Deciphering the role of CASY-1, an ortholog of mammalian Calsyntenins in the *C. elegans* locomotory circuit

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By

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DECLARATION

The work presented in this thesis entitled "Deciphering the role of CASY-1, an ortholog of mammalian Calsyntenins in the *C. elegans* locomotory circuit" has been carried out by me under the supervision of Dr. Kavita Babu at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or 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 acknowledgment 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

(Shruti Thapliyal)

Date:

Place:

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

(Dr. Kavita Babu)

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Dedicated To My

Mummy and Papa

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ABBREVIATIONS

Weights and measures

%	Percent
µmol, nmoles, mmoles,	micromole, nanomoles, millimoles,
°C	Degree centigrade
bp, kb, MB	Base pair, kilobase, Megabase
kDa	Kilodalton
O.D.	Optical density
Psi	Pounds per square inch
rpm	Revolutions per minute
RT	Room temperature
sec, min, h	Second, minute, hour,
µg, mg, g	microgram, milligram, gram
µl, ml, L	Microliter, milliliter, liter,
µM, mM, M,	micromolar, millimolar, molar
mV,V	Millivolt,Volt

Symbols

~	Approximately
=	Equal to
α	Alpha
β	Beta
γ	Gamma
Δ	Delta

Techniques

PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
FRAP	Fluorescence Recovery after Photobleaching
RNAi	RNA Interference
qPCR	Quantitative PCR
Y2H	Yeast two-hybrid

Chemicals

Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
DMSO	Dimethyl Sulphoxide
dNTPs	2'-deoxyadenosine 5'-triphosphate
DTT	dithiothreitol; Cleland's reagent
EDTA	Ethylenediamine-tetra-acetic acid
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2ethanesulfonic acid]
SDS	Sodium Dodecyl Sulphate
TEMED	N, N, N', N'-tetramethylethylenediamine

Tris	[Tris(hydroxymethyl)amino methane]
β-ME	β-Metcaptoethanol
PTZ	Pentylenetetrazol
ATR	all trans retinal
IPTG	Isopropyl-β-D-thiogalactoside
HA	Histamine dihydrochloride
BDM	2, 3-butanedione monoxamine
AT	3-Amino-1,2,4-triazole

Miscellaneous

aa	amino acids
ACh	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	Amyloid Precursor Protein
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium ion
CAM	Cell Adhesion Molecule
CGC	*C. elegans* Genetics Center
ChR2/ChRX2	Channelorhodopsin
CIP	Calf Intestinal Phosphatase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
C-terminal	Carboxy- terminal
DNA	Deoxyribonucleic acid
DNC	Dorsal Nerve Cord
EC domain	Extracellular cadherin domain
EPSC	Excitatory Postsynaptic Current
FP	Forward primer
GABA	gamma- aminobutyric acid
GFP	Green Fluorescent Protein
GPCR	G-protein-coupled receptor
GST	Glutathione S- transferase
HA	Hemagglutinin
HRP	Horseradish peroxidase
iGluSNFR	Intense GluSNFR
IPSC	Inhibitory Postsynaptic Current
LB	Luria Bertani
NBRP	National BioResource Project
NCBI	National Center for Biotechnology Information
NGM	Nematode Growth Medium
NMDA	N-methyl-D-aspartate
NMJ	Neuro-Muscular Junction
NPR	Neuropeptide Receptor
N-terminal	Amino- terminal
ORF	Open Reading Frame
RNA	Ribonucleic acid
ROI	Region of interest
RP	Reverse primer
SEM	Standard error mean

SL1	Splice Leader 1
SNB	Synaptobrevin
SP	Signal Peptide
SV	Synaptic vesicle
TBS	Tris-Buffered Saline
TBST	TBS-Tween 20
TE	Tris chloride and EDTA
TeTx	Tetanus toxin light chain
TMD	Transmembrane domain
TRP	Transient Receptor Potential
VNC	Ventral Nerve Cord
WT	Wild-type
YFP	Yellow Fluorescent Protein
YS	Yellow Stripe
LG/LNS	Laminin G/ Laminin-alpha, Neurexin and Sex harmone-binding globulin

SYNOPSIS

Introduction and background

Locomotion is a basic yet complex behavior in most organisms. Locomotion in *C. elegans* is regulated by neural circuits that allow for coordinated dorso-ventral sinusoidal bends. Locomotion behavior is synchronized at multiple levels and involves integration of diverse sensory cues that are processed by the interneurons and are manifested at the neuromuscular junctions (NMJ) (Bargmann, 2012; de Bono and Maricq, 2005). Despite an extensive understanding of the development and functioning of the locomotory circuit, the mechanisms controlling motor coordination are still elusive.

Many cell adhesion molecules (CAMs) are concentrated at synaptic sites in neuronal axons and dendrites serving as dynamic regulators of synaptic function. They maintain specific connections between the neurons that are responsible for orchestrated neural networks and circuitry of the brain (Biederer et al., 2002; Chih et al., 2005; Goda, 2002) . The *C. elegans* CASY-1 is an ortholog of the mammalian Calsyntenin genes. It is a type-I transmembrane protein that belongs to the cadherin superfamily of cell adhesion molecules. It is characterized by the presence of two cadherin-like tandem repeats, an LG/LNS domain in the extracellular region and an intracellular region that carries two kinesin light-chain binding domains (Hoerndli et al., 2009; Ikeda et al., 2008). All these regions are conserved in the three human Calsyntenin genes; *clstn1*, *clstn2* and *clstn3* that are highly enriched in the central nervous system (Hintsch et al., 2002; Vogt et al., 2001). Mammalian calsyntenins are implicated in diverse neuronal functions such as trafficking of various axo-dendritic components (Hintsch et al., 2002; Konecna et al., 2006; Ponomareva et al., 2014; Ster et al., 2014), in synaptic plasticity (Lipina et al., 2016; Preuschhof et al., 2010) as well as in development and functioning of excitatory and inhibitory synapses (Pettem et al., 2013). However, despite all these studies it has been very difficult to deduce the cellular and molecular mechanisms and implications of these proteins on animal behavior and function.

With a 302- neuron nervous system and simple neural wiring *C. elegans* has emerged as an excellent invertebrate model system for understanding the molecular basis of synapse development and function. Like its mammalian orthologs, *C. elegans* CASY-1 expression was also observed in most head neurons and some other tissues like intestine and gonadal sheath cells (Hintsch et al., 2002; Ikeda et al., 2008). CASY-1 has previously been found to be

essential for multiple forms of learning (Hintsch et al., 2002; Ikeda et al., 2008). Functional rescue experiments by expressing human CLSTN2 in *C. elegans casy-1* mutants were able to rescue the learning defects highlighting conservation of function.

We were interested to understand the molecular mechanisms for CASY-1-mediated functions in synaptic transmission. With this background, the following objectives were framed:

1. Deciphering the role of CASY-1 at the *C. elegans* NMJ.

2. CASY-1 mediated regulation of motor circuit dynamics and behavior stemming from sensory and interneurons.

Results and Conclusions

Deciphering the role of CASY-1 at the *C. elegans* neuromuscular junction (NMJ)

The *C. elegans* NMJ consists of body-wall muscles that receive synaptic inputs from both excitatory cholinergic and inhibitory GABAergic pre-synaptic motor neurons. An intricate balance between the excitatory and inhibitory signaling is responsible for the sinusoidal locomotion in *C. elegans* and any defect in this balance could result in altered synaptic function (reviewed in (Richmond, 2005; Seifert et al., 2006)).

Previously an RNAi screen was performed using the acetylcholine esterase inhibitor, Aldicarb, to identify the function of cell adhesion molecules at the *C. elegans* NMJ [(Babu et al., 2011) and Babu K. and Kaplan J.M.; unpublished data]. The presence of Aldicarb causes acute paralysis due to accumulation of acetylcholine at the NMJ. It has been shown that mutations that increase synaptic transmission results in Aldicarb hypersensitivity, while mutations that decrease synaptic transmission results in Aldicarb resistance (Mahoney et al., 2006; Sieburth et al., 2005; Vashlishan et al., 2008). RNAi against the *casy-1* gene showed hypersensitivity to Aldicarb in this screen. To validate the results of RNAi, two mutant alleles of *casy-1* (*tm718; hd41*) were obtained and tested, both alleles showed hypersensitivity to Aldicarb.

The *casy-1* locus in *C. elegans* encode for three isoforms. CASY-1A, a 984 residue full-length protein contains all the conserved domains of mammalian Calsyntenins. CASY-1B and CASY-1C are truncated proteins encoding 167 and 160 residues respectively and lack most of the N- terminal of the Calsyntenin gene. To understand the role of these isoforms in

synaptic functioning, an isoform specific rescue experiment was performed in which each isoform was expressed under its native promoter. All three isoforms fully restored the Aldicarb hypersensitivity of *casy-1* mutants to wild-type (WT) levels.

Expression analysis revealed that *casy-1a* is highly enriched in the head and tail neurons, while *casy-1b* and *casy-1c* show a more limited expression in the head neurons. However, both *casy-1b* and *casy-1c* are strongly expressed in the motor neurons, which is not seen with *casy-1a*. Since *casy-1a* shows no expression in motor neurons it is conceivable that the shorter *casy-1* isoforms which have the conserved C-terminal of mammalian calsyntenins are functioning in motor neurons to regulate synaptic signaling at NMJ. Our domain mapping experiments further established that the C-terminal of CASY-1 is required to rescue the Aldicarb hypersensitivity in *casy-1* mutants. Further, expressing CASY-1 isoforms in GABAergic motor neurons rescued the Aldicarb hypersensitivity of *casy-1* mutants while no rescue was observed upon expression in cholinergic motor neurons or muscle.

Our results go on to show that *casy-1b* and *casy-1c* function in GABAergic motor neurons to regulate GABA signaling. Analysis of GABAergic GFP-tagged SNB-1 (homolog of mammalian synaptobrevin) showed subtle but significant decrease in fluorescent intensity suggesting less GABA vesicles at the synapse. Postsynaptic $GABA_A$ receptors showed no change in fluorescent intensity suggesting the defect to be presynaptic. Also, experiments using the pharmacological antagonist of the $GABA_A$ receptor, PTZ, established pre-synaptic GABA defects in *casy-1* mutants. Further, electrophysiological recordings from *casy-1* NMJ showed significant reduction in endogenous Inhibitory post synaptic current (IPSC) frequency indicating that the release of GABA is reduced in *casy-1* mutants. However the IPSC amplitude remain unchanged suggesting that the muscle responsiveness to GABA remained unaffected.

We next performed several experiments to confirm the presence of less GABA vesicles at the synapse. SNB-1 tagged to pHluorin, a pH sensitive fluorescent tag revealed less GABA vesicles fusing at the synapse in *casy-1* mutants. Fluorescence recovery after photobleaching (FRAP) analysis for GABAergic SNB-1::GFP also revealed lower mobility of GABAergic synaptic vesicles (SVs) at the synapse. Additionally, optogenetic stimulation of GABAergic neurons results in less relaxation in *casy-1* mutants (GABA stimulus results in muscle relaxation). Finally, we monitored the transport characteristics of GABAergic SNB-1::GFP tagged vesicles along the commissures. This analysis revealed significant reduction in

anterograde vesicular flux, however the velocity of the vesicles remained unaltered. These mutant phenotypes were completely rescued by expressing the shorter CASY-1C isoform specifically in GABAergic motor neurons. Experiments performed in cholinergic motor neurons revealed no significant change in SV dynamics.

The above results suggest the possibility that fewer GABAergic SV precursors are trafficked to the NMJ. UNC-104/KIF1A is a Kinesin-3 motor reported to have a conserved role in trafficking SV precursors from the cell body to the synapse. Our Aldicarb and FRAP analysis revealed that the two proteins interact genetically. Further, translational CASY-1C::GFP reporter sequesters in the cell bodies in the *unc-104* mutant background presenting UNC-104 to be a potential candidate required for trafficking of CASY-1 to the synapse. We also examined the physical interaction of the CASY-1 C- terminal with several UNC-104/KIF1A domain constructs. Our data suggests that a weak interaction occurs between the cytoplasmic tail of CASY-1 and the C-terminal of UNC-104/KIF1A that largely includes the stalk region and the Pleckstrin Homology (PH) domain.

Collectively, we show that the two shorter isoforms of CASY-1, that only have the conserved C-terminal region and lack the entire extracellular N-terminal region, function in the GABAergic motor neurons to regulate GABA release by mediating the trafficking of GABA SV precursors via their interaction with the UNC-104/KIF1A motor protein.

Analysis of CASY-1A-mediated regulation of neural circuit dynamics and behavior stemming from sensory and inter-neurons.

Patch clamp recordings from *casy-1* NMJ revealed a significant increase in the endogenous Excitatory post synaptic currents (EPSC) frequency suggesting increased acetylcholine release at the NMJ. However, the Aldicarb sensitivity of *casy-1* mutants was not rescued by CASY-1 expression in cholinergic motor neurons. This raises the possibility that *casy-1* functions in higher levels of the locomotory circuit to regulate acetylcholine release. We first investigated the role of CASY-1A in sensory neurons. Previously, CASY-1A has been shown to function in sensory neurons to regulate synaptic plasticity in *C. elegans* (Hoerndli et al., 2009; Ikeda et al., 2008). Expression of CASY-1A in sensory neurons using *odr-4* promoter completely rescued the Aldicarb hypersensitivity of *casy-1* mutants. Since locomotion in *C. elegans* reflects the motor circuit activity, we also monitored the speed of *casy-1* mutants on

food, and found a significant increase in speed. The locomotion defects of *casy-1* mutants were also rescued by expressing CASY-1A in sensory neurons.

Most of the sensory neurons in *C. elegans* are glutamatergic i.e. they release glutamate as the neurotransmitter. We next investigated if increased glutamate release from sensory neurons is responsible for increased locomotory circuit activity in *casy-1* mutants. Removing glutamate vesicular transporter *eat-4*/VGLUT as well as several different ion channels required for sensory transduction completely abolished the Aldicarb hypersensitivity of *casy-1* mutants. Analysis of genetically coded glutamate sensor iGluSnFR also revealed increased glutamate release from sensory neurons. These results were further supported by optogenetically activating sensory neurons using light –activated cation channel Channelrhodopsin (ChRX2). Optogenetic stimulation of sensory neurons resulted in Aldicarb hypersensitivity in otherwise WT animals. Removing glutamate receptors subunits (*glr-1* and *nmr-1*) or silencing command interneuron (interneurons downstream of sensory neurons and directly synapse onto cholinergic motor neurons) signaling using Histamine gated chloride channels also abolished the hypersensitivity in *casy-1* mutants, thus validating that increased activity of sensory neurons and hence increased glutamate release is responsible for increased motor circuit activity in *casy-1* mutants.

Some of these behavioral phenotypes shown by *casy-1* mutants have been reported earlier for a G –protein coupled receptor mutant *npr-1*. *npr-1* mutants show sensory evoked enhancement of cholinergic transmission at the NMJ. Our aldicarb results support genetic interaction between the two genes. Just like *npr-1* mutants, the enhanced motor circuit activity and locomotion rate in *casy-1* mutants can be restored to WT levels by expressing CASY-1A in the RMG sensory circuit, thereby illustrating one mechanism by which *casy-1* exerts control over locomotion circuit from the sensory level.

Besides sensory neurons, CASY-1A also expresses in several interneurons. Our interneuron-specific rescue experiments revealed two interneurons RIM and AVE where CASY-1A functions to regulate motor circuit activity. Using optogenetic approaches and silencing vesicle release using Tetanus toxin light chain (TeTx), we first established that CASY-1A is required for the activity of these two neurons. Both these neurons are peptidergic, i.e. alongwith neurotransmitters they also release neuropeptides, small peptides that can modulate neural circuits from distance. Removing neuropeptide-processing enzymes *egl-3* and *egl-21* completely eliminates the Aldicarb hypersensitivity of *casy-1* mutants, suggesting the

involvement of neuropeptides in this function. We identified two neuropeptides *flp-1* and *flp-18*, secreted from interneurons AVE and RIM respectively that act antagonistically to modulate motor circuit in *casy-1* mutants. Finally, we went on to identify the potential neuropeptide receptors through which *flp-18* might be functioning to regulate cholinergic signaling at NMJ. Our results report the role for *npr-5*, a G- protein coupled receptor in regulating locomotion circuit downstream of *flp-18*.

Our study with *C. elegans* CASY-1 represents an interesting model where the three isoforms of the same gene are expressed in a spatially differential manner to regulate entirely different synaptic signalling processes. Full length CASY-1A functions in sensory and interneurons to regulate glutamate and neuropeptide release, which then modulates acetylcholine release at the NMJ. On the other hand, the shorter isoforms CASY-1B and CASY-1C, functions in GABAergic motor neurons to regulate GABA release dynamics via mediating trafficking of GABA-specific SV precursors. This thesis work highlights an interesting isoform-specific diversity in functions of a gene in *C. elegans*.

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CHAPTER 1:

Review of Literature

Review of Literature

GENERAL INTRODUCTION

This thesis deals with the mechanism by which a cell adhesion molecule (CAM) belonging to the cadherin superfamily, CASY-1 regulates excitation-inhibition balance in the *C. elegans* locomotion circuit in an isoform-specific manner. To introduce each of these aspects, I have divided the general introduction into three parts. Part 1: Specification of synaptic connectivity by cell adhesion molecules, Part 2: Locomotion circuit in *C. elegans* and Part 3: Isoform-mediated spatial and functional diversity in gene function.

Finally, I discuss the aims and the objectives of the present study.

Part 1: Specification of synaptic connectivity by cell adhesion molecules

A remarkable feature of the nervous system is the specific connections between the neurons, which is responsible for orchestrated neural networks and circuitry of the brain. Many cell adhesion molecules (CAMs) are concentrated at synaptic sites in neuronal axons and dendrites, serving as dynamic regulators of synaptic function. Considering the complexity of the nervous system, a tightly controlled spatial and temporal regulation of several different classes of CAMs is essential.

1.1. Basis of Synaptic transmission

Synapses are the asymmetric intercellular junctions that mediate synaptic transmission. They constitute the fundamental units of brain circuitry that enable execution of complex behaviors. It is at these specialized sites where the axon terminal of one neuron comes in contact with the target cell and allows chemical synaptic transmission in which small molecules move across the synaptic cleft to convey information from the presynaptic ("sending") neuron to the postsynaptic ("receiving") neuron. The specificity and plasticity of the synapses forms the structural and functional basis of the neuronal circuits.

Synapses comprise of several specialized domains, including the pre-synaptic compartment, the synaptic cleft and the post-synaptic terminal (Figure 1.1). The pre-synaptic terminal is characterized by the presence of hundreds of synaptic vesicles (SVs) filled with neurotransmitters (Qu et al., 2009; Takamori et al., 2006). A few synaptic vesicles are in physical contact with the plasma membrane at release sites

called active zones. The arrival of an action potential causes calcium influx via voltage-gated calcium channels resulting in depolarization of the plasma membrane. Upon this pre-synaptic depolarization, synaptic vesicles fuse with the plasma membrane releasing their contents into the synaptic cleft via a highly controlled process (Südhof, 1995; Sudhof, 2004). A synaptic cleft is a narrow gap approximately 20 nm in width between the pre- and postsynaptic neurons. It is packed with electron-dense material (Lucić et al., 2005; Zuber et al., 2005) including many different classes of cell adhesion molecules that support the exact juxtaposition of presynaptic and postsynaptic cells (Thalhammer and Cingolani, 2014). Released neurotransmitters diffuse across the synaptic cleft and bind to their receptors anchored directly apposed to the active zone. This region contains an electron dense meshwork of scaffolding and signaling proteins at the postsynaptic membrane that contributes to the integrated signaling (Sheng and Hoogenraad, 2007; Sheng and Kim, 2011). The neurotransmitters can cause either an excitatory or inhibitory response in the postsynaptic cell by binding to the diverse receptors on that cell. This neurotransmitter-receptor binding can then cause either further transmission of the signal (excitation) or can prevent the signal from spreading (inhibition) into the postsynaptic cell (Purves et al., 2001).

Precise regulation of excitatory and inhibitory synaptic transmission is crucial, as an intricate balance between the two allows for coordinated neuronal function. There are diverse classes of synaptic proteins that are concentrated at presynaptic and postsynaptic sites, but how they directly function to regulate excitatory and/or inhibitory synaptic transmission is not fully understood (Yi and Ehlers, 2005). Moreover, an imbalance in this synaptic signaling has shown considerable involvement in the pathogenesis of neurodegenerative disorders such as Parkinson's or Alzheimer’s disease, as well as in neurological disorders such as epilepsy and mental retardation (Ardley et al., 2005). Thus, understanding the genetic factors involved in controlling signaling at neuronal synapses is essential for better understanding of the pathogenesis of these disorders.

1.2. Cell adhesion molecules in synaptic function

A highly precise pattern of connectivity between the neurons is a key factor that determines the flow of information in a neural circuit. The nervous system employs multiple mechanisms to accomplish this challenging task (Sanes and Yamagata,

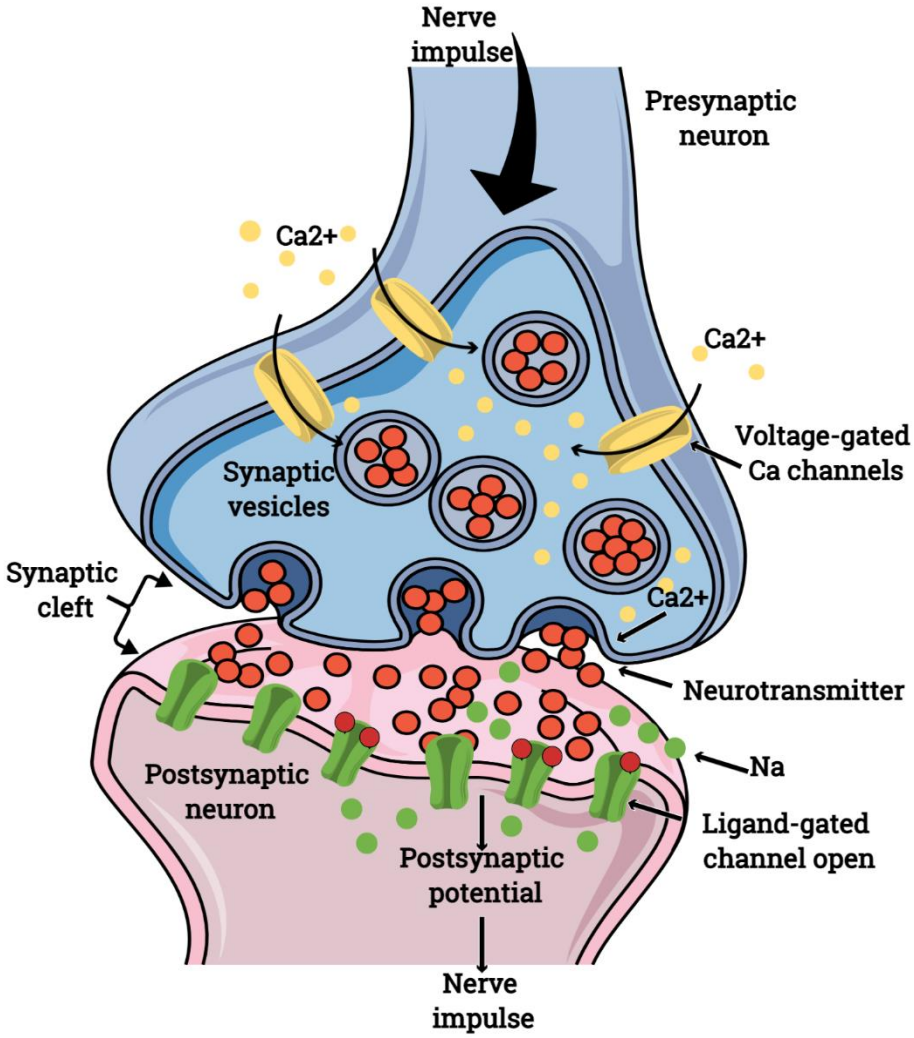


Figure 1.1: Schematic representing events in synaptic transmission at the synapse. (1) Action potential arrives at axon terminal (2) Voltage-gated Ca^{2+} channels open (3) Ca^{2+} enters the presynaptic neuron (4) Ca^{2+} signals to synaptic vesicles (5) Vesicles move to the membrane and dock (6) Neurotransmitter released via exocytosis (7) Neurotransmitters bind to receptors (8) Signal initiated in postsynaptic cell.

