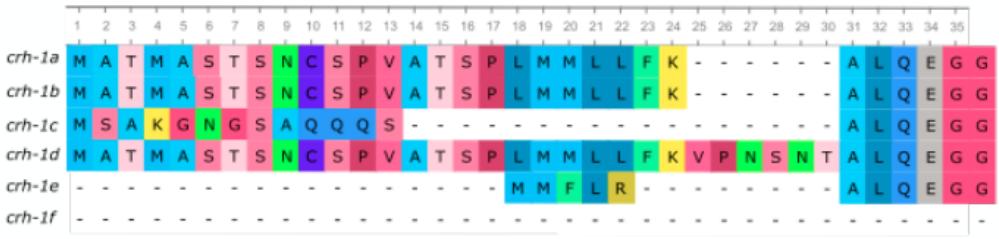

Figure 1.7: Isoforms of CRH-1 (6 of 7 isoforms have been shown). Isoform CRH-1b has additional two amino acids at 88th position post alignment when compared to CRH-1a. Adapted from WormBase.

Seven isoforms of CRH-1 have been identified, out of which isoform CRH-1f is truncated i.e. lacks a transactivation domain and isoform CRH-1g is a putative protein (Fig 1.7, Wormbase). Isoforms CRH-1a, CRH-1b, CRH-1c, CRH-1d and CRH-1e have a conserved DNA binding domain and differ in few amino acids in the transactivation domain towards the N-Terminal. To explain the difference in function of the CRH-1 isoforms, Yogesh Dahiya devised an associative learning paradigm that involved pairing chemoattractant isoamyl alcohol and high temperature (acts as a repulsive cue) and looked at their expression. He found that only *crh-1* isoforms CRH-1c and CRH-1e are necessary for long term memory formation (unpublished data). Through rescue experiments, he further narrowed down

the specific interneurons where the expression of *crh-1* was necessary for memory formation.

Our preliminary results showed that chemotactic behaviour of *crh-1(tz2)* mutants were different from that of the wildtype worms. We hypothesised that the observed memory defect in *crh-1* mutants could be partly due to innate defects in chemotaxis. The main aim of my thesis was to parse out the difference between innate chemotaxis defects and memory defects in *crh-1* mutants and investigate the role of different CRH-1 isoforms in both behaviours.

2 Objectives

2.1 Characterisation of innate olfactory preference

Studies in mice and Drosophila have showed that CREB is necessary for olfactory memory formation, but they haven't explored its role in innate behaviour. *crh-1* mutants have been shown to be athermotactic (Nishida et al. 2007), habituation variant (Timbers and Rankin, 2011) and memory formation defective (Amano and Maruyama, 2011; Lakhina et al. 2015). There are no previous reports of innate olfactory behavioral defects in *crh-1(tz2)* animals. Given the pleotropic role of CREB as a transcription factor and preliminary results, we suspected that *crh-1* mutants might show defects in olfaction or integration of sensory cues.

First Objective: To characterise innate response of *C.elegans crh-1* mutants towards isoamyl alcohol using multiple chemotaxis paradigms

2.2 Localisation of AMPA receptors subunit GLR-1

AMPA receptor trafficking and localisation have been shown to be critical for long term memory formation (Whitlock et al. 2006, Schmitt et al. 2003). Inhibition of CREB in hippocampal neurons in mice reduces the levels of AMPAR subunit GluA1 within the post synaptic density and causes defects in fear conditioning (Middei et al.2013). However, there are no previous reports of defects in AMPA receptor localisation in *crh-1* mutants in *C. elegans*.

Second Objective: To characterise defects in localisation of AMPA-type glutamate receptor subunit (GLR-1) in *crh-1* isoform specific mutants by visualising GLR-1:GFP expression in the posterior ventral nerve cord.

3 Methods

3.1 Strains

Standard laboratory methods for *C. elegans* as described by Brenner (Brenner et al. 1974). *crh-1(tz2)* and *tax-2*(ok3403) were obtained from Caenorhabditis Genetics Center(CGC). Other transgenic strains were generated by Yogesh Dahiya. Isoform specific deletion lines were constructed using the CRISPR- CAS genome editing technique (Fig 3.1). The strains used are mentioned in the appendix.

