Chapter II

Evaluation of memory in Caenorhabditis elegans against bacterial pathogens
Learning and memory are closely associated concepts that are indispensable for human beings. Learning is a process that helps to acquaint to the environment. Continuous learning process leads to memory retention. However for a memory system to function properly it has to undergo vigorous training. The capacity of memory system is also judged by the how long it can store information (both inconsistent and consistent) that has been learned (Patil et al., 2016). The choice of preferring an environment in vertebrate and invertebrate model depends on the memory it retains about that environment. As a consequence of experiences, animals change their behaviour pattern according to the changing environmental cues. This alters the response of animals to various conditions they are present in, which in turn benefit their survival. Hence, memory and learning are deliberated as related phenomena. Understanding the principles of such behavioral plasticity presumably leads to constructive inspirations in the field of neurobiology (Hasani et al., 2017). The simple nervous system of C. elegans, which comprises of 302 uniquely identifiable neurons, provides an opportunity to investigate behavioural changes in perspective with the memory. The mechanism of such behavioral plasticity is an important issue in neurobiology and is often investigated by using model organisms with a simple nervous system such as the nematode Caenorhabditis elegans (Fenk and Bono, 2017; Torayama et al., 2007; Tomioka et al., 2006). C. elegans feeds on bacteria and has sensory receptors to detect odors, pheromones, noxious chemicals and pathogenic bacteria (Peckol et al., 2001; Kauffman et al., 2010). Besides this, the nematode also set forth behavioral changes through its developmental process. In addition, C. elegans navigates its environment through neuron-specific detection of pathogenic/ non-pathogenic bacteria they encounter. Erstwhile, reports suggest that chemotaxis behaviour of C. elegans largely depend on the olfactory and chemosensory neurons to determine the pathogenic nature of the bacteria (Troemel et al., 1997; Meisel et al., 2014). Also, C. elegans is considered to be a convenient model to
determine various behavioral parameters related to memory. The memory storage of *C. elegans* depends upon how well they have been trained/ habituated to an environment; the memory capacity is therefore classified into two types, short term and long term memory (Ardriel and Rankin, 2010). Other than metabolism and longevity, cyclic AMP response element binding protein (CREB) was also shown to be essential for most long term memories and was found to be conserved across many species (Bernabeu et al., 1997; Josselyn 2005; Kauffman et al., 2010). *crh-1* gene which is a homolog of CREB attributes to long term memory in *C. elegans*. CREB was the very first transcription factor that was regulated as a result of phosphorylation. They induce expression of genes from promoters containing the cAMP response element (CRE) enhancer and through certain growth factor signals (Mayr and Montminy, 2001). CREB has been presumably shown to work as intracellular second messenger and marshals signals to downstream regulators. It is also shown that the neurotransmitters such as dopamine and octopamine modulate the CREB at molecular level (Suo et al., 2006). Dopamine was thought to involve in sensing bacteria inside the nematode. The neurotransmitters dopamine and octopamine are of the same pathway, wherein, activation of dopamine leads to inhibition of CREB through octopamine signaling. However, the absence of dopamine signaling leads to initiation of CREB transcription factor (Suo and Ishiura, 2013). Among all these, the cholinergic neurons were speculated to involve in the memory storage of the nematodes. Acetylcholine (ACh) was the first cholinergic molecule to be ascertained as neurotransmitter and has been related with nematode neuromuscular movement (Del Castillo et al., 1967). ACh induced the CREB by modulating the dopamine level thereby helping in spatial learning (Lakhina et al., 2015). Aldicarb generally causes neuromodulation by inhibiting the enzyme AChE. The accumulation of ACh at the synapse leads to false positive results because of enzymatic hydrolysis of AChE. This phenomena
causes paralysis and as well disturb other physiological and behavioural activities of the nematode (Mahoney et al., 2006; Dabbish and Raizen 2011).

With this milieu, the present study aims to detail the role of *crh-1* in the nematode’s short term memory storage. The nematode’s memory of a pathogen (an opportunistic pathogen *Proteus mirabilis* and a virulent pathogen *Staphylococcus aureus*) which is exposed with a given training period was assessed. The current study observes for the first time, CREB is needed for short term memory. Further, the memory mechanisms involve activation of neurotransmitter and differentiate the pathogen from non-pathogen. The observations further present *C. elegans* as an impeccable model to study short term olfactory associative memory through molecular studies. The study also demonstrates that mutation in *crh-1* leads to the loss of memory in host even after following a conditioning paradigm. The impact of neuromodulation was analysed in this work by introducing a carbamate molecule, aldicarb and the disturbances caused in the acetylcholine signaling.