2009). Many cell adhesion molecules are assembled at synaptic sites (Figure 1.2). Existing literature suggests that neuronal CAMs are not only important for adhesion but are also required for various aspects of synapse development and function (Cavallaro and Dejana, 2011; Dalva et al., 2007; Missler et al., 2012; Shapiro et al., 2007; Sheng et al., 2013). Cell adhesion molecules include the cadherin family, neurexin/neuroligin family, integrins, SynCAM, NCAM, SALMs (synaptic cell adhesion-like molecules) (Togashi et al., 2009) and the newly discovered LRRTMs (leucine rich-repeat transmembrane molecules) (Linhoff et al., 2009). During brain development, CAMs play crucial roles in neuronal migration, axon fasciculation and neurite outgrowth (Chih et al., 2005; Cremer et al., 1997; Graf et al., 2004; Hirano and Takeichi, 2012; Ko et al., 2009; Pettem et al., 2013; Siddiqui et al., 2010; Südhof, 2008; Togashi et al., 2002, 2006; Walsh and Doherty, 1997; Yasuda et al., 2007). In adult brain, they play essential roles in the regulation of synaptic plasticity as well as in axon regeneration in an injured nervous system (Blackmore and Letourneau, 2006; Hansen et al., 2008; Nacher et al., 2013; Tessier-Lavigne and Goodman, 1996; Walsh and Doherty, 1997). Among these molecules, the cadherin family is one of the best studied and will be discussed in greater detail.

1.3. Cadherin superfamily of cell adhesion molecules

Cadherin superfamily comprises a large family of transmembrane proteins that include classical cadherins, desmosomal cadherins, T-cadherins, 7D-cadherins protocadherins, CDH-15 & 23, FAT and Dachsous, Flamingo/CELSRs, Calsyntenins and Ret (Figure 1.3) and (Takeichi, 2007; Yagi and Takeichi, 2000). These members are characterized by the presence of tandem repeats of approximately 110 aa long motifs called ‘EC domain’ (Takeichi, 1988). However, the number of EC domains vary greatly among cadherin superfamily members (Takeichi, 2007). Cadherins were originally identified to mediate Ca^{2+} -dependent homophilic cell-cell adhesion (Takeichi, 1977, 1988; Yoshida and Takeichi, 1982).

Most studies are conducted on the classical cadherins which are transmembrane proteins characterized by the presence of five EC- domains in the extracellular region and a unique cytoplasmic domain that can bind β- catenin and p120-catenin (Takeichi, 1988, 2007). The extracellular EC- domains bear seven β strands each forming two β sheets, a topology very similar to the immunoglobulin domain (Overduin et al., 1995; Shapiro et al., 2007). Ca^{2+} binds at the interface of these EC- domains resulting in the

formation of stable rod-like structures which then mediates formation of strand dimers in *cis* or adhesion dimers in *trans* (Nagar et al., 1996). The classical cadherin subfamily can be further subdivided into type I and type II based on the differences in their adhesive properties (Patel et al., 2006; Takeichi, 2007). Mostly, type I classical cadherins typically mediate homophilic interactions, whereas type II classical cadherins form both homo- and heterophilic interactions (Shimoyama et al., 2000). Cadherin- mediated adhesion is reported to be involved in various neuronal developmental processes like neural tube and neuroepithelial formation, cell migration and differentiation, neurite elongation and branching, axon guidance and fasciculation. They also mediate synaptogenesis and synaptic plasticity. Moreover, dysfunction of classical cadherins is considered a cause for neurological disorders like schizophrenia and other mood disorders (Hirano and Takeichi, 2012).

Besides classical cadherins, members belonging to nonclassical cadherins like Protocadherins, Cadherin 23, Celsr/Flamingo, Fat and Dachsous have been reported to regulate different aspects of brain morphogenesis (Hirano and Takeichi, 2012). This thesis focuses on the functioning of *C. elegans* ortholog of mammalian Calsyntenins, a non-classical cadherin and will be discussed in greater details in next section.

1.4. Mammalian Calsyntenins

1.4.1. Structure and Expression pattern of Calsyntenins

Calsyntenins are type I transmembrane proteins that belongs to the cadherin superfamily of cell adhesion molecules. They were first discovered in a screen for proteins secreted from the developing spinal cord motor neurons of chick embryo (Vogt et al., 2001). Purification and cDNA sequencing revealed a transmembrane protein with a large extracellular N-terminal region and a short cytoplasmic C-terminal, called calsyntenin-1 (*clstn1*) (Vogt et al., 2001). A follow-up study reported the presence of two other calsyntenins in mouse, calsyntenin-2 (*clstn2*) and calsyntenin-3 (*clstn3*) (Hintsch et al., 2002). All three calsyntenins are characterized by the presence of two cadherin- like tandem repeats, an LG/LNS domain in the extracellular region and an intracellular region that carries two kinesin light-chain binding domains (Hintsch et al., 2002; Vogt et al., 2001). The intracellular region also contains clusters of acidic amino acids that can bind calcium ions with high affinity and hence can regulate intracellular calcium signaling (Vogt et al., 2001). The

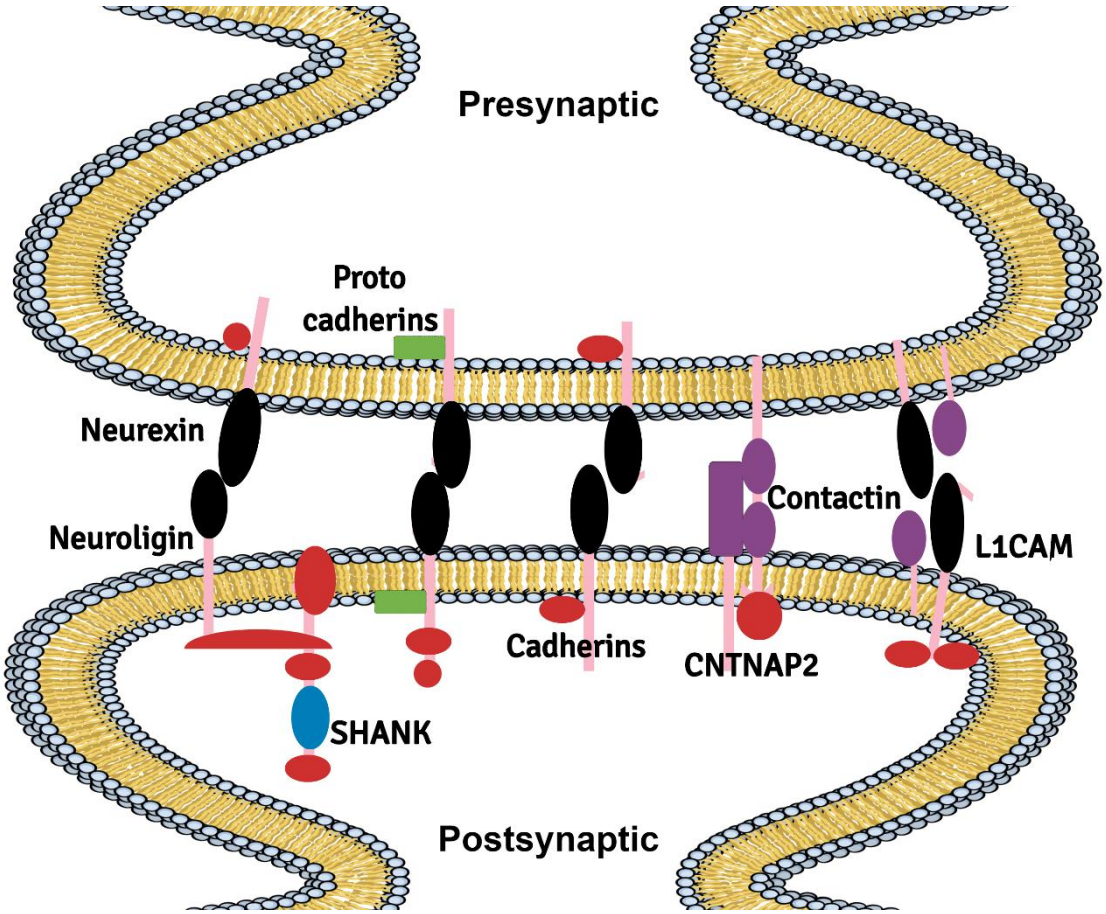


Figure 1.2: Schematic representing cell adhesion molecules localized at synaptic sites in neuronal axons and dendrites. These molecules bridge pre- and postsynaptic specializations and provide a mechanical link between cells. They mediate signaling necessary to accumulate and recycle presynaptic vesicles, components of exo- and endocytic zones, and postsynaptic receptors. CAMs also contribute to regulation of synaptic efficacy and plasticity, via direct interactions with postsynaptic neurotransmitter receptors and presynaptic voltage-gated ion channels (Image modified from Betancur et al., 2009).

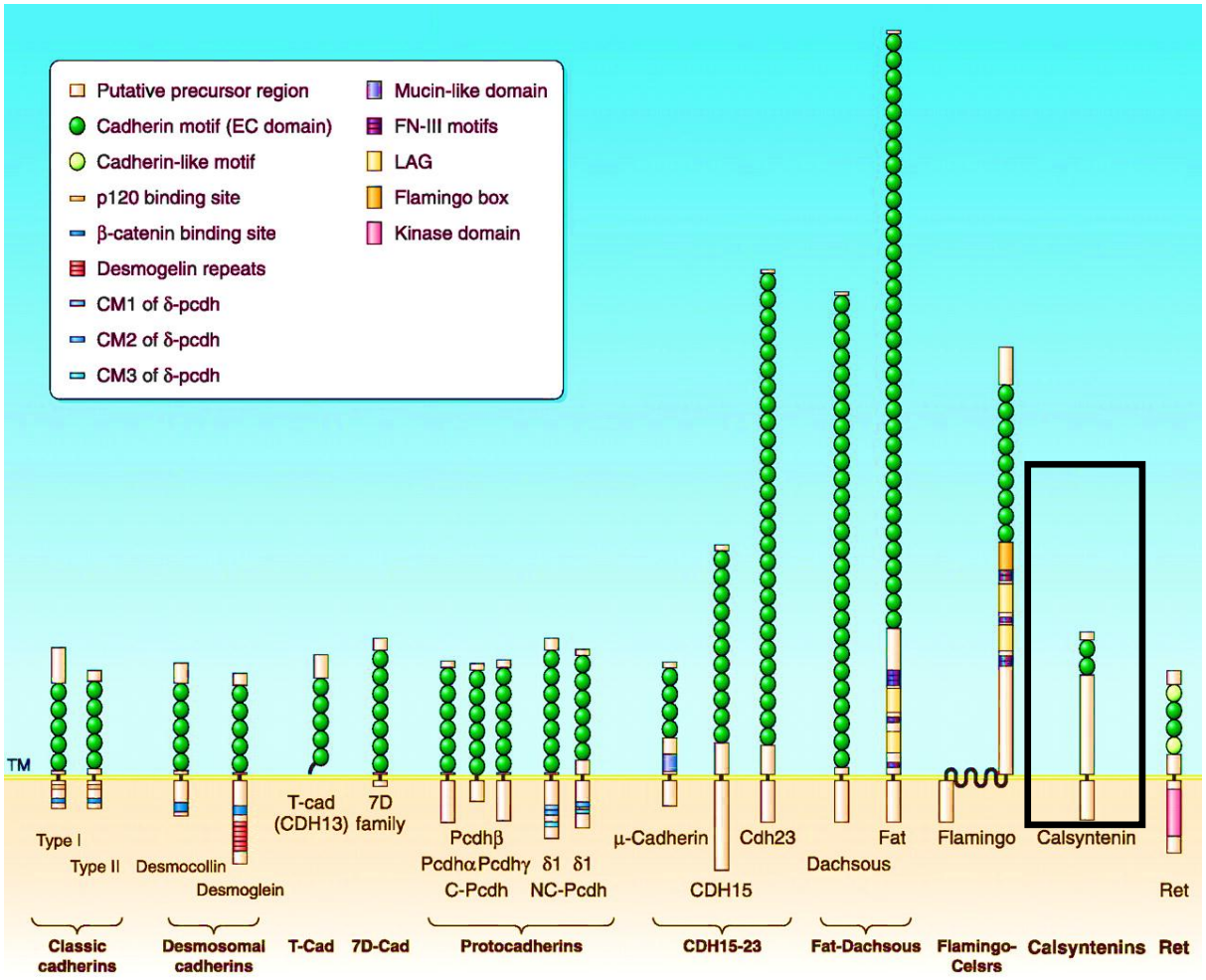


Figure 1.3: Schematic representing cadherin superfamily in mammals. The overall structure of the cadherin molecules are diverse among the members, although they all share 'cadherin motifs' or 'EC domains' in their extracellular domain. Highlighted with the box is the mammalian calsyntenin gene (Image modified from Hirano and Takeichi, 2012).

cadherin domains in calsyntenins do not cover the complete signature sequence ([LIV]-x-[LIV]-x-D-x-N-D-[NH]-x-P) of the classical cadherins (Takeichi, 1990). *clstn2* and *clstn3* show 58% and 52% identity with *clstn1* respectively (Figure 1.4). The three calsyntenins are highly conserved in their extracellular domains. However, variability exists in the C- terminal Ca^{2+} binding domains where the total number of acidic amino acids as well as the size of the contiguous acidic residue clusters is quite variable with longest in *clstn1* and shortest in *clstn3* (Hintsch et al., 2002). This variability suggests that Ca^{2+} binding affinity could be lower in *clstn2*, while *clstn3* is unlikely to bind any calcium at all. Bioinformatics analysis revealed that calsyntenins are highly conserved across species: mammals have three related calsyntenins, while single calsyntenin genes are found in *Drosophila* (*cals*) and *C. elegans* (*casy-1*) (Hintsch et al., 2002; Vogt et al., 2001).

Expression analysis revealed that all three calsyntenins are highly enriched in the brain, although *clstn1* also shows some expression in skeletal muscle, heart, kidney and lung tissue (Rindler et al., 2008; Vogt et al., 2001). *In situ* hybridizations in adult mouse brain sections revealed distinct neuronal expression patterns for the three calsyntenins with *clstn1* expression more uniform throughout the brain compared to the expression of *clstn2* and *clstn3*. *clstn1* is expressed in most brain regions but more strongly in the hippocampus and cortex. In the hippocampus, *clstn1* is expressed highly in CA1-3 regions while moderate expression is seen in dentate gyrus granule cells (Vogt et al., 2001). *clstn2* and *clstn3* are highly expressed in the GABAergic interneurons of the cerebral and cerebellar cortex (Hintsch et al., 2002). *clstn2* is expressed highly in the CA2-CA3 region and some scattered interneurons in pyramidal cells of CA1 layer and moderately in dentate gyrus granule cells. *clstn3* is strongly expressed in pyramidal neurons in the CA2-CA3 region and some interneurons in the pyramidal layer of the CA1 region. *clstn3* also expresses in a small population of interneurons in the hilus (Hintsch et al., 2002).

As mentioned earlier, the N-terminal portion of *clstn1* was originally isolated as a secreted protein, suggesting peptidases-mediated proteolytic cleavage in the synaptic cleft just juxtaposed to the membrane (Vogt et al., 2001). Further analysis revealed that N-terminal portion resulting from cleavage is released in to the cerebrospinal fluid while the C-terminal stump is internalized and accumulates within the spine apparatus on the spine synapses or on the subsynaptic membranes of the shaft synapses (Vogt et al., 2001).

1.4.2. Functions of Calsyntenins

Mammalian Calsyntenins show a high degree of spatial and temporal regulation of expression indicating a possible diversity of functions.

Calsyntenin-1: CLSTN1 has been reported to perform kinesin-1 motor-dependent transport of various axo-dendritic components in the brain (Konecna et al., 2006; Steuble et al., 2010; Uchida and Gomi, 2015; Vagnoni et al., 2011). Kinesins are motor proteins that transport axonal cargo along microtubules towards the plus end. Kinesin-1 consists of two light chains (KLC) and two heavy chains (KHC) (Hirokawa et al., 1989). Initially, a direct interaction of the C-terminal fragment of CLSTN1 used as bait with KLC1 in a yeast-two-hybrid screen was discovered (Konecna et al., 2006). Further analysis showed that CLSTN2 could also bind to KLC1, while binding of CLSTN3 was weaker. Binding between CLSTN1 and KLC1 is mediated by an interaction between the tetratricopeptide repeats (TPR) in KLC1 and by two "WDDS" motifs in CLSTN1, termed KLC1 binding segment 1 and 2 (KBS1, KBS2). Further, CLSTN1 associates with the scaffold proteins X11/X11L, which then associates with the amyloid precursor protein (APP) forming a tripartite complex in the brain (Araki et al., 2003, 2004; Ludwig et al., 2009). CLSTN1 has been shown to interact with the kinesin light chain protein resulting in blocking of the transport of APP-containing vesicles and thus causing more β- amyloid generation, suggesting a possible role of these molecules in the pathogenesis of Alzheimer's disease (Steuble et al., 2012; Uchida and Gomi, 2016; Uchida et al., 2011, 2013; Vagnoni et al., 2012; Yin et al., 2009). Developmentally, CLSTN1 has been shown to be required for the trafficking of NMDA receptors at the synapse, essential for neuronal maturation during early embryonic development (Ster et al., 2014). Further, CLSTN1 also regulates axon branching and endosomal trafficking during sensory neuron formation (Ponomareva et al., 2014). More recently, CLSTN1 has been shown to mediate trafficking of axon guidance receptors at spinal cord choice points (Alther et al., 2016) and in regulating microtubule polarity during sensory axon arbor development (Lee et al., 2017). CLSTN1 has also been reported to mediate hepatitis C virus replication (Awan et al., 2016).

Calsyntenin-2: In contrast to the roles of CLSTN1, which appears to mediate trafficking functions during nervous system development, CLSTN2 has been reported to be required for modulating synaptic plasticity. In a genome-wide screen aimed to identify human hippocampus based-episodic memory genes, a single nucleotide

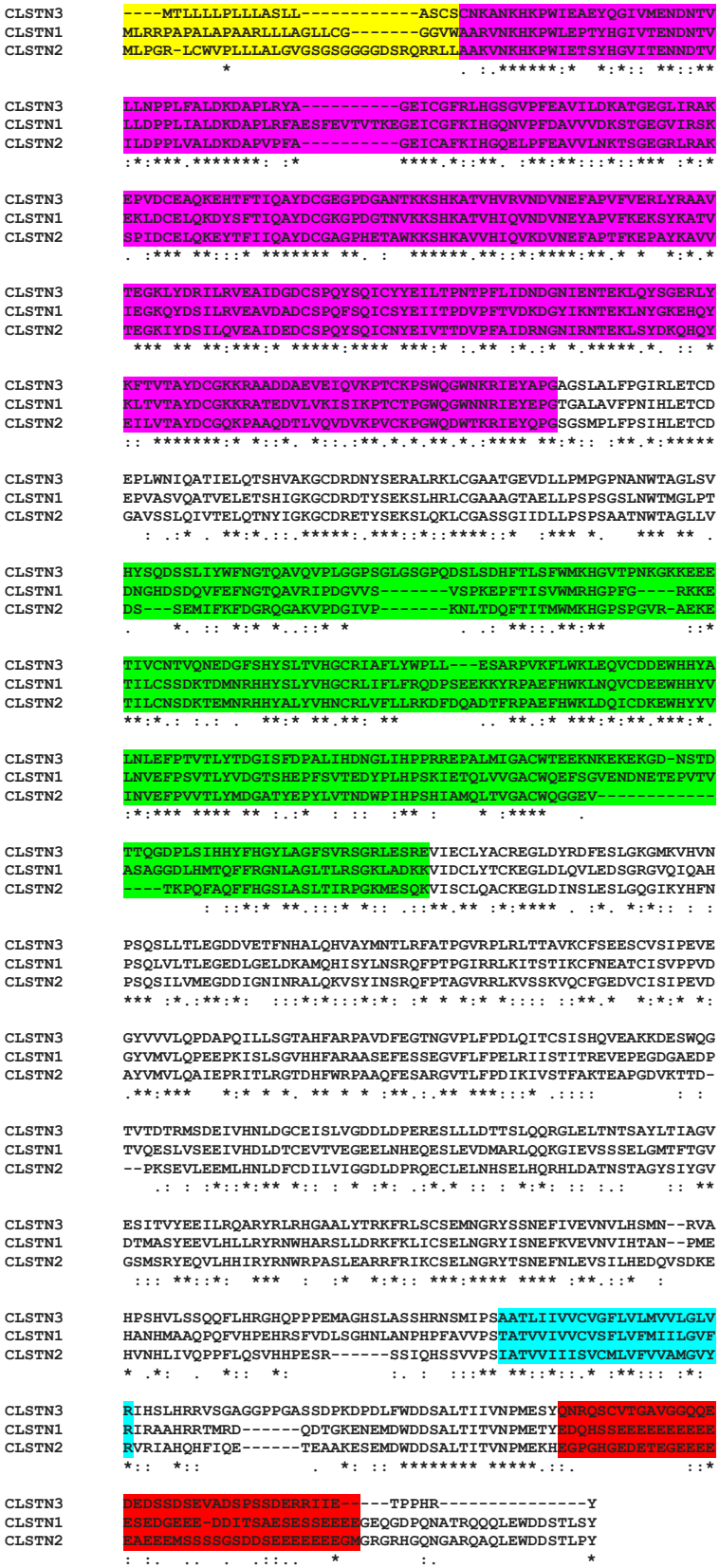


Figure 1.4: Clustal omega alignment of protein sequences from three human Calsyntenin genes- CLSTN1, CLSTN2 and CLSTN3. Predicted domains are shown with colored amino acid sequences- Signal peptide (yellow), Cadherin domains (pink), LG/LNS domains (green), Transmembrane domain (Blue) and Acidic region (red). CLSTN2 and CLSTN3 show 58% and 52% identity with CLSTN1 respectively.

polymorphism (SNP) in CLSTN2 was identified as a target that could be involved in human memory performance (Boraxbekk et al., 2015; Jacobsen et al., 2009; Papassotiropoulos et al., 2006; Preuschhof et al., 2010). This SNP consists of a common substitution (T -> C) in the first intron of *clstn2*, with the "C" allele conferring enhanced cognitive performance in memory tasks; this enhancement was related with increased activity in the hippocampus (Jacobsen et al., 2009). CLSTN2 knock-out mice are hyperactive with cognitive deficits due to reduced GABAergic neurotransmission (Lipina et al., 2016; Ranneva et al., 2017). The molecular basis of how CLSTN2 regulates GABA neurotransmission is however still unknown.

