"NMDA RECEPTORS MEDIATE ODOUR PREFERENCE ADAPTIVE LEARNING IN *Caenorhabditis elegans*"

A DISSERTATION SUBMITTED

BY

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DECLARATION

I, **Pratheesh. KV**, hereby declare that I had personally carried out the work depicted in the thesis entitled, **"NMDA RECEPTORS MEDIATE ODOUR PREFERENCE ADAPTIVE LEARNING IN *Caenorhabditis elegans*"** under the direct supervision of **Dr. Anoopkumar Thekkuveettil, Scientist G and Head, Molecular Medicine Division**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Science and Technology, Thiruvananthapuram, Kerala, India.

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CERTIFICATE

This is to certify that the dissertation entitled **"NMDA RECEPTORS MEDIATE ODOUR PREFERENCE ADAPTIVE LEARNING IN *Caenorhabditis elegans*"** is a bonafide work done by **Mr. Pratheesh. KV** in partial fulfilment for the degree of Master in Philosophy under my supervision and guidance at **Molecular Medicine Division**. Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Science and Technology, Thiruvananthapuram, Kerala, India.

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The Thesis entitled

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LIST OF FIGURES

TABLE OF CONTENTS

SYNOPSIS

Learning and memory are closely related to each other. Acquisition of knowledge can be termed as learning, while expression of the acquired knowledge is memory. Memory can be classified to be short term and long term, depends on how long it retains. It is very difficult to study this intricate pathways in higher animal models. The nematode *Caenorhabditis elegans* (*C. elegans)* is a perfect model to decipher the codes of neuro-biology. *C elegans* can sense and store memories as higher organisms does. In the present study we show *C. elegans* can sense and adapt to different volatile odourants namely butanone, benzaldehyde and isoamyl alcohol based on different paradigms of conditional stimulus. Among them starvation conditioned stimulus is more significant. *C. elegans* have the ability of distinguishing different odourants, and the selectivity is assessed through cross adaptation. It is well known that Ca^{2+} ions are essential part in many signaling pathways. In view of that, the role of calcium in olfactory adaptation was checked by blocking extracellular calcium ions and found that isoamyl alcohol adaptation is independent of calcium ion concentration. In addition, various calcium channels are participating in nervous system signaling pathways. Of these an important receptor is NMDA receptor. We selected nmr-1 and nmr- 2 subunit mutants of *C elegans* to find whether it affects worm adaptation towards odourants. Our results showed both NMDA receptor mutants are defective in adaptation and cross adaptation towards odourants we checked. It was believed that AWC sensory neurons are responsible for odourant sensing and adaptation. Perhaps result shows interneurons may also play some critical role in adaptation since nmr- 1 and nmr-2 are present only in interneurons and not in AWC neuron.

CHAPTER - 1

INTRODUCTION

1.1 LEARNING AND MEMORY

The concepts learning and memory are closely related and often overlaps. By definition, the acquisition of information is learning, and expression of the acquired information is memory. The whole process of learning and memory occurs in brain and there is huge body work has been conducted to decipher the processes involved. Human brain can recall learned information within a fraction of time, often faster than a super computer. Due to the promising leads in the process involved in synaptic plasticity and better understanding in neuronal connectivity in brain of both mammals as well as various model organisms, research in learning and memory got its momentum and has been one of the highly pursued area of research. (Okano et al. 2000).

Memory can be classified into two: short-term and long-term memory (Cowan et al., 2009). Short-term memory (or "primary memory") is the ability of brain for holding of information, for few minutes to hours. The extended memory which lasts for days to years is termed as long term memory. Based on types information stored, long term memory is again classified into two types: declarative memory and procedural memory (Gorpa et al 1999). For example, declarative memory consists of facts and events which can be consciously recalled. On the other hand, procedural memory are unconscious memories of acquired skills (example, riding a bike). Both these memories are independent because, patients having impaired declarative memory are found to have

normal procedural memory. Probably there must be independent mechanisms for each type of memory and also may be separate processing areas of brain. (Okano et al., 2000)

1.2 CONDITIONAL LEARNING

One of the interesting learning pathways is conditional learning, where one can create a conditioned reflex in an organism. The best example for conditional learning is the classical Pavlovian experiment. In this experiment, the dog was presented with food (Conditional Stimulus) first and sound of a bell (Unconditioned Stimulus). Salivation of dog was taken as the learning indication. When both CS and US was presented simultaneously, the dog learned to link the US with CS and start salivating when US alone was presented (Fig.1). This classical experiment brought the first evidence that

conditional responses are adaptive because they allow the animal to learn. (Mazur et al., 2001)

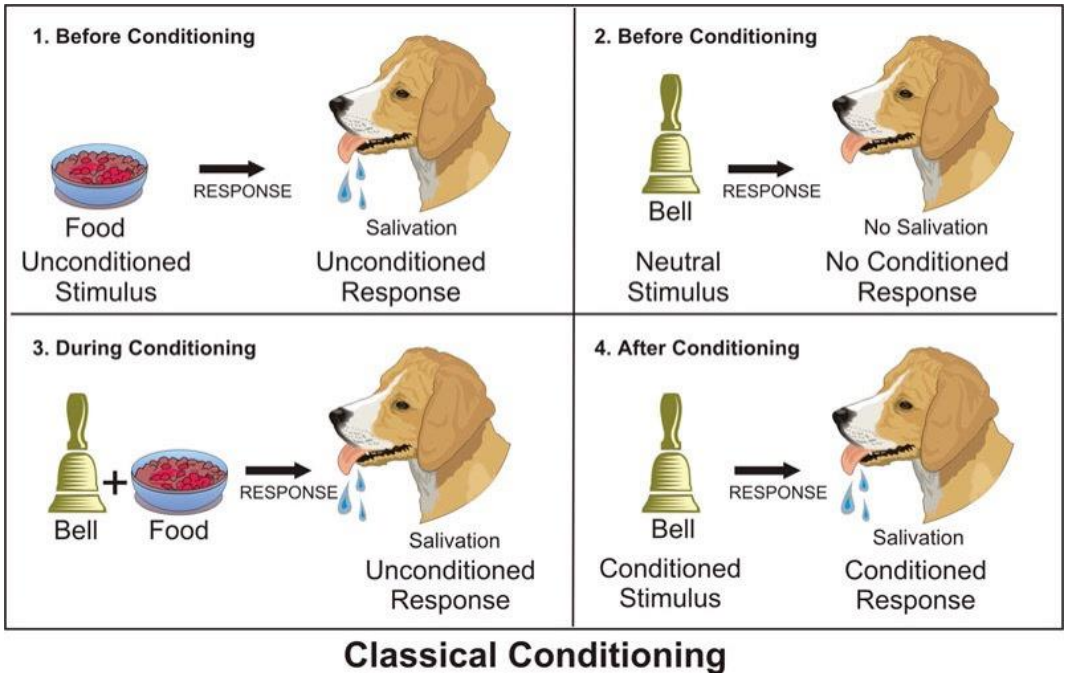

Figure 1: Classical conditioning - Pavlovian Experiment (https://oshepsyche.wordpress.com)

Conditional adaptation has been found in in various other model organisms (Borszcz, 1995). One of the easiest and very powerful model organism used for the studies of learning and memory are the soil nematode *C. elegans*.

1.3 C.ELEGANS AS A MODEL ORGANISM

In 1965, Sydney Brenner proposed research on *C. elegans* as a model organism. Major advantages of *C. elegans* are it has short life cycle, transparent body, small size (1.5-mm length), compact genome, ease of propagation and maintenance (Brenner S). The whole organism can develop into adult from egg in 3 days. The fecundity rate of the

organism is 300-350 progeny per animal, which makes it easy to propagate. Besides, both the genome and nervous system of this organism are well mapped.

Nervous system of *C. elegans* consists of 302 neurons. Considering the simplicity of the nervous system Sydney Brenner envisioned that the complete neural circuit could be determined by serial-section electron microscopy, and his vision was realized 20 years later (White *et al.*, 1986)

Being having a transparent body, position of each cells in the organism have been mapped (Sulston *et al.*, 1988). This also allows to modify the organism using various molecular biological techniques such as transposon-tagging, germ-line DNA transformation, laser microsurgery, and siRNA knockout of transcripts (Vander slice *et al.*, 1976)

1.4 *C. ELEGANS* LIFE CYCLE AND DEVELOPMENT

C. elegans have four larval stages starting from L1 to L4. It takes 8 hours to reach from egg to L1 stage. Developing from L1 to egg laying adult will take 2 days at 20^0C. There is an intermediate stage called dauer larvae, when the organism faces conditions like starvation it enters into this stage and can survive for few months without food. Once

all the environmental conditions favourable, dauer larvae will develop to L4 stage and then to adult. (Fig. 2)

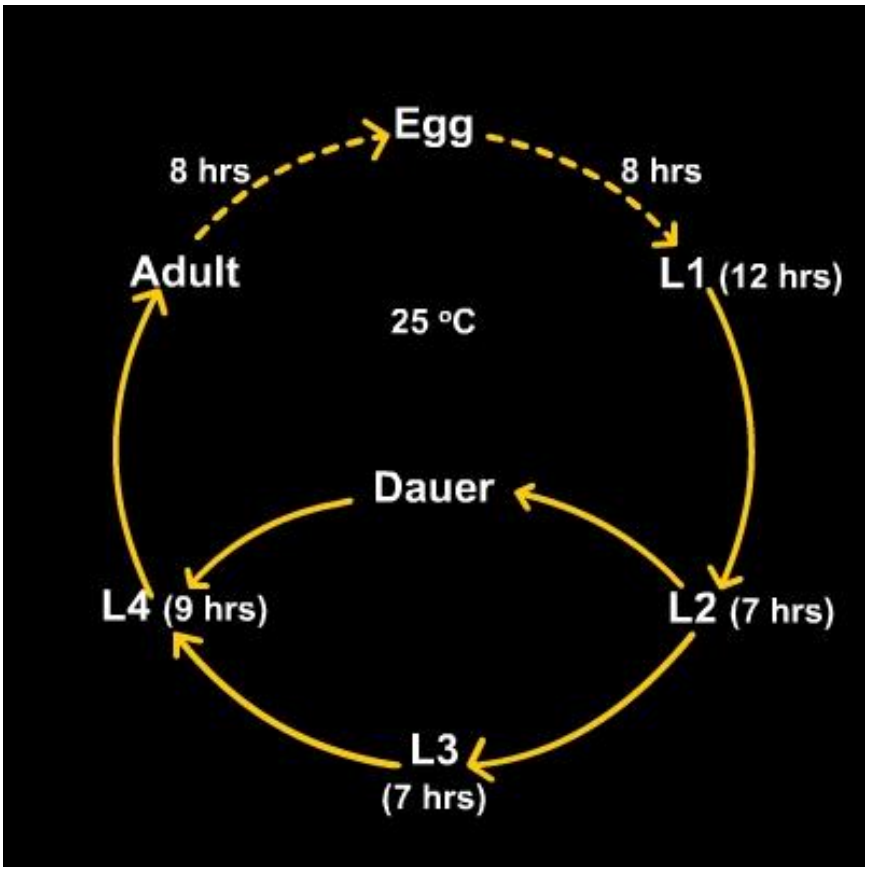

Figure 2: C.elegans life cycle (Wormbook.org)

This survival strategy of *C. elegans* has been extensively studied. One theory suggests that "*r*-selection versus *K*-selection," principle. According to this, reproduction of an organism is closely linked to environmental stability and mortality rates. In unfavourable conditions, which induces high mortality rates, then the organism favours *r*-selection (resulting in early reproduction and rapid development); on the other hand,

favourable environment allowing low mortality rates, favours *K*-selection (this results in slower development, longer life span and reproductive period. (Gray., 1988)

The nervous system of *C. elegans* consists of 302 neurons and 56 glial cells equal to 37% of the somatic cells in a hermaphrodite (Hobert 2005 wormbook)

Since the complete neuronal connectome of the organism has been mapped (White *et al*., 1986), it is one of the best model to understand behavioural response to external cues like the mechanosensation, chemo sensation etc. Besides, it is easy to tag Green Fluorescent Protein (GFP) with neuronal proteins and visualize the connectome of the organism.