![Figure 2.1. The training/conditioning protocol followed for the study.](image-url)
MATERIALS AND METHODS

Nematode cultivation

*C. elegans* were grown at 20 °C in NGM seeded with *E. coli* OP50 using standard methods *(Brenner, 1974)*. Nematodes were synchronized by hypochlorite solution and tested for learning and memory at L4 stage. The maintenance of *C. elegans* was done as described earlier in the Chapter I

Nematode and bacterial Strains

Wild type: (N2 Bristol); mutant strains: YT17 (*crh-I(tz2)* III) were obtained from the CGC, (University of Minnesota, Minneapolis, MN). *S. aureus* (ATCC 11632) and *P. mirabilis* (ATCC 7002) were obtained from the American Type Culture Collection (ATCC). The *E. coli* OP50 (laboratory food source of *C. elegans*) was obtained from CGC. All assays were performed with a constant bacterial inoculum (0.3 OD at 600 nm) i.e 12 x 10^9 cells L^{-1} for *P. mirabilis* and 18 x 10^9 cells L^{-1} for *S. aureus*.

Short term conditioning of nematodes with pathogens

The L4 stage nematodes were allowed to swim in M9 buffer for 2 h soon after it was washed thoroughly from NGM plates. The same sets of nematodes were subjected to *S. aureus* conditioning (20% inoculum) for 2 h. Subsequently, the nematodes were transferred to *E. coli* OP50 (20% inoculum) for 2 h and again to *S. aureus* for 2 h. The same set up was followed for the *P. mirabilis* conditioning too. To avoid any chronic infection related stress during the assay, the conditioning period of nematodes with pathogens was limited to 2 h with a single interval of *E. coli* OP50 exposure. The control nematodes were exposed to *E. coli* OP50 continuously for 8 h as the conditioning time of nematodes with the pathogens totally covered 8 h. This procedure was followed for all the assays performed in this study.
Chemotaxis Assay

The cultures of *P. mirabilis* or *S. aureus* and *E. coli* OP50 were spotted at an equal distance from the center of NGM plates (60 mm). Approximately twenty wild-type or *crh-1* mutant *C. elegans* were washed from the *E. coli* OP50 lawn and conditioned as mentioned before were placed at the center of the plate. Another set of nematodes were treated with aldicarb and subsequently conditioned with pathogens for short time were also taken for the assays and were placed in the center of the plate. The number of nematodes in zone A and zone B were calculated after 0.5, 1, 1.5 and 2 h intervals. The assay included controls in which both zone A and zone B were spotted with OP50. Chemotaxis index was calculated as (number of nematodes at test - number of nematodes at control) / total number of nematodes assayed. Each assay was done in triplicates.

Nematode Survival assay

Survival assay was performed by raising the *crh-1* mutant nematodes to synchronized age by bleaching and were allowed to develop till L4 stage. In a 24 well culture plate containing the pathogen *P. mirabilis* or *S. aureus* (20 % inoculum) twenty number of age-synchronized young adult nematodes were transferred. The nematodes that were exposed to *E. coli* OP50 alone served as control. The plates were incubated at 20°C and life-span changes were monitored for every 6 h during the assays. Nematodes were scored as dead when they ceased pharyngeal pumping and did not respond to prodding with a platinum wire. The assays were carried out in triplicates.

Gene expression analysis

The control and conditioned samples of *C. elegans* were washed with M9 buffer to remove adhered bacteria and were flash frozen for gene expression analysis. Total RNA and cDNA synthesis were performed as previously described by Durai et al., 2014. Quantification
of expression of each gene was performed in a single well format using 96-well standard PCR plates in which C. elegans candidate gene-specific primers and the primers for housekeeping gene (β-actin) with their qPCR mix (SYBR Green kit, Applied Biosystems Foster City, CA, USA) were combined separately at a predefined ratio. The reaction was ensured to lie within the linear range of amplification of the respective genes and the PCR cycle number (40 cycles) was titrated by following manufacturer’s protocol. The steady-state levels of candidate genes mRNA levels were assessed from the cycle threshold (Ct) values relative to the Cq values of β-actin of the respective samples. The expression levels were calculated using the values obtained from software (Applied Biosystems 7500) and comparative $2^{-\Delta \Delta Ct}$ method was used to represent the relative expression level of the gene. Gene expression assays were performed in triplicates for at least three biological replicates.

