Investigating the role of EXP-1, a cation selective excitatory GABA receptor in chemotaxis towards volatile attractants

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

Indian Institute of Science Education and Research Mohali

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Certificate of Examination

This is to certify that the dissertation titled "Investigating the role of EXP-1, a cation selective excitatory GABA receptor in chemotaxis towards volatile attractants" submitted by Ms. Aiswarya Joy M (Reg. No. MS11063) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rajesh Ramachandran **Dr. Shravan Mishra** **Dr. Kavita Babu**

(Supervisor)

Dated April 20, 2016

Declaration

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

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

Aiswarya Joy M

(Candidate)

Dated: April 20, 2016

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

Dr. Kavita Babu

(Supervisor)

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Abstract

C. elegans can sense a variety of volatile as well as gustatory cues through its highly developed chemosensory system. In this project, the role of a cation selective excitatory GABA receptor in chemotactic response to various volatile chemicals was investigated. Interestingly it was found that loss of function mutants of *exp-1* avoided isoamyl alcohol, an attractant for wild-type worms. When the response to other volatile attractants was investigated it was noticed that the particular set of attractants sensed through AWC amphid sensory neuron was being avoided. Upon performing pan-neuronal (using promoter for rab-3) rescue for *exp-1* mutants phenotypes, it was to found that *exp-1* mutant related phenotypes were partially rescued which confirmed that altered chemotactic response of *exp-1* mutant was a neuronal defect. Further other behaviours those are triggered by AWC neuron – reversals and omega turns (Ω) were observed. They were also found to be defective confirming the essential role of EXP-1 in AWC neuron-dependent phenotypes.

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Chapter 1

Introduction

1.1. BASIC THEORY

C elegans: Model organism in Neurobiology

Caenorhabditis elegans is a widely used model organism in various fields of biological research such as genomics, cell biology, aging and neuroscience. Major advantages of choosing *C. elegans* as a model organism are its short life cycle, compact genome, stereotypical development, ease of propagation and small size. Its body plan is quite simple with 959 somatic cells.[1] In past few years, *C. elegans* emerged as a favoured model organism for neurobiology due to its compact and transparent nervous system that provides for an easier approach to study various aspects of neuronal signaling and behaviour. It has 302 neurons of which, 32 are chemosensory, 28 are mechanosensory and above all has the majority of neurotransmitters found in mammals. They can learn from the mechanosensory and chemosensory inputs they encounter and manifests it in the form of locomotory behaviour .[2]

Learning and Memory in *C. elegans*

C. elegans can learn and remember chemical and mechanical cues that it encounters in its environment. In 1986, John White performed electron microscopic studies of serial sections and reconstructed the nervous system in *C. elegans.* The wiring diagram constructed from the studies predicted all putative neuronal synapses.[3] This led to the belief that 302 neurons in the organism are hard wired. But in 1990, the first paper focused on learning and memory in worms changed this belief and recognized the behavioural plasticity in the worms.[4] *C. elegans* can learn from the stimuli and approaches tastes and odours that indicate the presence of food and avoid those that indicate harmful chemicals or the

environment. They exhibit non-associative forms of learning like habituation, repeated stimuli decreases the response to that stimuli and sensitization. [5]

Worms also exhibit associative learning, where two events are coupled and the response is dependent on both stimuli. Studies also indicate that worms have the ability to form short-term as well as long-term memory. [2]

Multiple neurotransmitters/neuromodulators are known to be involved in learning and memory formation. Different roles of multiple neuromodulators in learning and memory formation could be well conserved. [6]One such neurotransmitter is dopamine. Abnormal dopaminergic signaling is associated with several memory disorders in humans. Dopamine receptive neurons are localized to morphologically plastic dendritic spine regions. [7] In *C. elegans*, dopamine has been reported to be involved in associative learning of chemical cue and starvation and habituation to mechanical stimuli modulated by presence of food.[8]

Dopamine and neuropeptide signaling was also shown to be essential for behavioural modulation towards non associative odor learning (chemotaxis) in *C. elegans.*[9]

In this study I performed preliminary studies to identify proteins basically cell adhesion molecules those showed co-expression in dopaminergic neurons so as to identify any cross connection between the specific cell adhesion molecules and dopamine auto-receptor, DOP-2 with respect to their role in associative learning.

Dopamine and dopamine receptors

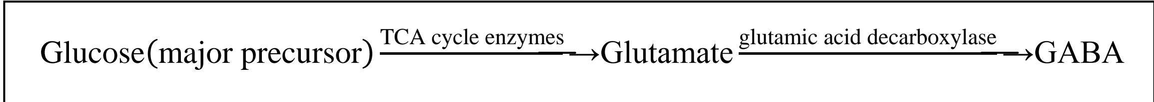
Four biogenic amines in *C. elegans* are octopamine, tyramine, dopamine, serotonin. They modulate behaviour in *C. elegans* by acting at neurons as well as muscles and are known to be involved in egg laying, pharyngeal pumping, locomotion and learning. Dopamine and serotonin have receptors and downstream signaling in *C. elegans* is similar to those in mammals and therefore is an excellent model system to study biogenic amine signaling in the brain. In *C. elegans* dopamine synthesis occurs in eight neurons: two anterior deirid neurons (ADE), two posterior deirid neurons (PDE) and four cephalic neurons.[3] Dopamine synthesis involves the enzymatic pathway as shown here:

In humans, receptors for dopamine are seven transmembrane G protein coupled receptors categorized as D1- and D2- like receptors. Activated D1- like receptors couple to $G\alpha_s$ and activate adenylcyclase, and D2- like receptors tend to act antagonistically to D1 receptors mediating signal transduction through $G\alpha_i$. In *C. elegans,* the genome is known to code for six dopamine receptors: *dop-1, dop-2, dop-3, dop-4, dop-5, and dop-6.* DOP-1 is classified as D1-like receptor and DOP-2 and DOP-3 are classified as D2-like receptors. DOP-4 is an invertebrate specific. Loss of function mutants of *dop-1 and dop-2* show phenotypes such as fast habituation and deficit in associative memory formation respectively. [10,11,12] DOP-2 receptor is unique in the sense that it is present in dopamine synthesising neurons and acts as an autoreceptor, regulating amount of dopamine being released. They are localized pre-synaptically.[13]

In this study we were interested in delineating the signalling pathway for DOP-2 receptor during associative learning. Initially we screened for cell adhesion molecules with expression overlap in dopaminergic neurons (Table. 1). However upon performing associative learning assay it became evident that none of these proteins showed any defects in associative learning whereas one of the other dopaminergic neuron expressing protein, EXP-1 showed an interesting phenotype

EXP-1: An excitatory GABA receptor

EXP-1 is an excitatory cation selective GABA (γ-aminobutyric acid) receptor. GABA receptors are members of ligand gated ion channel that mediate fast inhibitory neurotransmission in both vertebrates and invertebrates. [14]When GABA, the neurotransmitter binds the receptor, it opens the anion selective channel (Cl^- channel) which leads to hyperpolarisation and thereby inhibitory action on post synaptic cell. The biosynthesis of GABA is as follows:



In adult brain in vertebrates, GABA is majorly inhibitory. But there are reports where GABA functions as an excitatory neurotransmitter. Although excitatory

GABA receptor was not identified at molecular level until 2003, when *exp-1* was identified through genetic screens for expulsion defective mutants in *C. elegans* and characterised as an excitatory cation selective receptor.[14] The *exp-1* gene encodes a protein that is homologous to ligand-gated ion channel subunits. The predicted protein comprises of a large extracellular domain with a conserved disulphide bond, four transmembrane domains (M1-M4) and a large intracellular loop between M3 and M4. EXP-1 shares 21% identity with the human β2 subunit of GABA receptor. Alignments with GABA receptors indicate residues that line the GABA binding pocket are conserved. EXP-1 consists of residues that interact with GABA, found in α and β subunits, indicating that it can act as homomeric GABA binding protein. When GABA binds to the EXP-1 receptor, it mediates enteric muscle contraction in *C. elegans* required for defecation. In *C. elegans,* EXP-1 is expressed in intestinal and anal depressor muscles and PDA, RID, ADE, SABD neurons along with two unidentified neurons in the head ganglion.

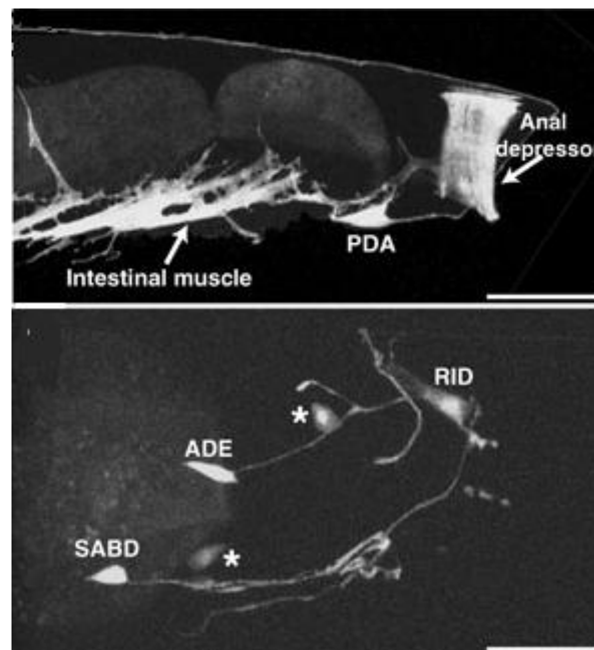


Figure 1 Expression pattern of exp-1 gene (i) Expression of EXP-1 in intestinal muscle, anal depressor muscle and PDA (ii) Expression of EXP-1 in head neurons(Beg & Jorgensen, *Nature* 2003)

Functions of this receptor, other than in enteric muscle contraction in *C. elegans* are not known. In this study, we found an interesting phenotype of loss for function mutants of *exp-1*. The mutants were found to move away from established attractant (isoamyl alcohol) of wild-type worms. . This lead us to investigate the role of EXP-1 in chemosensation.

Chemosensation

In *C. elegans,* chemosensory system is highly developed to sense a variety of volatile as well as gustatory cues associated with food, danger or other animals. 5% of its genes encode for those proteins that are required for recognition of environmental chemicals. It senses these chemicals through sensory cilia of the chemosensory neurons-amphid, phasmid and inner labial neurons that penetrate through cuticle to expose it to the environment.[3] Chemosensory neurons are generally found as bilaterally symmetric pairs in which left and right pairs are structurally similar.

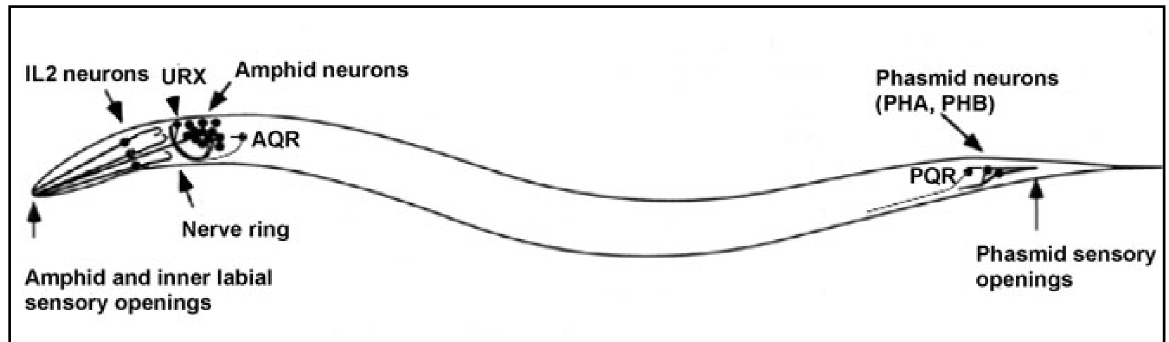


Figure 2 Major chemosensory neurons (Source: http://www.wormbook.org/)

A characteristic set of attractant/ repellents is sensed by particular receptors expressed on these neurons. About 500-1000 GPCRs are present on these chemosensory neurons. Downstream to these GPCRs are mainly two signal transduction pathways, one that uses cyclicGMP to open cGMP-gated channels and other that uses transient receptor potential channels. cGMP channels are encoded by *tax-2* and *tax-4* and are essential for functioning of ASE neurons that sense gustatory cues, AWC that sense volatile odours, AWB that detect repellents and AFD that mediate thermotaxis.[15] The plausible pathway of signal transduction is:

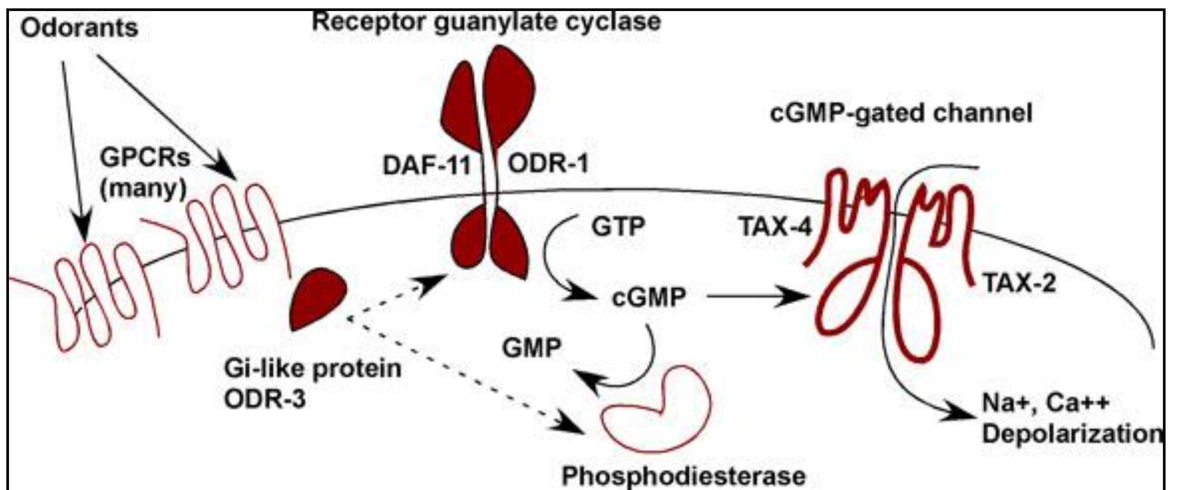


Figure 3 Potential signal transduction pathway for odor detection in AWC cilia (Source: http://www.wormbook.org/)