1.5 ADAPTIVE LEARNING IN C ELEGANS

Adaptive learning in *C. elegans* is well established. For example, the organism can adapt to various olfactory stimuli, salts and tapping (Kano *et al.,* 2008, Bargmann *et al.,* 1995). These conditional learning patterns can be short term or long term depends on the training paradigm applied (Ardiel *et al.,* 2010). Among these, olfactory adaptation and learning are the strongest. Olfactory adaptation, whether acting as an attractant or repellent, is odourant specific (L'Etoile *et al.,* 2000). Among the 32 sensory neurons in amphid, phasmid and inner labial organs, the 12 amphid neurons (Fig.3) are associated with chemo or themo sensation (Ward *et al*., 1975; Ware *et al*., 1975). Among these amphid neurons, the AWC sensory neuron has been the one responsible for the detection of five odourants namely butanone, benzaldehyde, isoamyl alcohol,

diacetyl, and pyrazine (Bargmann *et al.*, 1995). Olfaction in *C. elegans* allows the organism to seek food and avoid the danger.

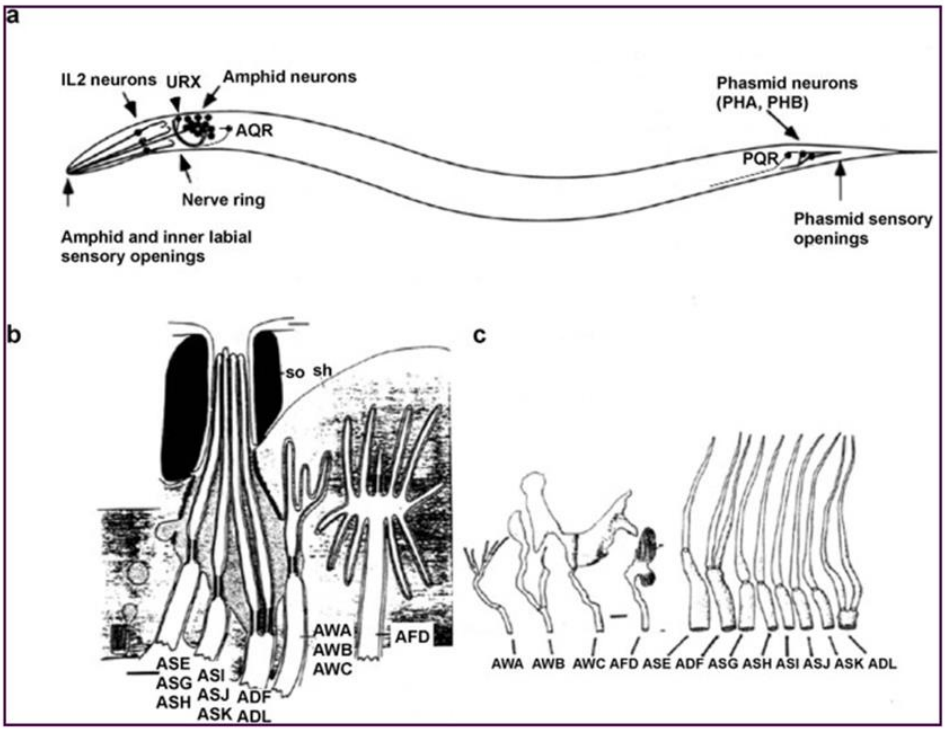

Figure.2. Structure of chemosensory organs in *C.elegans* (a).Arrangement of chemosensory neurons. Each of two amphids contains 12 associated chemosensory or thermosensory neurons. Each of two phasmids contains 2 chemosensory neurons, PHA and PHB. Six inner labial organs present each contains one IL2 chemosensory and one IL1 mechanosensory neuron. There are two URX neurons, one PQR and one AQR neuron and the endings of these are not exposed. (b). Complete structure of amphid sensory opening showing the socket (so), sheath (sh), and ciliated nerve endings. The AWA, AWB, AWC, and AFD endings are buried in the sheath and not exposed through the amphid pore. (C). Detailed cilia structure of the 12 classes of amphid neurons. (Perkins 1986)

Table 1: Functions and molecular properties of chemosensory neurons (Wormbook.org)

Neuron	Function	Receptors; G proteins	Signal transduction	Regulators
ASE	Water-soluble chemotaxis	Receptor guanylate cyclases?; *gpa-3*	*tax-4, tax-2, daf-11*, cGMP	*osm-9, gpc-1, tax-6, ttx-4, adp-1*
AWC	Volatile chemotaxis, Lifespan, Navigation	GPCRs *(str-2); odr-3* (major), *gpa-3, gpa-2, gpa-5, gpa-13*	*tax-4, tax-2, daf-11, odr-1, odr-4, odr-8*, cGMP	*osm-9, egl-4, grk-2, tax-6, ttx-4, sdf-13, adp-1, let-60, goa-1*
AWA	Volatile chemotaxis, Lifespan (minor)	GPCRs *(odr-10); odr-3* (major), *gpa-3, gpa-5; gpa-13; gpa-6*	*osm-9, ocr-2, fat-3*, PUFA, *odr-4, odr-8*	*egl-4, grk-2, tax-6, ttx-4*
AWB	Volatile avoidance	GPCRs; *odr-3*	*tax-4, tax-2, daf-11, odr-1*, cGMP	*kin-29*
ASH	Nociception: Osmotic avoidance, Nose touch avoidance, Chemical avoidance, Social feeding	GPCRs; *odr-3* (major), *gpa-3* (major), *gpa-11, gpa-1, gpa-13, gpa-14, gpa-15*	*osm-9, ocr-2, fat-3*, PUFA, *qui-1* (chemical only), *osm-10* (osmo only), *odr-4*	*grk-2* (chemical, partial osmo), *tax-6, ttx-4, gpc-1, kin-29*
ASI	Dauer formation, Chemotaxis(minor), Navigation	GPCRs; *gpa-1, gpa-3, gpa-4, gpa-5, gpa-6, gpa-10, gpa-14*	*tax-4, tax-2, daf-11*, cGMP, *odr-1, odr-4*	
ADF	Dauer formation, Chemotaxis (minor)	GPCRs; *odr-3, gpa-3, gpa-10, gpa-13*	*osm-9, ocr-2, odr-4*	
ASG	Dauer formation (minor), Lifespan, Chemotaxis (minor)	GPCRs; *gpa-3*	*tax-4, tax-2*, cGMP, *odr-4*	
ASJ	Dauer formation and recovery, Chemotaxis (minor), Lifespan	GPCRs; *gpa-1, gpa-3, gpa-9, gpa-10, gpa-14*	*tax-4, tax-2, daf-11, daf-21*, cGMP, *odr-1, odr-4*	
ASK	Avoidance (minor), Chemotaxis (minor), Lifespan, Navigation	GPCRs; *gpa-2, gpa-3, gpa-14, gpa-15*	*tax-4, tax-2*, cGMP, *odr-1, daf-11, odr-4*	*kin-29*
ADL	Avoidance (minor), Social feeding	GPCRs; *gpa-1, gpa-3, gpa-11, gpa-15*	*osm-9, ocr-2, odr-4*	
URX, AQR, PQR	Oxygen/aerotaxis, Social feeding	Soluble guanylate cyclases (*gcy-35, gcy-36); gpa-8*	*tax-4, tax-2*, cGMP	*npr-1*
PHA, PHB	Avoidance (antagonistic)	GPCRs; *gpa-1, gpa-2, gpa-3, gpa-6, gpa-9, gpa-13, gpa-14, gpa-15*	*osm-9, ocr-2, odr-4*	

A strong adaptive learning has been observed in these nematodes, when conditional stimulus (CS) like food and odorant (unconditional stimulus, US) were presented. If food is used as CS the worm learn to become attractant to an odorant and whereas starvation as CS will make the organism to learn to avoid the odorant US (Bargmann *et al.*,1995, Van der Kooy *et al.*, 2012). This learning behaviour is as evident when animals are starved in the presence of naturally attractive odours, the animal show a repelling behaviour to the ordour when tested after conditioning (Bargmann *et al.*, 1995). Recent evidences suggest that the aversive learning pathway involves additional inter-neurons like AIB, AIY etc. (Bargmann, *et al.*, 2016)

1.6 ROLE OF CALCIUM CHANNELS IN LEARNING AND MEMORY

Neurons communicate each other through neurotransmitters and ion channels (Lovinger *et al.*, 2008). Among them Ca^{2+} have a critical role in developing action

potential based on the stimulation induced neurotransmitter release (Berridge *et al.*,1998). Ligand gated calcium channels are localized in the post synaptic terminals of neurons, and this include AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), kainite, NMDA (N-methyl-D-aspartate receptor) and metabotropic receptors (mGluR) (Purves, 2001). These channels allows positive ion influx

(Ca^{2+}/Na+) into the neurons to propagate the action potential by depolarizing the neurons.