Table 2.1: List of primers used for the study

<table>
<thead>
<tr>
<th>S. No</th>
<th>Gene name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-actin</td>
<td>ATCGTCCTCGACTCTGGAGATG</td>
<td>ACGTCCAGCCAAGTCAAG</td>
</tr>
<tr>
<td>2</td>
<td>crh-1</td>
<td>TCCAATCACCTGACTCCCTCT</td>
<td>TCCACTGAGGCTCCCCGTA</td>
</tr>
<tr>
<td>3</td>
<td>egl-30</td>
<td>TCAGAAAGGCGGAAGTGGAT</td>
<td>GCTTCTCGGTGTCACACTCG</td>
</tr>
<tr>
<td>4</td>
<td>goa-1</td>
<td>TGTTCGATGTGGGAGGTCAA</td>
<td>TCGTGCATTCGGTTTGGT</td>
</tr>
<tr>
<td>5</td>
<td>tbh-1</td>
<td>ATGAGAAGTGGCGTTGCTCTTC</td>
<td>TTACTCATAATTATCATATTC</td>
</tr>
<tr>
<td>6</td>
<td>tax-6</td>
<td>TGGGGCACTCATAATCTGC</td>
<td>GGGGCTCTGGAATCTGT</td>
</tr>
</tbody>
</table>

Aldicarb sensitivity assay

Aldicarb sensitivity assay was performed by fostering the crh-1 mutant nematodes to synchronized age by bleaching and were allowed to develop till L4 stage. Twenty numbers of L4 stage nematodes were transferred to a 24 well culture plate containing the aldicarb (Supelco analytical, Bellefonte, USA) with different (10, 15 and 20 µM) concentrations in a liquid medium both in the presence and absence of food. The plates were incubated at 20 °C
and life-span changes were monitored every half an hour for a 6 h period during the course of assays. Nematodes were scored as paralysed when they cease the thrashing movement. The assays were carried out in triplicates.

**Immunodetection**

The nematodes (wild-type N2 and *crh-1* mutants) that were conditioned with *S. aureus* and *P. mirabilis* strains were sonicated briefly in solution phosphate buffered saline (PBS) containing protease inhibitor cocktail (Sigma St. Louis, MO, USA). The supernatant containing the total proteins was estimated by Bradford’s method using Biorad protein assay kit (Bio Rad Inc.). 60 µg of protein sample was loaded and separated by 12% SDS-PAGE and transferred to a polyvinylidenedifluoride (PVDF) membrane (Whatman, GE Healthcare, Chicago, IL, USA). The membrane was then probed with a 1:2000 anti-p-CREB (SAB4504375; Sigma) and β-actin (A1978; Sigma) as internal control at 4 °C for 6 h, and then with an alkaline phosphatase (AP) -conjugated secondary antibody with 1: 1000 for 4 h at 4 °C. Finally, the membrane was developed using solution containing substrate nitro-blue tetrazolium (NBT) (HiMedia Laboratories, Mumbai, India) and 5-bromo-4-chloro-3’-indolylphosphate (BCIP) (HiMedia Laboratories) until intense violet bands appeared in the membrane.

**Measurement of acetylcholine esterase level**

The activity of acetylcholinesterase (AChE) was measured by the methods described by Ellman et al., 1961). Briefly, aldicarb was exposed (not more than 5 minutes) to nematodes and conditioned with the *S. aureus* and *P. mirabilis* separately. The nematodes were washed thoroughly with M9 buffer and homogenized with Phosphate buffered saline (pH 7.4). The supernatant was collected by centrifuging the homogenate at 10,000 rpm for 10 min. Accurately, 250 µg of protein was estimated using Bradford's protein estimation method.
and was added together with 200 µl of 156 mM 2,5-dithio-bis-2-nitrobenzoic acid (DTNB) and 40 µl of 156 mM acetylthiocholine iodide (ATCI). The change in absorbance was measured spectroscopically at an OD of 405 nm. The enzyme activity was calculated based on molar extinction coefficient at 13,600 M⁻¹ cm⁻¹.