Transient receptor potential channel (TRP) is encoded by *osm-9* and *ocr-2*. The OSM-9/OCR-2 channel appears to be the transduction channel downstream of GPCRs and ODR-3 G protein signaling in AWA and probably ASH neurons.[1617]

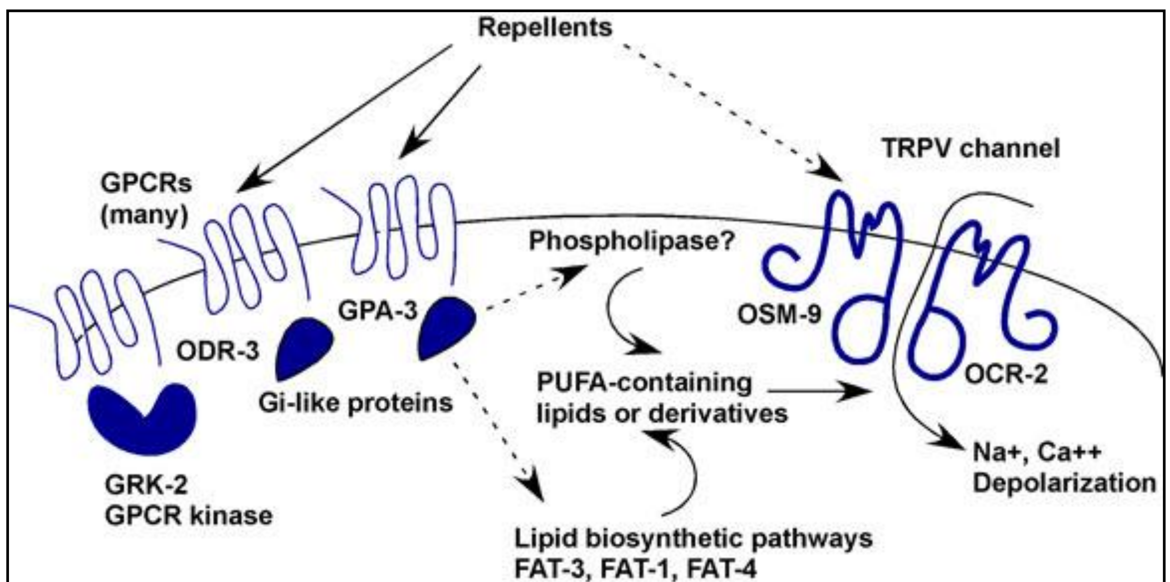


Figure 4 Potential signal transduction pathway for nociception in ASH cilia. (Source: http://www.wormbook.org/)

Chemosensory cues can elicit a variety of behavioural responses like chemotaxis, rapid avoidance, changes in overall motility and entry into and exit from the alternative dauer developmental stage. The response to volatile cues is both sensitive and diverse compared to that of water-soluble attractants. *C. elegans* can detect volatile odours in nano molar range. Many volatile compounds act as attractants and they are mostly products of bacterial metabolism in nature. Two

pairs of amphid sensory neuron that detect volatile odours are AWC and AWA. AWC detects benzaldehyde, butanone, isoamyl alcohol, 2,3-pentanedione, and 2,4,5-trimethylthiazole as attractants. [18]AWA detects diacetyl, pyrazine, and 2,4,5-trimethylthiazole. AWB senses volatile repellents like nonanone.[18]

Other functions of AWC neuron are in local search behaviour, turns and thermotaxis.[19,20]

Reversals

C. elegans explores its environment by forward movement and occasional reversal and turns. Reversals are changed in the mode of locomotion from forward to backward by changing the direction of propagation of sinusoidal waves. It also makes turns to change its direction of motion. The largest change in direction is generated in a sharp omega turn during which the animal's body shape resembles the Greek letter Ω.

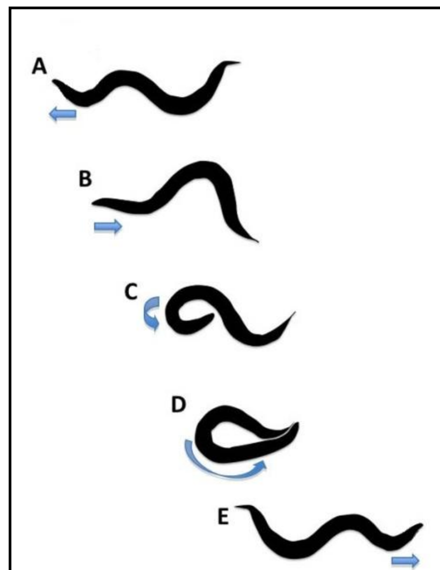


Figure 5 Reversals and omega turn A-B: reversal C-E: omega turn(Ω) (Alkema et al., 2005)

Reversals can be in response to acute stimulus (like touch) or spontaneous. Spontaneous reversals can occur as a result of the integration of various factors related to the environment as well as the internal state of the worm. Therefore, it can be a sensitive measure of behavioural state. Once the worm is removed from food, local search behaviour is triggered by AWC olfactory neurons, ASK gustatory neurons, and AIB interneurons. This is followed by dispersal. During

dispersal, reversals and turns are suppressed by ASI gustatory neurons and AIY interneurons. Downstream to interneurons AIB and AIY are other interneurons and motor neurons that determine certain aspects of reversals and turns such as amplitude, frequency, directionality etc. SMD motor neurons specify the steep amplitude of omega turns, RIV motor neurons determine the ventral bias of turns that follow a reversal, and SMB motor neurons encode the amplitude of sinusoidal movement.[19]

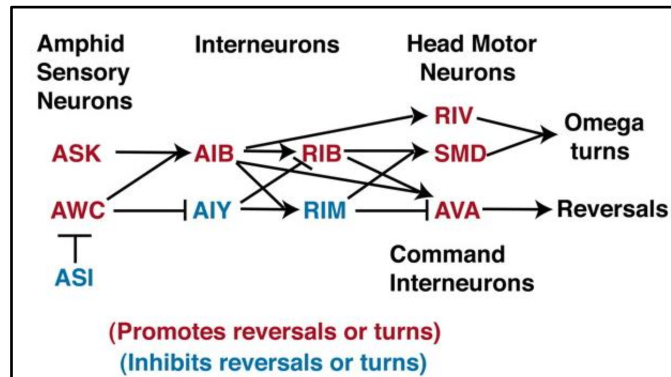


Figure 6 Neurons in navigation circuit[19] (Gray et al, 2005)

Chemotaxis and thermotaxis behaviours also involve the most of these sensory neurons, interneurons and motor neurons and therefore this circuit is common in many navigation behaviours.