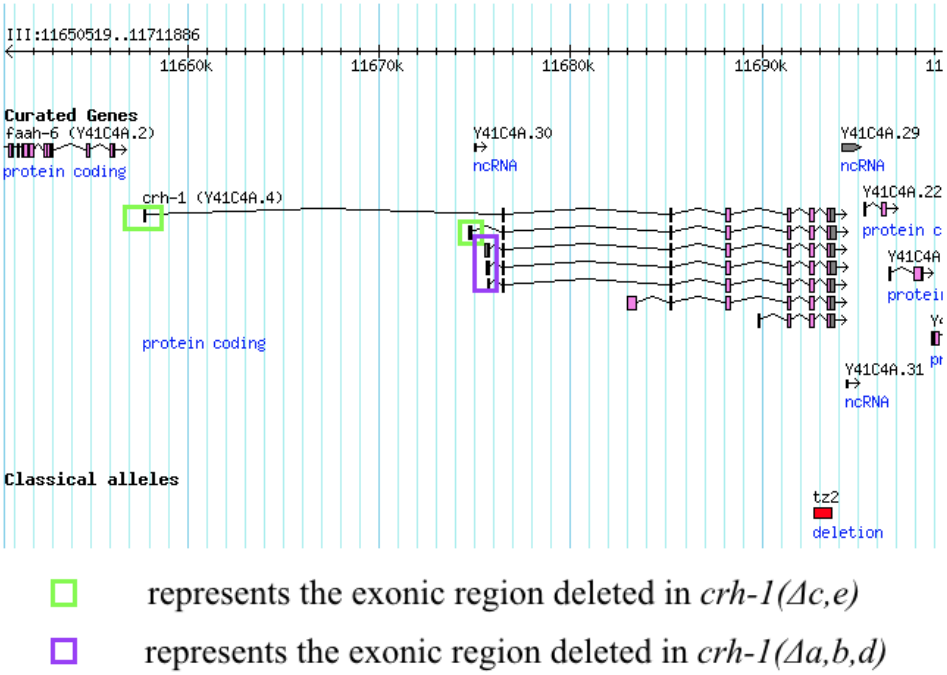

Figure 3.1: Exonic regions deleted in isoform specific deletion lines.

3.2 Confocal imaging

Confocal images of the posterior ventral nerve cord were collected on a Leica HC PL APO 63x/TCS SP8 confocal microscope (Leica Microsystems). Images were collected with a 40x oil immersion objective, at 1024*1024 pixels, with 0.5 micron Z-steps. For fluorescence analysis, maximum intensity projections were

obtained, followed by Bensen local thresholding using Image J software. Images from 15-25 animals per strain were obtained and used to plot the graph.

3.3 Behavioural assays

All behavioral assays were performed on young adults at 20 degrees on NGM-Agar plates unless stated otherwise. Worm populations were synchronised through bleaching.

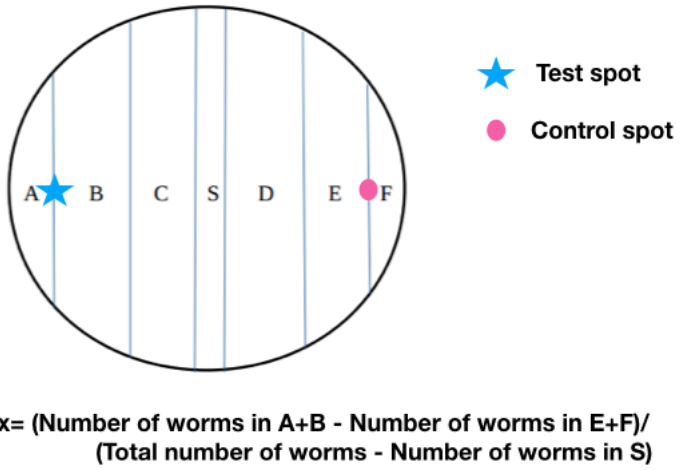

Figure 3.2: Conventional Chemotaxis Experimental Setup designed by Bargmann

3.3.1 Conventional chemotaxis assay

Standard procedure as described by Bargmann (Bargmann et al. 1993). 50-80 young adults were placed on 90 mm NGM agar plates at the bottom corner and 1 microlitre of isoamyl alcohol (or its dilutions) was pipetted on one side with the diluent on the diametrically opposite side along with 1 microlitre of 1M sodium azide which acts as a paralysing agent (Fig 3.2). The ratio of the difference between the number of individuals in 2 cm range of test spot/control spot to total number of individuals was used as the chemotaxis index (C.I).

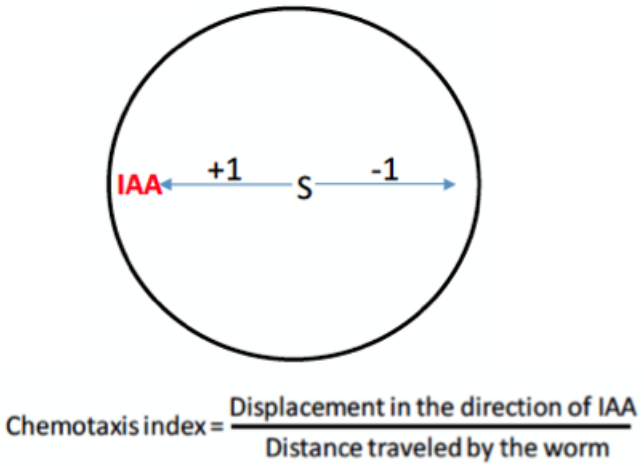

Figure 3.3: Revised Chemotaxis Experimental Setup designed by Yogesh Dahiya

3.3.2 Revised chemotaxis assay

The revised chemotaxis assay was devised by Dr. Yogesh Dahiya. 4-5 young adults were placed on 90 mm NGM agar plates in the center and 1 microlitre of isoamyl alcohol (or its dilutions) was pipetted on one side with the diluent on the diametrically opposite side (Fig 3.3). A Mightex USB 3.0 Monochrome 5 Megapixel Camera was used to record video of the worms for 10 minutes. The locomotory trails of the worms were measured for each individual worm and the displacement (in the direction of IAA) to distance ratio was adopted as the chemotaxis index (C.I.). A positive chemotaxis index (C.I.) value indicates attraction while a negative value indicates repulsion.