RESULTS

**Conditioning of N2 with pathogens stores memory for short time**

The short term memory of nematode after conditioning with two different pathogens was assessed by the chemotaxis analysis. The N2 nematodes were conditioned with *S. aureus* and *P. mirabilis* separately and then placed in the centre of the plate. The movement of the nematodes was monitored for every half an hour up to 2 h. Since earlier reports from this lab suggested that there was no mortality of nematodes even after continuous exposure of *S. aureus* and *P. mirabilis* up to 8 h the assays were carried out with an inoculum volume of 20% (Jebamercy et al., 2011; Jebamercy et al., 2013). In both OP50/*S. aureus* spotted plate and *S. aureus/*P. mirabilis spotted plate the N2 nematodes that were conditioned with *S. aureus* migrated towards the same pathogen (Fig 2.2A). In the similar way, the N2 nematodes that were trained with *P. mirabilis* preferred *P. mirabilis* over OP50 in both OP50/ *P. mirabilis* spotted plate and *S. aureus/*P. mirabilis spotted plate (Fig 2.2B). This inclination towards the conditioned pathogen implies that the nematode might possess memory of the conditioned pathogen. However, the memory retention does not last long and was found to decline after 2 h restricting it to a short term. Lau et al., 2012 study also supported that habituation to tap stimuli exhibited short term memory induced by chemosensory conditioning.
Figure 2.2. Chemotaxis index of wild type nematodes: (A) Conditioned with *S. aureus* in OP50/ *S. aureus* and *S. aureus/ P. mirabilis* plates. (B) Conditioned with *P. mirabilis* in OP50/ *S. aureus* and *S. aureus/ P. mirabilis* plates.

CREB mutant can resist pathogen up to conditioning time (8 h)

CREB is a transcriptional factor that a nuclear protein that modulates the transcription of genes with cAMP responsive elements and regulates neural plasticity from invertebrates to mammals (Kandel and Tauc, 1965; Silva et al., 1998). Prior to the conditioning assay, the CREB (*crh-1(tz2)*) mutants should be subjected to pathogens and checked for the survival. Hence, the survival and the time taken for the mutant nematodes to surrender itself to the pathogen were assessed before it was taken for chemotaxis analysis.

The complete killing of N2 nematodes was achieved at 96 h when exposed to *S. aureus*. Meanwhile, the (*crh-1(tz2)*) mutant nematodes exposed to *S. aureus* attained LT$_{50}$ at 24 ± 3 h and the complete killing was achieved at 72 ± 3 h. On the other hand the (*crh-1(tz2)*) mutant nematodes exposed to *P. mirabilis* and *E. coli* OP50 had 80 % survival at 72 h (Fig.
This result indicated that \textit{crh-1(tz2)} mutants were resistant to \textit{S. aureus} and \textit{P. mirabilis} for at least until 8 h. Based on this result, the effect of conditioning on \textit{crh-1(tz2)} mutant nematodes was evaluated.

\textbf{Figure 2.3.} Survival graph of \textit{crh-1(tz2)} mutant nematodes exposed to \textit{S. aureus} (\(P<0.05\)) and \textit{P. mirabilis}. Nematodes fed on \textit{E. coli} OP50 were considered as control. Data are presented as mean ± SD of three biological replicates. Statistical analysis was performed by one-way ANOVA followed by Duncan’s post hoc analysis.

\textbf{Conditioning did not alter the memory capacity of \textit{crh-1(tz2)} mutants}

The indulgence of CREB in memory storage was checked by chemotaxis analysis of \textit{crh-1(tz2)} mutant nematodes that was performed similar to N2. Earlier study indicated that defects in function of \textit{crh-1}, affects the BAG neurons that contribute to changes on the behaviour (Rojo Romanos et al., 2017). Yet another report confine that mutation in \textit{crh-1} has reduced responses to tap when compared to N2 nematodes (Timbers and Rankin, 2011). In the present study, chemotaxis analysis with \textit{S. aureus} conditioned \textit{crh-1(tz2)} mutant nematodes thoroughly avoided \textit{S. aureus} in both OP50/\textit{S. aureus} and in \textit{S. aureus} /\textit{P. mirabilis} (Fig 2.4A). In opposition, \textit{P. mirabilis} conditioned \textit{crh-1(tz2)} mutant nematodes
moved towards the *P. mirabilis* in both the cases (Fig. 2.4B). The probable reason could be that mutation in *crh-*1 gene failed to store the memory thereby displaying an aversive response towards virulent pathogenic *S. aureus* while in the case of *P. mirabilis*, it was contradictory owing to the character of opportunism. Thus, *C. elegans* with a mutation in *crh-*1 does not possess short term memory after the conditioning.

**Figure 2.4.** Chemotaxis index showing *crh-*1(tz2) mutant nematodes (A) conditioned with *S. aureus* in OP50/ *S. aureus* and *S. aureus*/ *P. mirabilis* plates. (B) Conditioned with *P. mirabilis* in OP50/ *P. mirabilis* and *S. aureus*/ *P. mirabilis* plates.