1.2 EXPERIMENTAL METHODS

Materials and methods

Maintenance of worms

C. elegans is maintained in the laboratory on Nematode Growth Medium (NGM) agar which has been aseptically poured into Petri plates (Brenner, 1974). Plates are seeded with bacterial strain *E.coli* OP50, which serves as food source for *C. elegans*. To seed the plate, a drop of OP50 culture is dropped at the centre and spread using a glass rod spreader. Then plates were incubated at 37°C for 12-13 hours.

Strains

Wild type animals used are *C. elegans* strain N2. The mutant strains used were *exp-1(ox276) II, dop-2(vs105) V, exp-1(ox276) II; dop-2(vs105) V*, pceh-10:: EXP-1 and prab-3::EXP-1. *exp-1(ox276) II; dop-2(vs105) V*, pceh-10:: EXP-1 and prab-3::EXP-1 were kindly given by Nagesh Kadam.

Setting up a cross

Males of a particular strain are generated (by crossing it with N2 males) and picked on a cross plate. Hermaphrodites of the other strain are put on the plate in 1:4 ratio with males. Generally 10-12 males are put along with 3 hermaphrodites. F1 progenies are singled out on 5 plates and genotyped. F2 progenies are then singled out from heterozygous F1 plate and genotyped to get the desired genotype.

Genotyping

1-3 worms are picked from the plate and lysed in lysis buffer by the following program.

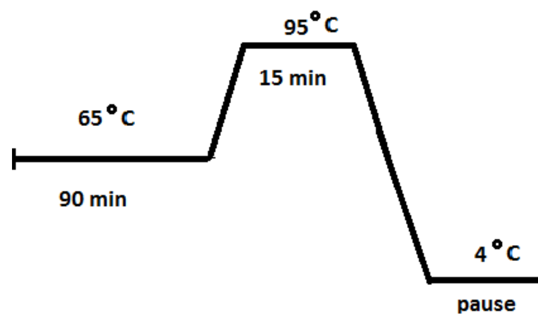


Figure 7 Thermal cycler- Worm lysis

The lysate is used as the template to put PCR. Two sets of primers are usually used – internal (designed such that one primer is from deletion region and the other, common primer) and external (one primer is out of deletion region and the other, common primer).

Behavioural assays

Chemotaxis assay

Well fed young adult worms were gently washed twice using the M9 buffer in an Eppendorf tube. Attractant (T) and control spot(C) was spotted on chemotaxis plate equidistant from the origin (O) spot as shown in the Figure 9. Sodium azide was also spotted at both the spots. Washed worms(~200) were dropped at the origin(O). The number of worms at test spot and control spot was counted after 90 minutes.[18]

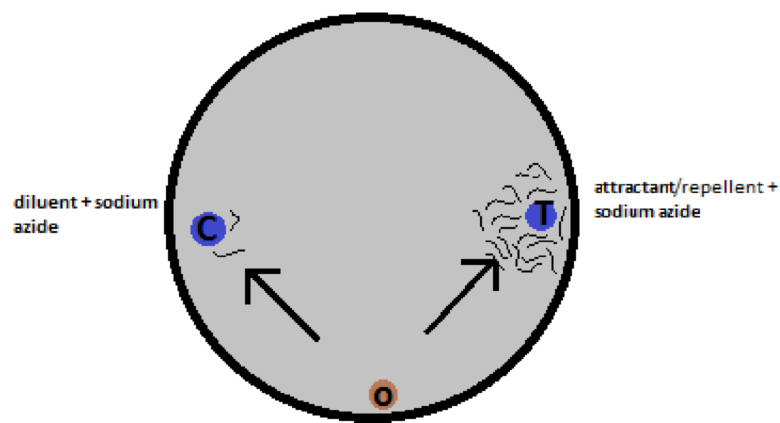


Figure 9 Chemotaxis assay plate (Bargmann et al., 1993)

Chemotaxis index was calculated as follows:

$$\text{Chemotaxis index} = \frac{\text{Number of worms at test spot (T) - Number of worms at control spot (C)}}{\text{Number of worms at test spot (T) + Number of worms at control spot (C)}}$$

Reversal assay

Well fed young adult worm was picked from a seeded plate (with food) to an unseeded plate (without food) and allowed to crawl for few seconds (10-15 sec). Then the worm was transferred to an unseeded plate. After 1 minute, the number of reversals and omega turns were counted for a duration of next 3 minutes and reversal rate and omega turn rate was calculated.

Generation of rescue constructs

1**. *exp-1* gene (genomic) is being cloned under its own promoter in vector pPD95.75**

Sites being used for *exp-1* gene: Kpn I and Xho I

Sites being used for *exp-1* promoter: Pst I and XbaI

2. ***exp-1* gene (genomic) is being cloned under *odr-1* promoter in vector pPD95.75 for AWC specific rescue.**

Sites being used for *exp-1* gene: Kpn I and Xho I

Sites being used for *odr-1* promoter: Pst I and XbaI

Results

Identification of Cell Adhesion proteins those function alongside Dopamine receptors in learning and memory

To shortlist the cell adhesion molecules we surveyed the proteins for their specific expression using the information available from Worm base as shown in Table. 1.

Table 1: Cell adhesion molecules showing expression overlap with dopamine receptors in *C.elegans*

Cell adhesion molecules	Expression overlap between cell adhesion molecules and dopaminergic receptors
cdh-11(casy-1)	**DOP-1**(VNC, tail neuron) **DOP-4**(intestine) DOP-3(VNC)
cdh-4	**DOP-1**(VNC) **DOP-4**(rectal gland cell, rectal epithelium, vulva) Dop-3(VNC)
cdh-6(fmi-1)	**Dop-2**(PDER, PDEL) **DOP-1**(PVQR, PVQL , VNC, cholinergic neuron, tail neuron) **DOP-3**(PVDR, PVDL, cholinergic neuron)
lad-1 (sax-7)	**Dop-1**(AVM, ALMR, ALML , PLMR , PLML,VNC) **DOP-3**(body wall musculature,VNC) **DOP-4**(CANR, CANL)
lad-2	DOP-1 (VNC) DOP-3(VNC)
F02G3.1(ncam-1)	**DOP-3**(NSMR, NSML) **DOP-4**(intestine)
F39H12.4(igcm-1)	**DOP-1**(tail neuron) **DOP-4**(CANR, CANL , intestine)
C26G2.1(syg-2)	**DOP-1**(ALNR, ALNL , PLNR, PLNL , RIML, RIMR) **DOP-3**(RICR , RICL , body wall musculature) **DOP-4**(vulva)
C33F10.5(rig-6)	**DOP-1**(ALMR, ALML, AUAR, RIBR, AUAL, RIML, RIBL , PLMR, PLML , RIMR, VNC) **DOP-2**(SIBVR, SIBVL SIBDR, SIBDL, SIAVR, SIAVL, SIADR, SIADL) **DOP-3**(RICR, RICL , SIAVR , SIAVL, SIADR SIADL, NSMR,NSML, VNC) **DOP-4**(CANR, CANL, rectal epithelium)
RIG-4	**DOP-1**(neuronal sheath cell, VNC) **Dop-2**(RIAR, RIAL) **DOP-4**(intestine, vulva)
WRK-1	**DOP-1**(socket cell, AUAR , AUAL , PLMR, PLML,

