The role of a TGF-beta family morphogen DAF-7 in modulating synaptic transmission at *Caenorhabditis elegans* neuromuscular junction.

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A dissertation submitted for the partial fulfillment of

BS-MS dual degree in science

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CERTICATE OF EXAMINATION

This is to certify that the dissertation titled "**The role of a TGF-beta family morphogen DAF-7 in modulating synaptic transmission at *C. elegans* neuromuscular junction**" submitted by Mr. Kuldeep Singh Tomar (Reg.No. MS09075) 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 and finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Samarjit Bhattacharya **Dr. Shravan K. Mishra** **Dr. Kavita Babu**

(Supervisor)

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 bona fide record of original work done by me and all sources listed within have been detailed in the bibliography.

Kuldeep Singh Tomar

(Candidate)

Dated: 24 April, 2014

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

Morphogens are signalling molecules with prominent effects in the development of an organism. Morphogens form a long range concentration gradient by diffusing from their source (Tabata and Takei, 2004). Fate of a cell responding to a particular morphogen depends upon its localization along the concentration gradient (Charron and Tessier-Lavigne, 2007) . Recent findings suggest that along with their role in development, morphogens also play a role in axon development and guidance during development of the nervous system (Sanchez-Camacho and Bovolenta, 2009). This project aims to study and find out the role of DAF-7, a TGF-beta family morphogen in modulating synaptic transmission at *C. elegans* neuromuscular junction.

CONTENTS

CHAPTER 1

1.1 NTRODUCTION

C. elegans is a free living transparent soil nematode about 1mm in length. Majority of these nematodes are hermaphrodites and frequency of males happen to be as low as one in a thousand. *C. elegans* is easy to maintain in the laboratory. Development to the adult stage takes around 46 hrs from hatching at 20 degree Celsius and the whole life span is 2 to 3 weeks long (Byerly et al., 1976). Developmental pattern of all the 959 somatic cells of *C. elegans* has been traced (Brenner, 1974). Nervous system of *C. elegans* contains only 302 neurons and 7000 synapses. Moreover, the entire connectivity of the nervous system has been reconstructed by John White and colleagues using serial electron micrographs (Chalfie et al., 1985; Ward et al., 1975; White et al., 1983, 1986).

1.1.1 MORPHOGENS

Development is a very powerful process involving genome transforming one egg cell into a fully developed organism. For this intricate process to happen successfully, cells in initial stages of development require some positional information specifying their fate and that could guide them through this process (Teimouri and Kolomeisky, 2014). Dividing cells manage to organise and assign themselves into a specific pattern creating spatiotemporal pattern and division of labour. Most of these early guidance cues provided to the dividing cells are in the form of signalling molecules known as morphogens (Teimouri and Kolomeisky, 2014).

Morphogens are small secreted signalling molecules that are produced in a restricted region within a tissue and they spread from their source forming a long range concentration gradient. Fate of a cell responding to a particular morphogen depends on its location along the concentration gradient of the morphogen (Wartlick et al., 2009).

The concept of morphogens in developmental biology dates back to the time of pioneering study in *Drosophila melanogaster* (fruit fly) done by the famous geneticist Thomas Hunt Morgan in early 20th century. The concept of morphogenesis was redefined by Lewis Wolpert in 1960s with his famous French flag model. In French flag model, French flag is used to represent the effect of morphogen on a cell differentiation. Morphogen affects cell states based on its location along the concentration gradient (Wolpert, 1969). These states are represented with the different colours of the French flag, high concentration activates a blue gene, low concentration activates a white gene and red colour presents default state in the cells below the necessary concentration threshold(Wolpert, 1969).

Although this concept of gradient of signalling molecules regulating differential gene expression in a concentration dependent manner provides a basis for understanding various patterning processes but it also raises some questions like how the distribution of a morphogen maintains the gradient of activity, how does concentration based differential gene activation happen that precisely, how is signal perceived and interpreted in a graded manner? (Ashe and Briscoe, 2006) Recent work in the field of developmental biology has shed a significant amount of light on these questions and has helped in understanding these mechanisms.

The first identified morphogen, Bicoid, was identified by Christiane Nüsslein-Volhard (Driever and Nusslein-Volhard, 1988). Bicoid was one of the transcription factors present in a gradient in the *Drosophila* syncytial embryo (Driever and Nusslein-Volhard, 1989). Labs of Gary Struhl and Stephen Cohen demonstrated that a secreted signalling protein, Decapentaplegic (the Drosophila homologue of Transforming Growth Factor Beta), act as a morphogen during later stages of Drosophila development (Ferguson and Anderson, 1992; Struhl, 1982; Zecca et al., 1995). Some of the earliest and best studied morphogens are transcription factors that diffuse within Drosophila making a concentration gradient and regulating its development. However, most of the morphogens are secreted proteins that travel between cells for conducting some vital signalling mechanisms.

There exists 6 major families of such molecules, named, Decapentaplegic/ TGF-beta, Hedgehog/ Sonic hedgehog, Wingless/ Wnt, Epidermal growth factor, Fibroblast growth factor and Retinoic acid (Tabata and Takei, 2004).

1.1.2 TGF-beta

Transforming growth factor beta is a superfamily of proteins which includes inhibins, activin, anti-mullerian hormone, bone morphogenetic protein, decapentaplegic and Vg-1 (Gumienny and Savage-Dunn, 2013). Ligands of TGF-beta family play vital role in development and physiology of animals including cell proliferation, differentiation and other vital functions. TGF-beta is a cytokine which plays a role in immunity, heart disease , bronchial asthema, diabetes, cancer (Blobe et al., 2000), Marfan syndrome (Cherubin, 1992; Habashi et al., 2006), Parkinson's disease, AIDS and other such deficiencies of human immune system.

Along with their role in some vital cell differentiation and development processes, TGF-beta family members are also involved in inducing apoptosis and regulating cell cycle in numerous cell types (Hanahan and Weinberg, 2000). TGF-β causes synthesis of p15 and p21 proteins, these proteins block the cyclin:CDK complex which is responsible for the Retinoblastoma protein (Rb) phosphorylation and thus blocks the advance through G1 phase of the cycle (Hanahan and Weinberg, 2000). In doing so, TGF-β suppresses expression of c-myc, a gene which is involved in G1 cell cycle progression.

TGF-beta is a secreted protein which exists in at least three isoforms, TGF-beta1, TGF-beta2, TGF-beta3 .