3.3.3 Associative learning paradigm

For the associative learning paradigm, late L4 worms were exposed to IAA for 2 minutes at 37 degrees Celsius followed by 8 minutes of no stimulus at 20 degrees Celsius. The cycle was repeated four times. The worms were tested for

chemotaxis towards IAA the next day.

3.4 Data analysis

Fiji was used for analysis of confocal images and chemotaxis videos. Statistical analysis was performed using R and plots were constructed using ggplot2. P values were identified by one-way or two-way ANOVA analysis across test populations, followed by post hoc analysis using two-tailed Student's t-test or Tukey's Honest Significant Difference test. We used non-parametric tests like the Mann-Whitney U-test for comparing two independent samples for cases where normality didn't hold. Differences were accepted as significant at $p < 0.05$.

4 Results

4.1 *crh-1* mutants show innate chemotaxis defects

The nematode worm *C. elegans* uses a biased random walk in the presence of an olfactory cue and increases pirouettes when moving away from the attractants by detecting changes in concentration over time (dC/dt) (Pierce-Shimomura et al. 1999). Wildtype worms showed a strong attraction towards isoamyl alcohol(IAA) while *crh-1* mutants showed a relatively decreased bias. To test the sensitivity of worms towards IAA, we subjected them to varying concentration of IAA diluted in ethanol.

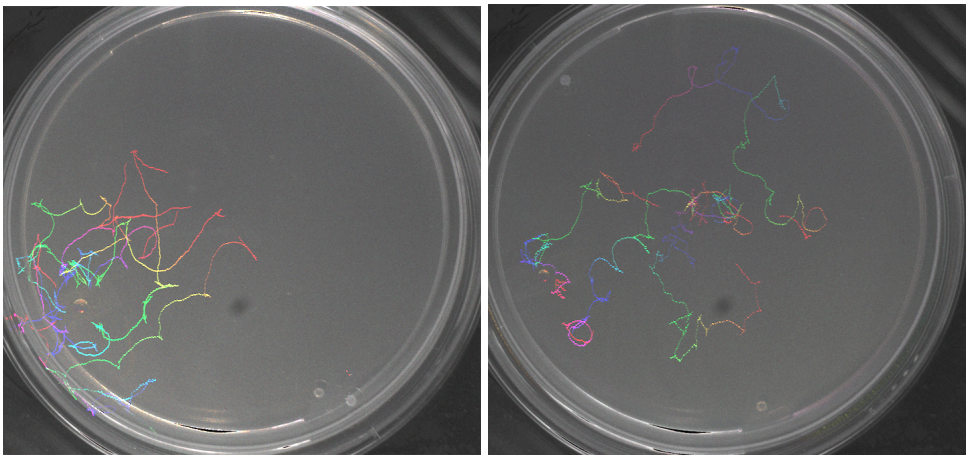

Figure 4.1: Chemotactic trails in the presence of IAA. Wildtype on the left, *crh-1(tz2)* on the right

As a negative control, we used *tax-2* mutants. *tax-2* encodes for the beta subunit of cyclic nucleotide gated cation channel that is expressed in the sensory neuron AWC (Wormbase). *tax-2* mutants are unable to sense IAA and don't show chemotaxis. We performed two-way ANOVA on the sensitivity data and found no interactional effects between genotype and concentration ($p = 0.798$, Two-way ANOVA). When compared to the wildtype worms, *crh-1(tz2)* and *tax-2* mutants show decreased chemotaxis index (Fig 4.2, Test 1: $p < 0.005$, Test 2: $p < 0.0005$, Tukey's Honest Significant Difference test (refer to Figure 4.3 for p-values)). The differences in C.I. between wildtype and isoform specific mutants (*crh-1(Δa,b,d)* and *crh-1(Δc,e)*) were also significant (Fig 4.2, Test 1: $p < 0.05$, Test 2: $p < 0.05$,