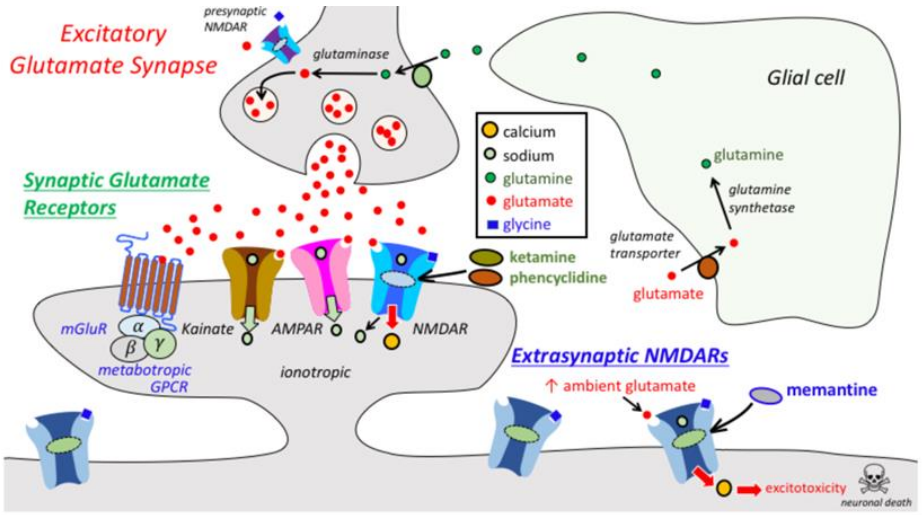

Figure.3: Glutamate neurotransmitter and its receptor signalling pathway at synapse.

Glutamate released from presynaptic nerve terminals, can bind to Kainate, AMPA, and NMDA ionotropic receptors or G-protein coupled metabotropic receptors (mGLuR) on the postsynaptic cells. Synaptic transmission can be terminated by the active transport of glutamate into neighbouring glial cells through glutamate transporters. Glutamate then converted to glutamine, and transported into glutamergic neurons by glial cells. Glutamergic neurons will convert intracellular glutamine to glutamate, and concentrates the neurotransmitter in presynaptic vesicles via vesicular transporter. The extrasynaptic NMDA receptors are coupled for different downstream signaling cascades compared to synaptic NMDA receptors. Excessive activation of extra synaptic NMDARs by elevated levels of ambient glutamate will result in excitotoxicity and cell death. Memantine is a selective blocker of extrasynaptic NMDA receptors.(Fig 4)

Among these ligand gated channels The N-methyl-D-aspartate (NMDA) receptor plays a vital role in neuronal plasticity, learning and memory etc. (Riedel *et al.*, 2003, Maleszka *et al*., 2000). In mammals, there are of seven NMDAR subunits namely NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B. Permutation combination of these receptor subunits found to alter the functional efficiency of NMDA receptors (Burnashev *et al.,* 2015). Moreover, activation of NMDA receptor is essential for the long-term potentiation (LTP) in the hippocampal, medial septum, and amygdala regions (Izquierdo *et al.*, 1994).

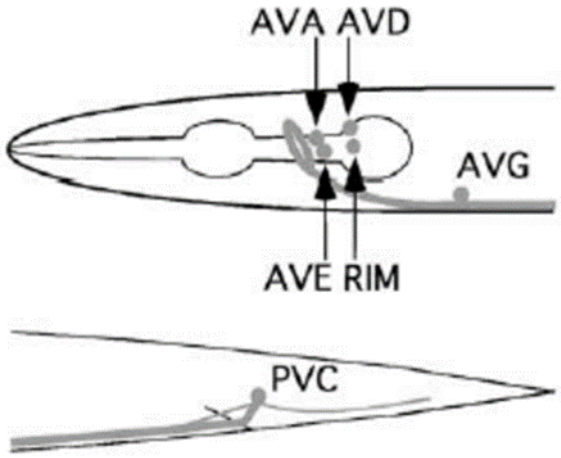

Figure.4: Expression of NMDA receptors in *C. elegans* neurons (P J Brockie, 2001)

The five interneurons AVA, AVD, AVE, AVG, PVC and the motor neuron RIM were the NMDA receptors are expressed in *C.elegans*. (Fig5)

Hypofunction of NMDA receptors in the brain has been found to hamper the learning and memory pathway. Besides, activation of NMDA receptors are dependent on various factors like level of AMPA receptors present, glycine and Mg^{2+} (Dongen *et al.*, 2009). Probably such molecular regulation are enforced to prevent accidental activation

of the receptors which could lead to excitotoxicity. In *C.elegans* two isoforms of NMDA receptor subunits present, nmr-1 and nmr-2 (Brockie *et al.,* 2001). There are evidences that NMDA receptors are essential in learning behaviour associated with NaCl paired with starvation. (Kano T *et al.,* 2008). NMDA receptor subunits of *C.elegans* (nmr-1 and nmr-2) are expressed in five interneurons namely AVA, AVD, AVE, AVG, PVC and one motor neuron RIM.

1.7 REVIEW OF LITERATURE

*C.elegans*has arised to one of the top model organisms in the field of biological research from the last fifty years. Comparing other laboratory animals, this nematode is considered as one of the easiest and simplest form of life. The life cycle of *C.elegans* is very simple and takes only three days to reach adult stage. *C. elegans* feeds on ephemeral bacterias in decomposing leaves and fruits. When unfavourable conditions like starvation they will enter into another stage called dauer stage. (Félix *et al.*,2015)

The major advantages of *C. elegans* as a model organism in neurobiology is its simplicity in the number of neurons and the ability of learning and memory. For example non-associative learning like mechanical vibratory stimulus causes changes in reversal reflex responses. This shows that studies of non-associative learning creates short term memory in *C. elegans* (Rankin *et al.*,1990).

The nervous system includes 302 neurons, and their pattern of synaptic connectivity is well deciphered. Having only five olfactory neurons they can respond to many attractive and repellent odours. Polymodal sensory neurons are able to detect a range of nociceptive signals and helps in the process of escaping responses (Bono *et al.*, 2005).

The knowledge available on the natural history of worms were that it could be found in humus and compost heaps (Hodgkin and Doniach, 1997).

The deterministic development of the nervous system of worms seems to limit the use of *C. elegans* as a model to study behavioural plasticity, but the worm shows extreme sensitivity to experience and show strong learning. Thus the deterministic development

becomes its greatest boon. Researchers can thus study neurons and behaviours of *C. elegans.* Thus the power of *C. elegans* as a model for neurobiological and behavioural studies led insights into the cellular and molecular pathways underlying learning behaviours. The genes with no such role in the development or primary functioning of neurons, were show to be requiring for learning in multiple paradigms, eg: asic-1, casy-1, glr-1, magi-1, and hen-1. This small organism shows a higher degrees of freedom in adapting and reflecting its experiences as behavior. (Evan *et al*., 2010).

C. elegans can sense at least five different odourants namely butanone, benzaldehyde, isoamyl alcohol, diacetyl and pyrazine. AWC sensory neuron is responsible for the detection and adaptation of various odourants. (Bargmann *et al.,* 1995)

The molecular mechanisms which mediates odourant adaptation were well depicted in various model species. But the types of adaptation which depends on neural circuits were unexplored. The Ras-MAPK pathway is essential for odour adaptation. In *C. elegans* this adaptation can be termed as early adaptation. *(*Hirotsu *et al.,* 2005).

C.elegans are capable of creating an association between the odourant benzaldehyde and the food content in its environment. When they were allowed one hour exposure to 100% benzaldehyde in the absence of food the attractive response get reduced, this olfactory adaptation is attenuated by the presence of food. Genetic studies revealed that effect of food in this paradigm were mediated by serotonergic signaling (Nuttley *et al.*, 2002)

Long-term memory of habituation in *C.elegans* depends on *glr-1*. Glr-1 is a homolog of non-NMDA glutamate receptors of mammalian system. Long-term memory

formation has been blocked by mutation in glr-1 gene. Green fluorescent protein (GFP) tagging were used to visualize *glr-1* expression in the (Nonet *et al.,* 1999)

Short term memory was not affected when glr-1 gene mutations in glr-1 has created but it blocked the long term memory.This shows that the receptor encoded byglr-1 is critical for long term memory in *C. elegans*.(Vianna *et al.,* 2000).

C.elegans uses olfactory stimulus to detect food. The responses to olfactory signals were shown to be well regulated in a dynamic. However prolonged exposure to odorant will leads to a decreased response towards odorant. Starvation effect can persist for several hours. (Bargmann *et al.,* 1997)

C.elegans was first observed to show adaptation, by mechanical tap in the year 1990. The parameters were allowed for this behavioural plasticity were then characterized which leads to the striking hypothesis that habituation is mediated through multiple mechanisms. Many tools were now present to easily do genetic manipulations *C. elegans* researchers that allow for relatively easy genetic manipulation. This leads to recent genetic advances to study the molecules which plays significant roles in the mechanisms of habituation. Some of these includes vesicular glutamate transporters, dopamine receptors, glutamate receptor subunit, and intracellular signaling molecules, such as kinases and G proteins. These researches has also leads to the studies on different phases of memory like short-term and long-term memory which were

triggered by various conditioning paradigms. The differences these memory pathways are also yet to be revealed (Andrew C. G *et al.,* 2009).

The non-associative form of learning, is also said to be the simplest form of learning. Cellular processes underlying its behavioural pathways were little known. *C. elegans* shows both learning with repeated training and long-term retention of training. Exposure to heat shock during the interblock intervals eliminates the long-term memory for habituation but not the accumulation of short-term memory over blocks of training. Analyses using laser ablation of identified neurons, and of identified mutants have shown that there are multiple sites of plasticity for the response and that glutamate plays a role in long-term retention of habituation training. (Rose *et al.,* 2001).

Cellular basis studies of learning and memory revealed several distinct phases of memory that can be identified by time course. Besides the traditional long-term memory short-term distinction, several other phases of memory has been also identified, types of intermediate-term memory, two distinct forms of long-term memory, and several forms of short-term memory. Through the behavioural, neural circuit and genetic analyses of habituation, research using this simple organism *C.elegans* has provided insights into different memory phases. Studying experience-dependent plasticity of this behaviour has not only provided corroborating evidence for the existence of short-, intermediate-, and long-term forms of memory, as have been demonstrated in both Aplysia and Drosophila, but also has revealed the possible existence of multiple forms of short-term memory. (Steidl *et al.,* 2003).

It is believed that short-term memory arises from changes in protein dynamics which increases the synaptic signaling strength, but many of the fundamental molecular

mechanisms remain unknown. The *C. elegans* assays provide olfactory short-term associative memory (STAM), in which the *C. elegans* can able to learn when food was associated with odour and can remember this association for over an hour. There are independent molecular characteristics for different phases of short term associated memory (STAM). In *C. elegans* STAM requires calcium and cyclic AMP signaling. Adaptation mutants show variable responses to short-term associative memory training. (Murphy *et al.,* 2014).