TGF-beta1 is a member of TGF-beta superfamily of cytokines. It performs many vital roles including cell proliferation, cell differentiation and apoptosis. TGF-beta1 in humans is encoded by a gene named TGFB1 (Border and Noble, 1994). TGF-beta1 was first identified in human platelets as a protein involved in wound healing with molecular weight of 25 kilodaltons (Munger et al., 1997).

TGF-beta2 is also a TGF-beta superfamily cytokine that has vital roles in the embryonic development. TGF-beta2 bears several alternative names like BSC-1 cell growth inhibitor, Glioblastoma-derived T-cell suppressor factor, G-TSF, Cetermin and Polyergin (Clark and Coker, 1998).

TGF-beta3 is believed to be involved during palate development in regulating molecules involved in cellular adhesion and extracellular matrix formation. Absence of TGF-beta3

gives rise to a deformity named cleft palate in mammals (Herpin et al., 2004; Taya et al., 1999).

In the nematode *C. elegans*, TGF-beta family consists of five ligands: *unc-129, dbl-1, daf-7, tig-2* and Y46E12BL.1 (Gumienny and Savage-Dunn, 2013). Role for three out of five, *unc129, dbl-1, daf-7* is known but the roles of two *tig-2* and Y46E12BL.1 are not yet known (Gumienny and Savage-Dunn, 2013).

Studies have shown that *unc-129* gene is required for the guidance of the pioneer motor axons along the DV axis (Ikegami et al., 2004). Mutation in *unc-129* gives rise to defects in the dorsally oriented trajectories of motor neurons. Signaling pathway for UNC-129 is still being elucidated because it does not seem to be acting through TGF-beta related receptors and Smads (Ikegami et al., 2004).

DBL-1 the another TGF-beta related ligand acts through Sma/Mab pathway and regulate body size and development of male specific sensory rays and copulatory spicules found in the tail of a male worm (Gumienny and Savage-Dunn, 2013).

DAF-7 is known to be involved in regulating the dauer formation in *C. elegans (Ren et al., 1996).* This thesis is based on my work done on DAF-7. Detailed information about DAF-7 is as follows:

1.1.3 DAF-7

DAF-7 is a ligand for conventional TGF-beta signaling pathway. In *C. elegans*, DAF-7 act through this pathway to regulate the normal growth of the worm (as shown in fig. 1.1) (Ren et al., 1996). DAF-7 binding leads to the recruitment of type I and type II receptors into a heteromeric complex and type II recptor phosphorylates the type I receptor and thus activates the type I receptor kinase. Activated type I receptor complex phosphorylates and thus activates the receptor regulated Smad or R-Smads. R-Smads form heteromeric complexs with Co-Smads and translocates to the nucleus. As shown in fig. 1.1, this complex supress the activity of DAF-5 DAF-3 complex and thus avoid dauer larvae formation and promotes non dauer growth of worms (Ren et al., 1996). Activity of DAF-7 is one of the reasons for normal growth of the worms under physiological conditions. However under stress conditions, be it overcrowding, be it starvation or be it unfavourable temperature, concentration of a particular Pheromone, known as dauer pheromone

released by worms, increase in the environment. This increased concentration can be sensed by the chemosensory receptors of the worm present in amphid neurons. Presence of this pheromone initiates a series of reactions which degrade DAF-7 and thus worm enters the dauer larvae stage (Ren et al., 1996).

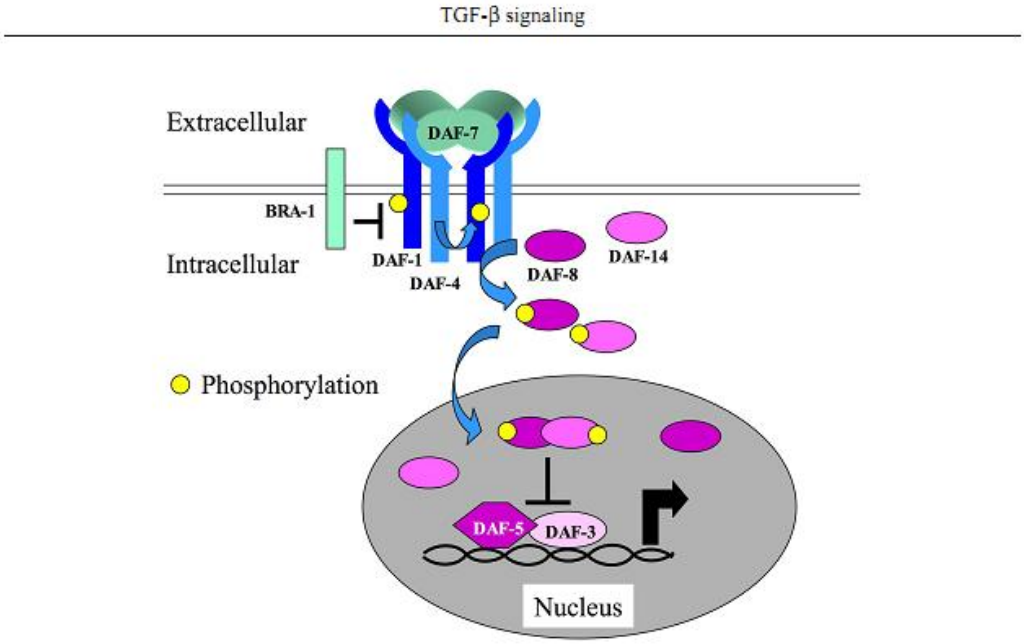


Fig.1.1 Dauer pathway, Image taken from Savage-Dunn, C. TGF-β signaling (September 9, 2005), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.22.1

Expression of DAF-7 was studied by Wendy S. Schackwitz et al. for the first time in 1996 (Schackwitz et al., 1996). They injected a pDAF-7::GFP (DAF-7 promoter was designed as 4.8 kb upstream to the ATG and 1.3 kb downstream to it) construct was injected in the wild type worm and expression was observed. Animals with this construct expressed pDAF-7::GFP in the cell body of one pair of bilaterally symmetric neurons in the lateral ganglia (Schackwitz et al., 1996). GFP was observed to be expressing right from L1 stage and expression went up in L2 stage, a stage right before dauer formation or a stage in which dauer formation is regulated. To corroborate that the fusion expresses in ASI neurons only, Schackwitz et al. killed both the ASI neurons in pDAF-7::GFP worms. In their study, four out of five worms did not show any fluorescence and the fifth animal

showed a weak fluorescence in the lateral ganglia in an unidentified cell which was not the cell body of ASI neurons (Schackwitz et al., 1996).

DAF-7 has homologs in other species as shown in table 1. CRE-DAF-7, a homolog of *C. elegans*'s DAF-7 in *C. remanei,* is a TGF-beta morphogen and is involved in the dauer pathway in *C. remanei*.