	excretory gland cell) **DOP-2**(PDA) **DOP-3**(RICR, RICL) **DOP-4**(intestine)
RIG-3	**DOP-1**(cholinergic neuron,) **DOP-3**(NSMR, NSML, cholinergic neuron) **DOP-4**(I1R, I1L , intestine)
SYG-1	**DOP-1**(RIS, RIML, RIMR, head muscle) **DOP-2**(SIBVR, SIBVL SIBDR, SIBDL, SIAVR, SIAVL, SIADR, SIADL) **DOP-3**(SIAVR, SIAVL, SIADR, SIADL) **DOP-4**(vulva)
C29A12.4(nrx-1)	Dop-6(nervous system, neuron)
ITX-1	**DOP-4**(intestine)
CRB-1	**DOP-4**(intestine)
EAT-20	**DOP-1**(ALNR, ALNL) **DOP-4**(R8BR, R8BL , R8AR, R8AL)
NLR-1	DOP-1 (VNC) DOP-3(VNC)
αINA-1	Dop-2 (male specific)
PAT-2	**DOP-1**(AVM, ALMR, ALML, PLMR, PLML, VNC, tail neuron) **DOP-3** (body wall musculature, VNC) **DOP-4**(vulva)
β pat-3	**DOP-4**(vulva)
DGN-1	**DOP-1**(ALNR, ALNL, PVQR, PVQL, PLMR, PLML) **DOP-4**(rectal gland cell, rectal epithelium, vulva)
SDN-1	**DOP-1**(VNC) **DOP-4**(vulva)
MUP-4	**DOP-3** (body wall musculature)

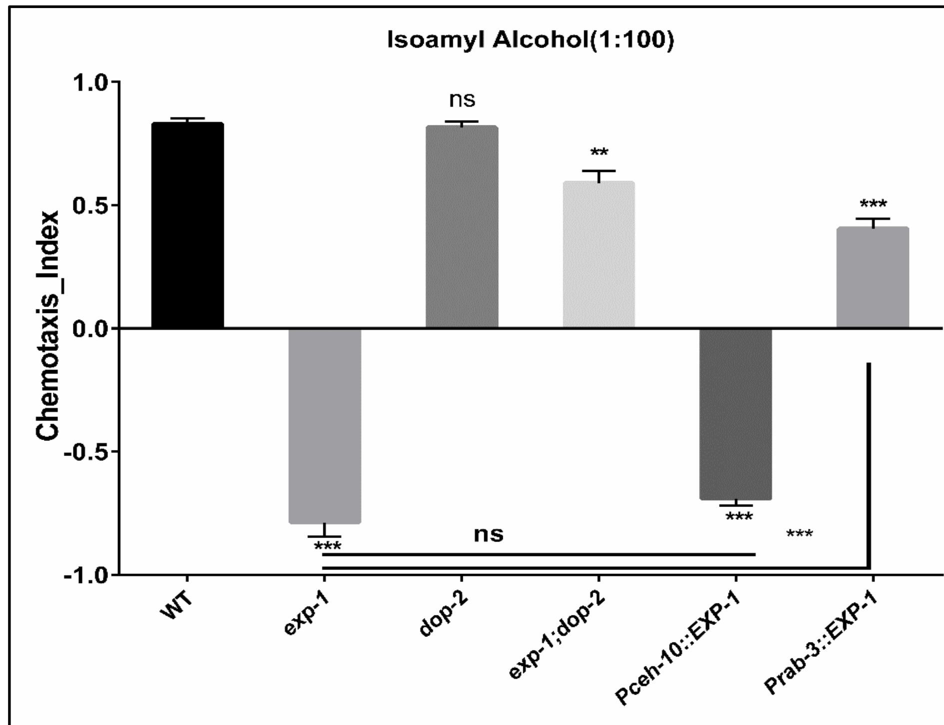


Figure 9 Response towards the attractant isoamyl alcohol (IAA)

Population chemotaxis assay with strains **WT, *exp-1(ox276) II, dop-2(vs105) V, exp-1(ox276) II; dop-2(vs105) V*, pceh-10:: EXP-1 and prab-3::EXP-1**. Asterisks indicate statistical significance.(* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test) . Error bars indicate standard error of mean (SEM). Number of animals per assay was around 200. Number of trials WT = 12, *exp-1*= 6, *dop-2* = 2, *exp-1; dop-2* = 3, pceh-10::EXP-1 = 3, prab-3::EXP-1 = 3. Isoamyl dilution was 1:100 in water

Chemotaxis assay was performed using attractant isoamyl alcohol. Our results indicated that *exp-1* loss of function mutants showed repulsion towards isoamyl alcohol which is an attractant for wild type (WT) worms. *dop-2* loss of function mutants did not differ significantly from wild type in chemotaxis towards IAA. The double mutant exhibited an intermediate phenotype. In order to establish the exp-1 mediated phenotype to be exp-1 dependent we performed chemotaxis using rescue lines where EXP-1 was expressed under the control of ceh-10 (RID neuron specific) and rab-3 (pan-neuronal) promoter. Pceh-10::EXP-1 is expressed in many neurons including RID(*exp-1* expressing neuron in wild type). This line was used as a negative control it did not rescue the defect in *exp-1* loss of function mutants. Prab-3::EXP-1 expresses EXP-1 in all neurons(pan neuronal). However, we have to perform rescue using few other Prab-3::EXP-1 transgenic lines but it is possible that incomplete rescue might be due to overexpression of EXP-1 since it has

been shown to be expressed in few specific neurons as compared to the wide expression obtained from Rab-3 promoter.

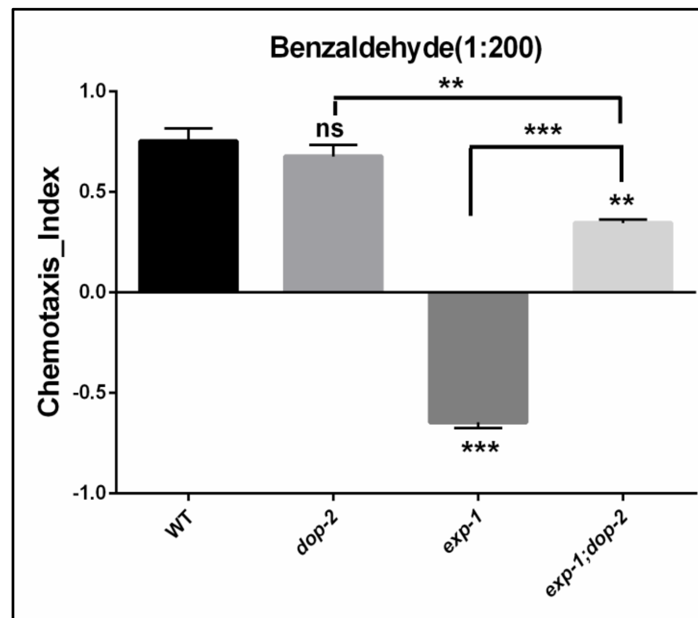


Figure 10 Response towards the attractant benzaldehyde

Population chemotaxis assay with strains **WT, *exp-1(ox276) II, dop-2(vs105) V and exp-1(ox276) II; dop-2(vs105) V***. Asterisks indicate statistical significance.(* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test) . Error bars indicate standard error of mean (SEM). Number of animals per assay was around 200. Number of trials WT = 5, *exp-1* = 4, *dop-2* = 2, *exp-1; dop-2* = 3. Benzaldehyde dilution was 1:200 in ethanol.