C.elegans exhibits behavior plasticity which appears to be corresponded to non-associative and associative learning, and short-term and long-term memory (Sasakura *et al.*, 2013).

In *C. elegans* memory is maintained in the order of minutes to hours. They can able to poses long lasting memory as in humans. Imprinting is also observed in *C.elegans*, where exposed to an attractive odourant at first larval stage in the presence of food leads to the attractive responses to the experienced odours during adult stage. (Remy *et al*, 2005).

Olfaction is a very versatile mechanism. In the case of detection of volatile odorants. *C. elegans* can able to detect many volatile chemicals, which can be attractants or repellents. Chemotaxis to volatile odorants requires different sensory indicating that *C. elegans* might have senses that corresponds to smell and taste. Each neurons have complex sensory properties. (Bargmann *et al*., 1993).

Calcium ions are universal second messengers which important activities of eukaryotic cells. Calcium have critical role in neurons as it is involved in transmission the

depolarizing signal and contributes thus for synaptic activity. Eukaryotes uses calcium ions as signal carriers to regulate various functions. Calcium as signaling molecule in nervous system have particular role, Such as synaptic transmission, which leads to the release of neurotransmitters, the process of learning and memory consolidation the long-term potentiation (LTP) or depression. All these processes are mediated in the neurons.

Glutamate receptors are abundant and important mediators for fast excitatory synaptic transmission in vertebrates. They also serves similar function in invertebrates system such as *Drosophila* and *C.elegans*. In *C. elegans,* a total of 10 different glutamate receptor subunits were identified. (Strutz *et al.*, 2003).

The N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors are important for the synaptic plasticity and the development and function of the nervous system. nmr-1, which is a subunit of *C. elegans* NMDA-type ionotropic glutamate receptor plays a major role in the movement and foraging behaviour. nmr-1 mutant worms showed a lower probability to switch forward to backward movement and showed a reduced ability to navigate through complex environments. Electrical

recordings from interneuron AVA showed that there is a selective disruption of NMDA-dependent currents in nmr-1 mutants. (Brockie *et al*., 2001).

NMDA receptors, are class of glutamate-gated cation channels having high calcium ion conductance. NMDA receptor mediates fast signal transmission and plasticity of excitatory synapses. (Sprenge *et al*., 1998).

The glutamate-gated ion channels N-methyl-D-aspartate receptors (NMDARs) were widely expressed in central nervous system. These receptors play important role in excitatory synaptic transmission. Since they are involved in numerous neurological disorders, NMDARs were targeted extensively. NMDARs exists as multiple subtypes which differs in its subunit composition and pharmacological properties. (Paoletti and Neyton, 2007).

N-methyl-D-aspartate (NMDA) receptors, are subset of ionotropic glutamate receptors, which play diverse role in nervous system, which includes the development and function of synapses, improvement of synaptic connections with synaptic plasticity (Dingledine *et al*., 1999).

Glutamate gated postsynaptic receptors of nervous system are essential for learning and memory both in the cases of vertebrates and invertebrates. Their genetics, biochemistry, and role in behaviour have been well studied *in vitro* and *in vivo*, but the structural aspects and molecular evolution were poorly understood. (Ryan *et al*.,2008).

Learning and memory are critical processes of both invertebrate and vertebrate systems which allow to survive in the environment and reproduce. Glutamate which is a neurotransmitter signals through ionotropic glutamate receptors (iGluRs). iGluRs are

important in learning and memory pathway.But the signaling pathways underlying these behaviours are still not understood. nmr-1 and nmr-2 are the subunits of NMDA receptors in *C. elegans* which are necessary for memory retention. nmr-2 deletion mutation in gene showed that like nmr-1, nmr-2 is very essential for NMDA-gated currents. (Kano *et al*., 2008).

Human NMDA receptors are heteromers which consists of NR1 and one or two types of NR2 subunits. *Xenopus laevis* consists of NR2A and NR2B proteins which are well conserved well conserved between species. (Rebecca *et al*.2009).

NMDA receptors are important in drug targets since these were related to pathophysiological conditions. It is important to know that whether other model organisms show similar properties and functions compared to human NMDA receptors. The amino acid sequence of human and rat NMDA receptor subunits were showed similarities. (Hedegaard *et al*.,2012).

It is difficult to point how a particular NMDA receptor subunit give synaptic function in *C. elegans*. (White *et al*., 1986). NMDA subtype of glutamate receptors are important for nervous system development, functioning and synaptic plasticity. nmr-1 subunits of *C. elegans* plays important role in the movement and foraging. nmr-1 mutants showed significantly lower probability of switching forward and backward movement. Inaddition interneuron electrical recordings from AVA were NMDARs expressed had showed that NMDA dependent currents were disrupted in nmr-1 mutants. Also showed

that a slowly desensitizing variant of non-NMDA receptor can rescue the nmr-1 mutant worms. (Penelope *et al*., 2001)

Since *C. elegans* can adapt to various odourants with conditional stimulus. This can be exploited to study the role of receptors participating in the adaptive learning pathway. Especially the ionotropic glutamate receptor like NMDA.

1.8 WORKING HYPOTHESIS

One of the fundamental function of brain is to recognize and adopt to various stimuli a living organism encounters. However, there is very limited insights on how this process works. There are two school of thoughts debating on number of neuronal connectome involved in the process.

- One argument is that since this process is the basic function of brain, a small subset of brain cells, probably a single neuron, could execute the storing and processing of information.
- The other argument is that, both sensing and adaptation are complex events and require a large set of interneurons to process the information.

This study tried to address this debate using a model system *C. elegans* and its adaptation towards olfactory signals.

1.9 AIM AND OBJECTIVES

Learning and adapting to various environmental stimuli has been considered as the basic functioning of brain. However it is not clear how the neurons detect the stimuli and process the information to adopt. This understanding is an essential step towards deciphering how brain acquire, store and retrieve information. This study used a simple model organism *C. elegans*, which has a strong sense of olfaction. Sensing various volatile solvents are confined to a subset of sensory neurons in this organism and hence makes it an interesting model to study how olfactory adaption alters based on conditional stimuli in a learning paradigm.

The Study objectives are:

1. Standardize a learning paradigm in which the organism can sense and adapt to different odourants under a conditional stimuli
2. Verify whether connectome is involved in the process of adaptation by
    a. Cross adaptation (Condition to one odourant and checking adaptation to other odourants.
    b. Blocking Ca^{2+}pathways
    c. Mutants lacking calcium channels like NMDA receptors.

CHAPTER - 2

MATERIALS AND METHODS

2.1 WORM MAINTENANCE AND STRAINS

C. elegans strains used for the study were maintained under standard conditions (Brenner. S, 1974). All the strains were grown and maintained at 20^0C, on Nematode Growth Medium (NGM) plates. Plates were seeded with *E. coli* OP-50 strain which is a uracil auxotroph, as the food source for *C. elegans.*

2.2 STRAINS USED

N2 Bristol (Wild Type).

VM-487: nmr-1 encodes an NMDA type ionotropic glutamate receptor subunit which affects the duration of forward movement, an important aspect during foraging behaviour. Also it affects the osmotic avoidance. An nmr-1 GFp fusion protein was shown to express in the interneurons AVA, AVD, AVE, AVG, DVA, PVC and a motor neuron PVC.

VC-2623: nmr-2 encodes an NMDA type ionotropic glutamate receptor subunit which is most similar to members of the NR2A subfamily of NMDA class of ionotropic glutamate receptors. nmr-2 is required for full memory retention of a learned avoidance behaviour, namely avoidance of NaCl after starvation conditioning. An nmr-2 GFP

fusion protein was shown to express in the AVA, AVD, AVE, AVG, PVC interneurons and RIM motor neurons.

All the strains were obtained from CaenorhabditisGenetics Centre (CGC), University of Minnesotta. All experiments were performed on the day-1 adult stage worms.

2.3 SYNCHRONISATION OF *C. ELEGANS*

Egg laying gravid adult worms were collected using M9 buffer in 1.5 ml centrifuge tube. Washed the worms 3 times using M9 buffer. Added 1ml of bleach solution (Sodium hypochlorite + NaOH). Vortexed the tubes in every 2 minute for next 10-12 minutes to dissolve cuticle of the worms completely. Since the eggs having thick cuticle they will not lyse easily. After the worms get lysed completely, centrifuged it at 7000 RPM for 1 minute. Removed the supernatant and washed it with M9 buffer 3 times to completely remove the bleach content. Removed the supernatant and mixed the pellet of eggs and were transferred to fresh OP-50 plates. Worms get hatched in 18-24 hours and all the worms hatched were in synchronised age. (www.wormbook.org)

2.4 CHEMOTAXIS ASSAY

Worms were collected from food plates using M9 buffer and transferred to 1.5 ml centrifuge tube. Washed the worms with M9 buffer 3 times to completely remove the food particles. Three spots were marked on the chemotaxis plate, the centre spot were the worms placed, a solvent and control spot on opposite edges having 1cm diameter. Worms were then pipetted onto the centre spot. Then added 3µlof 100mM NaN_3 on both spots marked solvent and control (Sodium azide anesthetize the worms reached the spots). Then added 3µl of ethanol on control spot (Since ethanol was used to

dissolve the solvents). Wiped out the excess M9 buffer from the worms using soft tissue paper. Then added 3µl of solvent in the spot marked close the lid and kept the plates for 20 minutes. Each experiments were done in triplicates. (Murphy C *et al*., 2011)

The chemotaxis index were calculated after 20 minutes by the following equation;

$$\text{Chemotaxis index(CI)} = \frac{\text{Number of worms in the solvent spot} - \text{number of worms in the control spot}}{\text{Total number of worms}}$$

2.5 ASSOCIATIVE LEARNING WITH FOOD AND WITHOUT FOOD

Wild type N2 worms were collected to 1.5 ml centrifuge tube using M9 buffer and washed 3-4 times with M9 buffer to remove all food content. Animals were given conditional stimuli (CS) for one hour with food and without food on an NGM plate. In the case of starvation conditioning (without food) worms were starved in NGM plates with 5µl solvent on lid, and in conditional stimuli with food, the worms were given with 400 µl OP-50 along with 5 µl solvent on lid, for 1 hour. Controls were treated in parallel were no solvent had given for conditioning. Chemotaxis assay was then conducted after 1 hour for 20 minutes. Concentration of solvents taken for conditioning and chemotaxis assay were 1/10 butanone and 1/100 isoamyl alcohol in ethanol. (Bargmann *et al.*, 1995)