Similarly, GDF-8, a homolog of DAF-7 in *D. rerio* acts specifically as a negative regulator of skeletal muscle growth and down-regulate muscle-specific transcription factors such as myod and myog (Acosta et al., 2005; Amali et al., 2004; Xu et al., 2003) and it expresses predominantly in muscle and in the adult pharynx, kidney, spleen, liver, gill, eyes, skin, swim bladder and ovary (Helterline et al., 2007; Radaelli et al., 2003).

GDF-11 is a homolog of DAF-7 in *H. sapiens* and is involved in specifying the positional identity along the anterior/posterior axis during development and it also play critical roles in patterning both mesodermal and neural tissues and in establishing the skeletal pattern (Gamer et al., 1999; McPherron et al., 1999). Expression and function of other DAF-7 homologs mentioned in table 1 is not yet known.

Species	Hit	Description	BLAST E-value	% Length
C.remanei	RP:RP12288	CRE-DAF-7	4.8e-85	98.3%
C. brenneri	CN:CN26842	gene CBN22190	1.8e-83	99.4%
C. briggsae	BP:CBP13064	CBR-DAF-7	5e-82	93.1%
C. japonica	JA:JA49255	gene CJA10692	1.1e-81	85.1%
A. caninum	TR:Q58J20	TGH-2	6.1e-53	93.4%
P. pacificus	PP:PP43974	gene PPA00676	9.2e-29	94.0%
D. rerio	SW:O42222	Growth/differentiation factor-8 (GDF-8)	5.1e-23	90.0%
H. sapiens	ENSEMBL:ENSP00000448390	GDF-11	1.2e-19	91.1%

Table 1. Homologs of DAF-7 in other species. (legacy.wormbase.org)

These are the things known about DAF-7 so far. This project is based on finding the role of DAF-7 in modulating synaptic transmission at the *C. elegans* neuromuscular junction. There are various evidences present in the literature indicating that morphogens also act as axon guidance cues. Netrins, Slits, Semaphorins and Ephrins are four major families of

guidance cues with prominent effects during development of the nervous system (Tessier-Lavigne and Goodman, 1996). Netrins, Slits and some Semaphorins are sectreted molecules whereas Ephrins and other Semaphorins are anchored to the cell surface (Tessier-Lavigne and Goodman, 1996). Slits, Semaphorins and Ephrins act primarily as repellents but depending on the context they can also act as attractants. Netrins can act as attractant or repellent. During the development of nervous system activity of these signaling molecules help a neuron in finding its partner (Tessier-Lavigne and Goodman, 1996). But the number of receptors and guidance cues seems to be very small when compared to the immense complexity of the nervous system and many guidance events during early development do not seem to be using these molecules (Tessier-Lavigne and Goodman, 1996). This raises the possibility of the involvement of other molecules in axonal guidance during development. Over the last few years, members of three secreted signaling molecule's (morphogen) families, Wingless/Wnt, hedgehog and BMP/TGF-beta, have been shown to act as guidance cues during the development of the nervous system (Tessier-Lavigne and Goodman, 1996).

Studies on commissural neurons in the CNS of Drosophila shows that wnt5 repels commissural axons from the posterior commissure (Bonkowsky et al., 1999) and wnt4 controls anterio-posterior guidance of commissural neurons (Lyuksyutova et al., 2003). Shh is known to act as a chemo attractant for commissural axons and a negative regulator of retinal ganglion cell axon growth in mice (Charron et al., 2003). BMPs are chemorepellents for commissural axons (Augsburger et al., 1999). Wnt in *C. elegans* is known to regulate the number of acetylcholine receptors on the post synaptic muscle at the neuromuscular junction (Turski et al., 1988).

1.2 MATERIALS AND METHODS

1.2.1 WORM HANDLING AND MAINTENANCE

C. elegans are grown on nematode growth medium agar plates and OP50 strain of *E. coli* is used as a food source (Stiernagle, 2006). OP50 is a uracil auxotroph which help in giving the limited growth of bacteria over the plate.

Throughout the experiments, worms were maintained at 20 degree Celsius.

Transferring of worms from old plate to new plate can be done either by chunking, bleaching or picking them from old plate.

Chunking involves cutting a portion from old plate and putting it on a fresh seeded plate followed by labelling of the plate correctly (Stiernagle, 2006).

Bleaching involves washing plates with M9 buffer and collecting worms in the 1.5 mL Eppendorf tube. Worms were then treated with bleaching solution for dissolving their cuticle so that eggs could come out. This step was followed by washing of the egg pellet, eggs were then dropped on a fresh NGM plate so that they could hatch (Stiernagle, 2006).

Plates were kept at 20 degree Celsius for further experiments.

For picking the worms from old plate, a specialised platinum worm pick was used.

1.2.2 ACQUISITION OF STRAINS

The strains used for this project included N2 (Wild type worms), RB2302 (*daf-7* mutant worms), KP5861 (pUNC-25::SNB-1::GFP), KP5865(pUNC-17::RFP), KP5866 (pUNC-29::SNB-1::GFP), ZDIS5 (pMEC-4::GFP) and CZ1931 (pUNC-25::GFP). N2 was acquired in the lab from Dr. Sandhya P. Kaushika from NCBS. RB2302 was acquired from CGC.

RB2302 is a strain of *C. elegans* that has a deletion mutation in the *daf-7* gene. *daf-7* in its wild type form in *C. elegans* is a 2 kb gene having 5 exons in it. RB2302 is having a deletion mutation in this gene as shown in fig.1.2.

Fig. 1.2 ok3125 mutation in wild type *daf-7* gene. As shown in figure, *daf-7* gene consists of five exons (each pink bar represents an exon) in its wild type form. However *daf-7* mutant worms were having deletion mutation in the three exons as shown in figure.

1.2.3 OUTCROSSING

Best way to study the function of a gene is studying the deformities in organisms carrying mutation for that particular gene. For doing such gene specific studies in *C. elegans*, mutant strains for gene of interest are ordered from CGC. Other than the mutation in the gene of interest, this ordered strain might be carrying other background mutations as well, having potential to alter the phenotype. To obviate any such possibility, worms were outcrossed before starting any phenotypic study on them. Outcrossing involves crossing mutant strain's worms with wild type worms and selecting homozygous mutants from progeny using genotyping techniques (Stiernagle, 2006). Theoretically one can go on outcrossing a strain for infinitely but practically only outcrossing a strain up to 4X is supposed to be good enough to remove unlinked background mutations.