**CREB crucial for short term memory**

The presumptive cascade members (*crh-*1, *egl-*30, *goa*-1, *tbh*-1 and *tax*-6) involved in the learning and memory of *C. elegans* were evaluated. There is already a report that long term memory of *C. elegans* was induced by CREB activity (Lakhina et al., 2015). So, the first and foremost analysis was done for *crh-*1 gene expression level in both N2 and *crh-*1 mutant nematode to weigh the importance of the CREB’s role during short term memory in regard with pathogens. There was a significant increase (***P < 0.005) in *crh-*1 gene in both the *S. aureus* conditioned N2 and *P. mirabilis* conditioned N2 (**P < 0.005) from the *E. coli* OP50
basal level. The same gene exhibited a down regulation in \( S. \text{aureus} \) conditioned \( crh-1 \text{tz2} \) (Fig 2.5). In the \( P. \text{mirabilis} \) conditioning, \( crh-1 \text{tz2} \) nematodes showed up regulation of \( crh-1 \) gene (Fig 2.5), \( egl-30 \) and \( goa-1 \) encode Gqα and Goα subunits respectively which is a part of G protein coupled receptor (GPCR) (Matsuki et al., 2006). Both the genes help in olfactory conditioned stimulus (Tomioka et al., 2006). The \( S. \text{aureus} \) conditioned and \( P. \text{mirabilis} \) conditioned N2 have showed increased regulation of \( egl-30 \) when compared to the \( S. \text{aureus} \) conditioned and \( P. \text{mirabilis} \) conditioned \( crh-1 \text{tz2} \) mutant nematodes (Fig 2.5). Noteworthily, \( goa-1 \) expression was down regulated in both \( S. \text{aureus} \) conditioned N2 (**\( P<0.005 \)) and \( P. \text{mirabilis} \) conditioned N2 (**\( P<0.005 \)). Similar kind of regulation was observed in in \( crh-1 \text{tz2} \) mutant nematode too (Fig 2.5). These data revealed that \( crh-1 \) probably express under the influence of \( egl-30 \), whereas \( goa-1 \) are inversely proportional to each other.

There is a great impact of neurotransmitter in the regulation of CREB (Suo and Ishiura, 2013). \( tbh-1 \) gene is required for the synthesis of octopamine and falls under the biogenic amine neurotransmitter category (Alkema et al., 2005). Both \( S. \text{aureus} \) conditioned N2 (**\( P<0.005 \)) and \( P. \text{mirabilis} \) conditioned N2 (**\( P<0.005 \)) showed an up regulation of \( tbh-1 \) mRNA. In addition to it, down regulation of \( tbh-1 \) in \( S. \text{aureus} \) and \( P. \text{mirabilis} \) conditioned \( crh-1 \text{tz2} \) mutant nematodes was observed suggesting that amine neurotransmitters are essential for the activation of CREB (Fig 2.5). Calcium signaling cascade is necessary for the CREB activation and memory retention, therefore level of \( tax-6 \) mRNA was analysed (Mair et al., 2011). \( tax-6 \) also plays an important role in behavioural memory (Li et al., 2015). Lakhina et al., 2015 has also reported that there is an increase in the calcium signaling upstream CREB in long term associative memory. As expected, there was a steep increase in the regulation of \( tax-6 \) in \( S. \text{aureus} \) conditioned N2 (**\( P<0.005 \)) and a down regulation in \( S. \text{aureus} \) conditioned \( crh-1 \text{tz2} \) mutants, wherein, \( P. \text{mirabilis} \)
conditioned N2 (*P< 0.005) and *P. mirabilis* conditioned crh-1(tz2) (*P< 0.005) showed no regulation in the former and down regulation in the later one (Fig 2.5). These results altogether imply that *egl-30, tbh-1* and *tax-6* might act as positive regulator of *crh-1* and inversely; it was likely that down regulation of *goa-1* could activate *crh-1*.

![Figure 2.5](image)

**Figure 2.5.** Relative mRNA expression level of genes involved in memory retention of nematodes (N2 and *crh-1(tz2)*) after conditioning with *S. aureus* and *P. mirabilis*. The expression of the candidate genes were normalized over the relative expression of housekeeping gene, β-actin. Data are presented as Mean (±s.d) of three biological replicates and the level of significance was analyzed by one-way ANOVA followed by Duncan's post hoc analysis (*P< 0.05, **P< 0.01, ***P< 0.005).