Chemotaxis was also performed using benzaldehyde, since the response in worms is mediated through AWC neuron as in the case of isoamyl alcohol. The phenotype observed was similar to isoamyl alcohol as shown in Fig. 10. *exp-1* loss of function mutants are showing repulsion towards benzaldehyde which is an attractant for wild type (WT). *dop-2* loss of function mutants does not differ from wild type significantly in chemotaxis towards benzaldehyde. However the *exp-1;dop-2* double mutant showed an intermediate phenotype.

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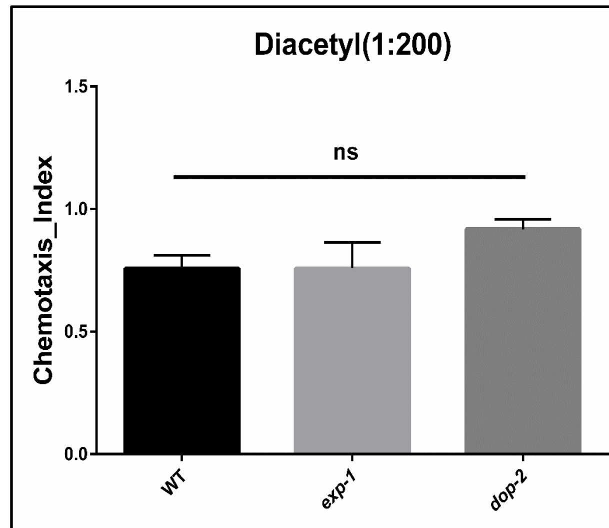

Figure 11 Response towards the attractant diacetyl

Population chemotaxis assay with strains **WT, *exp-1(ox276) II and dop-2(vs105) V.*** Asterisks indicate statistical significance.(* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test) . Error bars indicate standard error of mean (SEM). Number of animals per assay was around 200. Number of trials WT = 6, *exp-1*= 3 and *dop-2* = 2. Diacetyl dilution was 1:200 in ethanol.

Further we looked tested the response of the strains towards the other attractant that is sensed through other sensory neuron, AWB. There was no difference in chemotactic response towards diacetyl between wild type, *exp-1* loss of function mutants and *dop-2* loss of function mutants.

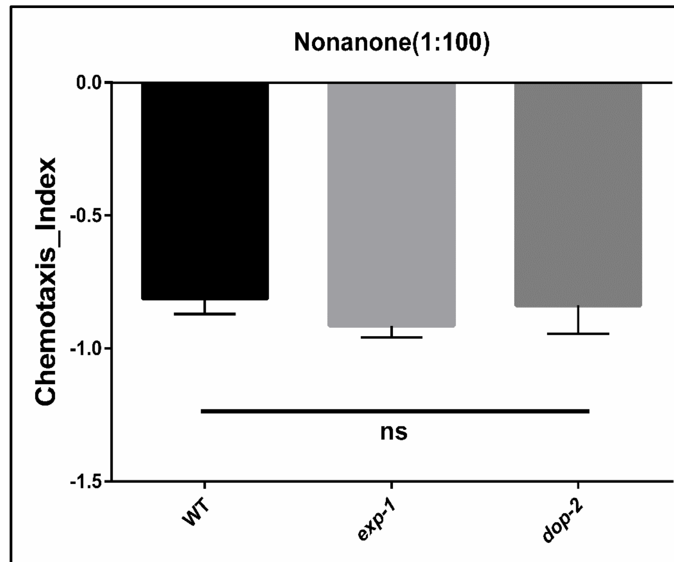

Figure 12 Response towards the repellent nonanone

Population chemotaxis assay with strains **WT, *exp-1(ox276) II and dop-2(vs105) V.*** Asterisks indicate statistical significance.(* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test) . Error bars indicate standard error of mean (SEM). Number of animals per assay was around 200. Number of trials WT = 6, *exp-1* = 3 and *dop-2* = 2. Nonanone dilution was 1:100 in water.

Next we wanted to study the response of *exp-1* towards the repellents. We decided to study the response towards the repellent nonanone that is sensed by AWA neuron. There was no difference in chemotactic response towards nonanone between wild type, *exp-1* loss of function mutants and *dop-2* loss of function mutants

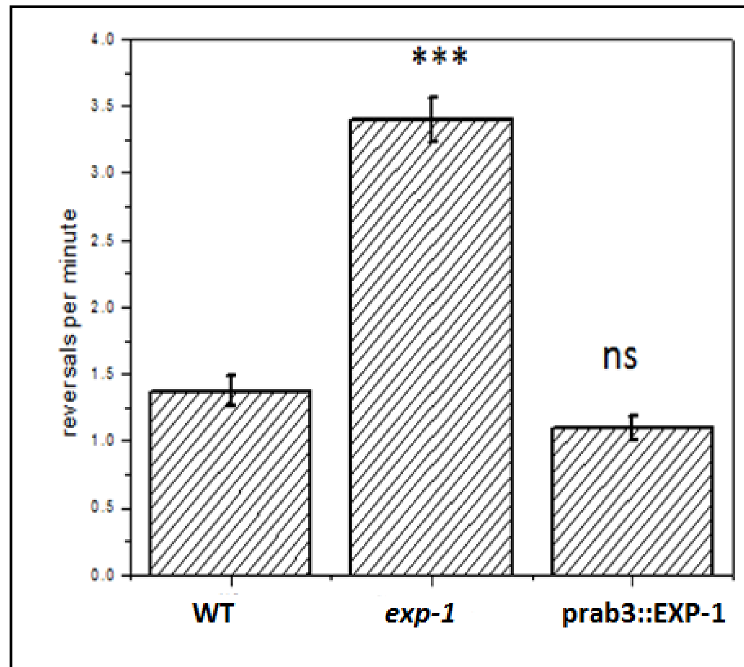

Figure 13 Reversals per minute is significantly higher in *exp-1* loss of function mutants

Reversal rate of **wild type (WT),** ***exp-1(ox276)II*** **) and prab-3::EXP-1**. Error bars indicate standard error of mean (SEM). Asterisks indicate statistical significance.(* $p<0.05$, ** $p<0.01$, ***$p<0.001$, ns $p>0.05$ (two sample t test). Number of worms used for the assay: WT =22, *exp-1*=25 and prab-3:: EXP-1=20.

Since our results indicate that the response of EXP-1 is linked specifically to AWC neuron thus we decided to look at the other phenotype that is mediated through AWC neuron. In our studies, we found that the reversal rate is significantly higher in *exp-1* loss of function mutants as compared to the wild type. Prab-3::EXP-1 expresses EXP-1 in all the neurons. This line could completely rescue the phenotype.