1.2.4 GENOTYPING

Genotyping is a tool for selecting homozygous mutant, heterozygous mutant or wild type worms. Genotyping involved a set of three primers, external forward primer, internal forward primer and reverse primer. Internal formal primer was deliberately designed in the region of gene that was missing in the *daf-7* worms. The genome of a homozygous mutant worm did not show any amplification when done PCR with internal forward primer and reverse primer. Heterozygous mutant showed the bands of two sizes, one equal to wild type and another smaller band subtracting the deletion with external primes. However wild type showed full amplification of the gene with external primers and a smaller band subtracting deletion in case of amplification with internal primers.

1.2.5 FREEZING

Caenorhabditis elegans can be frozen and stored indefinitely in liquid nitrogen (−196 °C). Freshly starved young larvae (L1-L2 stage) survive freezing best (Stiernagle, 2006).

Plates having freshly starved L1-L2 worms were washed with 750uL of S basal and transferred to a cryo vial (placed in an ice bucket).

750 uL of 30% glycerol was added to it (Stiernagle, 2006).

Vials were either placed in liquid nitrogen or at -80 degree Celsius according to need.

1.2.6 SEEDING

Using sterile technique, approximately 20 uL of *E. coli* OP50 liquid culture was dropped over 30 mm NGM plates and 50 uL over 90 mm NGM plates using a pipette and spreaded using a spreader. For making mating plates, 10 uL of OP50 liquid culture was dropped over 30mm NGM petri plate (Stiernagle, 2006).

1.2.7 ALDICARB ASSAY

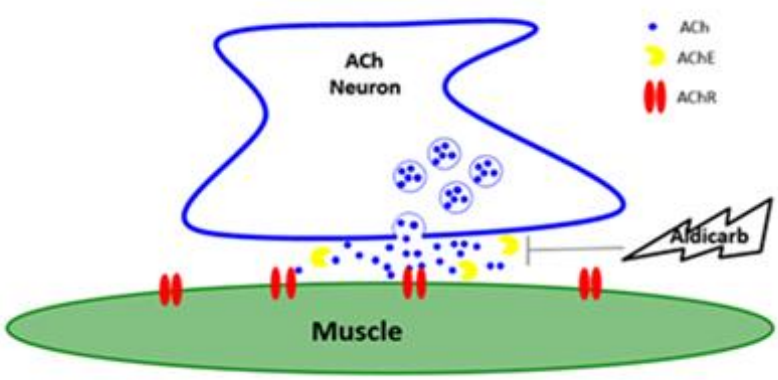


Fig. 1.3 Neuromuscular junction of worm and the way aldicarb acts there. (Image taken from Kavita Babu), Aldicarb assay was developed by Lewis, Hosono, Nonet and Rand Labs.

This is a widely used assay for assessing synaptic transmission at neuromuscular junction of *C. elegans*. Aldicarb is an acetylcholinesterase inhibitor (Mahoney et al., 2006). Acetylcholinesterase present in the synaptic cleft breaks down acetylcholine into acetate and choline and thus avoids the accumulation of acetylcholine. Presence of aldicarb inhibit the function of acetylcholinesterase and thus leads to the over accumulation of acetylcholine in the synaptic cleft, as shown in figure 1.3, which results in hyper contraction of muscle followed by paralysis or death in case of prolonged contraction (Mahoney et al., 2006).

As shown in figure 1.4, wild type worms maintain an optimum concentration of acetyl choline at the syanpses and during aldicarb assay, they show a certain pattern of paralysis but worms having mutation in some aspect of synaptic transmission show either increased excitation or decreased excitation (Mahoney et al., 2006). It make worms to either paralyze faster or slower depending upon the excitation. Any shift in phenotype from wild type phenotype can be because of a mutation.

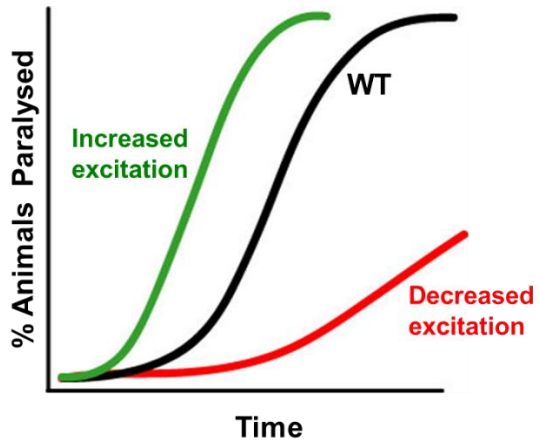


Fig. 1.4 Time course of aldicarb assay (Image taken from Kavita Babu). By maintaining optimum amount of acetylcholine in the synaptic cleft, WT worms show a certain pattern of paralysis. This pattern change according to the decreased or increased response to aldicarb in mutant worms

This assay has been used as a tool to find out any defect in the cholinergic neurons emanating from *daf-7* mutation.

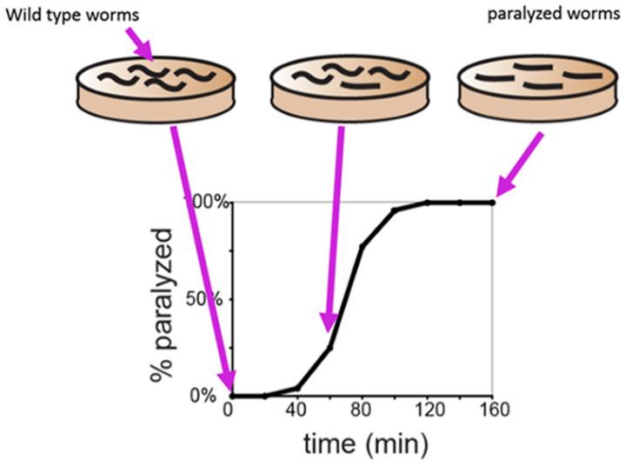


Fig. 1.5 Procedure of Aldicarb assay (Image taken from Kavita Babu)

As shown in figure 1.5, for doing this assay a fixed number of worms were transferred with the help of a worm pick to freshly prepared, seeded, aldicarb plates. Right after transferring the worms, percentage paralysis of worms was counted with time (Mahoney et al., 2006). This assay was done in a blind manner, where the name of strains were replaced with a code by someone randomly before starting the assay. Doing blind obviate any biasness that could have added because of some early assumptions.

Statistical analysis was done using Prism-graph pad software and unpaired two tailed students t-test was performed for calculating the p value.