CREB expression requires phosphorylation events for activation

In humans phosphorylation of CREB at ser-133 position leads to activation of this transcriptional factor. Analogous to the reports by Mayr and Montminy, 2001; Sakamoto et
al., 2011, it is predicted that CREB gets phosphorylated and activate other transcriptional factors associated with it. It is also determined that CREB phosphorylation at ser-133 position majorly contributed to its activation. The detection of phosphorylated level of CREB in all the experimental conditions were performed. The presence of p-CREB was observed in both *S. aureus* and *P. mirabilis* conditioned N2, wherein the band was below detectable level in *crh-1(tz2)* mutant nematode conditioned with *S. aureus* and *P. mirabilis* separately (Fig 2.6).

![Western blot](image)

**Figure 2.6.** (A) Western blot depicting the regulation of p-CREB proteins in wild type and *crh-1(tz2)* mutant nematodes conditioned with *S. aureus* and *P. mirabilis*. β-actin was used as a loading control

**Mild level of aldicarb is toxic to N2 and *crh-1(tz2)* mutant nematodes**

Aldicarb is an acetylcholinesterase inhibitor which causes deleterious effects to the host when exposed over an extended period of time (Mulcahy et al., 2012). Acute aldicarb exposure leads to time-course or dose-responsive paralysis in wild-type worms (Locke et al., 2008). The general notion that dopaminergic neurons are affected by aldicarb exposure through acetylcholine signaling was visualized. The sensitivity of N2 and *crh-1(tz2)* mutant nematodes towards different concentrations (10, 15 and 20 µM) of aldicarb in the presence and absence of *E. coli* was checked. At 20 µM concentration, complete paralysis of N2 was
achieved nearly at 300 min both in the presence and absence of food source (Fig 2.7A). Whereas, *crh-1*(tz2) mutant nematode attained paralysis at 270 min (30 min earlier when compared to N2) at 20 μM concentration of aldicarb in the absence of food source and paralysed at 240 min (60 min earlier when compared to N2) in the presence of food source (Fig 2.7B). Out of all the concentration 20 μM was found lethal to the nematodes.

**Figure 2.7. Paralysis graph of nematodes (N2 and *crh-1* (tz2)) after exposure of aldicarb**

**Aldicarb disturbs CREB activity through neurotransmitter signaling**

The influence of aldicarb on the memory retention was examined by chemotaxis analysis. The nematodes were found to move towards *S. aureus* up to 1 h then had an aversive response in OP50/*S. aureus* plate, whereas, the nematodes avoided *S. aureus* completely in *S. aureus*/P. *mirabilis* plate (Fig 2.8A). Opposing to the results obtained in *S. aureus* conditioned N2 plate, the *P. mirabilis* conditioned N2 preferred *P. mirabilis* in both OP50/*P. mirabilis* plate and *S. aureus*/P. *mirabilis* plate (Fig. 2.8B). Parallel to this, the chemotaxis assay was carried out in *crh-1*(tz2) mutant too, wherein, the *S. aureus* conditioned *crh-1*(tz2) showed attraction towards OP50 in OP50/ *S. aureus* and *S. aureus*/P. *mirabilis* plates (Fig. 2.8C). However, *P. mirabilis* conditioned *crh-1*(tz2) exhibited almost the same result as in *P. mirabilis* conditioned N2 (Fig. 2.8D).
These inconsistent results described above imply that aldicarb modifies the memory of nematode by probably modifying the acetylcholine signaling and intended us to quantify the levels of AChE. *S. aureus* conditioned *crh-1(tz2)* was observed to have maximum inhibition of AChE postulating that neuronal signaling is abrogated (Fig. 2.9). As expected, the aldicarb unexposed samples showed a very minimal level of AChE inhibition (Fig. 2.9).

**Figure 2.8.** Chemotaxis index showing (A) N2 nematodes exposed to aldicarb and subsequently conditioned with *S. aureus* in OP50/ *S. aureus* and *S. aureus/ P. mirabilis* plates. (B) N2 nematodes exposed to aldicarb and subsequently conditioned with *P. mirabilis* in OP50/ *P. mirabilis* and *S. aureus/ P. mirabilis* plates. (C) *crh-1(tz2)* mutant nematodes exposed to aldicarb and subsequently conditioned with *S. aureus* in OP50/ *S. aureus* and *S. aureus/ P. mirabilis* plates. (D) *crh-1(tz2)* mutant nematodes exposed to aldicarb and subsequently conditioned with *P. mirabilis* in OP50/ *P. mirabilis* and *S. aureus/ P. mirabilis* plates
Figure 2.9. The column chart represents percentage of AChE inhibition in the conditioned nematodes with prior exposure to aldicarb