2.6 ODOURANTS USED

Iso-amyl alcohol (1:100) (Central Drug house PVT LTD Mumbai), Butanone (1:10; 1:1000) and Benzaldehyde (1:200) (Spectrum Reagents and Chemicals PVT LTD Cochin)

2.7 CROSS ADAPTATION ASSAY

Animals were collected from food plate and washed using M9 buffer to remove all the food content. . Worms were then allowed for 1 hour starvation along with 5µl odourant on lid as conditional stimuli (CS). Animals conditioned with one particular odourant were then allowed to perform chemotaxis for other two odourants to detect cross adaptation. All the solvents were thus cross checked for adaptation. Cross adaptation to three solvents were checked on N2 (wild type), VM-487(nmr-1), VC-2623(nmr-2) strains. Each experiment were done in triplicates. (Bargmann *et al.,* 1995)

2.8 EGTA ASSAY

Nematode growth medium (NGM) plates were prepared with 50mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) in the absence of CaCl2. pH were maintained at 7.0. Wild type N2 worms were washed with M9 buffer and removed excess food. The animals were then starved for 1 hour along with 5 µl solvent on lid as conditional stimuli on the EGTA plates. Starvation without any solvent during conditioning were also checked in EGTA plates. Chemotaxis assay were carried out and CI values were plotted. (Bargmann *et al., 1995*)

2.9 STATISTICAL ANALYSIS

All statistical analysis and graphs were generated using Graph pad prism 6th edition (Graphpadprism.inc)

CHAPTER - 3

RESULTS AND DISCUSSION

3.1 RESULTS

C.elegans can adapt various odourants with a conditional stimulus (CS) such as food, starvation etc. Interestingly AWC sensory neuron has been found to detect five odourants includes butanone, benzaldehyde, isoamyl alcohol, diacetyl, and pyrazine (Bargmann *et al.*, 1995*)*. Modelling the cross adaptation of odourant stimuli (unconditional stimuli, US) with that of CS could be an interesting approach to uncover the molecular pathways in the neurons that recognizes various stimuli.

3.2 ADAPTATION BEHAVIOUR OF WORMS VARY TO CONDITIONAL STIMULI WITH DIFFERENT ODORANTS.

In order to verify the adaptation to odourants, butanone (1/10 dilution), and isoamyl alcohol (1/100 dilution), two known concentrations in which isoamyl alcohol act as a strong attractant (Bargmann *et al.*, 1995) and butanone as a repellent (Bargmann *et al.*, 1993) were tested. Under butanone, the worms showed repulsion under starvation ($p = 0.0012$) and learn to get attracted when food was given as a CS ($p = < 0.0001$) (Figure

6a). However, isomayl alcohol adaptation showed significant repellent behaviour both under CS stimuli of food and starvation (Fig 6b, p= 0.0008 and p= 0.0002 respectively).

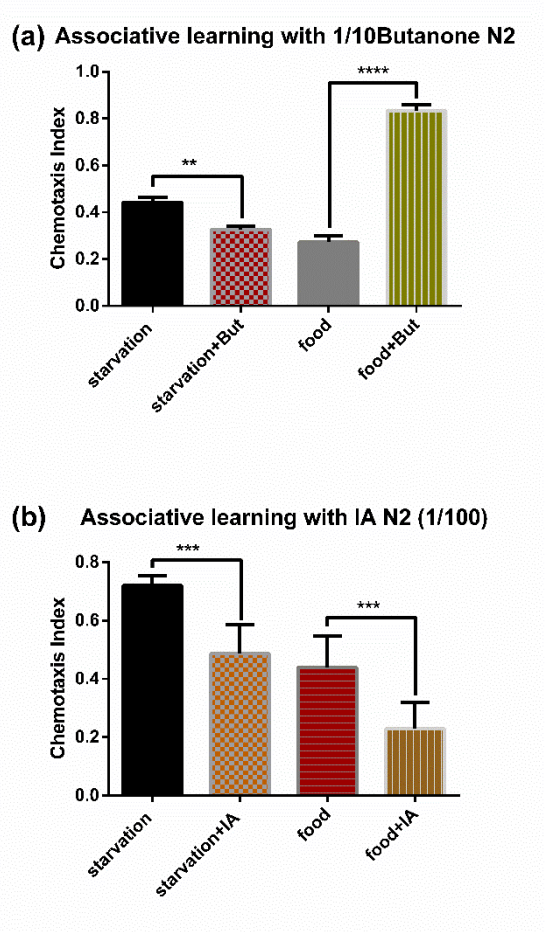

Figure 6: Chemotaxis index (CI) of N2 worms under conditional stimuli (CS) food and starvation with a) 1/10 Butanone and b) 1/100 Isoamyl alcohol as US. Each bar represents an average of 3-7 independent experiments. Number of worms 50-100 in each experiment. But-Butanone; IA-isoamyl alcohol one way ANOVA (Brown-Forsythe test). *p value ≤0.05 **p value ≤0.01 *** p value ≤ 0.001 ****p value ≤0.0001, Error values are ±SD

Most predictable adaptation was when starvation was used as a CS. Hence the study henceforth focused on starvation CS.

3.3 *C. ELEGANS* CAN ADAPT TO ODOURANTS ASSOCIATED WITH STARVATION

Since both butanone and isoamyl alcohol are detected by AWC neurons in the worm, solvent concentrations were standardized to make all of them strongly attractant for the naïve worms. Solvent concentrations butanone 1/1000 dilution, isoamyl alcohol 1/100 dilution and benzaldehyde 1/200 were found strong attractant (Figure.7). When the worms showed significant adaptation to starvation (CS) to all the US odorants (p value ≤ 0.0001, for all the three odorants).

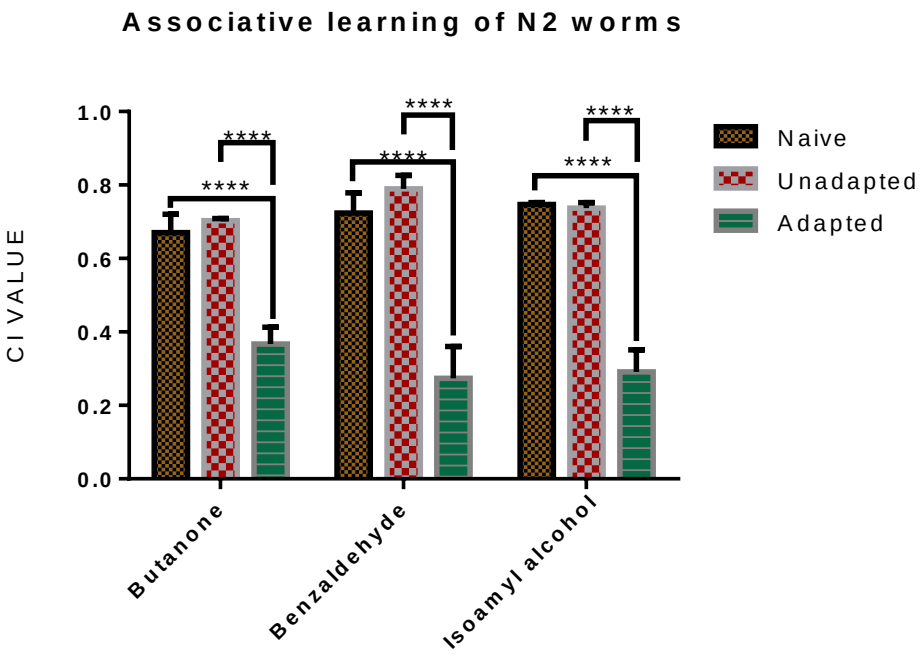

Figure7: Chemotaxis index (CI) of N2 worms under various odorants as US. Each bar represents an average of three independent experiments. Number of worms 50-100 were used in each experiment. But-Butanone; IA-isoamyl alcohol. **** p value (≤ 0.0001) 2-sided ANOVA (Turkey's multiple comparison). Error values are ±Mean with SD.

3.4 *C.ELEGANS* CAN CROSS ADAPT WITH BENZALDEHYDE AND ISOAMYL ALCOHOL BUT NOT WITH BUTANONE

Because all of these odours are detected by a single pair of sensory neuron, it is intriguing to know whether adaptation to one odorant will allow cross-adaptation to another. Additionally it will indicate the specificity of odourant adaptation as well. The test was conducted using worms that were conditioned with one odorant and checked for any adaptation towards other odorants.

Wild type N2 worms were adapted with 1/100 isoamyl alcohol for 1 hour along with starvation (CS) and tested chemotaxis index. The results showed that worms adapted with both 1/100 isoamyl alcohol and to benzaldehyde while no adaptation was shown toward butanone which behaved similar to that of naïve (Fig.8a). Similar results were obtained for 1/200 benzaldehyde (Figure 8b), suggesting both isoamyl alcohol and benzaldehyde share common signaling pathways and butanone signaling is unique. This was confirmed when 1/1000 butanone adaptation was carried out and checked for cross adaptation for isoamyl alcohol and benzaldehyde (Figure 8c). Unique recognition

pathway to butanone is interesting because it underscores that the AWC neuron has specific signaling pathway to a set of odorants both for adaptation and detection.