1.2.8 EXPRESSION OF VARIOUS MARKERS

To find out the morphology and integrity of the cholinergic and GABAergic neurons (Cell body and axon) and their synapses in mutant worms, following strategy was adopted-

4X outcrossed *daf-7* worms were crossed with males of the marker carrying strains. Marker strains were specific to cholinergic neurons (pUNC-29::GFP) [UNC-29 encodes a non-alpha subunit of the nicotinic acetylcholine receptor (nAChR) superfamily], GABAergic neurons (pUNC-25::RFP) [UNC-25 encodes the *C. elegans* ortholog of the GABA neurotransmitter biosynthetic enzyme, glutamic acid decarboxylase (GAD)], Cholinergic synapses (pUNC-29::SNB-1::GFP) [SNB-1 is synaptobravin, small integral membrane proteins of secretory vesicles making marker specific to the synapse],

GABAergic synapses (pUNC-25::SNB-1::GFP), and touch sensory neurons (pMEC-4::GFP) [*mec-4* codes for a touch sensory receptor found in the touch sensory neurons ALM and PLM]. Crossing mutant strain to these strains brought neuron or synapse specific marker into the *daf-7* strain.

1.2.9 CONSTURCTS

Though there are reports saying that *daf-7* expresses in the cell body of ASI chemosensory neuron. But it seems very unlikely for a morphogen to be that spatially restricted. To further corroborate these findings, a construct having *daf-7* promoter fused with NLS-GFP was designed (All the protocols for cloning were followed from "Molecular Cloning: A Laboratory Manual, Volume 1, 2, 3" by J. Sambrook and D.W. Russel). After successfully screeing the positive colonies of transformant, plasmid was isolated and construct thus obtained was injectd into the gonads of wild type *C. elegans*.

This construct was having 2.5 kb *daf-7* promoter fused with NLS-GFP.

2.5 kb *daf-7* promoter was amplified using primers YD87 and YD88. Fragment amplified thus was inserted into NLS::GFP vector.

For locating the expression of *daf-7*, tracking its movement and rescuing the mutant phenotype, a construct carrying 4.8 kb upstream to ATG start site of *daf-7* along with full length *daf-7* gene was fused to Dendra-2, a photo convertible protein. To make it a translational reporter gene construct, further 3' UTR of *daf-7* was also added to it. Primers KT01 and KT02 were used to amplify the *daf-7* gene with 4.8 kb upstream to ATG to the stop codone. 3' UTR of *daf-7* was amplified using primers KT03 and KT04.

Though green fluorescent protein and other such proteins have been excellent reporters in the past but recently introduced photo convertible proteins have completely revolutionised this field. Dendra-2 is an improved version of green to red switchable protein dendra. Dendra-2 converts from green to red upon irradiation with intense blue light. This photo conversion property of Dendra-2 will help me in tracking the movement of DAF-7 from one place to another place within the body of a worm, if it happens.

1.2.10 MICROINJECTIONS

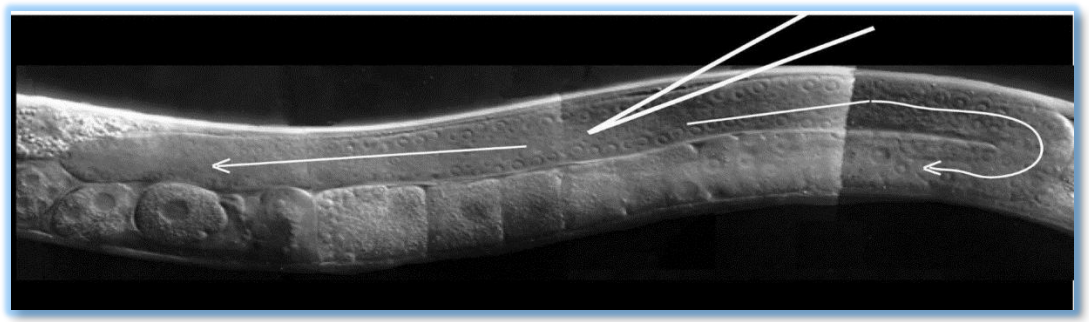


Fig.1.6 Microinjections image taken from (Evans, T. C, 2006). Image depicts the correct position of microinjection and the correct stage of worm at which it should be used for microinjection.

Microinjection is a very sophisticated technique used for making transgenic animals. Prior to injection, an injection mixture was prepared which carried mCherry as a head marker and PBS for maintaining optimum concentration of DNA along with the construct (Evans, T. C, 2006). Injection involves three major steps, First is fixing worms on agarose pads, second is breaking needle and third is microinjecions (Evans, T. C, 2006). Using a worm pick, worms were transferred to agarose pads, already having a drop of halocarbon oil dropped on it. Next step was breaking the needle. Around 2000 Pascal of pressure with a gentle tap gave very fine needle for microinjection (Evans, T. C, 2006). To inject the Injection mixture, first of all gonad of worm was located at 20X and worm as well as needle were brought to the same focal plane. At 100X, injection mixture was injected in to the gonads of worm using an Eppendorf Femtojet and Injectmen worm injector. As shown in figure 1.6, injection mixture can be seen going in both the directions when injected properly. Nikon Eclipse Ti microscope was used for visualization of worms during microinjection. The needles for injection were pulled using a Sutter P1000 needle puller. Post injection, worms were suspended in M9 and then transferred to a fresh seeded plate for revival. Progeny of the worm was tracked for next few days for identification of transgenic worms.

1.2.11 IMAGING

Imaging is done for the purpose of visualisation of a marker or expression profile of a gene. Worm need to be paralyzed so that imaging could be done. For this purpose, prior to imaging, worms were suspended in BDM solution to paralyze them. Worms were fixed on 2% agarose pads once paralyzed and a cover slip was put on it. Generally, worms in either late L4 phase or in early adult phase were used for imaging.

1.2.12 PRIMERS

YD87

5’-AGTCTGCAGCCCGTCATCAGTGATAATTAC-3’

YD88

5’-AGTGGATCCAACACCGGGAGTGAAGATGC-3’

KT01

5'-AACTGCAGCTTTTTTTTTTTGGAAAACCCTCGA-3’

KT02

5'-GGGGTACCTGAGCAACCGCATTTCTTGGCGATC-3'

KT03

5’-GCGGCCGCATTCCACTTCCGATTGTCACAAATG-3’

KT04

5’-GCGGCCGCATAATTTATTCCTCAATCACAGTGT-3’

CHAPTER 2

2.1 RESULTS

2.1.1 *daf-7* (0x) worms showed a sensitive phenotype to the aldicarb assay.