DISCUSSION

Hitherto, it was thought that *C. elegans* lacks adaptive immunity, yet, as a substitute, innate immune system comes into action whenever there is an exposure of potential pathogenic encounter (Aballay, 2013). For any organism to remember the previous pathogenic exposure it should have a proper memory storage system. Remembrance and forgetting are two separate entities that are subdivided under the basic phenomena called memory. The status of memory depends on how well an organism could learn particular information. Elimination of unnecessary memories which that are continuous and that are constantly changing is prone to be forgettable (Kraemer and Golding, 1997). The memory of *C. elegans* can be made stronger through habituation and therefore it is subdivided into, short term and long term memory (Ardriel and Rankin, 2010). Short term memories are those that are vulnerable and are forgotten within hours while long-term memories are renewable and
get stored for a prolonged period (McGaugh, 2000). Kandel and Tauc, 1965 was the first one to study about learning and memory in the marine mollusc Aplysia. This research has been further extended in Drosophila melanogaster and C. elegans (Quinn et al., 1974; Rankin et al., 1990).

*C. elegans* is a free-living, non-parasitic nematode that senses its environment through variety of environmental cues thereby altering the preference to stay around any chemical odors, temperature or a pathogen (Kauffman et al., 2010). The neuronal system of the nematode passes sensory neurons to motor neurons which in turn get reflected as chemotaxis behaviour (Miller et al., 2005). Therefore, *C. elegans* is considered to be one of the best models to evaluate various behavioural parameters related to memory.

Even though *C. elegans* innate immune system does not store the previous memory, it was speculated that training the host in a given environment would acquire and retain the memory for at least a limited period. In an effort to bring out the involvement of CREB in the memory process nematodes were conditioned with bacterial pathogens. For a nematode to survive it has to consume required quantity of food. When there is scarcity of food, the behaviour of the nematode changes and seeks for an alternate food source present in that environment. Different kind of stimulus and the number of trials the nematode undergoes in that stimulus determines the period of memory stored (Inoue et al., 2013). *C. elegans* in its natural environment come across multiple niches of bacteria. Moreover they have also been extensively studied for host pathogen relationship with single bacteria like Pseudomonas aeruginosa (Vigneshkumar et al., 2012), Shigella sp (Kesika et al., 2015) and Klebsiella pneumonia (Kamaladevi and Balamurugan, 2015).

Current studies unleash that training/conditioning *C. elegans* with *S. aureus*, makes the nematode migrate towards the pathogen when left in a chemotaxis plate (Fig 2.2A and B).
However, the attraction does not last longer than 2 h intimating that *C. elegans* carry short term memory against the pathogen it was conditioned in. Study done by Jebamercy et al., 2011 conveyed that N2 reaches LT$_{50}$ at 90 h, thus, conditioning the nematodes for 8 h would not bother the immune system of host much. Although the discrimination between pathogenic and beneficial microbes is essential for host to survive, vigorous training would alter the preferences accordingly. Associative learning has been reported in *C. elegans* which was pre-exposed to an odor and was attracted to the same when compared to the un-exposed ones (Colbert and Bargmann, 1995). While, another study by Sacki et al., 2001, suggested that, nematodes conditioned with NaCl exhibited avoidance to the conditioned cue.