Calsyntenin-3: CLSTN3 has been reported to act as a synaptogenic cell adhesion molecule that forms functional complexes with neurexins to regulate inhibitory synapse development and function (Lu et al., 2014; Um et al., 2014). CLSTN3 has also been shown to promote excitatory and inhibitory synapse development in concert with neurexins (Pettem et al., 2013).

As members of the cadherin superfamily, it is imperative that Calsyntenins mediate cell-cell adhesion, although their adhesive roles have not been investigated profoundly. In Zebrafish, the extracellular cadherin domains of Calsyntenins have been shown to mediate homophilic adhesion as well as to serve as diffusible homo-or heterophilic ligands in the vertebrate nervous system (Ortiz-Medina et al., 2015).

However, regardless of all these studies that have allowed us to understand the functions of Calsyntenins, we are still far from elucidating the molecular and physiological underpinnings of this molecule. Despite considerable reports emphasizing the involvement of Calsyntenins in synapse development and function, it has been very difficult to deduce the cellular and molecular mechanisms and implications of these proteins on animal behavior and function. This raises the necessity to explore the functions of this molecule in simpler model systems like *C. elegans* to improve our understanding of the complexity of Calsyntenin- related brain functions.

1.5. *C. elegans* as a model organism for the study of neuroscience

In the 19th century, an intense debate was going on between the two prominent scientists of the era, George Cuvier (1769-1832) and Geoffroy Saint-Hilaire (1772-1844), as to define the nature of relationships between living organisms (Grene, 2001). Cuvier believed that animals should be divided into four distinct groups, as they share no common ancestry and similarity in body parts is an outcome of similar

functions. Geoffroy, on the other hand, proposed that all animals descended from a common ancestor and that there was a common structural blueprint that links all the animals together, that has modified between organisms based on functions. One of the Geoffroy's interesting theories was that the dorsal side of vertebrates, where the central nervous system is located, is analogous to the ventral side of invertebrates, which contains the nerve cord. Charles Darwin's (1809-1882) theories based on common descent and evolution by natural selection allowed a re-examination of these models. Today, in the era of molecular analysis, we see a lot of homology and conservation of proteins and functions across the species, for example; Notch family proteins control nervous system patterning from the fruitfly *Drosophila melanogaster* to mouse (Williams et al., 1995), conservation in components of neurotransmitter release (Südhof et al., 1989), neuropeptide signaling (Nässel, 2002) or the action of G-protein coupled receptors (GPCRs) (Fredriksson et al., 2005). This perspective gave a basis to the use of model organisms to understand biology.

Caenorhabditis elegans is a free-living, transparent nematode that was introduced as a model organism by Sydney Brenner (1965) to study developmental and behavioral genetics (Brenner, 1973). *C. elegans* is small and a fully grown adult is approximately 1 mm in length and 50-60 µm in diameter. They have a rapid three-day life cycle, under standard lab conditions, which allows a large number of worms to be grown easily in the laboratory. *C. elegans* passes through four larval stages, named L1, L2, L3 and L4, before a final molting to produce the mature adult. Under stress conditions, the animals can enter an alternate developmental stage known as dauer (Cassada and Russell, 1975), which is highly resistant to desiccation.

C. elegans is a self-fertilizing hermaphrodite, producing sperms and eggs separately. This aspect of worm biology is advantageous as it allows easy maintenance of isogenic populations. A single hermaphrodite lays up to 300 eggs allowing rapid population expansion. They also produce a low, spontaneous proportion of males resulting from an elegant developmental switch occurring in animals that carry a single copy of sex chromosome (Hodgkin, 1983; Hodgkin et al., 1979). Males are useful for conducting genetic crosses allowing the generation of double mutants, for backcrossing mutated animals and for conducting epistasis and complementation experiments.

C. elegans is diploid, so it is amenable to study lethal mutations and introduce balancers and genetic markers (Riddle et al., 1997). In 1998, the *C. elegans* genome

was fully sequenced and the fully annotated 100MB genome sequence is available online (C. elegans Sequencing Consortium, 1998; Hodgkin, 2005). This genome has been subsequently joined by sequences for four other *Caenorhabditis* species: *C. briggsae* (Gupta and Sternberg, 2003), *C. remanei*, *C. brenneri* and *C. japonica*; as well as data for other nematode species, which can be easily accessed online at wormbase.org (Harris et al., 2010b). These characteristics make *C. elegans* a powerful tool in genetic and genomic studies. Many large-scale genetic screens using classical genetics and RNAi techniques have been performed using *C. elegans* to identify new genes, phenotypes, and interacting proteins (Carpenter and Sabatini, 2004; De Stasio, 2012; Kamath and Ahringer, 2003; Norris et al., 2017; Parry et al., 2007; Schøler et al., 2012; Sieburth et al., 2005; Sin et al., 2014). Also, a large repertoire of gene knockouts are easily available from *C. elegans* Genetics Center, University of Minnesota or can be obtained from two gene knockout consortia at celeganskoconsortium.omrf.org or shigen.lab.nig.ac.jp/c.elegans/index. jsp.

Furthermore, *C. elegans* is transparent which allows for tracking cellular development and functioning at single-cell resolution *in vivo.* In fact, *C. elegans* research has proved instrumental to the development of green fluorescent protein (GFP) and confocal microscopy (Chalfie et al., 1994; White et al., 1987). They also have an invariant number of somatic cells and invariant cell lineages (every cell division occurs in the same order in each individual) (Sulston and Horvitz, 1977; Sulston et al., 1983) thus reducing variation between individuals during embryonic development.

Another advantage of *C. elegans* is an easy generation of stable transgenic lines by simply injecting exogenous genetic material into the syncytia of nuclei in the gonad. Direct injection of DNA into the gonad allows incorporation of that DNA into the developing egg. This DNA could be maintained as large extrachromosomal arrays that are heritable, generating stably transformed lines (Mello and Fire, 1995). Integrated lines could also be created by simply introducing double-stranded breaks by UV irradiation, followed by incorporation of extrachromosomal DNA in to the genome (Mello and Fire, 1995). More recently, targeted introduction of DNA at specific sites in the genome using the Mos transposase (Frøkjaer-Jensen et al., 2008) was the method of choice. However, the advent of CRISPR technology has revolutionized genome editing in the worm biology (Chen et al., 2014; El Mouridi et al., 2017; Farboud, 2017; Kim et al., 2014; Paix et al., 2017; Sugi, 2017).

1.6. The nervous system of *C. elegans*

The adult hermaphrodite has a 302-neuron nervous system and a completely mapped cell lineage from zygote to adult worm. The hard wired synaptic connections have been reconstructed based on the physical connections and structural characters of neurons examined by serial-section electron microscopy reconstructions (Riddle et al., 1997). The *C. elegans* neurons are simpler, with fewer branches and there are about 5000 chemical synapses, 2000 neuromuscular junctions, and 600 electrical synapses or gap junctions (White et al., 1986). While individual synapses may form differently from animal to animal, the overall structure of the nervous system is consistent.

Despite huge conservation in synaptic proteins and synaptic transmission machinery, some major differences exist in *C. elegans* nervous system as discussed below:

- Mammalian neurons have multiple projections for both axons and dendrites and each dendrite can receive multiple inputs. *C. elegans* neurons are simpler, with fewer branches.
- Synaptic transmission in mammals is mediated by a wave of depolarization generated by the opening of voltage-gated- sodium (Na^+) channels (Alberts, et al., 2008). However, no voltage-gated- sodium (Na^+) channels have so far been discovered in *C. elegans*. They use voltage-gated calcium (Ca^{2+}) channels to mediate synaptic transmission.
- Mammalian synaptic transmission is facilitated by an action potential, an 'all-or-none' response with a stereotypical waveform, which is independent of the waveform or amplitude of initial depolarization. However, *C. elegans* allows propagation of membrane potential in a regenerative graded response where the waveform and amplitude of graded potentials is directly coupled to that of the stimulus received by the cell (Goodman et al., 1998).

Recently, reports of action potentials in some *C. elegans* neurons (Mellem et al., 2008) have been indicated, although another report argues against it to describe it more as plateau potentials, in which neurons have two equally stable membrane potentials, (in this case at -70mV and -35mV), between which they can switch in response to depolarization (Lockery and Goodman, 2009; Lockery et al., 2009).

Although most neurons in mammalian nervous system utilize action potentials for synaptic transmission, the existence of graded potential for neurotransmitter release have also been reported. For example, ribbon synapses in the eye retina and auditory hair cells tonically release neurotransmitters based on membrane potential (Heidelberger, 2007).

1.6.1. Synaptic signaling in *C. elegans* is mediated by two types of synapses

C. elegans forms *en passant* synapses between neighboring parallel nerve processes, or between nerve processes or muscle arms (Figure 1.5) (White et al., 1986). At the *en passant* synapses, synaptic boutons are formed along the entire length of the axon shaft, in contrast to the *terminaux* synapses, in which synaptic boutons are formed at the end of axonal branches. Synaptic communication in *C. elegans* is mediated by two types of synapses: the chemical synapse and electrical synapse (Figure 1.6).

1.6.1.1. Chemical synapses

Chemical synapses are defined by the presence of vesicles containing neurotransmitters ready for release and signaling to the postsynaptic cells. Two types of vesicles are observed in electron micrographs of *C. elegans* synapses:

- Small synaptic vesicles: These are small, clear vesicles of about 35-45 nm in diameter (Takamori et al., 2006) usually packaged with small molecule neurotransmitters. The synaptic vesicles are produced in the Golgi and then trafficked to the synapse via kinesin motor- mediated transport (Hall and Hedgecock, 1991; Klopfenstein and Vale, 2004). Synaptic vesicles contain a vast array of proteins, many present in multiple copies.
- Dense core vesicles (DCV): The second class of vesicles observed in electron micrographs are the large, electron-dense vesicles that range from 40-55 nm in diameter. Unlike synaptic vesicles, DCVs are not concentrated at the synapse nor are they recycled. They are packed with large neurotransmitter molecules called neuropeptides or catecholamine neurotransmitters like dopamine and serotonin. Neuropeptides are short amino acid sequence molecules that can diffuse over long distances, and can function either directly or indirectly to modulate the neural circuits (Li and Kim, 2008).

1.6.1.2. Electrical synapses

Electrical synapses or gap junctions are direct electrical linkages between cells. They allow passage of small molecules such as inorganic ions, cyclic AMP, inositol 1,4,5 triphosphate (IP_3) through these channels (Kumar and Gilula, 1996) by directly coupling the electrical potentials of linked cells. There are an estimated 600 gap junctions in the *C. elegans* nervous system (White et al., 1986) comprising some 10% of connections in the nervous system, although their biological significance is not well understood.

GAP junctions in mammalian systems are composed of hemichannels formed of 6 subunits of connexin proteins, while innexins, a related protein family, are the structural components of GAP junctions in invertebrates (Phelan and Starich, 2001; Phelan et al., 1998). Several studies have reported that GAP junctions are important for locomotion in *C. elegans* (Chen et al., 2007; Starich et al., 2009).

1.6.2. Neurotransmitters in *C. elegans*

Most of the neurotransmitters in *C. elegans* are conserved across different animal species. Acetylcholine (Ach) is the major excitatory neurotransmitter whereas γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter (Figure 1.7a and b). Glutamate is commonly used neurotransmitter in sensory neurons and glutamate signaling is mostly excitatory (Figure 1.7c). *C. elegans* also use biogenic monoamines and neuropeptides as neurotransmitters.

1.6.2.1. Acetylcholine

Acetylcholine is the major excitatory neurotransmitter at the nematode neuromuscular junction (NMJ) and more than one third of the *C. elegans* neurons release acetylcholine (Figure 1.8) (Rand, 2007). However, ACh may operate as an inhibitory neurotransmitter as well (Pereira et al., 2015). ACh is synthesized from acetyl-CoA and choline by the enzyme choline acetyltransferase which is encoded by the *cha-1*(Abnormal choline acetyltransferase-1) gene (Rand and Russell, 1985). Newly-synthesized ACh molecules are then loaded into the synaptic vesicles by the vesicular ACh transporter (VAChT) UNC-17 (Uncoordinated-17) (Alfonso et al., 1993). ACh released into the synaptic cleft binds to ACh receptors (AChRs) on the postsynaptic membrane.

C. elegans expresses at least 36 genes that encode for AChR subunits (Rand, 2007). These AChR subunits were identified in genetic screens for resistance to N-AChRs agonists or by gene homology studies (Fleming et al., 1997; Jones and

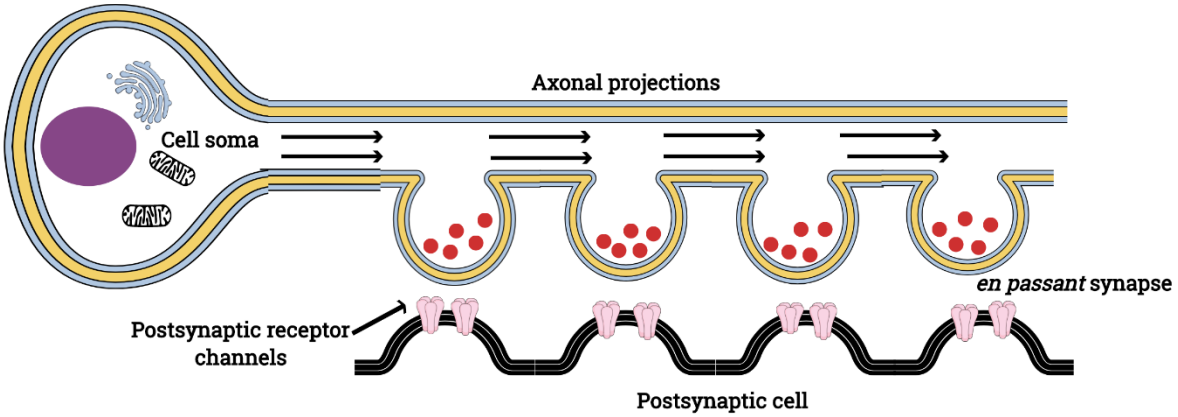


Figure 1.5: ***C. elegans*** **forms** ***en passant*** **synapses**—synaptic boutons are formed along the axonal shaft between neighboring parallel nerve processes, or between nerve processes and muscle arms. The presynaptic site is seen as a vesicle-filled varicosity containing the electron-dense presynaptic density in the membrane adjacent to the contact with the postsynaptic elements.

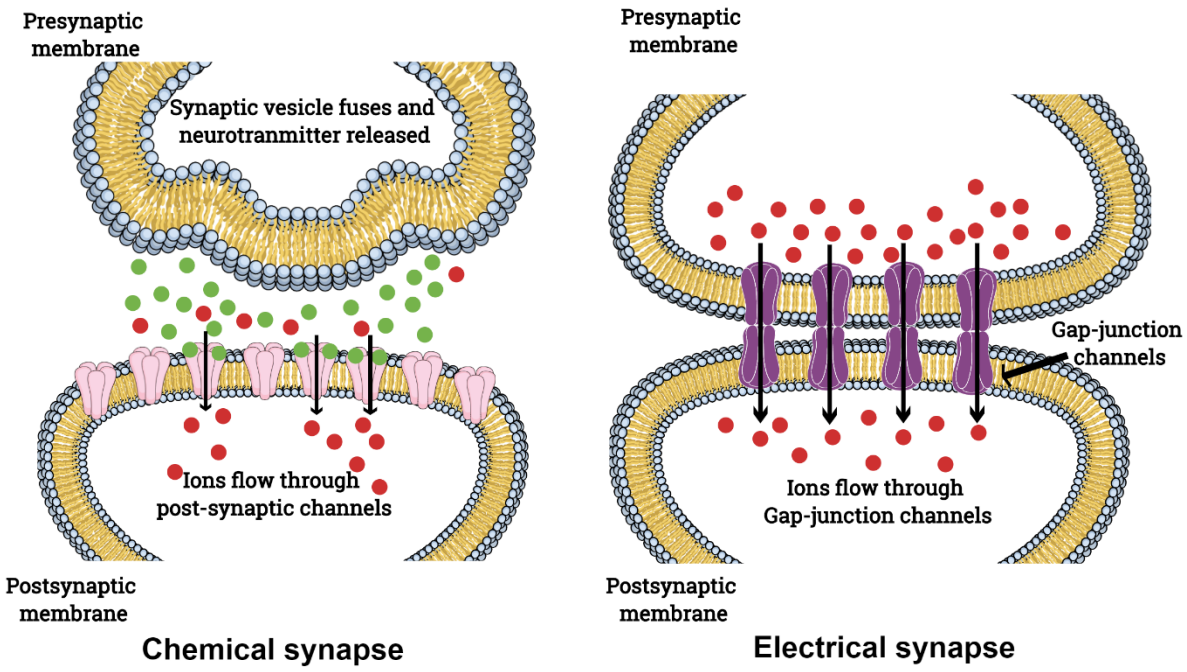
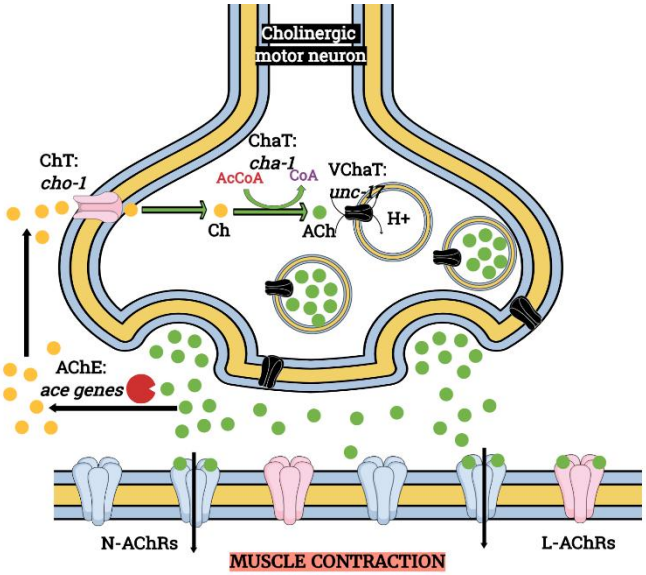
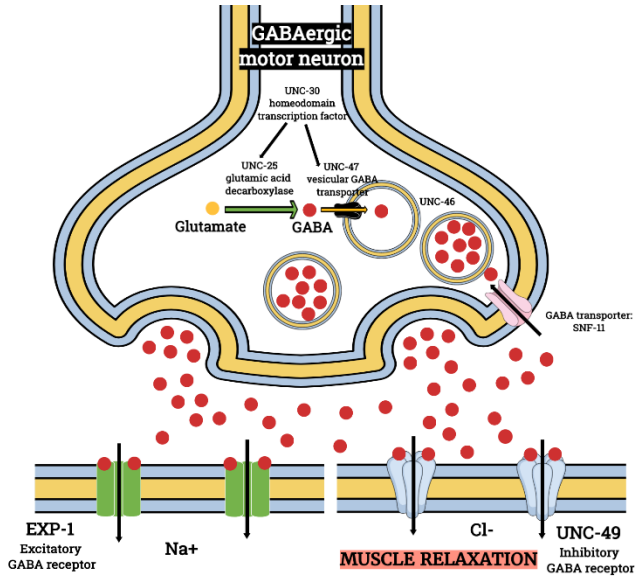


Figure 1.6: Types of synapses in ***C. elegans*****.** Chemical synapse- Action potential in the presynaptic neuron triggers the release of neurotransmitters. These neurotransmitters bind to postsynaptic receptors leading to signal initiation in the postsynaptic cell. Electrical synapse- Ions from the presynaptic neuron diffuse directly into the postsynaptic neuron.

(A)



(B)



(C)

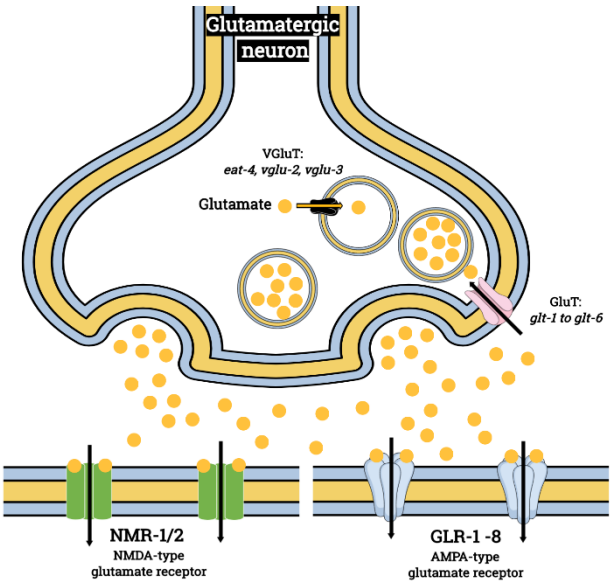


Figure 1.7: Schematic showing (A) Cholinergic, (B) GABAergic and (C) Glutamatergic neuron structure pre- and post synaptically.

Sattelle, 2004; Lewis et al., 1980). The best characterized *C. elegans* receptors are present on the muscle, and includes L-AChRs that responds to the levamisole and N-AChRs that respond to nicotine but not to levamisole (Richmond and Jorgensen, 1999). The nicotine sensitive receptors are homo-pentameric, ligand-gated ion channels that contain only the ACR-16 α-subunit (Francis et al., 2005; Touroutine et al., 2005; Unwin, 2005). The levamisole sensitive receptors on the body wall muscle are heteromeric, containing three essential subunits UNC-29, UNC-38 and UNC-63 along with two non-essential LEV-1 and LEV-8 subunits (Culetto et al., 2004; Fleming et al., 1997; Richmond and Jorgensen, 1999). Recently, a neuronal acetylcholine receptor *acr-2*, containing UNC-38, UNC-63, ACR-12 (α subunits) and ACR-2, ACR-3 (non-α subunits) has been reported to maintain excitation-inhibition balance at the NMJ (Jospin et al., 2009; Stawicki et al., 2013).

Apart from the nicotinic receptors on the muscle, the *C. elegans* genome contains three paralogous muscarinic AChR genes. *gar-2* (G-protein linked acetylcholine receptor-2) is expressed in ventral cord motor neurons (Lee et al., 2000) and regulates acetylcholine release at the synapse (Dittman and Kaplan, 2008; O'Hern et al., 2017). *gar-3* (G-protein linked acetylcholine receptor-3) has also recently been shown to maintain cholinergic transmission at the NMJ (Chan et al., 2012, 2013; Kalinnikova et al., 2013) and for spicule contraction during mating behavior (Liu et al., 2007).

Also, four genes encoding ACh-gated chloride channels were found in the *C. elegans* genome. Orthologs of these channels have not yet been identified in other species, nor have these channels been well characterized in *C. elegans* (Putrenko et al., 2005). Recently, they have been identified as potential anthelmintic drug targets (Wever et al., 2015).

ACh released into the synaptic cleft is degraded by the enzyme acetylcholinesterase. In *C. elegans*, there are four genes encoding this enzyme: *ace-1 – 4* (Abnormal acetylcholine esterase-1 – 4); and their quadruple mutants are lethal (Grauso et al., 1998; Johnson et al., 1981, 1988). Choline, one of the degradation product, is transported back into the neurons by the membrane choline transporter CHO-1 (Okuda et al., 2000).