Data obtained in blind fashion was tallied and codes were again replaced with the actual name of the strain. Data such obtained was plotted as percentage paralysis against time.

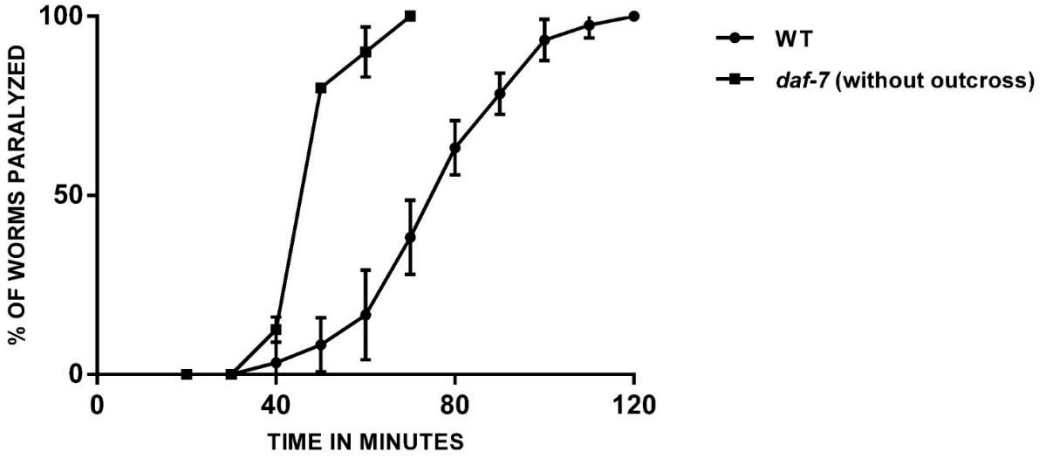
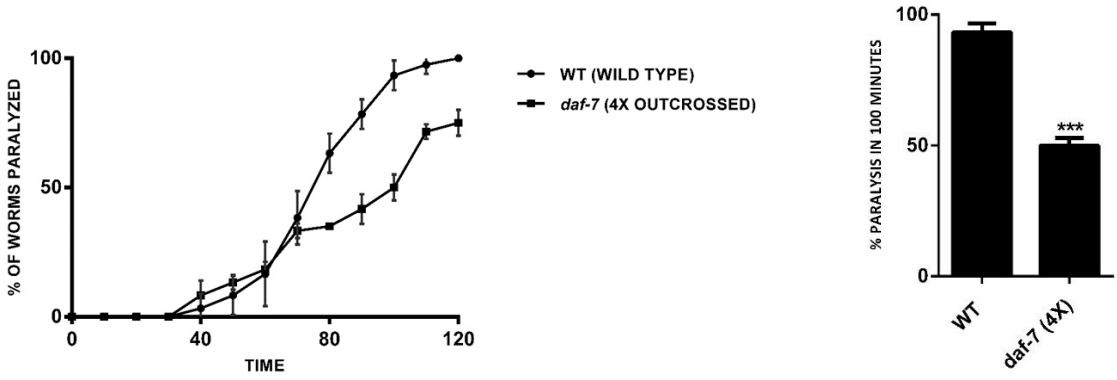


Fig. 2.1 Time - course of aldicarb - induced paralysis of wild type and *daf*-7 (without outcross) strain. Assay was done for 120 minutes on adult worms of both the strains in a blind fashion.

daf-7 (0X) worms showed a hypersensitive phenotype by getting paralysed very quickly. Almost all the daf-7 (0X) worms paralysed at around 70 minutes but even after 120 minutes, only around 70% of wild type worms showed paralysis.

2.1.2 *daf-7* (4x) worms showed a sensitive phenotype to the aldicarb assay.

Fig. 2.2 (A) shows the result of aldicarb assay done in triplicates. From graph it is evident that *daf-7* worms took longer time than wild type worms. This behaviour of *daf-7* worms show their resistant phenotype towards aldicarb.



(A) (B)

Key: ***: Highly Significant, p <0.001 (Students t-test)

Fig. 2.2 (A) shows time - course of aldicarb - induced paralysis of wild type and *daf-7* strain. Assay was done for 120 minutes with WT and *daf-7* (4X) worms in a blind fashion. Fig. 2.2 (B) shows percentage paralysis on a scale of 100 minutes.

Longer time taken by *daf-7* worms in paralysing indicates either slow build-up of acetylcholine in the synaptic cleft or there might be less number of receptors present on postsynaptic muscle. So even if there was regular build up, there was less contraction observed due to less number of postsynaptic receptors.

As shown in fig. 2.2 (B), on a scale of 100 minutes, only around 50% of *daf-7* worms were paralyzed whereas almost 100% of the wild type worms got paralyzed. This resistance indicates that DAF-7 might be involved in modulating the synaptic

transmission at *C. elegans* neuromuscular junction. Statistical analysis was done using Prism-graphpad software.

2.1.2 EXPRESSIONAL STUDIES

In October 1996, Wendy S. Schackwitz et.al published their study on *daf-7* in Neuron journal. They used 4.8kb DAF-7 promoter and fused it with GFP and injected it into the gonads of wild type worms to create transgenic line for this reporter gene construct. Their study revealed that *daf-7* expresses in the cell body of ASI chemosensory neurons only, located near pharynx as shown in figure 2.3.

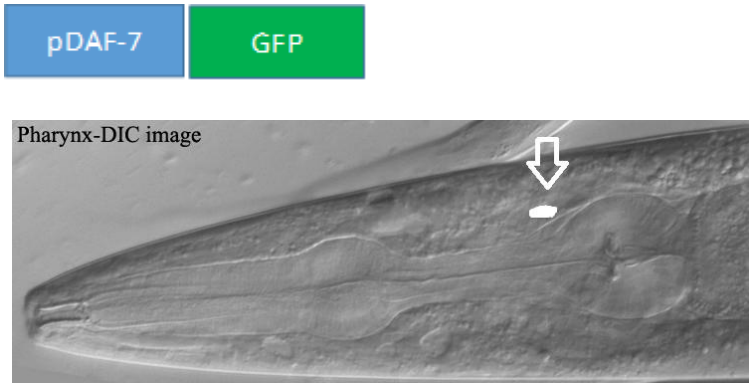
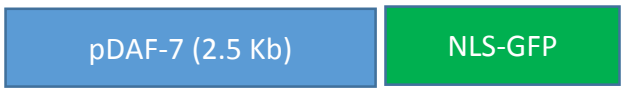


Fig. 2.3 Expression of *daf-7*, DIC image of pharynx was taken from reference [Altun, Z. F. and Hall, D. H. 2005. Handbook of C. elegans Anatomy. In WormAtlas. http://www.wormatlas.org/ver1/handbook/contents.htm]. White spot indicated in figure with an arrow represents the cell body of ASI chemosensory neuron, where daf-7 was reported to express.