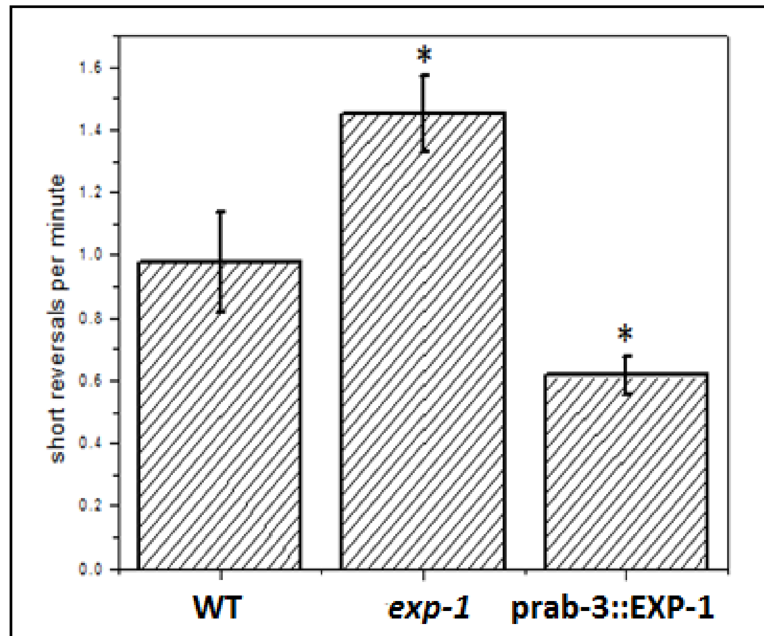

Figure 14 Short reversals (one body bend) per minute is significantly higher in *exp-1* loss of function mutants

Short reversals rate of **wild type(WT), *exp-1(ox276)II*) and prab-3::EXP-1**. Error bars indicate standard error of mean (SEM). Asterisks indicate statistical significance.(* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test). Number of worms used for the assay: WT =22, *exp-1*=25 and prab-3:: EXP-1=20.

We further characterized the reversals into different categories: short reversals (less than 2 body bends), long reversals (less than 4 body bends) and very long reversals (more than 4 body ends).Short reversals rate is significantly higher in *exp-1* loss of function mutants compared to wild type. Prab-3::EXP-1 expresses EXP-1 in all neurons. This line could rescue the phenotype.

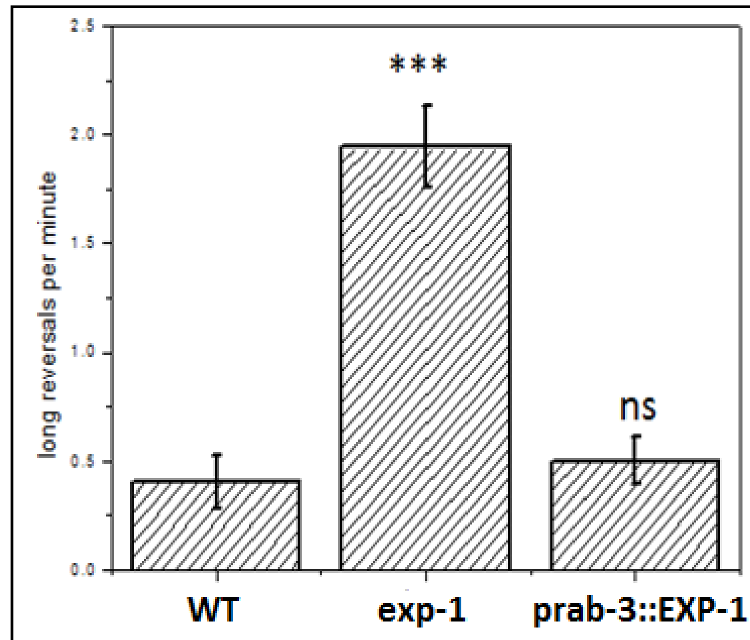

Figure 15 Long reversals (2or more than2 body bends) per minute is significantly higher in *exp-1* loss of function mutants

Long reversals rate of **wild type (WT), *exp-1(ox276)II*) and prab-3::EXP-1**. Error bars indicate standard error of mean (SEM). Asterisks indicate statistical significance.(* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test). Number of worms used for the assay: WT =22, exp-1=25 and prab-3:: EXP-1=20.

Long reversals rate is significantly higher in *exp-1* loss of function mutants compared to wild type. Prab-3::EXP-1 expresses EXP-1 in all neurons. This line could completely rescue the phenotype.

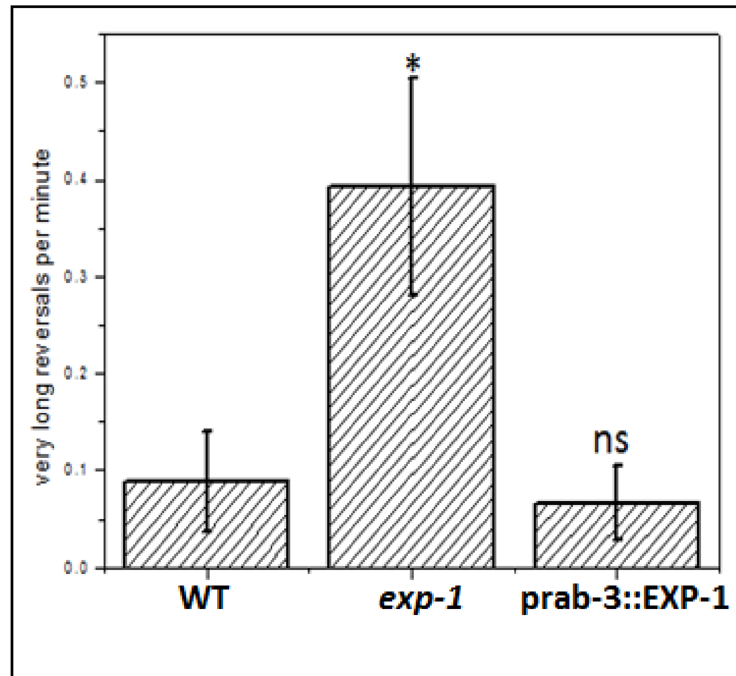

Figure 16 Very long reversals (3 or more than 3 body bends) per minute is significantly higher in *exp-1* loss of function mutants

Very long reversals rate of **wild type (WT), *exp-1(ox276)II*) and prab-3::EXP-1**. Error bars indicate standard error of mean (SEM). Asterisks indicate statistical significance. (* p<0.05, ** p<0.01, ***p<0.001, ns p>0.05 (two sample t test). Number of worms used for the assay: WT =22, exp-1=25 and prab-3:: EXP-1=20.

Very long reversals rate is significantly higher in *exp-1* loss of function mutants compared to wild type. Here again, we used Prab-3::EXP-1 rescue construct that expresses EXP-1 only in the neurons. This line could completely rescue the very long reversal phenotype obtained for *exp-1* mutants.