1.6.2.2. GABA

The *C. elegans* nervous system consists of 26 GABAergic neurons which include six DD and thirteen VD neurons that innervate the dorsal and ventral body-wall muscles respectively, four RME motor neurons that innervate the head muscles, the

AVL and DVB neuron that are pre-synaptic to the enteric muscles and RIS which is an interneuron (Schuske et al., 2004; White et al., 1986). GABA is the major inhibitory neurotransmitter in *C. elegans* and is used to control relaxation of body wall muscles during locomotion (Figure 1.8). Loss of GABA signaling has less impact on locomotion than the loss of acetylcholine signaling. The main locomotary phenotype in GABA mutants is defects in backward locomotion, as the animals exhibit a 'shrinker' phenotype (McIntire et al., 1993).

Several genes important for GABA signaling were identified in genetic screens (McIntire et al., 1993; Thomas, 1990). UNC-25 is the *C. elegans* glutamic acid decarboxylase (GAD) that converts glutamate into GABA (Jin et al., 1999). GABA molecules are pumped into the synaptic vesicles by the vesicular GABA transporter UNC-47 (VGAT) (McIntire et al., 1997). Synthesis of these proteins is controlled by UNC-30, a homeodomain transcription factor (Jin et al., 1994). UNC-46 is a vesicular transmembrane protein that is important for the localization of the UNC-47 on the vesicles (Schuske et al., 2007). GABA released into the synaptic cleft is cleared by the GABA transporter SNF-11 (Sodium - neurotransmitter symporter family-11) (Jiang et al., 2005; Mullen et al., 2006). *C. elegans* genome seems to encode a GABA transaminase, GTA- 1 and succinic semialdehyde dehydrogenase, ALH-7 required for GABA degradation; however, the proteins have not yet been functionally characterized (Wormbase release WS246).

During *C. elegans* locomotion, muscles on one side of the body are stimulated to contract by the action of acetylcholine, the opposite side relaxes due to the action of GABA on $GABA_A$ receptors, which encode GABA-gated chloride channels (UNC-49) (Schofield et al., 1987). This gene codes for three different splice variants resulting in the production of three distinct subunits of the $GABA_A$ receptor; the variants UNC-49B and UNC-49C form the receptor on the body wall muscle membrane (Bamber et al., 1999).

Interestingly, during defecation, GABA also controls an excitatory transmission event (Jorgensen, 2005), highly unusual for an adult organism, although an important part of GABA signaling during mammalian development (Ben-Ari, 2002). This function is mediated by EXP-1 (Expulsion defective-1) that encodes a GABA-gated cation channel required for normal defecation cycle in *C. elegans*. (Beg and Jorgensen, 2003; Thomas, 1990).

Protein	Function
CHA-1	Choline acetyltransferase
UNC-17	Vesicular ACh transporter
ACR-8, ACR-16, UNC-29, UNC-38, DEG-3	Nicotinic ACh receptor families
GAR-1 – 3	Muscarinic ACh receptors
ACC-1 – 4	ACh-gated Cl^- channels
ACE-1 – 4	Acetylcholine esterase
CHO-1	Choline transporter

Protein	Function
UNC-25	Glutamic acid decarboxylase
UNC-30	Homeodomain transcription factor
UNC-47	Vesicular GABA transporter
UNC-46	Accessory transmembrane protein
UNC-49, LGC-38, GAB-1	GABAA receptor
EXP-1	GABA-gated cation channel
GBB-1 – 2	GABAB receptor
SNF-11	GABA transporter
GTA-1	GABA transaminase

Protein	Function
PYR-1	Pyruvate carboxylase
GLN-1,2,3,5	Glutamine synthetases
GLNA-1 -3	Glutaminases
EAT-4, VGLU-2, VGLU-3	Vesicular glutamate transporters
GLR-1-8	non-NMDA iGluR subunits
NMR-1, NMR-2	NMDA iGluR subunits
GLC-1-4, AVR-14, AVR-15	Glutamate-gated Cl^- channels
GLT-1 -6	Glutamate transporters
MGL-1-3	Metabotropic glutamate receptors

Figure 1.8: Table showing proteins involved in Cholinergic, GABAergic and Glutamatergic signaling in *C. elegans*.

The second class of GABA receptor are metabotropic $GABA_B$ receptors which are 7-pass transmembrane proteins, that act through trimeric G-proteins to either activate K^+ channels or inhibit the voltage-gated Ca^{2+} channels; both leading to an inhibition of the target neurons. These GPCRs, GBB-1 ($GABA_B$ receptor-1) and GBB-2, are expressed in cholinergic ventral cord motor neurons where they heterodimerize to regulate ACh release (Dittman and Kaplan, 2008).

1.6.2.3. Glutamate

In the vertebrate central nervous systems (CNS), fast excitatory synaptic transmission between cells is mainly mediated by glutamatergic signaling. *C. elegans* also use glutamate as a neurotransmitter (Figure 1.8) (Brockie and Maricq, 2006). Some previous studies have provided evidence showing that glutamate serves as an excitatory neurotransmitter utilized by several classes of sensory neurons, including mechanosensory neurons, and plays a critical role in regulating the worm's locomotion.

Glutamate metabolism in *C. elegans* has not been fully characterized. *C. elegans* genome encodes one pyruvate carboxylase (*pyr-1*) for de novo synthesis of glutamate; four glutamine synthetases (*gln-1*, *gln-2*, *gln-3*, *gln-5*) that convert glutamate to glutamine and three glutaminases (*glna-1*, *glna-2*, *glna-3*) that synthesize glutamate from glutamine (Hobert, 2013). Glutamate molecules are pumped into synaptic vesicles via vesicular glutamate transporters (VGluTs), encoded by three genes *eat-4*, *vglu-2* and *vglu3*. Of these three, the *eat-4* vesicular transporter functions are best characterized (Giles and Rankin, 2009; Lee et al., 2008, 1999; Ohnishi et al., 2011; Rankin and Wicks, 2000; Rose et al., 2002; Serrano-Saiz et al., 2013). The *C. elegans* genome also encodes six glutamate plasma membrane transporters (*glt* genes/ SLC1 family) that are expressed in multiple distinctive cell types (Mano et al., 2007).

Ionotropic glutamate receptors (iGluRs) are a major class of heteromeric ligand-gated ion channels. In *C. elegans*, ten iGluR subunits have been identified (Brockie and Maricq, 2003; Brockie et al., 2001a). They consists of non-NMDA class (GLR-1 – GLR-8) most similar to the mammalian AMPA or kainate subfamilies and NMDA subunits (NMR-1 and NMR-2), that resemble the NR1 and NR2A subfamilies respectively. The amino acid sequence of *C. elegans* subunits show many similarities with the vertebrate iGluR subunits (Brockie et al., 2001a). The extracellular ligand (glutamate) -binding domains, the four transmembrane hydrophobic domains, and the pore region are highly conserved in *C. elegans*. Infact, the amino acid residues that

influence the functional properties of vertebrate iGluRs are also significantly conserved in *C. elegans* subunits, that include the "Q/R" site present in non-NMDA receptors (Seeburg et al., 1998) and an asparagine residue that regulates calcium permeability through NMDA receptors (Burnashev et al., 1992). The amino acid motif SYTANLAA that is present at the C-terminus of TM2 is also conserved in *C. elegans* subunits and appear to be essential for receptor gating.

The first iGluR subunit characterized in *C. elegans* was the AMPA-subtype encoded by *glr-1*. Mutations in *glr-1* gene have been reported to affect multiple behavioral paradigms: sensory integration of environmental cues (Campbell et al., 2016), reversal responses to nose touch stimulation (Hart et al., 1995; Maricq et al., 1995), avoidance responses to hyperosmotic stimuli (Mellem et al., 2002) and to the volatile repellent octanol (Chao et al., 2004; Hart et al., 1999), the 'spontaneous' shift from forward to backward movement (Brockie et al., 2001a; Zheng et al., 1999), foraging behaviors (Hills et al., 2004) and in long-term memory (Morrison and van der Kooy, 2001; Rose et al., 2003). NMDA receptors, on the other hand are required for sensitivity to the anesthetic nitrous oxide (N_2O) (Nagele et al., 2004), osmotic avoidance (Baidya et al., 2014; Mellem et al., 2002), in regulating frequency of reversals during locomotion (Brockie et al., 2001b) and during synaptic plasticity (Amano and Maruyama, 2011; Kano et al., 2008; Lau et al., 2013; Nishijima and Maruyama, 2017).

C. elegans genome also encodes a unique family of glutamate-gated chloride channels (GluCl) that are permeable to Cl^- ions and are sensitive to anthelmintic drug ivermectin (Cully et al., 1994; Yates et al., 2003). About 6 genes encodes GluCl subunits in *C. elegans* - ***glc-1*, *glc-2*, *glc-3*, *glc-4*, *avr-14/gbr-2*** and ***avr-15*** (Cully et al., 1994; Dent et al., 1997, 2000; Horoszok et al., 2001; Laughton et al., 1997; Vassilatis et al., 1997). GluCls have been reported to be involved in pharyngeal pumping, sensory perception and locomotion (Dent et al., 1997, 2000; Keane and Avery, 2003; Lee et al., 1999; Mellem et al., 2002; Pemberton et al., 2001).

C. elegans also contains three metabotropic glutamate receptors encoded by the genes *mgl-1*, *mgl-2* and *mgl-3* that functions to determine glutamate-dependent modulation of feeding in pharyngeal neural circuits (Dillon et al., 2006, 2015).

1.6.2.4. Monoamines

In *C. elegans* four monoamine neurotransmitters are present: serotonin and octopamine (Horvitz et al., 1982), dopamine (Sulston et al., 1975) and tyramine

(Alkema et al., 2005). Of these, tyramine is nematode-specific, while the others are found in mammalian systems (Chase and Koelle, 2007).These neurotransmitters act through a large number of receptors, identified by homology to mammalian receptors or through screens for mutants (Chase and Koelle, 2007; Komuniecki et al., 2004; Schäfer et al., 1996).

Mutants display a wide range of phenotypes ranging from egg-laying defects, accumulation of body fat (Noble et al., 2013; Oakes et al., 2017), misregulation of locomotion responses such as slowing in presence of food (Churgin et al., 2017; Donnelly et al., 2013; Luedtke et al., 2010; Suo et al., 2009, 2009; Yoshida et al., 2014), changes in pumping (Lemieux and Ashrafi, 2015), changes in nociception and aversive behaviors (Guo et al., 2015; Harris et al., 2010b; Mills et al., 2012; Wragg et al., 2007), lifespan (Suo et al., 2009) and modulation of learning and memory-associated behaviors (Hukema et al., 2008).

1.6.2.5. Neuropeptides

Neuropeptides are short stretches of amino acids that modulate neural synaptic activity either directly or indirectly. They can act as short-range signals, released close to the site of action or as hormones, acting on tissues outside the nervous system, thereby enhancing the intricate complexity of the activity of neural circuits. Large repertoire of neuropeptides in *C. elegans* functions to fine-tune the neural networks involved in diverse functions and also increases the complexity of behavioral outcomes of a worm. The *C. elegans* genome contains 113 neuropeptide genes that encode over 250 distinct neuropeptides. These are subdivided into three major families based on the presence of conserved sequence motifs. The first family constitutes the FMRFamide-like peptide (*flp*) gene family (Phe-Met-Arg-Phe-NH2) that consist of 70 different peptides encoded by 33 precursor genes (Li, 2005; Li et al., 1999). The second family comprises about 40 insulin-like peptides encoded by 40 precursor genes (Li et al., 2003; Pierce et al., 2001). The third family consists of peptides with no sequence similarity to either FMRFamide or insulin-like peptides, which are called neuropeptide-like proteins (*nlp*). This consists of the largest group of about 155 neuropeptides encoded by 46 precursor genes (Janssen et al., 2010; Nathoo et al., 2001).

Similar to mammals, mature neuropeptides in *C. elegans* are generated by posttranslational processing and modification of precursor molecules (Figure 1.9).

Typically, the neuropeptide precursor is first cleaved at the C-terminal to mono, di, or tribasic residues flanking the peptide sequence by proprotein convertase enzymes (EGL-3/ KPC-2). EGL-3 expresses in most neurons in the nervous system and loss of *egl-3* functions results in defects in mechanosensation, egg-laying and locomotion (Kass et al., 2001) indicating that EGL-3-cleaved precursors peptides perform varied functions. Basic amino acids are eliminated from the peptide sequences by carboxypeptidases E (EGL-21) to generate pro-neuropeptides. *egl-21* null mutants show defects in egg laying, mechanosensation, locomotion and defecation as well as extremely low FMRFamide immunoreactivity (Jacob and Kaplan, 2003). To prevent degradation, neuropeptides are usually modified at the N- or C-terminus by addition of specific motifs (such as amide groups) to yield mature peptides (Sieburth et al., 2005).

These active neuropeptides are stored in dense-core vesicles, which may be released either at synaptic sites or extrasynaptically in a hormonal fashion. Several proteins show involvement in the transport and release of dense-core vesicles. These include the motor protein UNC-104 (Jacob and Kaplan, 2003; Schinkmann and Li, 1994; Zahn et al., 2004), the calcium-dependent activator protein UNC-31 (Cai et al., 2004), the protein kinase I PKC-1 (Sieburth et al., 2007), and the protein tyrosine phosphate-like receptor IDA-1 (Zahn et al., 2004). After the release of the vesicles contents, neuropeptides are cleared from the synaptic cleft by the action of proteolytic enzymes, like NEP-1 neprilysin (Sieburth et al., 2005). Hence, neuropeptides are synthesized *de novo* in the cell body and transported to the synapse unlike small molecule neurotransmitters, which can be recycled at the synapse and re-loaded into the synaptic vesicles.

The neuropeptides show widespread expression in *C. elegans* nervous system as well as in non-neuronal tissues. For instance, above 160 neurons, that represents over half of the 302 neurons in the *C. elegans* nervous system, express one or more FLPs (Kim and Li, 2004). Similarly, the expression of the *ins* (Pierce et al., 2001), *daf-28* (Li et al., 2003), and *nlp* (Nathoo et al., 2001) genes is extensive in the nervous system. Second, there is a considerable expression pattern overlap. Although each gene expresses in a distinctive set of neurons, a single neuron can express multiple neuropeptide genes, thus considerably increasing the diversity of neuropeptide expression.

Based on the expression pattern of the neuropeptides, neuropeptide signaling has been demonstrated to modulate several different behaviors in *C. elegans*, that includes dauer formation, locomotion, egg-laying, and mechano- and chemosensation (Artan et al., 2016; Banerjee et al., 2017; Braeckman and Vanfleteren, 2007; Chalasani et al., 2010; Chang et al., 2015; Chen et al., 2016, 2013; Cheong et al., 2015; Cornils et al., 2011; Delaney et al., 2017; Harris et al., 2010a; Hu et al., 2011, 2015; Hung et al., 2014; Kao et al., 2007; Laurent et al., 2015; Leinwand and Chalasani, 2013, 2014; Li et al., 2013; Lin et al., 2010; Nagy et al., 2014; Nath et al., 2016; Nathoo et al., 2001; Nelson et al., 2013; Pierce et al., 2001; Stawicki et al., 2013).

1.6.2.6. FMRFamide-related peptides

An important class of neuromodulators in *C. elegans* are the FMRFamide-related peptides (FaRPs) also called FLPs. This family includes about 33 precursor genes that encodes over 70 peptides and functions though at least 11 known receptors (Nelson et al., 1998a). These peptides are generally 4-20 amino acids in size and are characterized by the presence of a common C-terminal motif of Arg-Phe-NH_2. Several FaRPs have also been identified in vertebrates, where they have been associated with the regulation of pain responses (Dong et al., 2001; Panula et al., 1999; Yang et al., 1985) and feeding behavior (Chartrel et al., 2005; Murase et al., 1996).

FLP neuropeptides have been reported to regulate a plethora of functions in *C. elegans* (Li and Kim, 2014). Here, I will discuss the role of FLP-1 and FLP-18 in *C. elegans* locomotion as these two neuropeptides are discussed in the thesis work.

Wild-type *C. elegans* moves on solid surface generating dorso-ventral sinusoidal bends. FLP-1 has been reported to modulate locomotion as *flp-1* mutants show an exaggerated waveform on solid NGM surface and overexpression results in flattening of the waveform and extreme sluggishness (Nelson et al., 1998b). More recently, FLP-1 and FLP-18 have been shown to modulate the excitation-inhibition imbalance generated in a nicotinic acetylcholine receptor subunit, ACR-2 gain of function mutant. *acr-2 (gf)* have increased cholinergic excitation and decreased GABAergic inhibition resulting in spontaneous convulsions (Jospin et al., 2009). In a screen for potential neuropeptides that might be affecting the convulsive phenotype, *flp-1; flp-18* mutants have been shown to enhance convulsions by inhibiting GABAergic neurotransmission at NMJ (Stawicki et al., 2013). These results support a role of these two neuropeptides in preventing the overexcitation of the locomotion circuit.

1.6.2.7. FLP peptides function through NPR-1 (Neuropeptide Receptor), an NPY receptor homolog in *C. elegans*

The *npr-1* gene encodes a predicted G- protein coupled receptor that is homologous to mammalian NPY receptors (de Bono and Bargmann, 1998). *npr-1* expression is mainly seen in the nervous system, where it is largely concentrated in the neurons of the sensory circuit (de Bono and Bargmann, 1998; Coates and de Bono, 2002). Expressed in the sensory circuit, NPR-1 regulate diverse sensory behaviors. *npr-1* mutants have been reported to display exaggerated sensory responses to multiple environmental cues like hyperoxia and certain types of pheromones that alter the social feeding behavior (Cheung et al., 2005; Gray et al., 2004; Luo et al., 2015; Macosko et al., 2009; Srinivasan et al., 2012) and pathogen susceptibility (Martin et al., 2017; Reddy et al., 2009; Styer et al., 2008). NPR-1 mediated signaling also regulates nociception (Ezcurra et al., 2016) and lifespan (Abergel et al., 2017) in *C. elegans*. Blocking sensory transduction with mutations in genes that mediate sensory reception (CNG, TRPV channels) or ablation of sensory neurons completely abolish the exaggerated responses in *npr-1* mutants. These results support the role of NPR-1 in inhibiting the activity of sensory neurons, as inactivation of NPR-1 results in enhanced sensory activities. Role of NPR-1 in inhibiting the sensory circuit is further supported by the fact that, NPR-1 is coupled to Gi/Go proteins and its activation hyperpolarizes the membrane potential through G protein-activated inwardly rectifying K+ channels (GIRKs) (Rogers et al., 2003).

NPR-1 regulates diverse sensory responses and related behaviors by controlling the sensory transduction occurring in the RMG circuit. In the *C. elegans* head sensory circuit, the RMG interneurons form gap junctions with several classes of sensory neurons (Figure 1.10). NPR-1 expression has been reported in a subset of RMG circuit neurons including the RMG interneurons. Expression of NPR-1 specifically in the RMG sensory circuit or ablation of the RMG circuit neurons suppresses the exaggerated behavioral phenotypes seen in *npr-1* mutants (Macosko et al., 2009). Collectively, NPR-1 functions in a hub and spoke sensory circuit where it can regulate 'spoke' sensory neurons and related behavioral outputs by altering the activity of 'hub' RMG neurons.

More recently, NPR-1 was identified to function in the central sensory circuit to regulate behavioral quiescence in *C. elegans* (Choi et al., 2013; Nagy et al., 2014). During each larval molt, *C. elegans* experiences a period of quiescence called

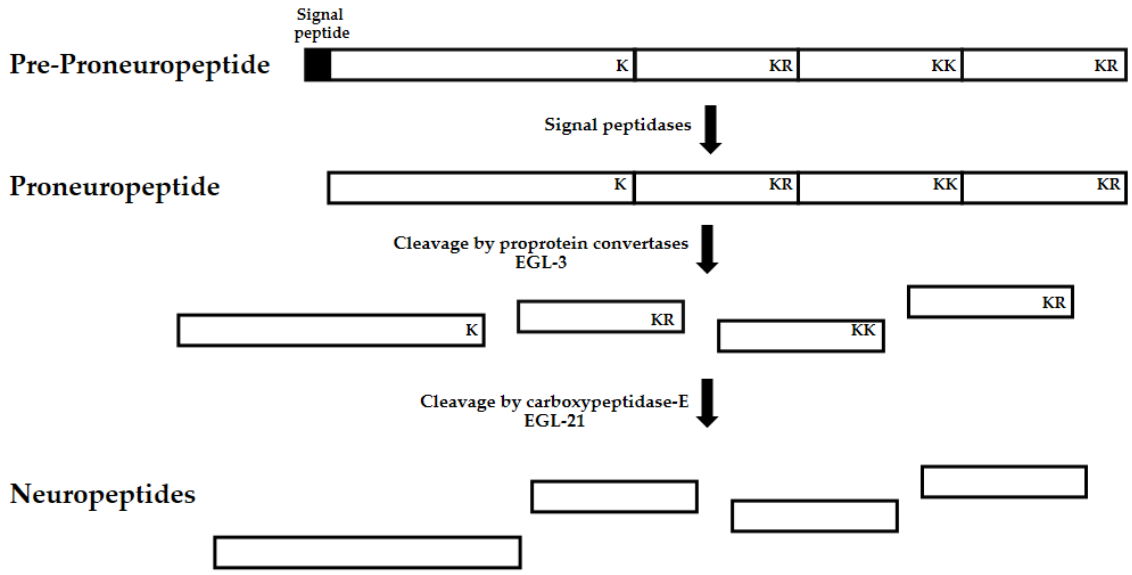


Figure 1.9: Processing of neuropeptide gene products. After translation, the pre-propeptide is cleaved by signal peptidases in the endoplasmic reticulum to release the signal peptide. The propeptide is further cleaved C-terminal to mono, di, or tri-basic residues (indicated by K and R) by proprotein convertases such as EGL-3/KPC-2. The basic amino acids are removed by carboxypeptidases E, such as EGL-21, to yield the basic neuropeptides. Further post-translational modifications to these peptides then yield mature neuropeptides.