Since there were not many reports about the expression pattern of *daf-7* and it seemed very unlikely for a morphogen be that restricted in one part only, I planned to do cell specific expressional study of *daf-7*. For this purpose, I designed pDAF-7::NLS-GFP construct and created transgenic worms using microinjections.



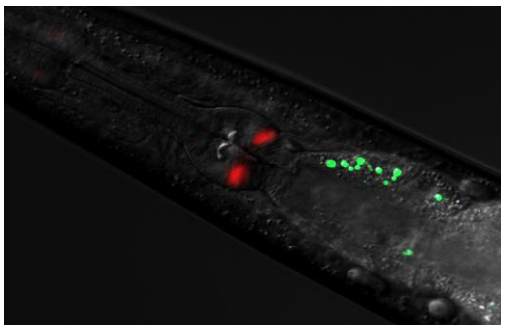


Fig. 2.4 expression of pDAF-7::NLS-GFP GFP in in Pharyngeal region

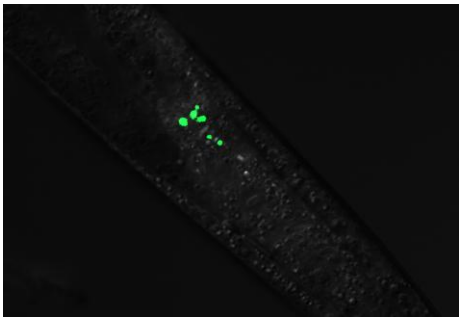


Fig 2.5 expression of pDAF-7::NLS-GFP tail region

Both the images were taken at 20X magnification.

Imaging of these worms revealed that *daf-7* is not exclusively localised to the cell body of ASI chemosensory neuron but it also expresses in tail and some other regions in the pharynx also, as shown in fig. 2.4 and fig. 2.5.

2.1.3 EXPRESSION OF VARIOUS MARKERS:

A) pMEC-4::GFP

MEC-4 encodes a touch sensory receptor in the ALM (Anterior lateral Microtubule) and PLM (Posterior Lateral Microtubule) touch sensory neurons of the worm. So pMEC-4::GFP expresses in the ALM and PLM touch sensory neurons only.

As shown in figure 2.6, two patterns of ALM were observed in wild type as well as in *daf-7* worms with normal one being the most frequent and extended one being the less frequent one. However PLM was observed with a particular pattern only in both wild type and *daf-7* worms. Purpose of looking at the pattern of these neurons is to find out whether mutation in *daf-7* brings any change in the polarity of these neurons or not.

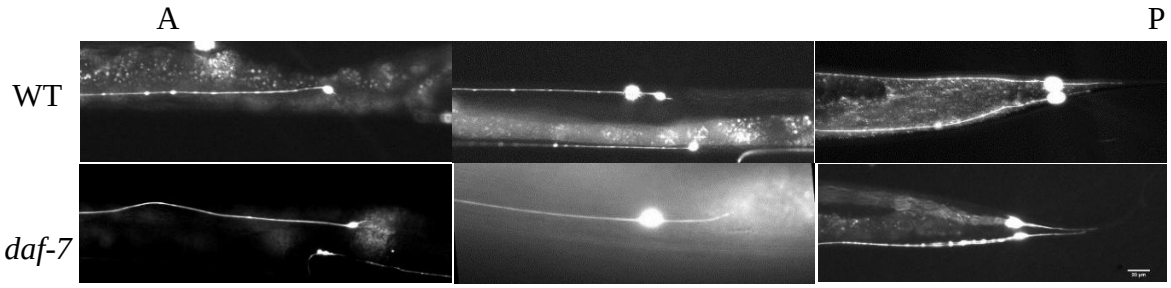


Fig. 2.6 Expression of pMEC-4::GFP in ALM and PLM neurons at 20X magnification. A refers to anterior and P refers to posterior whereas WT refers to wild type and *daf-7* refers to 4X outcrossed *daf-7* mutant worms.

	NORMAL		BRANCHED		EXTENDED	
	WT	*daf-7*	WT	*daf-7*	WT	*daf-7*
ALM	15	19	1	1	0	1
PLM	16	21	0	0	0	0

Table 2. Results of pMEC-4::GFP. Data was collected for 16 WT worms and 21 *daf-7* worms. WT refers to wild type worms and daf-7 refers to 4X outcrossed DAF-7 mutant worms.

Data provided in the table 1 indicates that there might not be any change in the polarity of these neurons emanating from mutation in *daf-7*. However more worms need to be imaged to get a consolidated data set. I am still in the process of imaging these worms to get a complete data set.

B) pUNC-25::SNB-1::GFP

UNC-25 encodes the *C. elegans* ortholog of the GABA neurotransmitter biosynthetic enzyme, glutamic acid decarboxylase which is present in GABAergic neurons only.

SNB-1 (synaptobrevin) are present in secretory vesicles as small integral membrane proteins. The fusion of these two with GFP make this marker specific for the regions of GABAergic synapses.

Fluorescent line in figure 2.7 shows dorsal nerve cord of the *C.elegans* and each dot along this line represents a region of synapse. Nothing can be concluded about the morphology of synapses from the preliminary data. For coming to any conclusion more number of worms need to be imaged and quantification need to be done. I am in the process of imaging pUNC25::SNB-1::GFP to get a complete data set for both WT and *daf-7.*