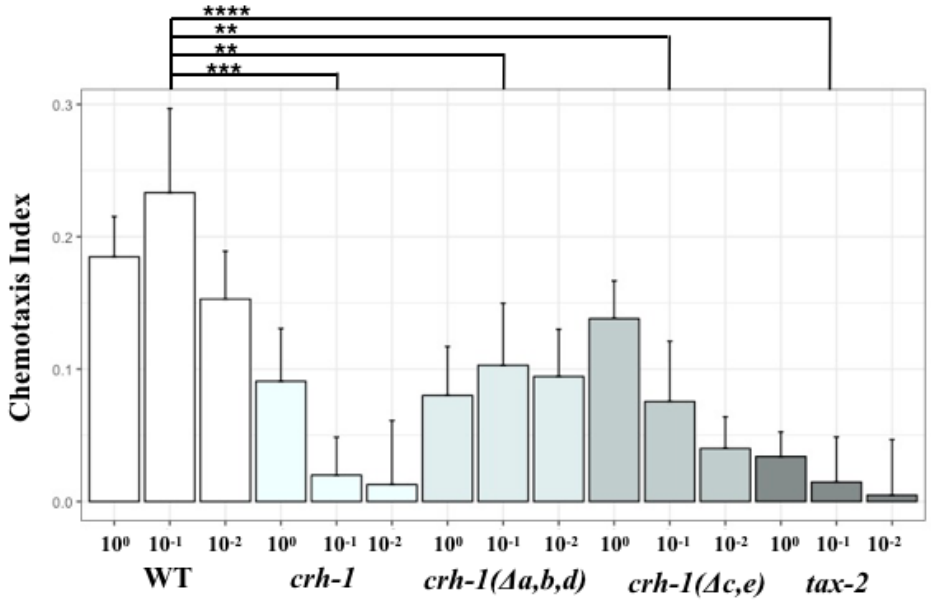

Figure 4.2: Quantification and comparison of chemotaxis index of worms subjected to varying concentration of IAA obtained from the experimental paradigm designed by Yogesh Dahiya (Mean + SEM). Concentration of IAA solution and strains used are represented on the x-axis. Concentration used are as follows: 10^{0}- 100% IAA, 10^{-1}- 10% IAA, 10^{-2}- 1% IAA.

Group 1	Group2	p-value
WT	*crh-1*	0.0004815
WT	*crh-1(Δa,b,d)*	0.0288166
WT	*crh-1(Δc,e)*	0.0379355
WT	*tax-2*	0.0000877
crh-1	*crh-1(Δa,b,d)*	0.7945708
crh-1	*crh-1(Δc,e)*	0.7644935
crh-1	*tax-2*	0.9119325
crh-1(Δa,b,d)	*crh-1(Δc,e)*	0.9999958
crh-1(Δa,b,d)	*tax-2*	0.3482600
crh-1(Δc,e)	*tax-2*	0.3242197

Figure 4.3: P-values obtained from pairwise comparisons of strains from Tukey's Honest Significant Difference test.

Tukey's Honest Significant Difference test). However, the effect of concentration of IAA across groups was not significant (Fig 4.2, p = 0.250, Two-way ANOVA).

crh-1 mutants do not produce functional forms of CRH-1 protein, but isoform specific mutants do have a basal expression of other isoforms. We speculate that the CRH-1 isoforms might work in conjuction to regulate basal internal processes that mediate chemotaxis behaviour.

The decreased bias towards IAA in *crh-1* mutants could be due defects in sensing IAA, interneuronal processing or locomotion of worms or any combination of three factors. There have been no reports of defects in locomotion in *crh-1* mutants in the absence of any cues except of slight decrease in reversal length. The main parameters that affect worm's trajectory are speed and reversal frequency (Zhao et al. 2003).

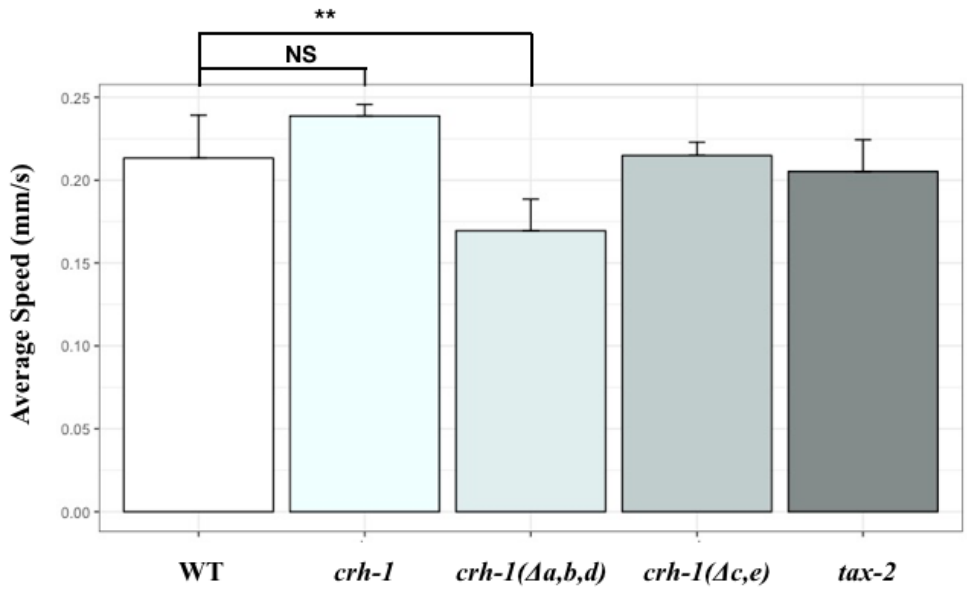

Figure 4.4: Quantification and comparison of average speed of worms in the presence of IAA (Mean + SEM).