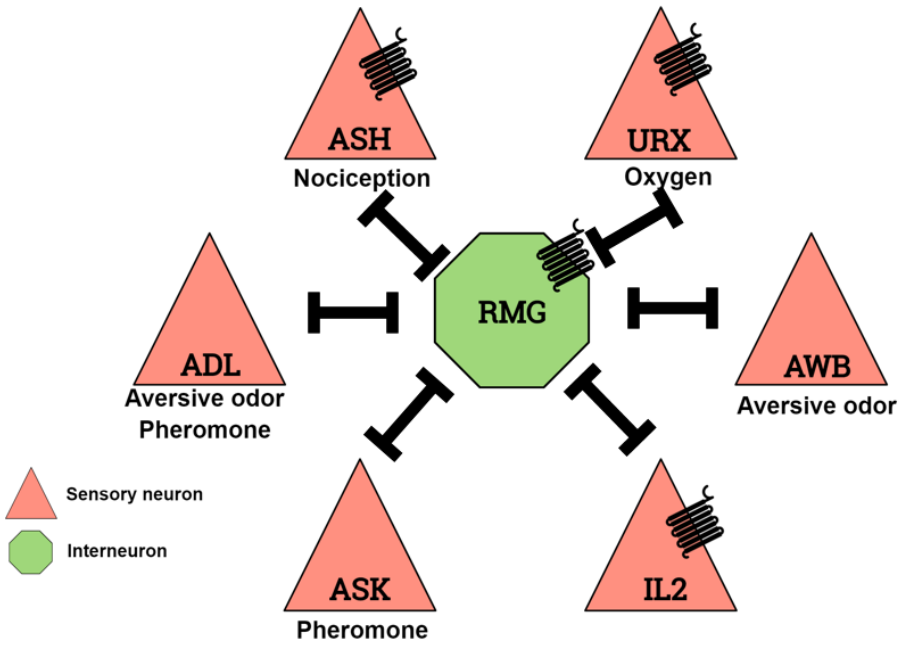


Figure 1.10: A schematic illustrating the RMG sensory circuit. Sensory neurons mediating diverse aversive responses form gap junctions with the RMG interneuron. The neurons expressing the NPR-1 receptor are indicated with seven-transmembrane channel (Image adapted from Macosko et al., 2009).

lethargus whereby locomotion and feeding behaviors are inactive for~ 2 hours (Cassada and Russell, 1975). NPR-1 mediated decrease in the central RMG sensory circuit results in less secretion of the arousal peptide Pigment dispersing factor-1 (PDF-1). This peptide enhances the sensitivity of peripheral mechanosensory neurons by binding to PDFR-1 receptor on these neurons. In *npr-1* mutants, the sensory circuit activity is enhanced resulting in increased secretion of PDF-1 from RMG interneurons. This in turn, mediates arousal in *npr-1* mutants during larval molts. Increased activity of the RMG sensory circuit in *npr-1* mutant adults also results in sensory evoked enhancement of cholinergic transmission and locomotion mediated by augmented glutamate and neuropeptide release (Choi et al., 2015).

1.7. Synaptic vesicle axonal transport in *C. elegans*

Presynaptic components including synaptic vesicle precursors (SVs), active zone proteins and mitochondria are synthesized and assembled in the neuronal cell body and move through axons to reach nascent synapses (Goldstein et al., 2008).

The axonal transport of synaptic vesicle precursors is a tightly regulated process involving cytoskeleton and motor proteins (Hirokawa et al., 2010). The motor proteins undergo cycles of conformational changes causing binding/unbinding with the cytoskeleton in an ATP-dependent manner (Liu et al., 2003). The cytoskeleton consists of actin filaments and microtubules. Three proteins families execute most of the transport in neuronal cells: myosins, dyneins and kinesins. Myosins utilize actin filaments as tracts, while dynein and kinesins use microtubules (Kapitein and Hoogenraad, 2011). Microtubule proteins are polarized structures with a minus-end facing the cell soma and plus-end directed to the distal part of the axons. Kinesins mediate anterograde axonal transport as they walk in the plus direction. While dyneins walk in the minus-directions and thus mediate retrograde movement of axonal cargo.

Active cargo transport in axons is characterized by either fast (~1-5 μm/s) or slow transport (~2-100 nm/s) (Brown, 2003). Fast axonal transport delivers synaptic vesicles consisting of synaptic vesicle precursors, mitochondria, and active zone precursors (Cai and Sheng, 2009), while slow transport mainly delivers cytoskeletal components (Roy, 2014).

unc-104/ KIF1A kinesin is one of the most studied motor family. *C. elegans unc-104* mutants show uncoordinated, slow body motion and growth rate. The primary defect is the failure to transport synaptic vesicles from the soma to the synapses,

resulting in the accumulation of the SVs in the cell somas (Hall and Hedgecock, 1991; Otsuka et al., 1991). UNC-104 is a neuron-specific motor protein which is required for the fast anterograde axonal transport of the SV precursors and some other associated proteins, like synaptobrevin, synaptotagmin, UNC-17 and Rab3, with a fast *in vivo* average velocity of 1.02 µm/sec (Lee et al., 2003; Zhou et al., 2001). Recent studies also report the role of UNC-104 ortholog in an organelle-specific dynein-driven retrograde transport mechanism (Barkus et al., 2008).

UNC-104 is a monomeric kinesin belonging to kinesin-3 family. It shows a globular structure and exists either as an inactive monomer (Hirokawa et al., 2009) or as an active homodimer (Rashid et al., 2005). UNC-104 consists of an N-terminal motor domain (aa 1–348) that functions as ATP hydrolyzing and force generating unit, a fork-head-homology (FHA) domain (aa 463–592), an elongated stalk region of unknown structure, and a C-terminal Pleckstrin homology (PH) domain (aa 1460–1560) responsible for membrane attachment and cargo binding. The PH domain has been reported to bind Phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) (Klopfenstein et al., 2002). PtdIns(4,5)P2 serve as the lipid target of several PH-domains and are implicated in regulating membrane trafficking events (Martin, 2001). It is also reported that PtdIns(4,5)P2 are concentrated in lipid rafts (Caroni, 2001; Simons and Ikonen, 1997) thus providing multiple binding sites for UNC-104 within a small surface area of the synaptic vesicles.

However, not all SV proteins use the *unc-104* motor while some non-vesicular proteins are using this motor protein (Rand and Nonet, 1997). This specificity between the synaptic cargoes and motor proteins requires different adaptors (Byrd et al., 2001; Hsu et al., 2011; Sakaguchi-Nakashima et al., 2007; Tien et al., 2011; Wagner et al., 2009; Wu et al., 2016; Zheng et al., 2014).

Another motor family that is well characterized in *C. elegans* is Kinesin 1 (Siddiqui, 2002). Kinesin proteins are usually characterized by three functional domains: the microtubule binding domain that acts as a force generator by a undergoing conformational changes requiring ATP hydrolysis, a domain for dimerization (each protein forms one leg), and a cargo binding domain. The kinesin-1 motor is a hetero-tetramer composed of two kinesin heavy chains and two kinesin light chains (KLC-2). The KLCs lack the motor domain and associate with the globular carboxyl terminals of the KHC (Cyr et al., 1991).

In *C. elegans*, *unc-116* gene encodes the only kinesin heavy chain (KHC) (Patel et al., 1993). Mutants display uncoordinated locomotion, paralysis, and poor sensitivity to touch, and also show a coiling phenotype as larvae.

In *C. elegans*, *klc-1* and *klc-2* genes encode the kinesin light chains. KLC-1 and KLC-2 encodes 516 and 561 amino acid residues, respectively, and show about 50% sequence homology to each other. Both KLCs contain an N-terminal α-helical domain that associates with the KHC stalk and also harbor multiple tetratricopeptide repeat TPR motifs (Diefenbach et al., 1998; Vale and Fletterick, 1997). Both KLCs interact with KHC to regulate axonal cargo trafficking in *C. elegans* (Sakamoto et al., 2005; Signor et al., 1999) and mutations in both seem to affect SV-like trafficking (Byrd et al., 2001; Sakamoto et al., 2005), but also general intracellular trafficking.

1.8. The cadherin superfamily in *C. elegans*

C. elegans genome has 12 genes that encode 13 cadherins (Pettitt, 2005). *C. elegans* contains representatives of all major cadherin families that are conserved between *Drosophila* and vertebrates (except Ret family of tyrosine kinases which are missing in *C. elegans*) - the classical, Fat-like, Flamingo and calsyntenin classes (Figure 1.11) (Hardin et al., 2013). The single classical cadherin HMR-1 occurs as two splice variants HMR-1A and HMR-1B via alternative promoter usage (Broadbent and Pettitt, 2002). HMR-1A expresses in epithelia and some unidentified set of neurons mainly functioning in embryonic morphogenesis (Costa et al., 1998; Grana et al., 2010). HMR-1B expression is confined to neurons functioning to regulate dorso-ventral axon navigation (Broadbent and Pettitt, 2002). The function of *C. elegans* cadherins are largely unknown as loss-of-function mutations are available only for few classes (Loveless and Hardin, 2012). Other than classical cadherins, a Fat-like cadherin, *cdh-3* and *cdh-4* and a Flamingo homolog, *fmi-1* have been functionally studied in *C. elegans*. The two Fat-like cadherins CDH-3 and CDH-4 are both essential for embryonic morphogenesis (Pettitt et al., 1996; Schmitz et al., 2008). CDH-4 also expresses in the nervous system and is required for proper axonal navigation and neuroblast migration (Schmitz et al., 2008; Sundararajan et al., 2014). The Flamingo cadherin, FMI-1 has been reported to regulate cell polarity during GABAergic neuronal development in *C. elegans* (Huarcaya Najarro and Ackley, 2013; Najarro et al., 2012). They have also been shown to regulate pioneer-dependent navigation of follower axons during neuronal development (Steimel et al., 2010). *C.*

elegans also expresses five nematode-specific cadherins that have no identified homologs in mammals.

C. elegans CASY-1, an ortholog of mammalian Calsyntenins is a non-classical cadherin that has been functionally characterized as a part of this study.

1.9. *C. elegans casy-1*, an ortholog of mammalian calsyntenins

C. elegans casy-1 is an ortholog of mammalian calsyntenins which was first discovered in a screen for mutants with altered phenotypes in salt chemotaxis learning assay (Ikeda et al., 2008). Just like its mammalian homologs, *casy-1* is also characterized by the presence of two cadherin- like tandem repeats, an LG/LNS domain in the extracellular region and an intracellular region that carries two kinesin light-chain binding domains. Protein sequence alignment with vertebrate calsyntenins revealed that CASY-1 is most similar to mammalian CLSTN2 (Hoerndli et al., 2009). Further characterization revealed that *casy-1* functions in a mature neural circuit to regulate learning and memory and is not required for neural development in *C. elegans*. CASY-1 is widely expressed in the *C. elegans* nervous system including most head neurons some of which send processes into the ventral nerve cord, some tail neurons and in some other tissues like intestine and the gonadal sheath cells (Ikeda et al., 2008). However, unlike mammalian calsyntenins which show postsynaptic localization, CASY-1 expression was seen in neuronal cell bodies both in intracellular membranes as well as on plasma membrane. Ectodomain cleavage of the extracellular N- terminal is conserved for the *casy-1* gene. Learning defects of *casy-1* mutants were completely rescued by expressing the released ectodomain of CASY-1 specifically in a single sensory neuron suggesting two points:

1) CASY-1 functions in sensory neurons to regulate synaptic plasticity, however, the mechanism is still unknown.

2) CASY-1 can modulate learning via autocrine and paracrine signaling.

Further, domain mapping experiments suggested the major requirement of LG/LNS domain in the extracellular N- terminal region of CASY-1 to modulate synaptic plasticity, while the cadherin domains may play an accessory role (Ikeda et al., 2008). The role of CASY-1 in learning and memory was further validated by a subsequent study which reported memory impairments in *casy-1* mutants in multiple learning paradigms (Hoerndli et al., 2009). *casy-1* mutants have been shown to have

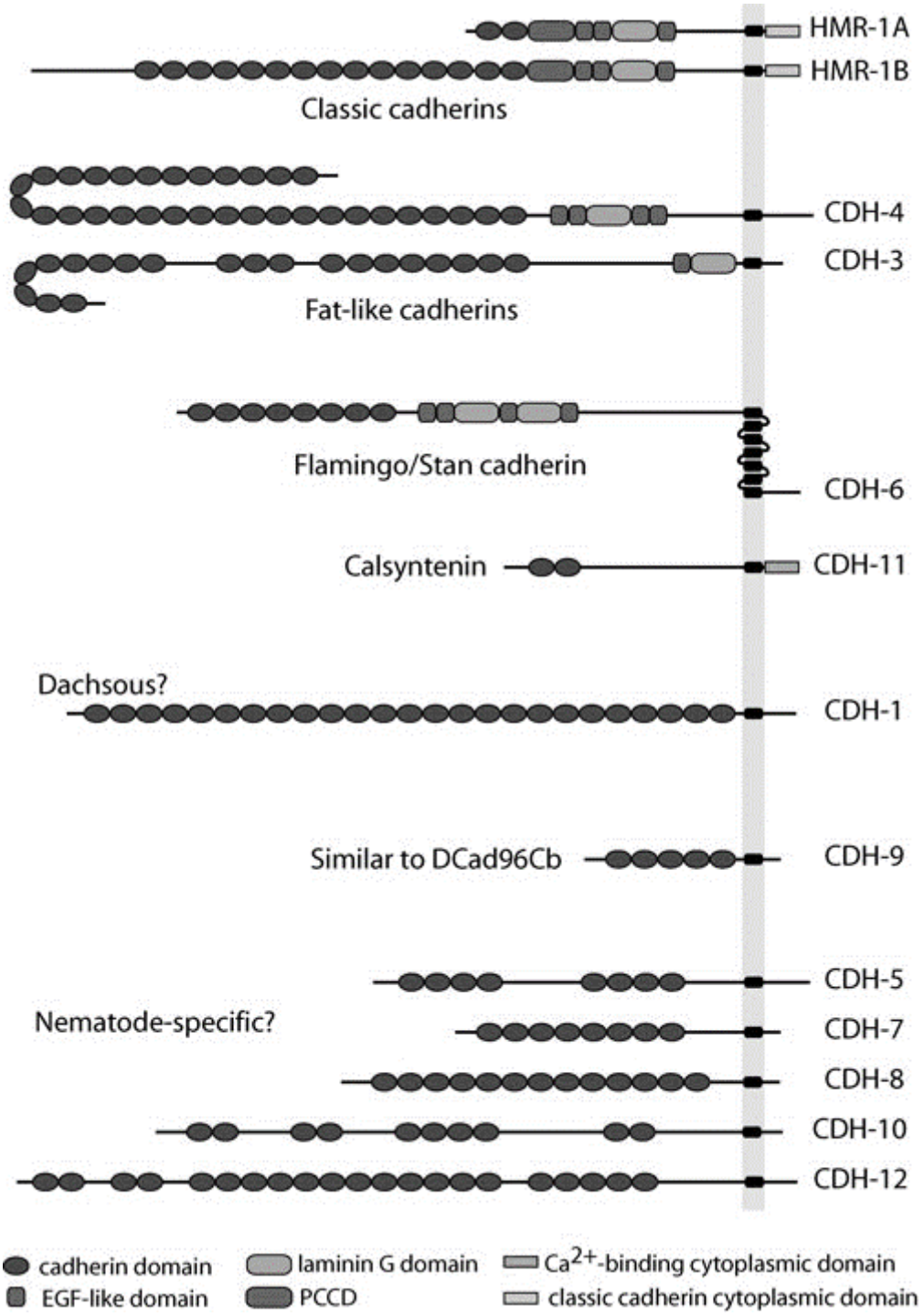


Figure 1.11: The *C. elegans* cadherin superfamily showing domain organization of all *C. elegans* cadherins. (Image modified from Pettitt, 2005).

normal sensory perception but might be defective in sensory integration and memory processing (Hoerndli et al., 2009; Ikeda et al., 2008; Li et al., 2011). Interestingly, the learning deficits of *casy-1* mutants were completely rescued by expressing human CLSTN2, suggesting conservation of function. Also, CASY-1 expression was found in several GLR-1 expressing cells, which are AMPA-like *C. elegans* glutamate receptors and increasing gene dosage of GLR-1, was also able to compensate for the behavioral deficits in *casy-1* mutants, signifying that CASY-1 may regulate GLR-1 signaling during learning and memory (Hoerndli et al., 2009).

More recently, mitogen-activated protein kinase regulated physical interaction of CASY-1 and kinesin motor protein KLC-1 was shown to be essential for translocation of a novel *daf-2* isoform, *daf-2c* to the synaptic region of a chemosensory neurons, to mediate behavioral learning in taste avoidance training paradigm (Ohno et al., 2014). This study highlights the possible conservation of function of *casy-1* in mediating trafficking of various synaptic proteins, which can be explored further. Also, *C. elegans casy-1* has recently been shown to interact with neurexin-related protein *bam-2* promoting fasciculation between sensory neurons required in mating circuits of the adult male (Kim and Emmons, 2017).

Collectively, reports till now have highlighted the role of *C. elegans casy-1* in modulating synaptic plasticity in a mature nervous system via functioning in the sensory neurons.

Part 2: Locomotion circuit in *C. elegans*

One of the fundamental goals of neuroscience research is to understand how neural circuits and genetic factors coordinate to generate behavior. Behavioral outcome of an organism results from efficient integration of several different sensory cues. Despite its simple neural wiring, *C. elegans* shows a variety of complex behaviors like locomotion, sleep- like state, mating and behavioral plasticity to a wide variety of learning paradigms (de Bono and Bargmann, 1998; de Bono and Maricq, 2005; Feng et al., 2006; Liu and Sternberg, 1995; Mori and Ohshima, 1995; Raizen et al., 2008).

Locomotion is one of the most prominent behavioral output in *C. elegans*. Neural circuits that produce coordinated dorso-ventral sinusoidal bends allow for normal locomotion in *C. elegans*. Locomotory behavior is synchronized at multiple levels and involves the integration of diverse sensory cues by sensory neurons that are processed

by the interneurons and ultimately cause changes at the neuromuscular junctions (NMJ) (Figure 1.12) (Bargmann, 2012; de Bono and Maricq, 2005).

In terms of spatial organization, sensory neurons and interneuron cell bodies form a cluster of neurons or ganglia in the head that can be considered as a rudimentary brain in *C. elegans*. Two parallel tracts run along the dorsal and ventral sides of the worm that constitute the dorsal and ventral nerve cord respectively. The dorsal and ventral nerve cord mainly contains the motor neurons that receive synaptic input from the command interneurons and relay information on to the muscles. The cell bodies of the motor neurons are localized on the ventral side of the worm, and some of these cell bodies send axonal projections called commissures along the dorsal side, which then synapse onto the dorsal nerve cord.

Based on function, the *C. elegans* locomotory circuit can be divided into three levels which are discussed in detail below.

1.10. Sensory neurons

C. elegans has 68 sensory neurons that explore its environment and helps to move to favorable surroundings detecting a wide range of environmental stimuli. These environmental cues include volatile and soluble chemicals (chemotaxis), temperature (thermotaxis), pH, oxygen (aerotaxis) as well as various forms of mechanical stimuli (Bargmann et al., 1993; Bretscher et al., 2011; Chalfie et al., 1985; Culotti and Russell, 1978; Hedgecock and Russell, 1975; Kaplan and Horvitz, 1993; Sassa et al., 2013; Ward, 1973).

In *C. elegans*, the cell bodies of most sensory neurons are embedded in the ganglia around the nerve ring in the head and in the tail. Their dendritic projections terminate in microtubule rich cilia (Ward et al., 1975; Ware et al., 1975). The amphids are the principal sensory organs in *C. elegans* wherein each amphid contains the ciliated dendritic endings of twelve sensory neurons, a sheath and a socket cell, which together forms a pore that opens to the exterior. The phasmids are similar but smaller sensory organs in the tail (Apfeld and Kenyon, 1999; White et al., 1986).

Sensory neurons in *C. elegans* express distinct sensory receptors that are involved in diverse functions such as in taste reception (Bargmann and Horvitz, 1991), olfaction (Bargmann et al., 1993), mechanosensation (Kaplan and Horvitz, 1993), thermal perception (Mori and Ohshima, 1995) and pheromone detection (McGrath et al., 2011). Most sensory receptors are metabotropic G-protein coupled receptors (GPCRs) that utilize different downstream heterotrimeric G- proteins to regulate the

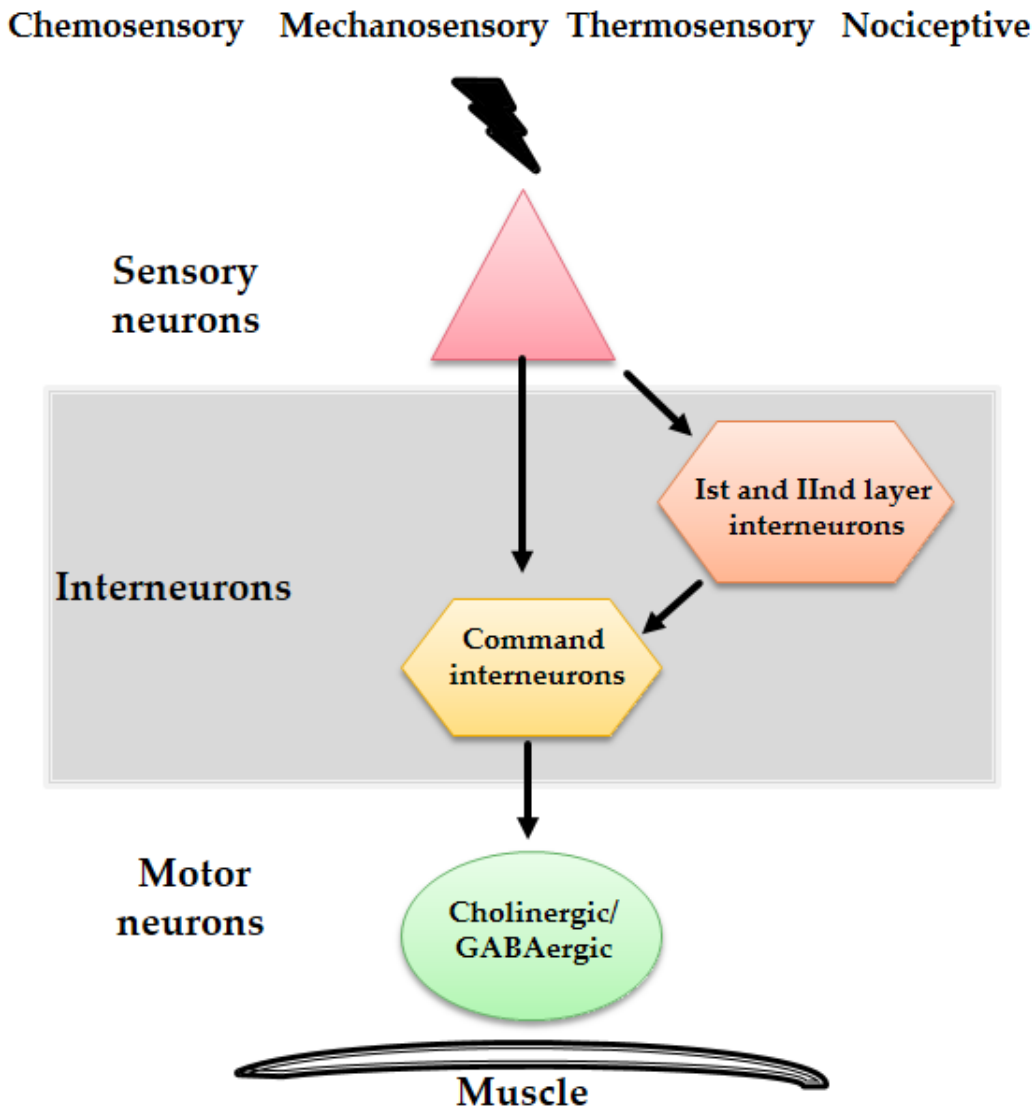


Figure 1.12: Schematic representing the *C. elegans* locomotor circuit. Diverse environmental cues sensed by the sensory neurons are processed and integrated at interneurons. The processed information reaches the command interneurons, the pre-motor neurons that decide the direction of locomotion and manifest the outcome at the neuromuscular junction by coordinated action of cholinergic and GABAergic motor neurons synapsing on to the body wall muscles.

opening of sensory transduction channels like TRP- or cGMP-gated channels (Bargmann, 2006; Jansen et al., 1999).