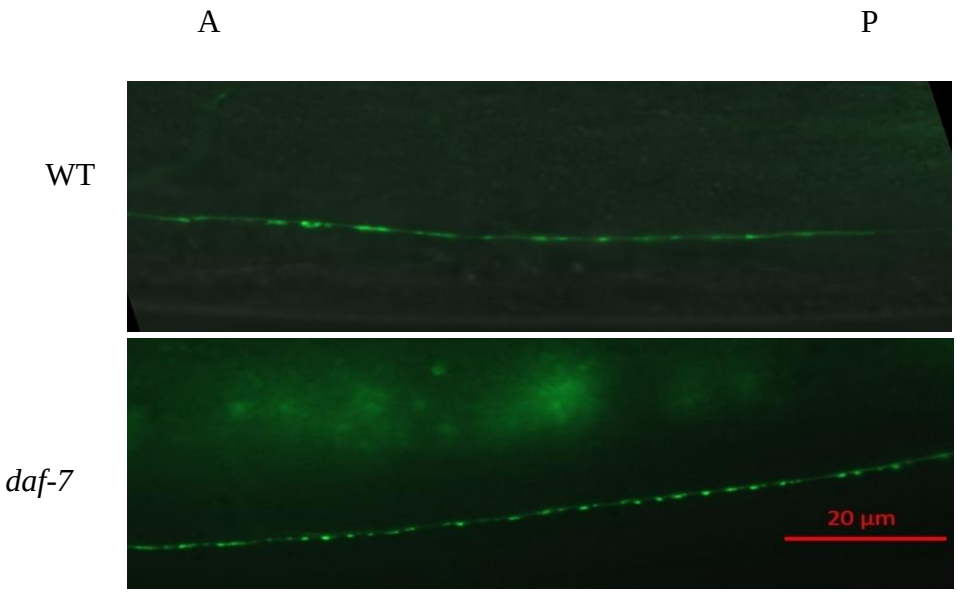


Fig. 2.7 Expression of pUNC-25::SNB-1::GFP in wild type and *daf-7* worms. Each puncta in these Images, taken at 63X, represents a region of GABAergic synapses. A refers to anterior and P refers to posterior whereas WT refers to wild type and daf-7 refers to 4X outcrossed DAF-7 mutant worms.

Following lines are also ready and I am in the process of imaging them.

1. pUNC-29::SNB-1::GFP – This marker shows expression in the Cholinergic synapses.

2. pUNC-25::GFP – This marker shows expression in the GABAergic neurons (Cell body and axon).

3. pUNC-17::RFP – This marker shows expression in the Cholinergic neurons (Cell body and axon).

daf-7 mutant has been crossed with the markers listed above and I am in the process of imaging.

2.1.4 RESCUE AND EXPRESSION

The construct, as shown in fig. 2.8, was designed with 4.8kb DAF-7 promoter, upstream to ATG start site with full *daf-7* gene till stop codone fused to Dendra-2 photo convertible protein followed by addition of DAF-7 3' UTR. This construct will serve dual purpose of rescue and expression.

Fig. 2.8 A fragment of 6.6kb having *daf-7* gene with its 4.8 kb promoter was amplified using primers KT01 and KT02. This fragment was cloned into a Dendra2 vector. Plasmid isolated from positive colonies was processed and then ligated with DAF-7 3' UTR

Because it is having full length *daf-7* gene in it so I will cross the males of this transgenic line with the hermaphrodites of *daf-7* strain. The progeny of this cross will give me a line in which resistant phenotype to aldicarb will be rescued if it was emanating from DAF-7 mutation only.

Since Dendra-2 is a photo convertible protein, it will help in locating the expression of daf-7 as well as in tracking the movement of DAF-7 from one place to another place within the body of organism.

2.2 DISUSSION AND FUTURE DIRECTIONS

C. elegans is a very simple yet powerful model system to work on. It possesses qualities like easy to culture, non-pathogenic, androdioecious, transparent, short life span that can easily be manipulated by fluctuating the temperature, property of giving clonal population and full neural circuitry discovered which make it even more fascinating for geneticists, developmental biologists as well as for neurobiologists. The transparent body of *C. elegans* make it easy to look at the expression of a marker.

Worm execute its diverse behaviours through its locomotion. Locomotion of worm involve continuous and co-ordinated firing of both GABAergic and cholinergic neurons. A defect in the locomotion of worm shows improper functioning at its neuromuscular junction. Proper formation and functioning of neurons require molecules like neurotrophins. Recent reports suggest that not only neurotrophins but morphogens are also involved in such processes. DAF-7 is one such morphogen that has exhibited some potential of altering the synaptic transmission at *C. elegans* neuromuscular junction based on some preliminary results. To confirm this phenotype, assay for synaptic transmission i.e. aldicarb assay, was done with *daf-7* (4X) worms. They showed a resistant phenotype towards aldicarb which indicates that mutation in this gene might have altered the synaptic transmission at C. elegans neuromuscular junction.

Interestingly when results of aldicarb assay done on *daf-7* (0X) are compared with that of *daf-7* (4X), figure 2.1 and figure 2.2 respectively, an interesting shift in the phenotype from sensitive phenotype to resistant phenotype can be observed. This shift occurred due to the outcross mediated removal of background mutation causing this phenotype.

Results of aldicarb assay indicated some defect in either the cholinergic neuron, in release of synaptic vesicles or at the postsynaptic level in form of the number of acetylcholine receptors present on the postsynaptic muscle. Whichever it might be but for the time being it became important to assess the morphology of synapses, cell body and axons of both cholinergic as well as GABAergic neurons.

Locating the expression of *daf-7* was the next quest in this project since my data showed some contradiction with the data obtained earlier by Wendy S. Schackwitz in 1996. Interestingly enough, this time the use of Dendra2, a photo switchable protein, as a

reporter for *daf-7* gene will definitely provide a bigger picture of expression pattern of *daf-7.* Transgenic line made with this reporter gene construct if crossed with *daf-7* mutant will also rescue the aldicarb phenotype if it was solely due to the mutation in *daf-7* gene only.

The pDAF-7::Dendra2::DAF-7 3'UTR, construct has been designed and it is in the process of sequencing. One sequence get confirmed, the microinjections with this construct can be started.

Though DAF-7 seem to be involved in modulating synaptic transmission at C. elegans neuromuscular junction but it is still not clear that whether it acts through some developmentally regulated pathways or through direct involvement in real time. This quest seems quiet intriguing and following experiment can be done to find it out-

DAF-7 is known to act through canonical TGF-beta pathway (Gumienny and Savage-Dunn, 2013), where it bind to the type I and type II tgf-beta receptors and activate downstream pathway, as shown in figure 1.1. A protein BRA-1 can inhibit the activity of DAF-7 by blocking the dimerization of type-I and type-II receptors and hence disrupting the whole pathway (Gumienny and Savage-Dunn, 2013). One more experiment that can be done to check the involvement of *daf -7* is by preparing a construct by fusing HSP-16::BRA-1 and inject it into the wild type worm. Giving heat shock to the worms will overexpress the BRA-1. Overexpression of BRA-1 will lead to the blockage of the canonical TGF-beta pathway and hence *daf-7* will not be able to act. Such adult worms will be subjected to the aldicrab assay. If these worms also show a resistant phenotype similar to *daf-7* (4X) worms then it suggests that DAF-7 is directly involved in modulating the synaptic transmission at NMJ.

These experiments come together will provide a clearer picture of the role of DAF-7.

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