We found that average speed of *crh-1* mutants is not significantly different from the wildtype worms in the presence of IAA (Fig 4.4, p = 0.257, Student's t-test). But, *crh-1* mutants show lower reversal frequency towards IAA than wildtype worms (data not shown). The circuit that determines reversal frequency is a point of integration for many sensory modalities like mechanosensation and chemosensation. Since the reversal frequency of *crh-1* mutants in the absence of IAA is similar to wildtype worms, we can deduce that the dissimilarity in the presence of IAA could be due to defects in sensory perception or interneuronal

processing. However, it becomes difficult to parse out if the difference in reversal frequency is due to defects in sensory or interneuronal processing.

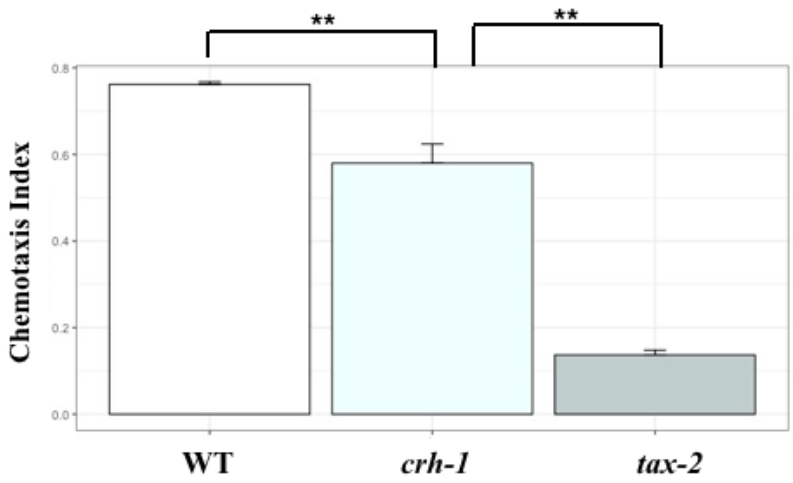

Figure 4.5: Quantification and comparison of chemotaxis index of worms subjected to 100% IAA obtained using the experimental paradigm designed by Bargmann (Mean + SEM).

If *crh-1* mutants are indeed defective in chemotaxis and this is not a artifact of the experimental paradigm, they must show similar behaviour in different chemotaxis paradigms. So, we did a population chemotaxis assay used in previous studies (Bargmann et al. 1993). We observed results similar to those obtained in the experimental paradigm designed by Yogesh Dahiya. The chemotaxis index of *crh-1* mutants was lesser than wildtype worms (Fig 4.5, $p < 0.05$, Mann-Whitney U-Test) and higher than *tax-2* mutants (Fig 4.5, $p < 0.05$, Mann-Whitney U-Test). However, the two assays differ in the following aspects:

1. Difference in density: Previous studies used 50-80 worms on a 90 mm NGM agar plate, while we used worms per plate. Crowding affects individual worm dispersal.

2. Addition of 1M sodium azide: Sodium azide, a paralysing agent is placed next to the IAA (test spot)/control spot in the method mentioned in previous studies. The worms traversing in the vicinity of IAA (test spot)/control

spot are paralysed. Our method doesn't involve sodium azide or any other paralysing agents and worms freely move throughout the time of observation

3. Time: Our method monitors the worms for ten minutes, while the one mentioned in previous studies monitors the worms after 30 minutes. In the absence of food, the worms initiate a local search behavior consisting of reversals and deep omega-shaped turns triggered by AWC olfactory neurons, ASK gustatory neurons, and AIB interneurons. After 30 minutes, the animals disperse, as reversals and omega turns are suppressed by ASI gustatory neurons and AIY interneurons (Grey et al. 2005).

4.2 Mutants in *crh-1* show learning defects

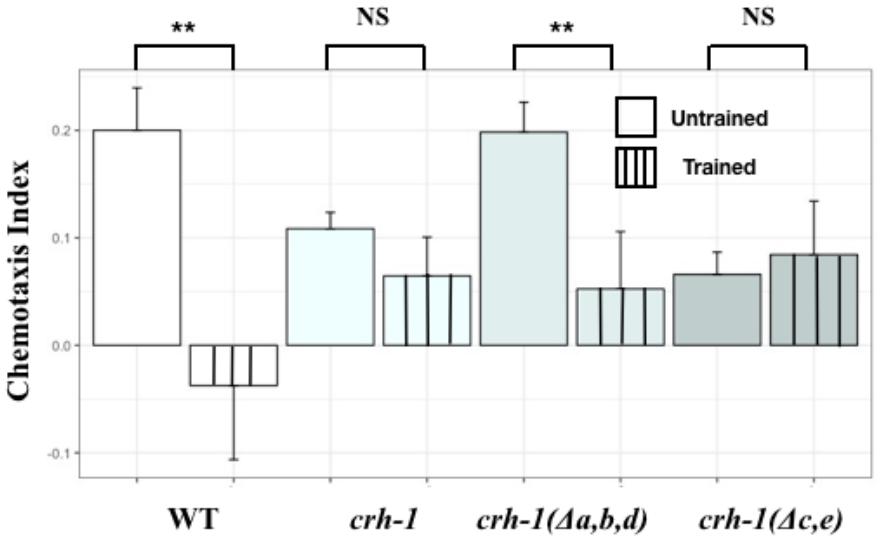

Figure 4.6: Quantification and comparison of chemotaxis index of *crh-1* mutants post-aversive conditioning (Mean + SEM). Adapted from Yogesh Dahiya.