1.11. Interneurons

Interneurons mediate information flow from sensory to motor neurons and are the site of sensory information integration and processing. Processed information from several layers of interneurons ultimately reaches five pairs of distinct pre-motor interneurons (AVA, AVB, AVD, AVE and PVC) that direct locomotory behavior and are called the command interneurons (Figure 1.13). These command interneurons govern the direction of movement and are unique as they are the only interneurons that directly synapse onto the excitatory motor neurons of the ventral nerve cord as well as span the entire length of the ventral cord (Chalfie et al., 1985; White et al., 1986). Their role in directing forward or backward locomotion was first assigned via laser ablation studies of command interneurons while dissecting out touch response neural circuit (Chalfie et al., 1985). These studies reported that AVA, AVD and AVE are required for backward locomotion or reversals in *C. elegans*, while activation of AVB and PVC promote forward locomotion. These results were further corroborated by downstream synaptic connections of these command interneurons with distinct motor neurons as discussed in the next section.

The advent of more sophisticated technologies like calcium imaging and optogenetics in behaving worms has further improved our understanding of neural control of locomotion by the command interneurons (Ben Arous et al., 2010; Boyden et al., 2005; Faumont et al., 2011; Gray et al., 2005; Guo et al., 2009; Kawano et al., 2011; Piggott et al., 2011; Rakowski et al., 2013; Tsalik and Hobert, 2003).

1.12. *C. elegans* neuromuscular junction (NMJ) as a model to study synaptic transmission

This thesis involves experiments that centered on the *C. elegans* neuromuscular junction (NMJ). At the *C. elegans* NMJs, muscles extend projections called muscle arms to the nerve cord where they form *en passant* synapses with axons of motor neurons. Alternating dorsal and ventral body wall muscle contractions propels worm in forward and backward directions. The body-wall muscles are innervated by five classes of motor neurons present on the ventral nerve cord (VNC); A, B, D, V and AS. The A-, B-, V- and AS- type motor neurons are excitatory and release acetylcholine as the neurotransmitter while D- type motor neurons are inhibitory and release γ-aminobutyric acid (GABA) as the neurotransmitter (Figure 1.13) (Lewis et

al., 1980; McIntire et al., 1993; Richmond and Jorgensen, 1999). Based on whether they innervate the dorsal or ventral body wall muscle, the A, B and D class are further subdivided (DA, VA, DB, VB, DD, VD). They are also subdivided based on development (born embryonically or post embryonically). Cell bodies of the dorsal motor neurons reside in the VNC that extend commissures dorsally to establish dorsal nerve cord.

Functional interaction between command interneurons and motor neurons drives the directionality of locomotion. The A- type cholinergic motor neurons receive synaptic inputs from AVA, AVD and AVE and are responsible for backward locomotion. While, the B-type cholinergic motor neurons receive inputs from AVB and PVC command interneurons and hence monitor forward locomotion (Chalfie et al., 1985; White et al., 1986). The axonal projections of A- and AS-type motor neurons are directed anteriorly while the B-type motor neurons have posteriorly directed processes. The D-type neurons are required for locomotion by acting as cross-inhibitors that prevent the dorsal and ventral muscles at the contralateral position from contracting simultaneously (McIntire et al., 1993). The D-type motor neurons are postsynaptic only to excitatory motor neurons and extend their axonal processes both anteriorly and posteriorly. Spatially, the DD motor neurons are postsynaptic to the VB and VA cholinergic motor neurons, whereas the VD motor neurons are postsynaptic to the DB, DA, and AS neurons (White et al., 1976, 1986).
The motor neurons innervate the 95 body wall muscle cells that are arranged anterioposteriorly into four quadrants arranged in interleaved patterns of two dorsal and two ventral rows (Sulston and Horvitz, 1977). Eighty-one muscle cells that make the body wall are developed embryonically while the remaining fourteen head muscles develop post embryonically (Sulston and Horvitz, 1977; Sulston et al., 1983). Adjacent muscle cells are electrically coupled by gap junctions (Liu et al., 2013).

At the NMJ synapse, ionotropic receptors cluster adjacent to the motor neuron release sites at the tip of the muscle arms. Two receptor classes mediate excitatory cholinergic transmission in muscle: the nicotine sensitive acetylcholine receptors (N-AChRs) and the levamisole sensitive acetylcholine receptors (L-AChRs). Inhibitory GABA transmission is mediated by single $GABA_A$ receptor on the muscle.

The locomotor circuit in *C. elegans* works through a "cross-inhibition model" where the excitatory cholinergic motor neurons form dyadic synapses onto both the ipsilateral muscle causing contraction as well as onto inhibitory GABAergic motor

Command interneuron	Direction of locomotion	Neurotransmitter/ Neuropeptide	Synapse onto motor neuron
AVA	Backward Locomotion	Acetylcholine, FLP-1, FLP-18	DA, VA
AVB	Forward Locomotion	Acetylcholine, NLP-3, NLP-9	DB, VB
AVD	Backward Locomotion	Acetylcholine	DA, VA
AVE	Backward Locomotion	Acetylcholine, FLP-1	DA, VA
PVC	Forward Locomotion	Acetylcholine	DB, VB

Motor neuron class	Function	Neurotransmitter	Origin
DB	Forward Locomotion	Acetylcholine	Embryonic
VB	Forward Locomotion	Acetylcholine	Postembryonic
DD	Forward/Backward Locomotion	GABA	Embryonic
VD	Forward/Backward Locomotion	GABA	Postembryonic
DA	Backward Locomotion	Acetylcholine	Embryonic
VA	Backward Locomotion	Acetylcholine	Postembryonic
VC	Egg laying behavior	Acetylcholine	Postembryonic
AS	Unknown	Acetylcholine	Postembryonic

Figure 1.13: Table showing command interneuron and motor neuron classes in *C. elegans.*

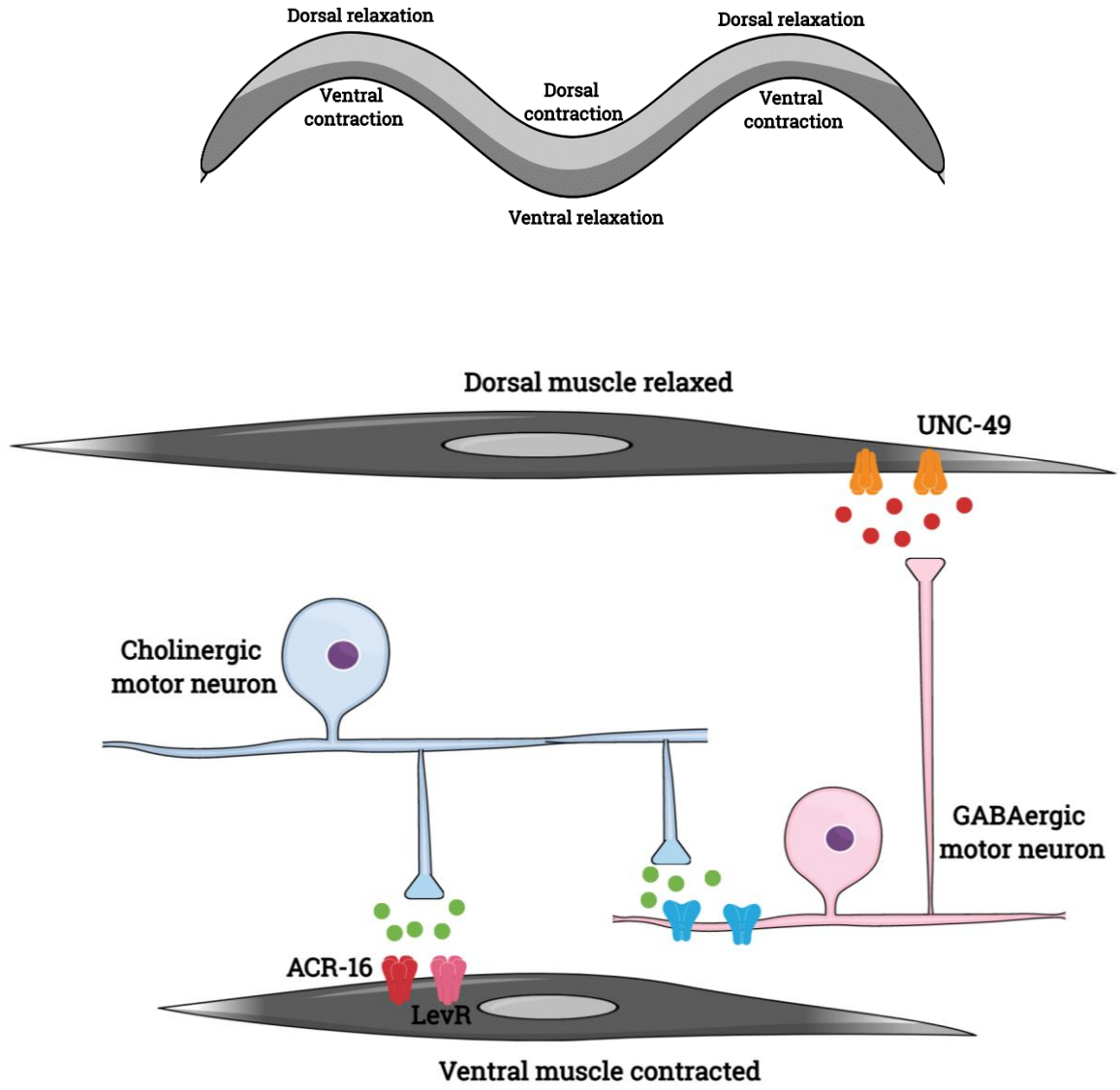


Figure 1.14: The locomotor circuit in *C. elegans*. This circuit works through a "cross-inhibition model" where the excitatory cholinergic motor neurons form dyadic synapses onto both the ipsilateral muscle causing contraction as well as onto inhibitory GABAergic motor neurons that then synapse onto the contralateral muscle causing relaxation. This alternating waves of contraction and relaxation along the worm body drives locomotion in *C. elegans*.

neurons that then synapse onto the contralateral muscle causing relaxation (White et al., 1976, 1986). The alternating wave of contraction and relaxation along the worm body drives locomotion in *C. elegans* (Figure 1.14). An important principle that maintains locomotion is a coordinated balance between the excitation (E) and inhibition (I) at the NMJ (Isaacson and Scanziani, 2011; Stawicki et al., 2013). The overall magnitude of the dorsal and ventral contraction must be equal for normal coordinated locomotion.

Part 3: Isoform mediated spatial and functional diversity in gene function.

A precise control of spatiotemporal regulation of gene expression along with cell/tissue-specific isoforms expression is a prerequisite for the development of multicellular organisms. This requires more efficient genome organization in model systems like *C. elegans* that have a relatively small genome size. The generation of cell/tissue-specific isoforms is commonly achieved by alternative splicing. Alternative splicing also provides a major mechanism for transcriptome and proteome diversity in an organism (Keren et al., 2010). Alternative splicing is achieved by six basic modes (Figure 1.15):

Exon skipping or cassette exon: An exon may be spliced out or retained in the primary transcript. This represents the most common mode of alternative splicing for mammalian messenger RNAs (pre-mRNAs).

Mutually exclusive exons: One of the exons is retained in the mature RNA, but not both.

Intron retention: An intron is transcribed into pre-mRNA and is retained in the final mRNA. This mode is rarely used in mammals.

Alternative 5'and 3'splice sites: An alternative 5' (donor site) or 3' (acceptor site) splice junction is used changing the 3' end of the upstream exon or 5'end of the downstream exon.

Alternative polyadenylation: Alternative poly (A) sites permits a single gene to encode multiple RNA transcripts by providing several different 3'end points for the mRNA. This changes the mRNA coding potential or effects the fate of mRNAs either by altering the availability of RNA binding protein sites or microRNA binding sites (Di Giammartino et al., 2011).

Alternative promoters: Promoters constitute the DNA sequences that contains a complex array of *cis*-regulatory elements required for accurate gene transcription and expression. They also constitute the prime target sites for diversity and flexibility of gene expression. Usage of alternative promoters for a gene provides versatile mechanisms to create diversity and flexibility in patterns of gene expression. It has been reported that more than half of the human genes (~ 53%) have alternative promoters (Kimura et al., 2006). Alternative promoters can generate diversity in gene expression in a variety of ways that includes: (1) developmental stage-specific (temporal) expression of a gene, (2) regulation of expression level by using promoters of different strengths or different responsiveness or generating mRNA isoforms with altered stability or translation efficiencies and (3) utilizing promoters resulting in tissue-specific expression of a gene (Ayoubi and Van De Ven, 1996; Schibler and Sierra, 1987).

Recent literature has verified the use of alternative promoters as a common mechanism for generating proteomic diversity at the genomic level (Bee et al., 2010; Das et al., 2012; Jacox et al., 2010; Sarda et al., 2017; Xin et al., 2008).

This thesis addresses the isoform-specific roles of CASY-1 in synapse function. So, the usage of alternative promoters in *C. elegans* will be discussed in detail in the section below.

1.13. Alternative promoter usage in *C. elegans*

Gene expression and mRNA processing have several distinct features in *C. elegans* (Choi and Newman, 2006):

- ~43% pre-mRNAs can be both *cis*- as well as trans-spliced. *cis*- splicing occurs between two exons (intron removal) and *trans*-splicing between an SL1 (Splice Leader 1) leader sequence provided by an SL1 mRNA and an exon. The molecular mechanism of trans-splicing is quite similar to that of *cis*-splicing (Blumenthal, 2005; MacMorris et al., 2007; Saldi et al., 2007).
- About 15% genes are found in operons.
- Only 14.2% of the genes are alternatively spliced in *C. elegans.*
- Quantitative RNAseq meta-analysis revealed that about 84% of *C. elegans* protein-coding genes are *trans*-spliced (Tourasse et al., 2017).

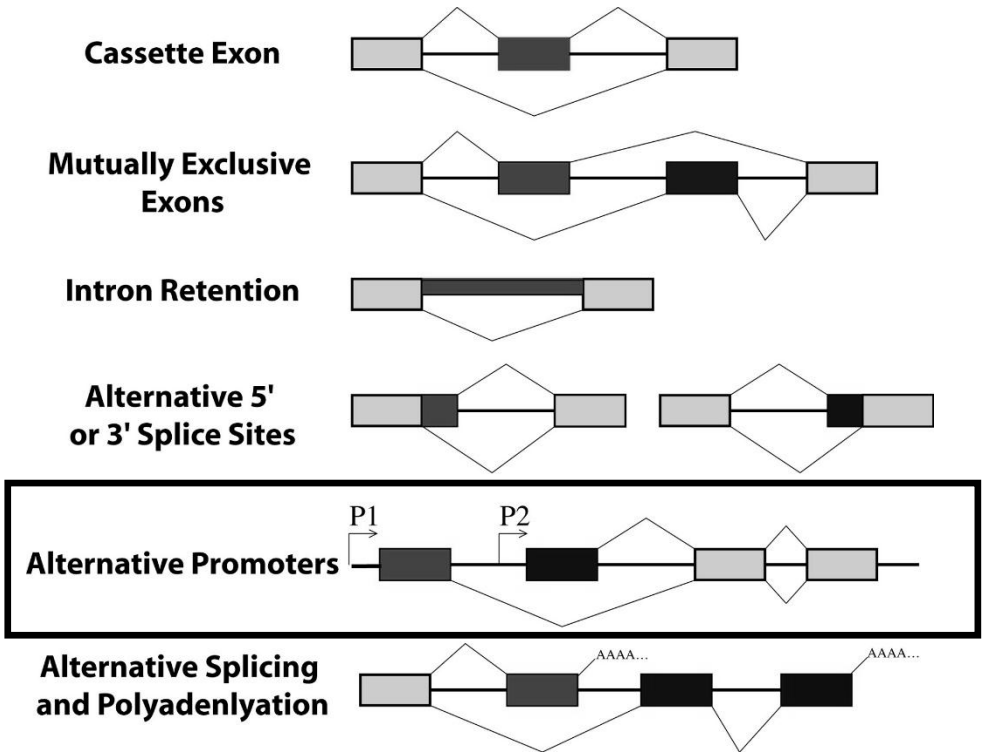


Figure 1.15: Schematic showing types of alternative splicing. Exons are represented by boxes and introns by lines. Exonic regions included in the messenger RNA by alternative splicing are colored black while constitutive exons are shown in gray. Promoters are indicated with arrows and polyadenylation sites with AAAA. Box highlights the mechanism of alternative promoter usage discussed in detail (image modified from Zahler, 2005).

C. elegans has two major classes of spliced leader sequences, SL1 and SL2. Both sequences are 22- nucleotide long and possess a trimethyl guanosine cap that protects the 5'-ends of mature mRNAs and also facilitate the efficient binding of the translational machinery (Huang et al., 2007).Trans-splicing of the 5'-ends of pre-mRNAs primarily utilizes the SL1 leader sequence . While SL2 is exclusively required for the trans-splicing of downstream genes (non-leading, or distal genes) within operons, although few downstream genes in some operons are also trans-spliced to SL1 (Spieth et al., 1993; Zorio et al., 1994). The operon phenomenon in *C. elegans* was solved with the discovery of SL2 trans-splicing (Huang and Hirsh, 1989; Spieth et al., 1993).

Choi and Newman, 2006 proposed 'two-promoter system' as a mechanism of gene regulation in *C. elegans* where a single gene has multiple isoforms which differs in length as well as the sequence in their N- terminals. Usually, the expression of the longer isoform of a gene is controlled by the 5′ upstream regulatory sequences (a conventional upstream promoter) while that of the shorter isoform requires sequences that are present in the upstream introns (an intronic promoter coupled to an internal SL1) of the longer isoform. They suggested four prerequisites for isoforms of a gene being expressed by their independent promoters:

1) Transcription start sites of the shorter isoform which undergo trans-splicing to an internal SL1 sequence must be in immediately upstream introns of the trans-splice sites so that the upstream 5'splice site is not included in the transcripts.
2) Internal SL1-coupled introns could function as separate promoters.
3) These SL1-coupled introns will be significantly longer than other introns as they act as internal promoters.
4) Isoforms expressed by 'two-promoter system' are generally spatially regulated.

Since the discovery of 'two promoter system' several studies have reported the presence of multiple isoforms of gene regulated by internal promoters performing diverse functions (Chen et al., 2015; Hurd et al., 2010; Keiper et al., 2000; Kwon et al., 2010; Lockhead et al., 2016; Mohamed and Chin-Sang, 2011; Pinan-Lucarré et al., 2014).

1.14. Alternative isoforms for *C. elegans* CASY-1

C. elegans genome encodes sole ortholog CASY-1, compared to three Calsyntenin genes in mammals. However, the *casy-1* locus in *C. elegans* is predicted to encode three isoforms based on EST evidence (Reboul et al., 2001). CASY-1A, a 984 residue full-length protein contains all the conserved domains of mammalian Calsyntenins- two cadherin- like tandem repeats, an LG/LNS domain in the extracellular region and an intracellular region that carries two kinesin light-chain binding domains. **(**Illustrated in Figure 1.16**)**. CASY-1B and CASY-1C are truncated proteins encoding 167 and 160 residues respectively and lack most of the N- terminal of the Calsyntenin gene. CASY-1B and CASY-1C differ by just seven amino acids present at the extreme N-terminal of CASY-1B which are absent in CASY-1C (sequence is FVNILEM). All three isoforms are type I transmembrane proteins. Multiple sequence alignment of the three isoforms revealed that the C- terminal of all three isoforms is 100% identical.

1.15. Aim of the present study

Our lab is involved in understanding the functional roles of cell adhesion molecules (CAMs) at the synapse. We are particularly interested in characterizing the non- adhesive, non –classical roles of CAMs in synapse development and function.

CAMs belonging to diverse proteins families are concentrated at both presynaptic and postsynaptic compartments at the synapse. CASY-1, a non-classical cell adhesion molecule has been reported to show abundant expression in the *C. elegans* nervous system. Mammalian homologs of *C. elegans casy-1* have been emphasized to have a role in pathogenesis of several neurological disorders. Thus, it is imperative to gain functional insights into the cellular and molecular underpinnings of this molecule.

Based on the background knowledge and literature we accordingly formulated the objectives of the present thesis as follows:

1. Deciphering the role of CASY-1 at the *C. elegans* NMJ.

2. CASY-1-mediated regulation of motor circuit dynamics and behavior stemming from sensory and interneurons.

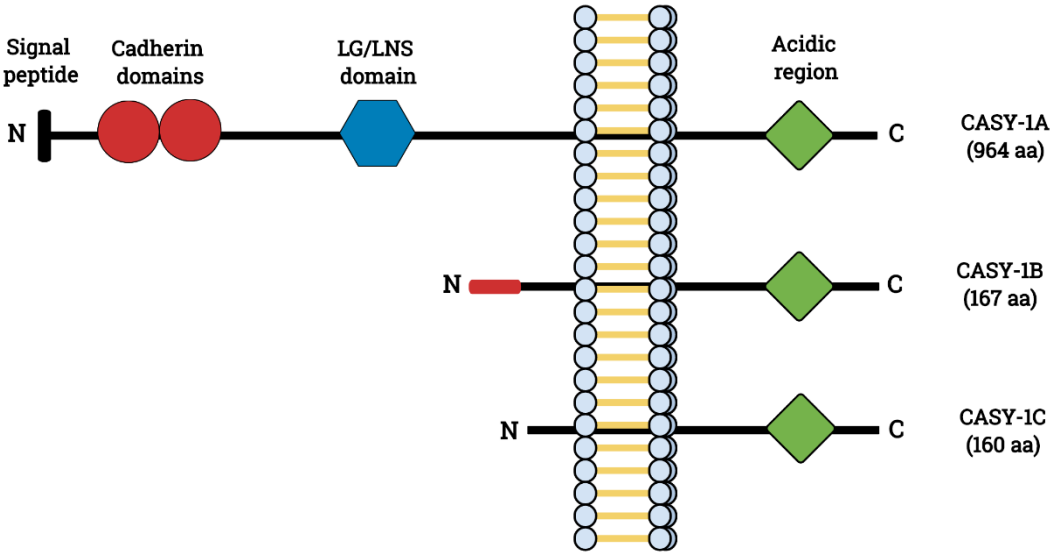


Figure 1.16: A schematic of the CASY-1 isoforms expressed in *C. elegans*. CASY-1A is a full-length protein with all the conserved domains- signal peptide (SP), two tandem cadherin domains, LG/LNS domain, transmembrane region and cytosolic acidic region. CASY-1B and CASY-1C are truncated proteins lacking the extracellular LG/LNS domain as well as cadherin repeats. CASY-1B and CASY-1C differ in just 7 amino acids at the N- terminal region, which are extra in CASY-1B.