Defects in *crh-1* gene affect the memory of *C. elegans* by perturbing the neural synapses (Nishida et al., 2011). In this context, the present study examined if *crh-1*(tz2) mutant nematodes could restrain the short term memory against conditioning with *S. aureus* and *P. mirabilis*. Preceding to that, it was made certain that *crh-1*(tz2) mutant nematode could survive over the conditioning time. As predicted, the results suggested resistance of *crh-1*(tz2) mutant nematode towards *S. aureus* by initiating the death of the nematode only after 12 h of continuous exposure. On the other hand, *P. mirabilis* exposure to the *crh-1*(tz2) mutant nematode initiates death only after 30 h (Fig. 2.3). Subsequently, *crh-1*(tz2) mutant nematodes were conditioned with *S. aureus* and *P. mirabilis* separately and put into the chemotaxis set up done similar to N2. The *crh-1*(tz2) mutant nematodes put forward the failure of the nematodes to recognize the pathogen (*S. aureus*) after conditioning (Fig 2.4A). Albeit the fact that *crh-1*(tz2) mutant nematodes moved towards *S. aureus* was considerable, the *P. mirabilis* failed to display same results (Fig 2.4B). The pathogenicity (opportunist) status of *P. mirabilis* could be the probable reason for this observation making it move towards the bacteria (JebaMercy et al., 2013).
Since the results obtained from chemotaxis assay seemed to propose the prominence of \textit{crh-1} gene, the contribution of \textit{crh-1} to short term memory of \textit{C. elegans} was assessed. To refine more insights at molecular level, genes responsible for the memory retention was analysed by q-PCR. To start with the role of \textit{crh-1} gene was assessed and was found to be up regulated and supports the chemotaxis results (Fig 2.5). CREB was already reported to produce increased expression for long term memory by Lakhina et al., 2015. Next, the transcript levels of the molecular targets that activate the CREB transcriptional factor were also assessed. \textit{egl-30} kindle production of diacylglycerol which enable neurotransmitter release (Lackner et al., 1999). It was also predicted that, release of acetylcholine from cholinergic motor neurons was performed by \textit{egl-30} (Bastiani et al., 2003). We observed an increase in relative mRNA fold of \textit{egl-30} and down regulation of \textit{goa-1}, an upstream regulator of \textit{egl-30} in \textit{S. aureus} conditioned N2 and \textit{P. mirabilis} conditioned N2 apparently specifies conflicting roles in CREB activation (Fig 2.5). \textit{goa-1} acts more often in serotonin signaling and decreases the profusion of diacylglycerol at nerve endings (Nurrish et al., 1999).

\textit{tbh-1} codes for tyramine beta hydroxylase which is analogous to epinephrine in vertebrates and this tyramine is synthesized by RIM neurons (Jin et al., 2016). \textit{tbh-1} aids in synthesis of octapamine neurotransmitter that can act as learning cues (Alkema et al., 2005). The transcript levels of \textit{tbh-1} was significantly higher in pathogen conditioned N2 and have decreased in pathogen conditioned \textit{crh-1(tz2)} mutant nematodes clearly representing that amine neurotransmitters are imperative for the stimulation of CREB (Fig 2.5). Other than that the \textit{tax-6} regulation was also investigated which exhibited a decreased regulation in \textit{crh-1(tz2)} mutant nematode conditioned with the pathogens when compared to N2 (Fig 2.5). Evidences show that \textit{tax-6} is very much essential for the calcineurin signaling that help in
thermotaxis behaviour and was expressed largely in AFD neurons (Hobert et al., 2003; Li et al., 2015).

Although it was agreeable that CREB at the transcript level interferes in the short term memory of nematode, the protein level data has to be scrutinized for a clearer theory. Hence, immunobloting technique was executed. The results clearly demonstrate the presence of p-CREB in the N2 conditioned with S. aureus and P. mirabilis and absence of the same in the crh-1(tz2) mutant nematode (Fig 2.6). Thus, the present study define there is connectivity between short term memory against pathogens and CREB. Given that CREB functions under the influence of neurotransmitters, further experiments were sought to identify the consequence of aldicarb, an AChE inhibitor on the memory of nematode. As well, it was also reported that maintaining the integrity of CREB relies on acetylcholine signaling (Suo and Ishiura, 2013). The aldicarb toxicity was checked prior to the behavioural assay. The observations suggested that 10 µM (~5 min of exposure time) of aldicarb was enough to arrest the motor neurons of the nematode in both presence and absence of food (Fig 2.7A and 6B). Concomitantly, the effect of aldicarb on the memory of nematode was also tested. Initially, S. aureus conditioned N2 preferred S. aureus and later it showed an aversive response (Fig 2.8A). By contrast, the P. mirabilis conditioned N2 chose P. mirabilis instead of OP50 (Fig 2.8B). crh-1(tz2) mutant nematodes also showed similar results (Fig 2.8C and D). The reason for the nematodes to have opted P. mirabilis is still remains unclear. As the above observations put forth that acetylcholine signaling to be the primary reason for the aversive nature of N2 and crh-1(tz2) mutant towards S. aureus, we intended to check the levels of AChE inhibition. It was found that in all the aldicarb exposed samples the AChE inhibition level was higher, hence weakening the acetylcholine signaling (Fig 2.9).
The current study unfolds the active participation of CREB in short term memory of nematode against pathogens. The intense understanding of the food/pathogen preferences reinforces the importance of CREB in being a link between neuronal circuits and behaviour. Overall, these findings advance the wealth of genetic resources available for study of \textit{C. elegans} in the neuro-immune behaviour and plasticity attached with it. In addition, this work opens up an exciting new field in which \textit{C. elegans} can be utilized to examine immune responses and the memory network in relation to the environmental cues and harmful pathogens.