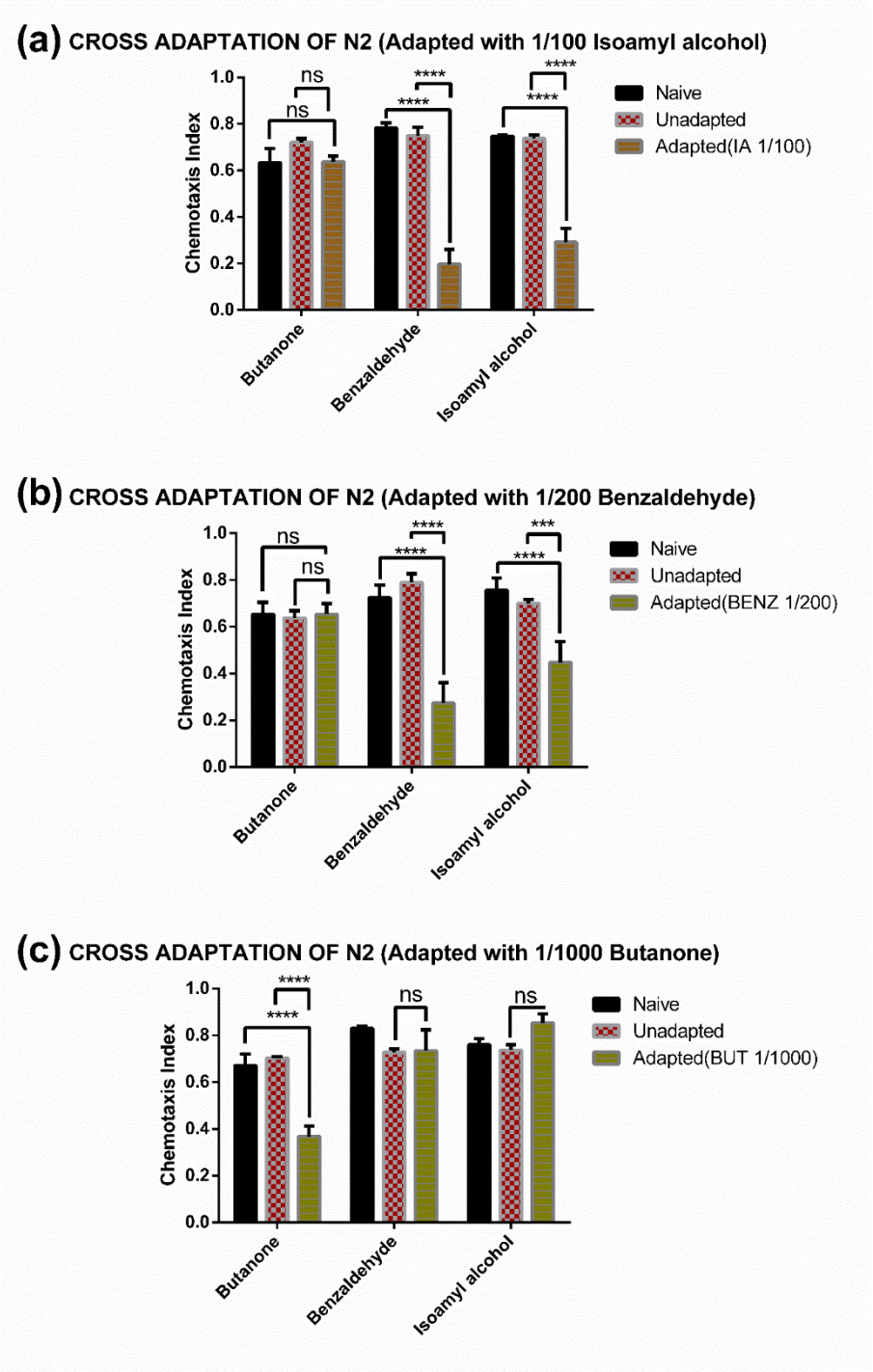

Figure 8: Cross adaptation assay – a) adapted with 1/100 isoamyl alcohol b) 1/200 Benzaldehyde and c) 1/1000 butanone. Each bar represents an average of three independent experiments. Number of worms 50-100 in each experiment. But-Butanone; IA-isoamyl alcohol.).. *p value ≤0.05 **p value ≤0.01 *** p value ≤ 0.001 ****p value ≤0.0001, 2-way ANOVA (Turkey's multiple comparison test). Error values are ±SD. ns - not significant

Adaptive learning has various signaling pathways including synaptic plasticity, connectome variations, alterations in neurotransmitter release pattern etc. (Kandel *et al.*, 2001). Since all these events occur at synaptic level, understanding the role of calcium in these adaptive pathways is essential. Calcium is one of the critical signaling molecules in the neuron. Both ligand gated and voltage gated channels to allow these positive ions to enter the neurons to initiate potentiation and cell-cell communication. Hence the role of calcium in this adaptive learning was tested further.

3.5 CALCIUM IONS ARE NOT ESSENTIAL FOR THE ADAPTATION TO ISOAMYL ALCOHOL

To find whether calcium influx into sensory neurons is responsible for adaptation to odorants, worms were assayed in EGTA plate. EGTA is a known chelator for Ca^{2+}, with low affinity to Mg^{2+} (Bargmann *et al.,* 1995).

Interestingly, the results showed that for isoamyl alcohol adaptation calcium ions are not essential while both butanone and benzaldehyde adaptations are calcium (dependent) (Figure 9).This result was surprising because the aversive learning pathway involves additional inter-neurons like AIB, AIY etc (Bargmann *et al.*,2016)

and involves ionotropic calcium channels (Kano *et al.*, 2009). To probe further, mutants of NMDA receptor an ionotropic calcium channel was tested.

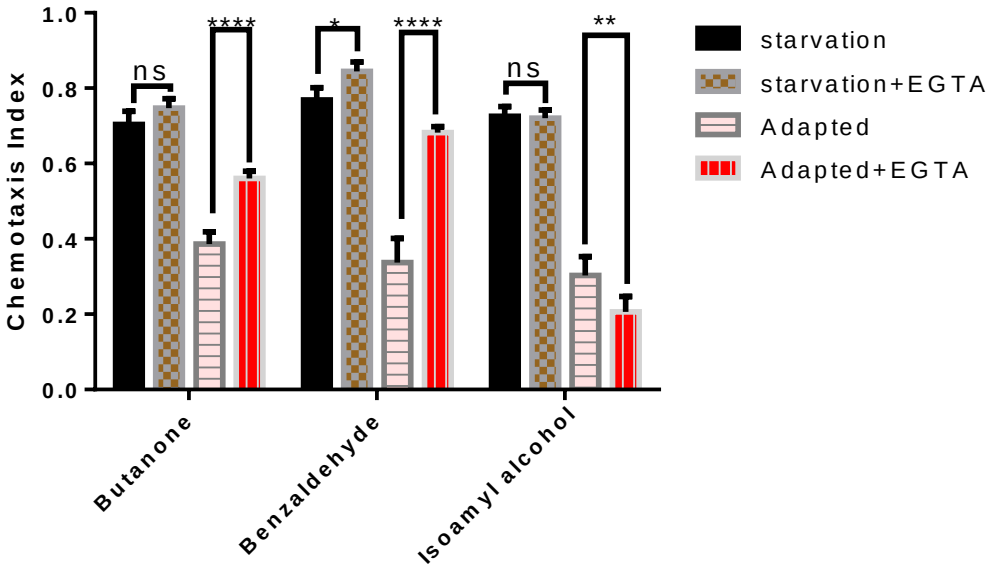

Figure 9: Chemotaxis index (CI) of N2 in the presence of EGTA. Each bar represents an average of three independent experiments. Number of worms 50-100 in each experiment.).*p value ≤0.05 **p value ≤0.01 *** p value ≤ 0.001 ****p value ≤0.0001, 2-way ANOVA (Turkey's multiple comparison test). Error values are ±SD. ns - not significant

3.6 NMDA RECEPTOR MUTANTS ARE ADAPTATION DEFECTIVE TO BUTANONE, BENZALDEHYDE AND ISOAMYL ALCOHOL

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels widely expressed in the central nervous system that play key roles in excitatory synaptic transmission (Paoletti *et al.*, 2006). NMDA receptor is composed of two subunits in *C.*

elegans namely, nmr-1 and nmr-2. Olfactory adaptation were checked for both nmr-1 and nmr-2 mutant worms.

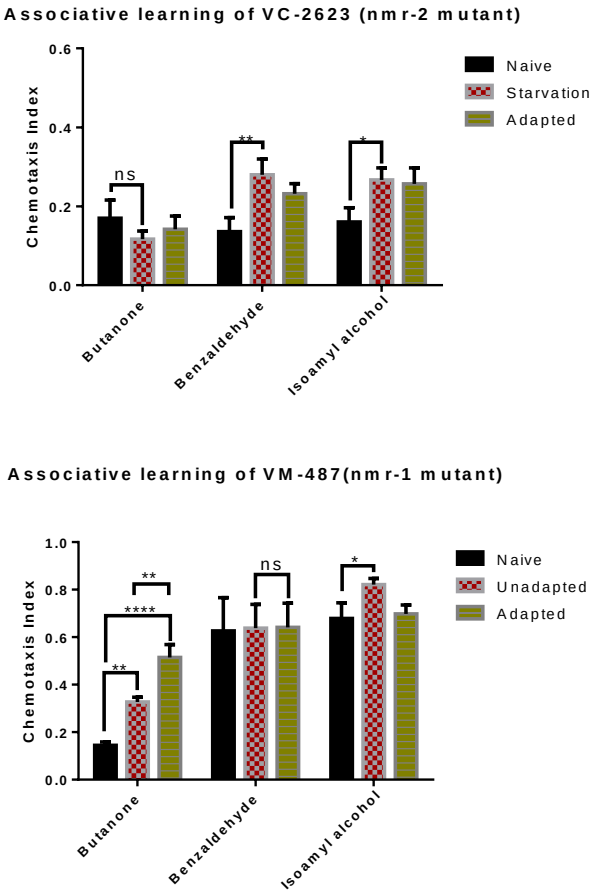

Figure 10 Associative learning assay in a) nmr-1 and b) nmr-2 mutants. Each bar represents an average of three independent experiments. Number of worms 50-100 in each experiment, *p value ≤0.05 **p value ≤0.01 *** p value ≤ 0.001 **** p value (≤ 0.0001) 2-way ANOVA (Turkey's multiple comparison test). Error values are ±SD. ns - not significant.

Both the mutants were shown defective in adaptive learning (Figure 10). The attraction towards the solvents were significantly lower in nmr-2 mutants compared to N2 (see Figure 7) and nmr-1 mutants (CI value was 0.2 in naïve compare to 0.7 in N2 and nmr-1). The mutant nmr-1 naïve worms however showed significant low attraction towards butanone compared to the other two solvents (Figure 10). On exposure to odorants

there is mild increase in attraction but the adaptive learning pattern lacked in both the mutants. This result confirms that interneurons expressing NMDA receptors play a critical role in learning pathway for the selected odorants. Significantly low attraction to solvents in nmr-2 mutants is an unexpected result. This suggest that NMDA receptors are involved in odour detection which makes the whole pathway more complicated than expected. One possibility is that because worm keeps a record of all the experiences (Bargmann *et al*., 2016), activation of interneurons could be essential for recognition pathway. This is significant since the odour recognition is not a passive act but rather a complex process. In addition in learning and adaption pathway, the organism has to decide whether to avoid or attract to the signal; past experiences could be playing a critical role in odour recognition.

Since the worms were showing cross adaptation to odorants, both nmr-1 and nmr-2 were tested to see how cross adaptation alter with lack of channel receptors.

3.7 NMR-1 AND -2 MUTANT WORMS ARE DEFECTIVE IN CROSS ADAPTATION

Both nmr-1 and nmr-2 mutant worms were showed significantly low cross adaptation to solvents tested (Figure 11 a-f). In nmr-2 mutant showed an adaption to benzaldehyde, but had not shown cross adaptation to isoamyl alcohol. However CI value of nmr-2 mutants to benzaldehyde was significantly lower than the N2 control worms (see figure 8). These data suggest that NMDA receptors have critical roles in the odorant detection

as important as adaptation. Besides, it also indicates AWC neurons are not a decision making cells in *C. elegans* as earlier thought (Kano *et al.*, 2009).