CHAPTER 2:

Materials and Methods

SECTION A: MATERIALS

2.1. CHEMICALS AND REAGENTS

All chemicals used in the present study were either of analytical or molecular biology grades and were obtained from commercial sources. Media components, fine chemicals and reagents were purchased from Sigma Aldrich USA, HiMedia India, Merck India Ltd India, USB Corporation, USA or Difco, USA. Oligonucleotides (primers) were designed either manually or using ApE- A plasmid Editor Software and were purchased from Sigma India and IDT. Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Phusion* DNA polymerase, *Taq* DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA and protein molecular weight markers were purchased from New England Biolabs Inc, USA or MBI Fermentas, USA. Gel-extraction kits and plasmid miniprep columns were obtained from Qiagen, USA or Bioneer, Korea. DNA extraction kits were purchased from Invitrogen, USA and RNA isolation kit were procured from Qiagen, USA. Aldicarb PESTANAL, Tetramisole hydrochloride (Levamisole), Pentylenetetrazole (PTZ), Muscimol, 2,3-butanedione monoxamine (BDM), all trans- Retinal (ATR), Isopropyl-β-D-thiogalactoside (IPTG) and Histamine dihydrochloride (HA) were obtained from Sigma-Aldrich, USA. The 0.1µm polystyrene microspheres was purchased from Polysciences, USA. Hybridization nitrocellulose membrane (filter type 0.45µm) and Luminata^{TM} forte Western HRP substrate was obtained from Millipore, India. Anti-HA mouse monoclonal antibody (26183) and Anti-Myc mouse monoclonal antibody (MA1-980) were procured from Thermo Fisher Scientific, USA and horse anti-mouse HRP linked antibody was procured from Cell Signalling Technology, USA.

2.2. STRAINS AND PLASMIDS

A complete list of plasmids and strains utilized in this study is given in Tables 2.1 to 2.4. Most worm stocks were obtained from the *Caenorhabditis* Genetics Center (CGC supported by National Institutes of Health - Office of Research Infrastructure Programs). *casy-1(tm718)* mutant allele was procured from National BioResource Project (NBRP), Japan (shigen.nig.ac.jp/c.elegans). Strains requested from labs are mentioned in the table. Plasmids obtained from Addgene (www.addgene.org) or requested from labs are mentioned in the table.

Escherichia coli OP50 and HT115 were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). The *E.coli* strain DH5α was used as a cloning host and grown at 37^0C.

Table 2.1. List of integrated lines used in the study

S. no.	Plasmid	Integrated Line number	Source and reference
1	P*unc-17*::MCHERY	*nuIs321*	Josh Kaplan Lab (Babu et al., 2011)
2	P*unc-25*::GFP	*juIs76*	Yishi Jin Lab (Baran et al., 2010)
3	P*unc-129*::SNB-1::GFP	*nuIs152*	Josh Kaplan Lab (Babu et al., 2011; Sieburth et al., 2005)
4	P*unc-25*::SNB-1::GFP	*nuIs376*	Josh Kaplan Lab (Babu et al., 2011)
5	P*unc-129*::SYD-2::GFP	*nuIs160*	Josh Kaplan Lab (Babu et al., 2011; Sieburth et al., 2005)
6	P*unc-25*::SYD-2::GFP	*hpIs3*	CGC
7	P*myo-3*::ACR-16::GFP	*nuIs299*	Josh Kaplan Lab (Babu et al., 2011)
8	P*myo-3*::UNC-49::GFP	*nuIs283*	Josh Kaplan Lab (Babu et al., 2011; Sieburth et al., 2005)
9	P*unc-25*::*mCherry*	*lhIs6*	Erik A. Lundquist Lab (Norris and Lundquist, 2011)
10	P*unc-25*::*snb-1*::*supereclipticpHluorin*; *lin15(+)*] *IV*; lin-15(*n765ts*)	*oxIs155*	Erik Jorgensen Lab (Ernstrom et al., 2012)
11	P*casy-1a*::GFP	*sIs10330*	CGC
12	Pan- neuronal promoter::UNC-104::GFP; *unc-104* (*e1265*)	*tBIs147*	Sandhya Koushika Lab (Kumar et al., 2010)
13	P*flp-18*::FLP-18::*sl2*::GFP	AX1444	Mario de Bono Lab (Cohen et al., 2009)
14	P*myo-3*::UNC-29::GFP	*akIs38*	Andres Villu Maricq Lab (Francis et al., 2005)
15	P*unc-129*::GELSOLIN::VENUS	*nuIs169* (KP5862)	Josh Kaplan Lab (Babu et al., 2011)
16	P*unc-25*::GELSOLIN::VENUS	KP5926	Josh Kaplan Lab

Table 2.2. List of transgenes and arrays used in the study

S. no.	Plasmid	Plasmid number	Array number
1	P*casy-1a*:: *mCherry*::CASY-1A (injected into *casy-1*)	BAB353	*IndEx190*
2	P*casy-1b*::mCherry::CASY-1B (injected into *casy-1*)	BAB354	*IndEx192*
3	P*casy-1c*:: mCherry::CASY-1C (injected into *casy-1*)	BAB355	*IndEx193*
4	P*casy-1b*::mCherry::CASY-1B (injected into *nuls376*)	BAB354	*IndEx1007*
5	P*casy-1c*::mCherry::CASY-1C (injected into *nuls376*)	BAB355	*IndEx1009*
6	P*casy-1c*::CASY-1C::mCherry (injected into *casy-1*)	BAB363	*indEx1015*
7	P*casy-1c*::CASY-1C::GFP (injected into N2)	BAB384	*indEx1033*
8	P*casy-1c*::CASY-1C::GFP (injected into *casy-1*)	BAB384	*indEx1034*
9	P*casy-1b*::NLS-GFP (injected into N2)	BAB351	*IndEx118*
10	P*casy-1c*::NLS-GFP (injected into N2)	BAB352	*IndEx119*
11	P*unc-17*::CASY-1A (injected into *casy-1*)	BAB364	*IndEx188*
12	P*unc-17*::CASY-1B (injected into *casy-1*)	BAB365	*IndEx181*
13	P*unc-17*:: CASY-1C (injected into *casy-1*)	BAB366	*IndEx180*
14	P*unc-25*::CASY-1A (injected into *casy-1*)	BAB367	*IndEx189*
15	P*unc-25*::CASY-1B (injected into *casy-1*)	BAB368	*IndEx182*
16	P*unc-25*:: CASY-1C (injected into *casy-1*)	BAB369	*IndEx184*
17	P*unc-25*::CASY-1A (ΔCt) (injected into *casy-1*)	BAB350	*IndEx1011*
18	P*myo-3*::CASY-1A (injected into *casy-1*)	BAB335	*IndEx185*
19	P*unc-30*::CASY-1C(injected into *casy-1*)	BAB383	*IndEx1038, IndEx1039*
20	P*unc-25*:: CASY-1C (injected into *casy-1(tm718); zxIs3*)	BAB369	*IndEx1002, IndEx1012*
21	P*unc-25*:: CASY-1C (injected into *casy-1(tm718); nuls376*)	BAB369	*IndEx1012*
22	P*casy-1c*::CASY-1C::mCherry(injected into *nuls376*)	BAB385	*IndEx1017*
23	P*casy-1c*::CASY-1C::mCherry(injected into N2)	BAB385	*IndEx1056*
24	P*casy-1c*::CASY-1C::GFP (injected into *unc-104(ok913)*)	BAB384	*indEx1035*
25	P*casy-1c*::CASY-1C::GFP (injected into *klc-1(ok2609)*)	BAB384	*indEx1036*
26	P*gcy-13*:: nCRE (injected into N2) alongwith pKN22	BAB346	*IndEx1046*
27	P*gcy-13*:: nCRE (injected into N2) alongwith pKN24	BAB346	*IndEx1049*
28	P*gcy-13*:: nCRE (injected into *casy-1*) alongwith pKN24	BAB346	*IndEx1050*
29	P*odr-4*:: nCRE (injected into N2) alongwith pKN22	BAB387	*IndEx1051*
30	P*odr-4*:: nCRE (injected into N2) alongwith pKN24	BAB387	*IndEx1052*
31	P*odr-4*:: nCRE (injected into *casy-1*) alongwith pKN24	BAB387	*indEx1053*
32	P*nmr-1*::*HisCl* (injected into N2)	BAB395	*indEx1043*
33	P*nmr-1*::*HisCl* (injected into *casy-1*)	BAB395	*indEx1044*
34	P*nmr-1*::CASY-1A (injected into *casy-1*)	BAB396	*IndEx174*
35	P*gcy-13*::CASY-1A (injected into *casy-1*)	BAB337	*IndEx176*
36	P*opt-3*::CASY-1A (injected into *casy-1*)	BAB397	*IndEx171*
37	P*rig-3*::CASY-1A (injected into *casy-1*)	BAB338	*IndEx1004*
38	P*nmr-1*::mCherry (injected into *sls10330)*	BAB398	*IndEx1028*

39	P*gcy-13*::mCherry (injected into *sls10330)*	BAB367	*IndEx1029*
40	P*opt-3*::mCherry (injected into *sls10330)*	BAB368	*IndEx1054*
41	P*rig-3*::mCherry (injected into *sls10330)*	BAB369	*IndEx1055*
42	P*gcy-13*::CASY-1B (injected into *casy-1*)	BAB344	*IndEx1031*
43	P*gcy-13*::CASY-1C (injected into *casy-1*)	BAB345	*IndEx1032*
44	P*flp-21*::CASY-1A (injected into *casy-1*)	BAB386	*IndEx1048*
45	P*flp-1*::FLP-1 (injected into N2)	BAB388	*IndEx1058*

Table 2.3. List of strains used in this study

S. no.	Genotype	Strain number	Source and reference
1	*casy-1(tm718)*		Yuichi Iino Lab (Ikeda et al., 2008)
2	*casy-1 (hd41)*		Yuichi Iino Lab (Ikeda et al., 2008)
3	*casy-1(tm718);indEx190*	BAB1068	This study
4	*casy-1(tm718);indEx192*	BAB1070	This study
5	*casy-1(tm718);indEx193*	BAB1071	This study
6	*casy-1(tm718);indEx188*	BAB1066	This study
7	*casy-1(tm718);indEx181*	BAB1059	This study
8	*casy-1(tm718);indEx180*	BAB1058	This study
9	*casy-1(tm718);indEx189*	BAB1067	This study
10	*casy-1(tm718);indEx182*	BAB1060	This study
11	*casy-1(tm718);indEx184*	BAB1062	This study
12	*casy-1(tm718);indEx1011*	BAB1102	This study
13	*casy-1(tm718);indEx185*	BAB1063	This study
14	*indEx118*	BAB174	This study
15	*indEx119*	BAB175	This study
16	*casy-1(tm718); nuls321*	BAB133	This study
17	*casy-1(tm718); juls76*	BAB1046	This study
18	*casy-1(tm718); nuls152*	BAB131	This study
19	*casy-1(tm718); nuls376*	BAB132	This study
20	*casy-1(tm718); nuls160*	BAB164	This study
21	*casy-1(tm718); nuls321*	BAB163	This study
22	*casy-1(tm718); nuls299*	BAB160	This study
23	*casy-1(tm718)*; Ex[*hsp16.2p*::*casy-1*, *myo-3*p::venus].	JN442	Yuichi Iino Lab (Ikeda et al., 2008)
24	*casy-1(tm718)*; Ex[*ins-1p*::*casy-1*(RYV, ΔNt), *myo-3*p::venus]	JN1440	Yuichi Iino Lab (Ikeda et al., 2008)
25	*casy-1(tm718)*; Ex[*ins-1p*::*casy-1*(RYV, delta Ct), myo-3p::venus].	JN1442	Yuichi Iino Lab (Ikeda et al., 2008)
26	*casy-1(tm718);indEx1038*	BAB1138	This study
27	*casy-1(tm718);indEx1039*	BAB1139	This study
28	*casy-1(tm718); zxIs3; IndEx1001*	BAB1087	This study
29	*casy-1(tm718); zxIs3; IndEx1002*	BAB1088	This study
30	*nuls321; sls10330*	BAB176	This study
31	*nuls321; IndEx118*	BAB177	This study
32	*nuls321; IndEx119*	BAB1001	This study
33	*lhIs6; sls10330*	BAB1048	This study
34	*lhIs6; IndEx118*	BAB1014	This study
35	*lhIs6; IndEx119*	BAB1013	This study
36	*IndEx1007*	BAB1098	This study
37	*IndEx1009*	BAB1100	This study
38	*IndEx1017*	BAB1109	This study
39	*unc-104(ok913)*	RB992	CGC
40	*lin-15B&lin-15A(n765) X; zxIs3 [[unc-47p::ChR2(H134R)::YFP + lin-15(+)]*	ZX388	CGC
41	*casy-1(tm718); zxIs3*	BAB1012	This study

42	*casy-1(tm718); oxIs155*	BAB1112	This study
43	*IndEx1019*	BAB1111	This study
44	*IndEx1017*	BAB1109	This study
45	*indEx1033*	BAB1133	This study
46	*indEx1035*	BAB1135	This study
47	*indEx1036*	BAB1136	This study
48	*indEx1037*	BAB1137	This study
49	*IndEx1023*	BAB1116	This study
50	*casy-1 (tm718)* II; Ex[*odr-4p::casy-1, myo-3p*::venus]	JN485	Yuichi Iino Lab (Ikeda et al., 2008)
51	*casy-1 (tm718)* II; Ex[*glr-1p::casy-1, myo-3p*::venus]	JN484	Yuichi Iino Lab (Ikeda et al., 2008)
52	*eat-4 (ky5)*	MT6308	CGC
53	*ocr-2 (ak47)*	CX4544	CGC
54	*tax-4 (p678)*	PR678	CGC
55	*mec-4 (u253)*	TU253	CGC
56	*eat-4 (ky5); casy-1(tm718)*	BAB1158	This study
57	*ocr-2 (ak47); casy-1(tm718)*	BAB1085	This study
58	*tax-4 (p678); casy-1(tm718)*	BAB1159	This study
59	*mec-4 (u253);casy-1(tm718)*	BAB1156	This study
60	*kyEx4787 (Prig-3::IntenseGluSNFR, unc122p::dsRed)*	CX14652	C. Bargmann Lab (Marvin et al., 2013)
61	*kyEx4787; casy-1 (tm718)*	BAB1168	This study
62	*IndEx1046*	BAB1151	This study
63	*IndEx1049*	BAB1157	This study
64	*IndEx1050*	BAB1160	This study
65	*IndEx1051*	BAB1161	This study
66	*IndEx1052*	BAB1162	This study
67	*indEx1053*	BAB1163	This study
68	*glr-1 (n2461); nmr-1(ak4)*	BAB1084	This study
69	*glr-1 (n2461); nmr-1(ak4); casy-1(tm718)*	BAB1164	This study
70	*indEx1043*	BAB1143	This study
71	*indEx1044*	BAB1144	This study
72	*nuIs24* (P*glr-1*::GLR-1::GFP)		J. Kaplan (Rongo et al., 1998)
73	P*nmr-1*::NMR-1::GFP	VM484	CGC
74	BAB504; *casy-1(tm718)*	BAB168	This study
75	VM484; *casy-1(tm718)*	BAB170	This study
76	*IndEx174*	BAB1052	This study
77	*IndEx176*	BAB1054	This study

78	*IndEx171*	BAB1049	This study
79	*IndEx1004*	BAB1091	This study
80	*IndEx1028*	BAB1125	This study
81	*IndEx1029*	BAB1126	This study
82	*IndEx1054*	BAB1165	This study
83	*IndEx1055*	BAB1166	This study
84	*IndEx1031*	BAB1130	This study
85	*IndEx1032*	BAB1131	This study
86	*egl-3 (ok979)*	VC671	CGC

87	*egl-21 (n476)*	KP2018	CGC
88	*egl-3 (ok979); casy-1 (tm718)*	BAB1148	This study
89	*egl-21 (n476); casy-1 (tm718)*	BAB1083	This study
90	*IndEx1048*	BAB1155	This study
91	*pdf-1(tm1996)*	LSC27	CGC
92	*pdfr-1(ok3425)*	CX14295	CGC
93	*pdf-1(tm1996) ; casy-1 (tm718)*	BAB1149	This study
94	*pdfr-1(ok3425); casy-1 (tm718)*	BAB1150	This study
95	*pdf-1(tm1996)* III; lstIs1 [*pdf-1p::pdf-1*::3'UTR + *elt-2p*::GFP]	LSC90	CGC
96	*flp-1 (ok2811)*	RB2126 (outcrossed 3X)	CGC

97	*flp-18 (gk3063)*	VC2016 (outcrossed 3X)	CGC
99	*flp-1 (ok2811); casy-1 (tm718)*	BAB1172	This study
100	*flp-18 (gk3063); casy-1 (tm718)*	BAB1092	This study
101	*flp-1 (ok2811); flp-18 (gk3063)*	BAB1178	This study
102	*flp-1 (ok2811); flp-18 (gk3063); casy-1 (tm718)*	BAB1173	This study
103	P*flp-18*::FLP-18::*sl2*::GFP; *casy-1 (tm718)*	BAB1086	This study
104	*npr-1 (ok1447)*	RB1330 (outcrossed 3X)	CGC
105	*npr-1 (ok1447); casy-1 (tm718)*	BAB1121	This study
106	*lndEx1058*	BAB1184	This study
107	*lndEx1058; casy-1 (tm718)*	BAB1185	This study
108	*kyEx4787; casy-1 (tm718);* Ex[*odr-4p*::*casy-1, myo-3p*::venus]	BAB1183	This study
109	*unc-25 (sa94)*	JT94	CGC
110	*akIs38; casy-1 (tm718)*	BAB199	This study
111	*nuis169; casy-1 (tm718)*	BAB165	This study
112	KP5862; *casy-1 (tm718)*	BAB162	This study
113	*lndEx1056*	BAB1179	This study
114	*lndEx1056; hpIs3*	BAB1186	This study
115	*klc-1 (ok2609)*	RB1975	CGC
116	*klc-2 (km11)*		Sandhya Koushika Lab (Yandell et al., 1994)
117	*klc-1 (ok2609); casy-1 (tm718)*	BAB1094	This study
118	*klc-2 (km11); casy-1 (tm718)*	BAB1107	This study
119	*lndEx1036*	BAB1136	This study
120	*nrx-1 (ok1649)*	VC1416	CGC
121	*nlg-1 (ok259)*	VC228	CGC
122	*nrx-1 (ok1649); casy-1 (tm718)*	BAB173	This study
123	*nlg-1 (ok259); casy-1 (tm718)*	BAB172	This study

Table 2.4. List of other plasmids used in this study

Name	Description	Source
pGBDU-C1	Yeast-2-hybrid vector-Gal4 DNA Binding Domain	James 1996 (James et al., 1996)
pGAD-C1	Yeast-2-hybrid vector-Gal4 Activation Domain	James 1996 (James et al., 1996)
BAB342	pGBDU-C1 *casy-1* C- terminal minus acidic region	This study
BAB373	pGAD-C1 UNC-104 (1-360 aa)	This study
BAB374	pGAD-C1 UNC-104 (460-633 aa)	This study
BAB375	pGAD-C1 UNC-104 (623-1105 aa)	This study
BAB341	pGAD-C1 UNC-104 (1087-1628 aa)	This study
BAB399	pGAD-C1 KLC-1 (1-536 aa)	This study
BAB400	pGAD-C1 KLC-2 (1-540 aa)	This study
pKN22	P*rab-3*::*inverse Chr2*C128S*::SL2::GFP	K. Norman (Fry et al., 2014)
pKN24	P*rab-3*:: *inverse TeTx*::SL2::GFP	K. Norman (Fry et al., 2014)
pKN27	P*ver-3*::*nCre*	K. Norman (Fry et al., 2014)
pNP471	P*rig-3*::*HisCl1*::SL2::mCherry	C. Bargmann (Pokala et al., 2014)
BAB393	pET-23A UNC-104 (1087-1628 aa)- HA	This study
BAB380	pGEX-KG *casy-1* C- terminal (48-160 aa)	This study

2.3. OLIGONUCLEOTIDES

Table 2.5. List of primers used for genotyping and Real time PCR

Mutant genotyped	Primer number	Primer sequence	Mutation
casy-1 (tm718)	ST150 ST151 ST152	gacgggtgatggaatgaaag tcaaagcttctcctcccaga cagcacgctcctacaacaag	Deletion
casy-1 (hd41)	ST156 ST157 ST158	tcgtgctggacacgttactc accatccagcaaaggatacg ggcgtatgattctggtcgag	Deletion
unc-104(ok913)	ST415 ST416 ST417	cgaaggaaataaagcgagg cgacggtcggtggctgttcgg ctcttcatctctatcgagaacc	Deletion
lin-15b(n744)	ST176 ST177 ST175	gtctgacgcattccccac gtctgacgcattccccat cattgtcgagagctcagc	Substitution
eri-1(mg366)	ST173 ST588 ST174	aaacttcggaacatatggggc ttcgataaagtgcctgttttttt tgggtaaggaatcgaagacg	Deletion
eat-4 (ky5)	ST509 ST510 ST511	gacgggtgggacccagcg ggtgcagcacccccacagc ctgagtgactaagtacccg	Deletion
tax-4 (p678)	ST512 ST513 ST514	ccagcggccaccggtgggc ccagcggccaccggtgggt tcacaaattttcatatgtggg	Substitution
ocr-2 (ak47)	ST515 ST516 ST517	cactagcagcatttaactgg cgttcgccaaattctgcacc gcttcatcaacagcttaccc	Deletion
mec-4 (u253)	ST635 ST636 ST637	cctctctgattgacattcttcc cctggctcaaaaaatgctcc catctgctcacggaattccc	Deletion
glr-1	AB037 AB038 AB039	acctttcggctccgacttg acctttcggctccgactta attgaaatgaccataccacc	Substitution
nmr-1(ak4)	ST366 ST367 ST368	gatgtgaggtgtccatgggc gaagagtgcagtccagaggg attcccttcaagcacctgctc	Deletion
egl-3 (ok979)	ST625 ST500 ST626	ggtgattgtactcgagtcc agacaccgttgtcacgggcgg caacgtctaccctctcccc	Deletion

egl-21 (n476)	ST496 ST497 ST498	cagcataggacttagcgagcc cgaggaatttcaaacactaacc ggtgacgtggcagctggctg	Deletion
pdf-1(tm1996)	ST649 ST650 ST651	ctgaacctgtgttcttgggc gagggagcaacacgcccac atgaatacgatccggtggc	Deletion
pdfr-1(ok3425)	ST652 ST653 ST654	ggtgaaagaggcggtgtacg ccgtatccagcaatgaaataggg gcaggcttattgcccaagcg	Deletion
flp-1 (ok2811)	ST372 ST373 ST374	gtcctcgtcaagcacgtc ctcgatgctctccaccatgg aaccggctttctttcccagcg	Deletion
flp-18 (gk3063)	AB113 AB114 AB115	aggacggaaattacctgtgc gcttcgggaaacgctcatat ttattctttcttgtcggggcc	Deletion
npr-1 (ok1447)	AB116 AB117 AB118	acctgtcacttttacgccgg tgatttcgttccagttgaacg gaaccttcacttctcctgtg	Deletion
npr-4 (tm1782)	AB119 AB120 AB121	agctgttgtctccttccagg cgatttccgatgaggaaacc cacagcttctaataggaaaggg	Deletion
npr-5 (ok1583)	AB122 AB123 AB124	gcacgacgaactgcaaattt tccttgagttttctgggatg aggcatttttggaaacggcg	Deletion
nrx-1 (ok1649)	ST372 ST373 ST374	cgcgggatagtgacgaaa ccatgctcaacgagagaagc cctccggccatcaactatc	Deletion
nlg-1 (ok259)	ST372 ST373 ST374	gtggatccgttccgaaga gagagccccttattccactg gatggacaggtgggttgaag	Deletion
casy-1a	ST408 ST152	ggttgttctccgccgtgaagctgg cagcacgctcctacaacaag	Real time PCR
casy-1b	ST411 ST244	atgttcgtgaacattctgg tcccactcaagacctccaac	Real time PCR
casy-1c	ST241 ST244	cggtggtgcaattgatgagt tcccactcaagacctccaac	Real time PCR
act-1	ST066 ST067	tgcgacattgatatccgtaagg ggtggttcctccggaaagaa	Real time PCR