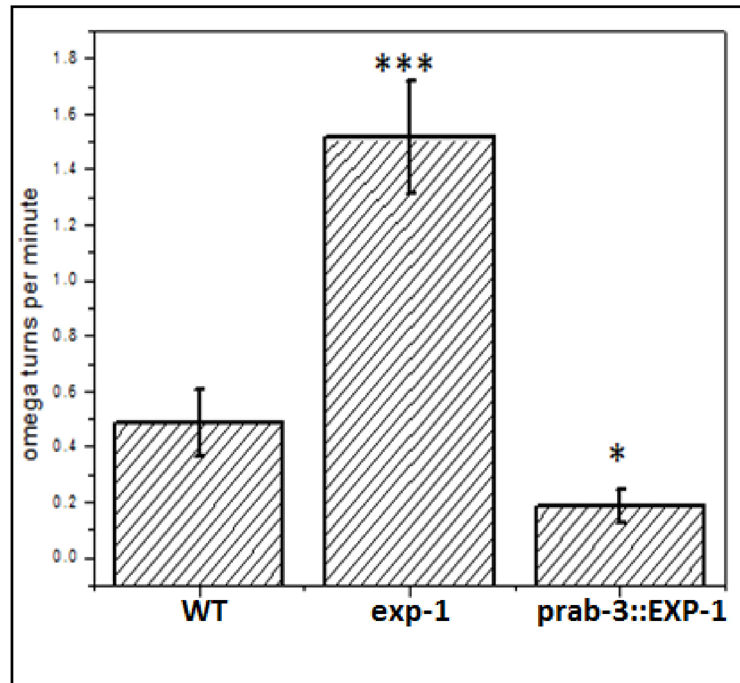

Figure 17 Number of omega turns per minute is significantly higher in *exp-1* loss of function mutants

Number of omega turns of **wild type (WT), *exp-1(ox276)II*) and prab-3::EXP-1**. Error bars indicate standard error of mean (SEM). Asterisks indicate statistical significance.(* $p<0.05$, ** $p<0.01$, ***$p<0.001$, ns $p>0.05$ (two sample t test). Number of worms used for the assay: WT =22, exp-1=25 and prab-3::EXP-1=20.

Number of omega turns is significantly higher in *exp-1* loss of function mutants compared to wild type. To identify if the effect was specifically related to EXP-1 we performed rescue using Prab-3::EXP-1 construct. We found that exp-1 reversal phenotype was completely rescued.

Chapter 2

Summary & Conclusions

2.1 Concluding remarks

DOP-2, a D2 like autoreceptor for dopamine in *C. elegans* and was previously shown to be involved in associative learning.[21,8] Initially we identified few cell adhesion molecules those showed expression overlap with DOP-2. Associative learning assays were performed to identify the possible functional connection between these cell adhesion molecules and DOP-2. One of the other molecules that showed expression overlap with DOP-2 is EXP-1, a cation selective excitatory GABA receptor[14]. Upon performing associative learning assay, it was quite surprising to find that EXP-1, instead of moving towards isoamyl alcohol was moving away from this attractant. Further, the assay was performed using different volatile attractants and repellents sensed by different sets of neurons- IAA and benzaldehyde sensed by AWC, diacetyl sensed by AWA, nonanone sensed by AWB . Pan-neuronal rescue was done to check if this is a neuronal phenotype. Reversal and omega turn frequency was also observed since chemotaxis and thermotaxis behaviours also involve most of the sensory neurons, interneurons and motor neurons involved in reversal and omega turn.

Chemotactic response of *exp-1* loss of function mutants were analysed and interestingly, they avoided the isoamyl alcohol, which is an attractant for wild type worms. *dop-2* loss of function mutants did not differ significantly in chemotaxis towards isoamyl alcohol. Chemotaxis index (CI) can vary from +1(perfect attraction) to -1(perfect repulsion). For exp-1 mutant chemotaxis index was -0.78 and in contrast for wild type, where it was +0.8. For *dop-2* mutants, CI was similar to wild type (+0.81). When EXP-1 was expressed pan-neuronally using the rab-3 promoter, a partial rescue was observed and the CI was +0.4. This indicated that the phenotype observed was a neuronal phenotype. Incomplete rescue might be due to the reason that pan-neuronal expression would be an overexpression of EXP-1 since EXP-1 is not expressed in all the neurons and over expression of the receptor might have caused some nonspecific changes causing defects in chemotactic behaviour.

Further assays were performed using other known attractants and repellents in

C. elegans –benzaldehyde, diacetyl, nonanone. For benzaldehyde, an attractant the response was similar to what it was for IAA. The chemotaxis index of *exp-1* loss of function mutants for benzaldehyde was -0.6 and in contrast, for wild-type and *dop-2* loss of function mutants were +0.75 and +0.68 respectively. Whereas for diacetyl, an attractant and nonanone, the repellent the chemotactic response of both *exp-1* and *dop-2* loss of function mutants was similar to that of the wild-type.

A particular set of attractants/repellents is sensed by a particular neuron and this holds true for the attractants and repellents used in above experiments. IAA and benzaldehyde are sensed by AWC, diacetyl is sensed by AWA and nonanone is sensed by AWB. This led to the conclusion that the response of EXP-1 is associated with amphid sensory neuron, AWC is associated with the IAA/benzaldehyde avoidance phenotype.

Other behaviours are also initiated by this amphid sensory neuron AWC. Reversals and omega turns are triggered by AWC neuron. So in order to further strengthen the AWC dependent phenotype of *exp-1* mutant. the reversal phenotype of worm was scored. It was found that *exp-1* loss of function mutants were defective in this behaviour also. Reversal frequency was 2.5 times higher in *exp-1* loss of function mutants when compared to wild type. Omega turn rate was 4 times higher in *exp-1* loss of function mutants when compared to wild type. Another noticeable phenotype is that very long reversal frequency is very significantly higher in *exp-1* mutants with 4.5 times increased frequency, whereas short reversal frequency is just 1.4 times increased. These changes might be due to the neuronal defects caused due to loss of EXP-1, the excitatory cation selective GABA receptor. Pan neuronal rescue line with EXP-1 under pan neuronal promoter *rab-3* was able to rescue the phenotype. This confirmed that the defects in reversal and omega turn frequency were due to neuronal defects.

2.2 Future outlook

When EXP-1 was first identified and characterized in *C. elegans* the expression pattern was also checked. But certain head neurons which expressed EXP-1 could not be identified. So we are generating EXP-1::GFP expression constructs and the expression pattern will be studied in the worms The previous expression profile from Jorgensen lab gave us a strong inference that EXP-1 might be expressed in AWC

sensory neuron or other associated neuron nearby. In order to check and confirm that it is expressed in AWC neuron, RFP labeled AWC neuron (using odr-1::RFP), in the background of translational fusion *exp-1*::GFP line will be imaged. Further we are also generating constructs so as to perform AWC specific rescue. Rescue using EXP-1 endogenous promoter will also be performed.

Structural defects are often associated with defects in chemotactic response [22]. So, RFP labeled AWC neuron (using odr-1::RFP) will be imaged in *exp-1* loss of function mutant background.

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