A spaced interval training paradigm was adopted to train the worms to associate high temperature and IAA. The worms were considered to have learnt if chemotaxis index of the worms post-training was significantly lesser than innate

chemotaxis index. Untrained wildtype worms showed strong attraction towards IAA. Since high temperature acted as an aversive cue, the wildtype worms showed negative chemotaxis index and avoided IAA post-training (Fig 4.6, $p < 0.005$, Student's t-test). However, *crh-1 mutants* failed to associate these two cues and showed similar behaviour to that of untrained worms (Fig 4.6, $p = 0.424$, Student's t-test). Previously, Yogesh showed through isoform specific rescue experiments that isoforms CRH-1c and CRH-1e are necessary for memory formation. Accordingly, we see that *crh-1(Δa,b,d)* mutants, which lack isoforms CRH-1a, CRH-1b and CRH-1d, can learn (Fig 4.6, $p < 0.05$, Student's t-test) while *crh-1(Δc,e)* mutants, which lack isoforms CRH-1c and CRH-1e, cannot learn (Fig 4.6, $p = 0.659$, Student's t-test). Also, both the isoform specific strains show naive chemotaxis defect. With the above data, we hypothesise that isoforms CRH-1a, CRH-1b and CRH-1d might be involved in other homeostatic functions that don't involve memory formation, while isoforms CRH-1c and CRH-1e might perform basal functions along with memory related functions.

4.3 *crh-1* mutants show defective spatial localisation of ionotropic glutamate receptor subunit GLR-1

The majority of fast excitatory transmission in the brain is mediated by AMPA receptors which are glutamate-gated cation channels (reviewed in Henley et al. 2013). AMPA receptors are inserted at perisynaptic sites and translocated to synapses on LTP induction (Yang et al. 2008). AMPA receptor trafficking and localisation have been shown important for fear conditioning and memory formation (Whitlock et al. 2006, Schmitt et al. 2003). GLR-1 encodes for an AMPA type receptor subunit homolog in *C. elegans* and has been shown critical for long term memory formation in the context of habituation (Rose et al. 2003). Using GFP based translational reporter lines, GLR-1 has been shown to express in ventral cord interneurons punctata (group of synapses).

To test if the memory defects were due to improper glutamate localisation at synapses, we introduced the GLR-1::GFP marker in the isoform specific mutants and visualised their puncta using confocal microscopy. We compared the expres-

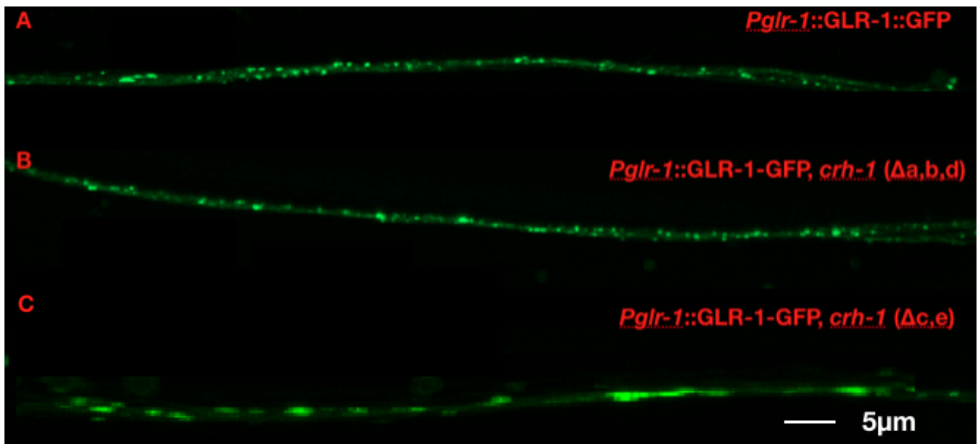

Figure 4.7: Confocal images of postsynaptic expression of glr-1 receptors in the posterior ventral nerve cord visualised with GLR-1::GFP