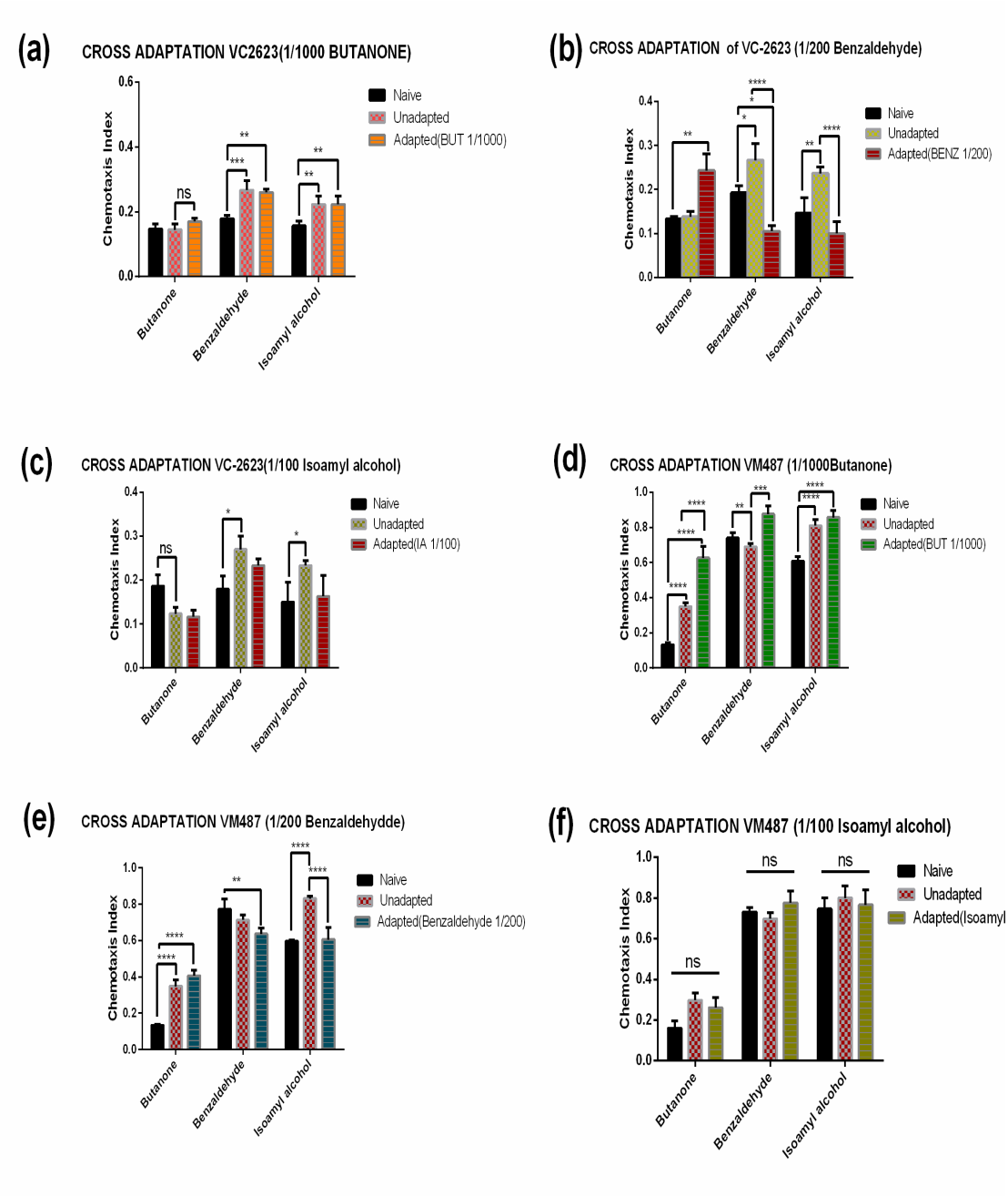

Figure 11: a-c: nmr-2 cross adaptation to various solvents; d-f: nmr-1 cross adaptation to various solvents. Each bar represents an average of three independent experiments. Number of worms 50-100 in each experiment. **** p value (≤ 0.0001) 2-way ANOVA. Error values are ±SD. ns - not significant

3.8 DISCUSSION

One of the challenging problems in biology is to decipher on how neuronal cells store and recall information. Understanding the pathways involved is critical because majority of the neurological diseases affect the memory pathways. Adaptive learning has been tested in various model systems including *Aplysia* a marine mollusc (Shekib *et al.,* 2007), Drosophila (Quinn *et al.,* 1974), Zebra fish (Roberts *et al.,* 2013), *C. elegans* (Bargmann *et al.,* 1995) and rodents (Jaramillo *et al.,* 2014). The data suggest that adaptive learning have the same molecular pathway in all these models.

The results presented in this thesis demonstrate that the organism follows olfactory adaptation in a unique manner. For example to sense different kinds of odourants and to adapt to those are found to have differences in calcium dependency. Cross adaptation to various volatile odours can be considered as response based on experience of the animal (Colbert and Bargmann, 1995). This experience also found to be unique as butanone exposure did not show recognition to other odours.

C elegans are shown to have change its behaviour based on its past experience. Studies have shown that the organism has a rapid adaption towards salt and touch responses (Kano T 2008). Touch response is a nonassocitive form of learning and has been studied to understand habituation pathways (Maguire *et al.,* 2011). *C. elegans* also seems to remember the temperature in which animals are raised as they preferentially move towards that specific temperature in a temperature gradient (Ramot *et al.,* 2009).

Studies have shown that *C. elegans* can adapt to odourants when associated with a conditional stimulus (Van der Kooy *et al.,* 2012). Recent studies have suggested that

attractive and repulsive learning behaviour involves different neuronal connectome (Bargmann *et al.*, 2016).

One of the aim of these studies to evaluate whether there is a discrete selection between odorant because all the three odourants tested are detected by a single pair of AWC neuron. The results showed that indeed there are specific pathways for each odour detection as well as adaptation. Worms can cross adapt with benzaldehyde and isoamyl alcohol but not with butanone which indicates that AWC neuron responsible for sensing these odourants have specific signaling pathways especially during adaptation. These results indicate that individual olfactory adaptation allows discrete changes in the signaling pathways of neurons, probably through selectively switching off/on those receptors or signaling pathways. For example for oxygen sensing in metazoans HIF plays a pivotal role (Kaelin and Ratcliffe 2008) by altering its level in the cell. Similarly in human it has been found that a single bout of exercise alters the levels of transcription factors MEF2, HDACs and NRFs (Egan and Zierath 2013).

During learning a series of signaling pathways gets initiated in neurons, including synaptic plasticity connectome variations, variations in neurotransmitter release patterns (Kandel E *et al.*, 2001). All these events occur at synaptic level. Among various players, calcium is an important candidate in regulating depolarising signals and providing synaptic activity (Brini *et al.*, 2014). Besides protein kinases and arrestins, which plays a critical role in olfactory adaptation in mammals, found to get activated (Chen and Yau 1994). One possibility is that all the signaling changes are occurring at AWC neuron or it might have a more complex neuronal integration with inter neurons. GPCR STR-2 found to have role in odour discrimination in *C. elegans* by randomly

expressing either left or right AWC neurons (Bargmann CI and Wes 2001). This interesting observation suggests that there is AWC^{on} and AWC^{off} neurons allows segregation of odours for their detection.

Chelating the extracellular calcium blocked adaptation to all odourants gets disrupted except for isoamyl alcohol, indicating these adaptations are distinct and partially overlapping. There are indications olfactory adaptation occurs are axon terminals which depends on the Ca^{2+}stores in Drosophila (Murmu *et al.*, 2011). However the results show that impairment of N-methyl-D-aspartate receptor (NMDAR)s could result in odour recognition and adaptation pathways. Interestingly in mammals functional NMDA receptors in olfactory bulb is essential in odour habituation (Chaudhury *et al.*, 2010). However in *C. elegans* NMDA receptors are not present in AWC neurons but in AVA, AVD, AVE, AVG, and PVC interneurons. This indicates that one or more of these interneurons are involved in odour recognition and adaption pathway. Killing AVA interneurons have found to affect avoidance behaviour towards isoamyl alcohol but killing AVE did not affect this behaviour (Yoshida *et al.*, 2012). AVA and AVD are command interneurons controls forward and backward locomotion (White *et al.*, 1986). AWC neurons are glutamatergic and have extensive cross talk with various

interneurons (Chalasani *et al.*, 2010) but has a single synapse with AVA which expresses NMDA receptor (see Fig 12, 13 and 14).

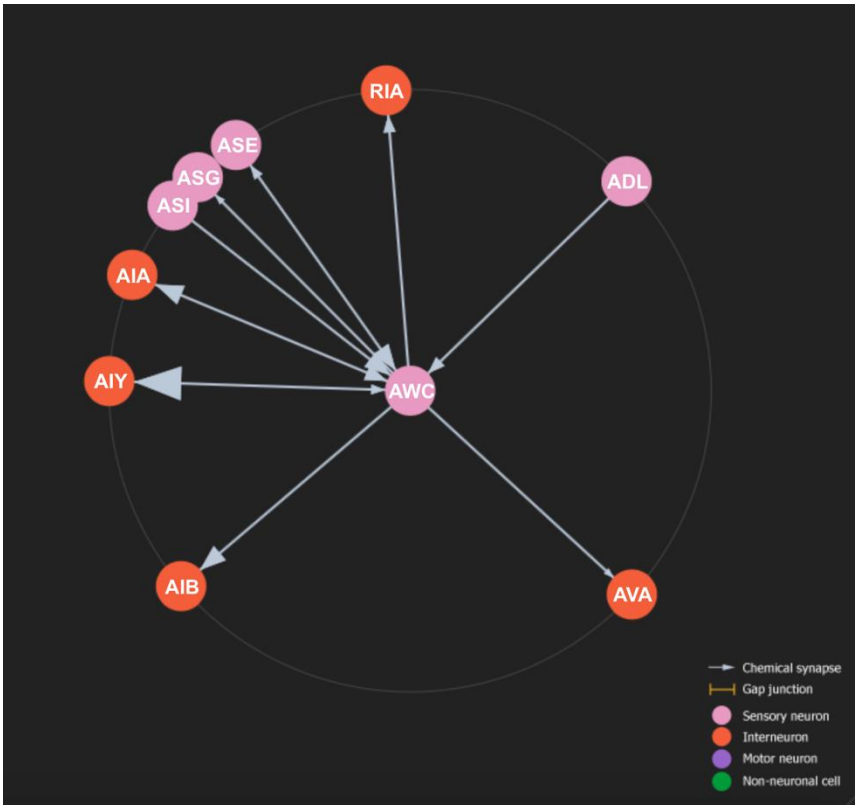

Figure 12: AWC connectome map using http://wormweb.org/. AWC has a synaptic link to AVA interneuron.