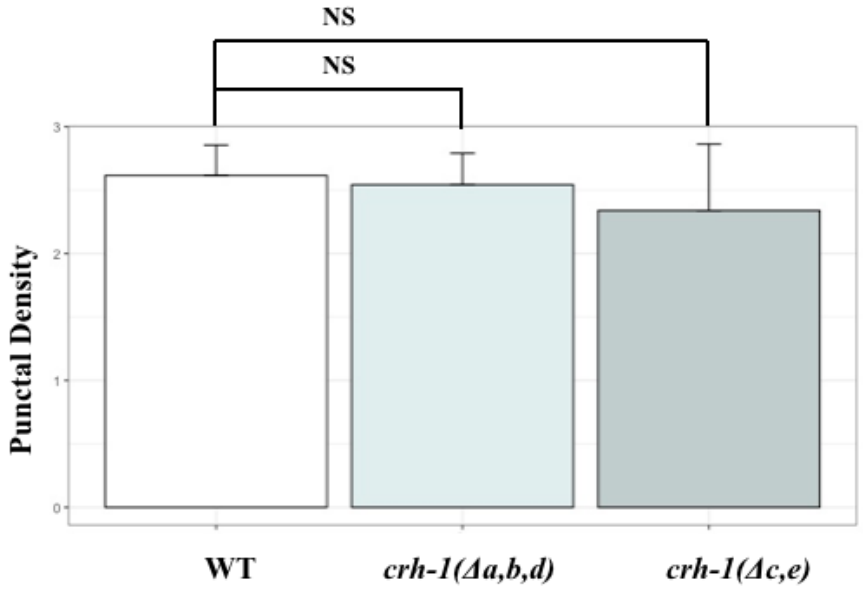

Figure 4.8: Quantification and comparison of punctal area of strains with differential isoform deletion (Mean + SEM).

sion of GLR-1::GFP in the ventral cord of wildtype worms and mutants worms through two proxies: punctal density (number of puncta/10 micrometer) and punctal area. We observed no significant difference in punctal density between the

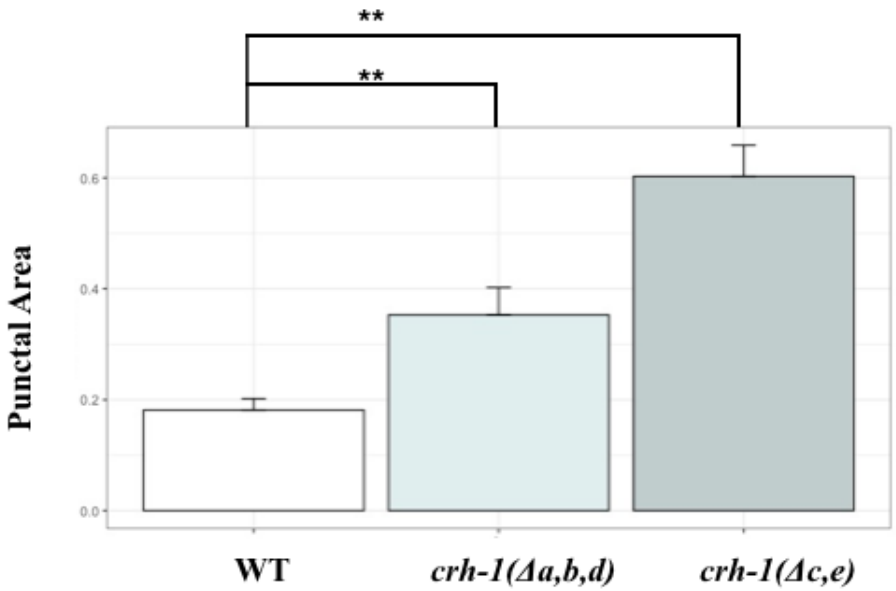

Figure 4.9: Quantification and comparison of punctal density of transgenics with differential isoform deletion (Mean + SEM).

wild type and the isoform specific mutants (Fig 4.8, Test 1: p = 0.7729, Test 2: p = 0.4555, Mann-Whitney U-Test) but *crh-1*(Δc,e) mutants show increased punctal area when compared to the wildtype worms (Fig 4.9, $p < 0.005$, Mann-Whitney U-Test). *crh-1*(Δc) and *crh-1*(Δe) show significant increase in punctal area are compared to the wildtype, but not as drastic as *crh-1*(Δc,e) indicating that isoforms CRH-1c and CRH-1e might work in conjunction i.e. there might be redundancy in function (Data not shown). The increase in punctal area can be due to an increase in receptor density or improper receptor tethering which could be responsible for the memory defects in *crh-1*(Δc,e). Since the levels of glr-1 mRNA in *crh-1* mutants were not significantly different from wildtype worms (data not shown), we propose the latter mechanism is more probable.

5 Discussion

C. elegans CREB homolog, CRH-1 has been important for long term memory formation in different paradigms (Amano and Maruyama, 2011; Lakhina et al. 2015). In this study, we show that CRH-1 isoforms CRH-1c and CRH-1e are necessary for memory formation. Along with memory formation, CRH-1 seems to have other homeostatic functions that mediate chemotaxis.

crh-1(tz2) mutants have a deletion mutation in the leucine zipper region, hence they don't produce functional forms of CRH-1 protein. They show innate chemotaxis defects and learning defects. Complementing Yogesh's preliminary results, our experiments show that isoform *crh-1(∆a,b,d)* mutants don't show learning defects but *crh-1(∆c,e)* mutants showed naive and learning defects. We hypothesise that CREB isoforms CRH-1a, CRH-1b and CRH-1d might be involved in homeostatic functions. This is re-enforced by the fact that the mRNA levels of *crh-1* isoforms CRH-1a, CRH-1b and CRH-1d are each greater in magnitude than CRH-1c and CRH-1e put together. We propose that CRH-1 isoforms function in two different neuronal modules: one for innate odour responses and other for associative learning of complex odorous information. Kobayakawa has demonstrated the existence of similar binary module system in mice (Kobayakawa et al. 2007).