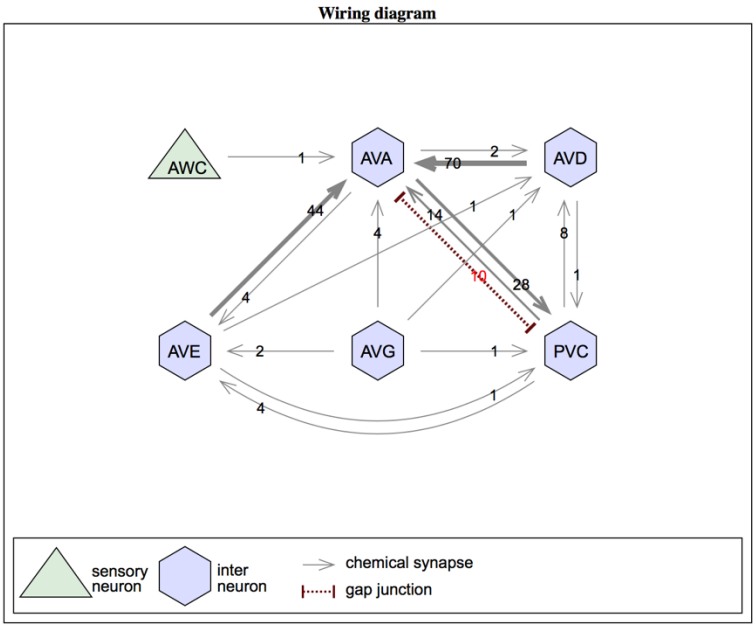

Figure 13: Synaptic connectivity data. The data was generated using *http://ims.dse.ibaraki.ac.jp/ccep-tool/*

Synaptic connectivity data

presynaptic neuron class	(A) somatic nervous system						(B) pharyngeal nervous system sources of data are the figures in [2]		
	(A-1) sources of data are the figures in [1]			(A-2) sources of data are the tables and the text in [1]					
	connection	chemical synapse	gap junction	connection	chemical synapse	gap junction	connection	chemical synapse	gap junction
AVA	AVAL(in) --> AVAR(in) AVAR(in) --> AVAL(in)	0 0	1 1	AVA(in) --> AVA(in) AVA(in) --> AVD(in) AVA(in) --> AVE(in) AVA(in) --> PVC(in)	3 2 4 28	4 0 0 10	no data	-	-
AVD	no data	-	-	AVD(in) --> AVA(in) AVD(in) --> AVD(in) AVD(in) --> PVC(in)	70 2 1	0 0 0	no data	-	-
AVE	AVEL(in) --> AVER(in) AVER(in) --> AVEL(in)	0 0	1 1	AVE(in) --> AVA(in) AVE(in) --> AVD(in) AVE(in) --> PVC(in)	44 1 1	0 0 0	no data	-	-
AVG	no data	-	-	AVG(in) --> AVA(in) AVG(in) --> AVD(in) AVG(in) --> AVE(in) AVG(in) --> PVC(in)	4 1 2 1	0 0 0 0	no data	-	-
AWC	AWCL(se) --> AVAL(in) AWCR(se) --> AWCL(se)	1 2	0 0	no data	-	-	no data	-	-
PVC	PVCL(in) --> AVAR(in) PVCL(in) --> AVDL(in) PVCL(in) --> AVDR(in) PVCL(in) --> AVER(in) PVCL(in) --> PVCR(in) PVCR(in) --> AVAL(in) PVCR(in) --> AVAR(in) PVCR(in) --> AVDL(in) PVCR(in) --> AVDR(in) PVCR(in) --> AVEL(in) PVCR(in) --> AVER(in) PVCR(in) --> PVCL(in)	2 3 2 1 2 1 3 2 1 1 2 1	0 0 0 0 1 0 0 0 0 0 0 1	PVC(in) --> AVA(in) PVC(in) --> AVD(in) PVC(in) --> AVE(in) PVC(in) --> PVC(in)	14 4 2 1	10 0 0 4	no data	-	-
	Total[*]	24	3	Total[*]	185	18	Total[*]	0	0

Figure14: **Synaptic connectivity data**. For the sum of chemical synapses, neuromuscular junctions are included when the connections to muscle ("MUSCLE", "VUL" and "m") exist on the chosen neuron classes. For the sum of gap junctions, the actual total number of them is shown. Since a gap junction is bidirectional, the actual total number is half of the sum. It should be noted that there are gap junctions within the identical neuron (class), such as in RIBL neuron. Columns A: In the paper on the somatic nervous system, the data presented in figures and that in tables are NOT complementary each other. In some cases, therefore, data mergence of the columns A-1 and A-2 would lead to double-counting of synapses. Being different from the columns A-1 (data presented in the figures), furthermore, the columns A-2 (data presented in the tables) describe the synaptic connectivity between neuron classes, not between neurons except for motorneurons. For example, a connection between neuron classes "AVA --> AVB" indicates one of the four combinations "AVAL --> AVBL", "AVAL --> AVBR", "AVAR --> AVBL" or "AVAR --> AVBR". Columns B: The columns B (synaptic data on the pharyngeal nervous system) and the columns A (synaptic data on the somatic nervous system) are complementary each other. The columns B, however, describe the synaptic connectivity between neuron classes, not between neurons. The number of neuromuscular junctions is ambiguous. 1. J. G. White, E. Southgate, J. N. Thomson and S. Brenner, "The structure of the nervous system of the nematode C.elegans", Phil. Trans. R. Soc. London B 314 (1986) 1-340.2. D. G. Albertson and J. N. Thomson, "The Pharynx of C.elegans", Phil. Trans. R. Soc. London B 275 (1976) 229-325.The data was generated using http://ims.dse.ibaraki.ac.jp/ccep-tool/

This suggest that in addition to AWC, AVA interneuron is critical in learning behaviour. Both the NMDA receptor mutants tested showed no defect in movements. It would be interesting to test whether these behavioural defects are confined to odour detection or extends to chemotaxis behaviours like attraction towards salt gradients.

Adaptive behaviour allows organism to learn from its past experiences. Complex molecular systems involved in it have similarity to visual systems of human where it learn to understand the low and high light levels (Stryker, 1998). The adaptive learning in *C. elegans* follows a similar pathway and allows it to learn from various clues from the environment.

CHAPTER - 4

SUMMARY AND CONCLUSION

The study aimed to decipher the olfactory sensing and adaptation of *C.elegans* to different volatile odourants. It was known that C. elegans can sense at least five odourants namely butanone, benzaldehyde, isoamyl alcohol, diacetyl, and pyrazine by a single pair of sensory neuron AWC.

We found that the adaptation behaviour of worm vary to conditional stimuli with different odourants. Based on the paradigm of starvation and food as conditioning stimulus, we found very strong adaptation on both cases. But most predictable adaptation was observed when starvation was used as the conditional stimulus. I specifically selected three attractive concentrations of odourants namely butanone, benzaldehyde and isoamyl alcohol and found that worms could adapt to these odours when starvation was used as the conditional stimulus.

Further, it is interesting to know whether adaptation to one odorant will make the worms cross adaptated another odour. The result showed that cross adaptation is limited to as set of odours, indicating there are specific pathways in each odour detection as well as adaptation. For example, worms could cross adapt to benzaldehyde and isoamyl alcohol while no cross adaptation was found towards butanone. This indicate the possibility

that the AWC neuron, which senses all three odorants tested, has specific signaling pathway towards these odours, especially during adaptation.

Since all these events occur at synaptic level, it was essential to understand the role of calcium in this adaptation pathway. Both ligand gated and voltage gated channels allow calcium ions to enter the neurons and initiate potentiation and cell-cell communication. When calcium was blocked, adaptation towards all the odorants were impaired except for isoamyl alcohol. This result again reiterated the fact that the recognition and adaptation to odours are more complex than anticipated.

To explore further, role of ionotropic calcium channel NMDA receptor in these adapations were probed. *C. elegans* have two NMDA receptor subunits namely nmr-1 and nmr-2 which are functionally similar to human NMDA receptors subunits. Our results showed that both nmr-1 and 2 NMDA receptors are essential for odour recognition as well as adaptation. Interestingly while nmr- 1 mutants were able to sense benzaldehyde and isoamyl alcohol, a complete knockout in odourant detection was observed in nmr-2 mutants. This observation is significant because it questions the earlier belief that recognition of odours are confined to the sensory neuron AWC. Since both nmr-1 and -2 are lacking in AWC neurons, and is present in interneurons like AVA, AVD, AVE, AVG, PVC, the odour recognition could be happening downstream to AWC. Besides, both nmr-1 and nmr-2 were found defective in cross adaptation pathway. Involvement of interneurons in odourant recognition pathway is a novel observation in this study. It would be interesting to study further the downstream connectome in odourant sensing and adaptation of solvents, so that a better

understanding on how neurons recognize signals and process it to decode the information.

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ANNEXURE

1. Nematode Growth Medium (NGM)

For 100 ml

NaCl	:	0.3 g
Agar	:	1.7 g
Peptone	:	0.25 g
Distilled water	:	97.5 ml

Cover it and autoclave for 50 min. Cool the flask in 55^{0}C for 15 min in water bath. Then add:

1 M CaCl2	:	0.1 ml
5mg/ml cholesterol	:	0.1 ml
1 M MgSO4	:	0.1 ml

1 M KPO4 buffer 2.5 ml

Mix well and pour into petriplates and allow to dry.

2. Chemotaxis medium

For 100 ml

Agar : 2g

Cover it and autoclave for 50 min. Cool the flask in 55^0C for 15 min in water bath. Then add:

1 M MgSO4 : 0.1 ml

1 M KPO4 buffer : 0.5ml

1 M CaCl2 : 0.1 ml

Mix well and pour into petriplates and allow to dry.

3. M9 buffer

For 1 litre

KH2PO4 : 3 g

Na2HPO4 : 6 g

NaCl : 5 g

1M MgSO4 : 1 ml

Mix well and make up the solution to 1 litre and sterilize by autoclaving.

3. Odourant Dilutions

1/1000 Butanone: Dissolve 1µl butanone in 999 µl ethanol.

1/200 Benzaldehyde: Dissolve 1µl benzaldehyde in 199 µl ethanol

1/100 Isoamyl alcohol: Dissolve 1 µl isoamyl alcohol in 99 µl of ethanol