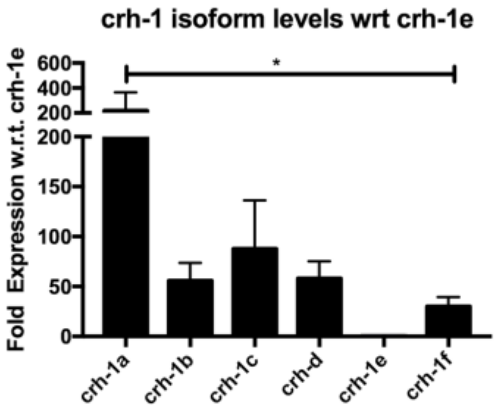

Figure 5.1: Relative mRNA levels of the crh-1 isoforms in *C. elegans*. Adapted from Yogesh Dahiya.

CREB's role in neuronal processes not related to learning and memory, have been studied thoroughly in other species. CREB has been shown to regulate transcription of a wide variety of genes including those involved in cellular metabolism, signal transduction, growth and neurotransmission (Lonze et al. 2002). CREB's crucial role in development has been shown through genetic studies. CREB null mice display severe growth defects in sensory neurons of the dorsal root and trigeminal ganglia along with important regions like corpus collosum and the anterior commissure (Bonni et al. 1999; Riccio et al. 1999; Walton et al. 1999). Drosophila Rutabaga mutants that are defective in the same pathway show defective neuro-muscular development (Chen et al. 2012). Several studies have highlighted CREB's role in neuronal outgrowth and differentiation (Redmond et al. 2002), circadian rhythms (Gau et al. 2002) and protection against harmful stimuli (Deak et al. 1998; Wiggin et al. 2002).

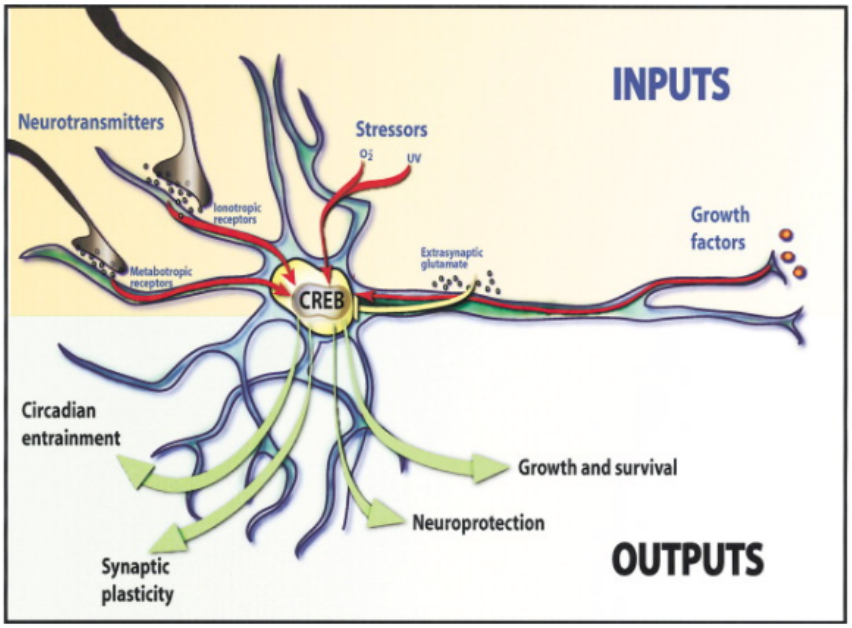

Figure 5.2: Schematic displaying various inputs of CREB and its function. Adapted from Lonze et al. 2002.

The striking caveat of our study is that we are not able to address which of the above processes is affected in *crh-1* mutants along with memory formation.

To confirm sensory processing defects, we could look at morphology of sensory neurons which can be observed through Dio Staining or quantify neural activity through GCaMP calcium indicators. To address defects in synapse formation, we can express of the synaptic vesicle marker synaptobrevin::GFP in neurons of interest or look at ultrastructure using electron microscopy (Baran et al. 2010). Rescue experiments have shown that expressing *crh-1* only in a subset of interneurons (AVA, AVD, AVE, and PVC) could rescue defects in memory formation. It would be interesting to look at gross morphology of these specific interneurons using markers and their activity. Given *crh-1* expression is found in multiple layers of neurons, a combination of these effects are possible.

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7 Appendix

7.1 Strains

Strains	Genotype
BAB709	*crh-1(Δa,b,d)*
BAB710	*crh-1(Δc,e)*
BAB504	glr-1p::GLR-1::GFP
BAB756	glr-1p::GLR-1::GFP; *crh-1(Δa,b,d)*
BAB757	glr-1p::GLR-1::GFP; *crh-1(Δc,e)*

Table 1: List of Strains