Table 2.6. List of primers used for cloning (FP: Forward primer; RP: Reverse primer)

Gene/ Promoter cloned	Primer no.	Forward/ Reverse primer	Primer Sequence	Vector backbone
casy-1a promoter	ST331 ST332	FP RP	ctctctcctgcaggccgtacttcctctgaatcgac ctctctggatccaatggtgatgtttggcgtaggagacgcc	pPD49.26
casy-1b promoter	ST324 ST325	FP RP	ctctctctgcaggagtcgagcactcctgacaccc ctctctggatccgcagtgaaataagtgtataaagaag	pPD49.26
casy-1c promoter	ST328 ST329	FP RP	ctctctcctgcaggctgaaaatctcttcactatacaacccg ctctctggatccctaaaagtataaacaattagattatgac	pPD49.26
casy-1a gene (N- terminal MCHERRY fusion)	ST334 ST335	FP RP	ctctctcccggggctgctcgagcgccaataatcaatctgc ctctctgctagcgacacgataagaacgagcgttcg	pPD49.26
casy-1b gene (N- terminal MCHERRY fusion)	ST357 ST335	FP RP	ctctctcccgggttcgtgaacattctggaaatggacc ctctctgctagcgacacgataagaacgagcgttcg	pPD49.26
casy-1c gene (N- terminal MCHERRY fusion)	ST288 ST335	FP RP	ctctctcctgcaggctgaaaatctcttcactatacaacccg ctctctgctagcgacacgataagaacgagcgttcg	pPD49.26
MCHERRY (N- terminal fusion)	ST404 ST406	FP RP	ctctctggatccatggtctcaaagggtgaagaag ctctctcccgggcttatacaattcatccatgcc	pPD49.26
MCHERRY (C- terminal fusion)	ST609 ST610	FP RP	ctctctgctagcgtctcaaagggtgaagaag ctctctggtaccctacttatacaattcatccat	pPD49.26
GFP (C- terminal fusion)	ST552 ST553	FP RP	ctctctggtaccagtaaaggagaagaacttttc ctctctcggccgctatttgtatagttcatcc	pPD49.26
casy-1a gene (C- terminal MCHERRY/GFP fusion)	ST599 ST597	FP RP	ctctctggatccgctgctcgagcgccaataa ctctctaccggtttgacacgataagaacg	pPD49.26
casy-1b gene (C- terminal MCHERRY/GFP fusion)	ST596 ST597	FP RP	ctctctggatccatgttcgtgaacattctgg ctctctaccggtttgacacgataagaacg	pPD49.26
casy-1c gene (C- terminal MCHERRY/GFP fusion)	ST598 ST597	FP RP	ctctctggatccatggacctcccgcgtcc ctctctaccggtttgacacgataagaacg	pPD49.26
casy-1b promoter transcriptional reporter line	ST305 ST306	FP RP	ctctct*cctgcagg*cgacggagtcaccactaccacatccacg a ctctctggatccctggaatacaagtatcaacttcagaattctg	pGC76
casy-1c promoter transcriptional reporter line	ST307 ST308	FP RP	ctctctcctgcaggctgaaaatctcttcactatacaacccg ctctctggatccccagaatgttctaaaagtataaacaattagatt atgac	pGC76
unc-17 promoter (cholinergic rescue)	ST363 ST318	FP RP	ctctctaccggtcatgacaaagtggtgacactgg ctctctcctgcagggactccaccgagttaccttaaa	pCFJ910 (later sub cloned into pPD49.26)
unc-25 promoter (GABAergic rescue)	ST373 ST374	FP RP	ctctctaagcttggtcaaaagccgaaatttaaagctag ctctctggatccgtggctcagttgtgtagttgct	pPD49.26
myo-3 promoter (muscle rescue)	ST466 ST467	FP RP	ctctctggatccagtgattatagtctctgttttcg ctctctgctagccatttctagatggatctagtgg	pPD49.26
unc-30 promoter (DD/VD neuron rescue)	ST621 ST622	FP RP	ctctctctgcagcgccattcggagcacgctcttcagc ctctctggatccgccggagggcgcctcaatcccc	pPD49.26
CASY-1A gene (for rescue)	ST336 ST162	FP RP	ctctctggtaccatgcgaactgcgtactttatttttgtcg ctctctactagtggggaaggagtgaaaaggac	pPD49.26
CASY-1B gene (for rescue)	ST337 ST162	FP RP	ctctctggtaccatgttcgtgaacattctgg ctctctactagtggggaaggagtgaaaaggac	pPD49.26
CASY-1C gene (for rescue)	ST338 ST162	FP RP	ctctctggtaccatggacctcccgcgtccg ctctctactagtggggaaggagtgaaaaggac	pPD49.26

CASY-1A truncated (ΔCt) (for rescue)	ST336 ST502	FP RP	ctctctggtaccatgcgaactgcgtactttatttttgtcg ctctctactagtttatggcattggggtgtctcgc	pPD49.26
unc-104 RNAi	ST443 ST444	FP RP	ctctctaccggttcctgaacacatgcaagagg ctctctaagcttcaacagctggttgagcacat	L4440
casy-1 RNAi	ST146 ST147	FP RP	ctctctactagtcggcaagagcactctcttct ctctctggtaccagacccttttctcgcaatcc	L4440
CASY-1 C- terminal minus acidic region (for yeast two hybrid assay)	ST447 ST486	FP RP	ctctctggatccaaaatgcgagacaccccaatgc ctctctgtcgacatcactgaactcatcaattgcacc	pGBDUC1
UNC-104 (1-360) (for yeast two hybrid assay)	ST449 ST611	FP RP	ctctctcccgggtcatcggttaaagtagctgtacg ctctctctgcagctattttgcatttggatcctc	pGADC1
UNC-104 (460-633) (for yeast two hybrid assay)	ST612 ST613	FP RP	ctctctcccgggagtccaaagaagttacccc ctctctctgcagctaatattcccttgtctgatg	pGADC1
UNC-104 (623-1105) (for yeast two hybrid assay)	ST614 ST615	FP RP	ctctctcccggggaacaaaaaatgtatcatc ctctctctgcagctaccacacaagaagatcatg	pGADC1
UNC-104 (1087-1628) (for yeast two hybrid assay)	ST481 ST450	FP RP	ctctctcccggggcaccaattcagaacaataacgc ctctctctgcagttatgaagcagcaattgaagatg	pGADC1
odr-4 promoter	ST638 ST639	FP RP	ctctctgcatgcgaagccgcatcagaaactc ctctctgctagcggattctgtaactggaattgc	pPD49.26 cloned with Cre recombinase
nmr-1 promoter	YD260 YD271	FP RP	atagcctgcaggaagtggacactgagagagagag agcaggtaccctgtaacaaaactaaagtttgtcgtgttc	pPD49.26/
opt-3 promoter	ST003 ST004	FP RP	ccccccgggattccctcgatcctcttctctc ccccccggggaggcggaattaattttatagttcggc	pPD49.26
gcy-13 promoter	ST005 ST006	FP RP	atcaggatccgtcctgaaaaattattgaaagtttgtaataag atcaggatccgtcctgaaaaattattgaaagtttgtaataag	pPD49.26
rig-3 promoter	ST007 ST008	FP RP	gcgtcgacaagtgacaccacgctcaca ccccccgggagctgtgaaatttttaggcagt	pPD49.26
flp-21 promoter	ST633 ST644	FP RP	ctctctgcatgcaactaggtccagtgaccgaaagtg ctctctcccgggtggagcaatgagagggtggg	pPD49.26
flp-1 promoter	ST375 ST376	FP RP	ctctctaagcttcacgatgagcaggaccaccg ctctctggatccccgtatccaatcttgaacccccc	pPD49.26
CASY-1A gene (for rescue)	ST336 ST162	FP RP	ctctctggtaccatgcgaactgcgtactttatttttgtcg ctctctactagtggggaaggagtgaaaaggac	pPD49.26
CASY-1B gene (for rescue)	ST337 ST162	FP RP	ctctctggtaccatgttcgtgaacattctgg ctctctactagtggggaaggagtgaaaaggac	pPD49.26
CASY-1C gene (for rescue)	ST338 ST162	FP RP	ctctctggtaccatggacctcccgcgtccg ctctctactagtggggaaggagtgaaaaggac	pPD49.26
FLP-1 gene (for rescue)	ST377 ST378	FP RP	ctctctcccgggatgactctgctctaccaagtagg ctctctgagctcttattttccgaaacgaagg	pPD49.26
mCherry	ST404 ST506	FP RP	ctctctggatccatggtctcaaagggtgaagaag ctctctcccgggcttatacaattcatccatgcc	pPD49.26
HisCl	ST001 ST002	FP RP	caggaggaccttggctagcatgcaaagcccaactagcaa gatgagacagcggtacctcataggaacgttgtccaat	pPD49.26 (amplified from pNP471)
Superecliptic pHluorin	ST606 ST607	FP RP	catacggaaaacttaccc gttgaacgcctccatcttc	pPD49.26

CASY-1 C-terminal (48-160 aa) (for GST- pull down experiment)	ST447 ST618	FP RP	ctctctggatccaaaatgcgagacaccccaatgc ctctctgtcgacttacagatcctcttctgagatgagtttttgttcgacacgataagaa	pGEX-KB
UNC-104 (1087-1628 aa) (for GST- pull down experiment)	ST619 ST620	FP RP	ctctctgctagcatggcaccaattcagaacaataacgc ctctctgagctcttaagcgtaatctggaacatcgtatgggtatgaagcagcaattg	pET-23A

2.4. MEDIA

All strains were maintained on nematode agar growth medium (NGM) plates seeded with OP50 *Escherichia coli* at 20^{O}C under standard conditions (Brenner, 1974). All the media, buffers and stock solutions were prepared using Millipore elix 3 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2-0.45 µm (for heat labile compounds). NGM plates utilized for various pharmacological assays and RNAi experiments were prepared fresh before the experiment and all the chemicals and antibiotics were added to molten NGM at 55^{0}C unless otherwise mentioned.

2.4.1. NGM (Nematode Growth) Medium	NaCl 3 g/l Peptone 2.5 g/l Agar 17 g/l H_2O 975 ml Above mix was autoclaved for 50 minutes and chemicals listed below are added. 1M $CaCl_2$ 1 ml 1M $MgSO_4$ 1 ml 1M KPO_4 pH 6.0 25 ml Cholesterol (5mg/ml in EtOH) 1 ml
2.4.2. LB (Luria–Bertani) Medium	Yeast extract 5 g/l Tryptone 10 g/l NaCl 10 g/l pH of the above medium was adjusted to 7.0 with 1N NaOH
2.4.3 LB Agar (Luria–Bertani) Medium	Yeast extract 5 g/l Tryptone 10 g/l NaCl 10 g/l Agar 15 g/l pH of the above medium was adjusted to 7.0 with 1N NaOH. Desired antibiotics were added after autoclaving, before plates were poured.
2.4.4. SD (Synthetic Defined) Medium	YNB 1.7 g/l (Yeast Nitrogen Base) (without amino acids and ammonium sulphate) $(NH_4)_2$ SO_4 5 g/l Dextrose 20 g/l Amino acids 50 mg/l (as required) pH was adjusted to 6.0–6.5.

2.5 BUFFERS AND STOCK SOLUTIONS

2.5.1. Aldicarb Stock Solution (100 mM)

100 mg Aldicarb was dissolved in 5.25 ml of absolute ethanol. It was stored at -20°C.

2.5.2 Levamisole Stock Solution (100 mM)

25 mg Levamisole was dissolved in 1 ml of deionized water. The solution is prepared fresh before experiment.

2.5.3 PTZ Stock Solution (100mg/ml)

The required amount of PTZ was dissolved in the required volume of absolute ethanol. The solution is prepared fresh before experiment.

2.5.4 Muscimol Stock Solution (100 mM)

12 mg Muscimol was dissolved in 1 ml of deionized water. It was stored at -20°C and used within one month of preparation.

2.5.5 all trans-Retinal Stock Solution

25 mg all trans-Retinal was dissolved in 900 µl of absolute ethanol. It was stored at -20°C in dark and used within one month of preparation.

2.5.6 2, 3-butanedione monoxamine Solution (BDM) (30mg/ml)

The required amount of BDM was dissolved in the required volume of M9 buffer. The solution is prepared fresh before imaging experiments.

2.5.7 Histamine-dihydrochloride Stock Solution (1 M)

9 mg Histamine-dihydrochloride was dissolved in 50 ml of deionized water. Aliquots were stored at -20°C.

2.5.8 Ampicillin Stock Solution (100 mg/ml)

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water, and was filter-sterilized using a 0.2 µm filter membrane. It was stored at -20°C.

2.5.9. Tetracycline Stock Solution (12.5 mg/ml)

The required amount of Tetracycline was dissolved in 1:1 volume of distilled water: ethanol and was stored at -20°C in aliquots.

2.5.10 Bleach Solution

5 N NaOH (20 gm NaOH dissolved in 100 ml distilled water) and Sodium hypochlorite solution were mixed in 1:2 ratio freshly before use.

2.5.11 Isopropyl-β-D-thiogalactoside (IPTG) (1M)

238 mg IPTG was dissolved in 1 ml of deionized water. Aliquots were stored at -20°C.

2.5.12. 50% Glycerol (used for preparing –80°C stocks of *E. coli)*

***Caenorhabditis elegans* buffers**

2.5.13. M9 buffer	KH_2PO_4 3 g/l Na_2HPO_4 6 g/l 1M $MgSO_4$ 1 ml Sterilized by autoclaving.
2.5.14. S-basal buffer	NaCl 5.85 g/l KH_2PO_4 6 g/l K_2HPO_4 1 g/l Sterilized by autoclaving. 1ml Cholesterol (5mg/ml in EtOH) was added.
2.5.15. Liquid freezing buffer	S-basal buffer (see above) Glycerine 30% Sterilized by autoclaving
2.5.16. Worm Lysis buffer (for genotyping)	KCl 50 mM Tris (pH 8.3) 10 mM $MgCl_2$ 2.5 mM NP-40 0.45% Tween-20 0.45%

2.5.17. Alkaline Lysis Buffers (Plasmid DNA preparation from *E. coli*)

a) Solution-I (Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution) (freshly prepared)	0.2 N NaOH (freshly diluted from a 10N stock) 1% SDS (freshly diluted from a 10% stock) Stored at room temperture.
c) Solution-III (Neutralization	5 M Potassium acetate 60 ml

Solution) (100ml)	Glacial acetic acid 11.5 ml Deionized water 28.5 ml The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA) (pH 8.0) **TE-RNAse (stock prepared at 10 mg/ml)**	10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0). Working stock 20µg/ml in TE Buffer, pH 8.0.
e) PCI (Phenol-chloroform-isoamyl alcohol) Solution (100ml)	Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] Chloroform 48 ml Isoamyl alcohol 2 ml Stored at 4°C in dark brown bottle.

2.5.18. Agarose Gel Electrophoresis Reagents

a) 1× TBE (Tris-Borate-EDTA) Buffer (per 1000 ml) (prepared from 50× TBE stock)	89 mM Tris pH 7.6 89 mM Boric acid 2 mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
b) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G 30% glycerol
c) 1-2% Agarose gel in 1× TAE	
d) Ethidium Bromide (10 mg/ml) Stock	Final working concentration used at 0.5 µg/ml.

2.5.19. Solutions for preparation of chemical competent *E. coli* cells (Nishimura et al., 1990)

a) Medium A	0.2% Glucose 10 mM $MgSO_4.7H_2O$* *added after filter sterilization.
b) Storage Solution B	36% Glycerol 12% PEG 8000 12 mM $MgSO_4.7H_2O$*

	LB broth (pH 7.0) (for volume make-up) * added after filter sterilization.

2.5.20. Yeast Transformation Solutions (*S. cerevisiae*) (Ito *et al.*, 1983)

a) 0.1 M Lithium acetate in TE (pH 7.5)

b) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

2.5.21. SDS-PAGE Solutions and Reagents (Sambrook, 1989a)

a) 30% Acrylamide Mix	29.2% (w/v) Acrylamide 0.8% (w/v) N, N'- methylenebisacrylamide Filtered before use.
b) Resolving (Lower) Gel Tris Buffer (Stock) (4×) 1.5 M Tris-HCl (pH 8.8)	18.18 g Tris pH adjusted to 8.8 with 6 N HCl and volume made up to 100 ml. (0.4% SDS can be added to the buffer itself).
c) Stacking (Upper) Gel Tris Buffer (Stock) 0.5 M Tris-HCl (pH 6.8)	6.06 g Tris pH adjusted to 6.8 with HCl and volume made up to 100 ml. (0.4% SDS can be added to the buffer itself).
d) 0.4% SDS	
e) TEMED (N, N, N, N-Tetramethylethylenediamin)	99%
f) APS (Ammonium persulphate)	10%
g) Tris-Glycine Gel Running Buffer (Laemmli Buffer) (pH 8.3)	25 mM Tris base 250 mM Glycine (electrophoresis grade) 0.1% SDS
h) 5× Sample Buffer/Gel Loading Buffer (pH 6.8)	0.15 M Tris-HCl (pH 6.8) 5% SDS 25% Glycerol 12.5% β-mercaptoethanol 0.006% Bromophenol blue
i) Gel Staining Solution	40% Methanol 10% Glacial Acetic acid 0.1% Coomassie Brilliant Blue (R250)
j) Gel Destaining Solution	40% Methanol 10% Glacial Acetic acid

Composition of SDS-PAGE Resolving (Lower) Gel (9%) (For 10 ml)

a)	Distilled water	4.35 ml
b)	Resolving (Lower) Gel Tris Buffer (Stock) (4×) 1.5 M Tris-HCl (pH 8.8) with 0.4% SDS	2.5 ml
c)	30% Acrylamide Mix	3.35 ml
d)	10% APS (Ammonium persulphate)	0.05 ml
e)	TEMED (N, N, N', N'-Tetramethylethylenediamine)	0.01 ml

Composition of SDS-PAGE Stacking (Upper) Gel (4%) (For 10 ml)

a)	Distilled water	6.1 ml
b)	Stacking (Upper) Gel Tris Buffer (Stock) 0.5 M Tris-HCl (pH 6.8) with 0.4% SDS	2.5 ml
c)	30% Acrylamide Mix	1.3 ml
d)	10% APS (Ammonium persulphate)	0.05 ml
e)	TEMED	0.01 ml

2.5.22. Immunoblotting (Western Blotting) Reagents (Sambrook, 1989a)

a) Transfer Buffer (pH 9.2)	48 mM Tris base 39 mM Glycine 0.037% SDS 20% Methanol
b) Stripping Buffer	62.5 mM Tris-HCl, pH 6.7 100 mM β-mercaptoethanol 2% SDS
c) Tris-Buffered Saline (TBS)	150 mM NaCl 20 mM Tris-HCl (pH 7.5) (Made as 10× stock and kept at 4°C)
d) Washing Buffer TBS-Tween 20 (TBST) Buffer	0.1% Tween 20 in TBS (pH7.5)
e) Blocking Agent	5% Skim milk in TBST.
f) Ponceau S Staining Solution	0.5% Ponceau S 1% Glacial Acetic acid

SECTION B: METHODS

2.6. *C. elegans* strain maintenance

All strains were maintained on nematode agar growth medium (NGM) plates seeded with OP50 *Escherichia coli* at 20^{O}C under standard conditions (Brenner, 1974). The *C. elegans* Bristol strain, N2 was used as the wild-type (WT) control. Strains were synchronized by hypochlorite treatment followed by allowing *C. elegans* to grow for approximately 2.5 days at 20^{O}C. All experiments were carried out with young adult hermaphrodites at ~ 23^{O}C, unless otherwise mentioned. Occasional contamination which was observed on plates was also removed by treatment with bleach, as eggs are resistant to bleach. For long term storage strains were maintained in liquid nitrogen using freezing media (composition in Section 2.5.15).

2.7. *C. elegans* genomic DNA isolation

Worms were grown on OP50 plates until food was depleted. Worms were washed from the plates using M9 buffer (composition in Section 2.5.13) and transferred to 1 ml eppendorf tubes. Worms were successively (3-4 times) washed using M9 buffer by gently pelleting them using low spin, throwing the supernatant and resuspending the worm pellet in fresh M9 buffer. Washing with M9 buffer facilitated complete removal of bacteria.

The worm pellet was finally suspended in 500 µl of worm Lysis solution (100mM Tris pH 8.5, 100mM NaCl, 50mM EDTA, 1% SDS, and proteinase K 100µg/ml). Worms suspended in lysis solution were incubated at 60ºC for 90 minutes. RNaseA (20µg/ml) was then added and the tube was incubated at 37ºC for 30 minutes. The tube was spinned in a microfuge and the supernatant transferred to new tube and an equal volume of Phenol: Chloroform was added. It was gently mixed which was followed by centrifugation at 13000 rpm for 3 minutes. The aqueous phase obtained was extracted. To this extract 2.5 volumes of absolute ethanol was added. Whole mixture was spinned at 13000 rpm for 15 minutes. The pellet was washed with 70% ethanol to remove salt. The pellet was then re-suspended in 100µl TE buffer (composition in Section 2.5.17) and was stored at -20ºC.

2.8. *C. elegans* RNA isolation

Worms were grown on OP50 plates for a mixed-stage population. Worms were washed from the plates using M9 buffer in an RNase-free Eppendorf tube. Worms

were pelleted by spinning briefly at 14,000 rpm. Supernatant was removed leaving 100µl of solution on top of the sample. In the hood, 400 µL of Trizol reagent was added followed by vortexing for about 2 minutes. Samples were freeze-thawed thrice in liquid nitrogen. Again, 200 µL of Trizol reagent was added and samples were allowed to sit at room temperature for 5 minutes. To this 140µl of chloroform was added followed by vigorous shaking for 15 seconds. Samples were incubated at room temperature for 2 minutes. Further, samples were centrifuged at 12,000xG for 15 minutes in the cold room (4°). The aqueous phase was transferred to a new 1.5 ml tube and slowly an equal volume of 70% EtOH was added. The mixture was taken in a Qiagen RNeasy spin column. Samples were spun at maximum speed for 15 seconds. This was followed by washing with 700µl of buffer RW1 and 500µl of buffer RPE (twice). Everytime centrifugation was done at maximum speed for 15 seconds. After final wash, columns were transferred to a new 1.5 ml tube, and RNA was eluted in 50µl of RNAse-free water.

2.9. Transgenic strains and constructs

Tables 2.2 and 2.4 lists all the plasmids and constructs used in this study. Table 2.5 and 2.6 lists the primers used to make the different transgenes used in this study. Tables 2.1 and 2.3 lists are the transgenic lines used in the study. All the plasmids were generated using standard restriction digestion based cloning strategy and sequenced before use in the experiments. Previously described microinjection techniques were used to generate stable transgenic *C. elegans* lines carrying extra-chromosomal DNA arrays using either p*myo-3*::mCherry, p*myo-2*::GFP or p*opt-3*::mCherry as co-injection markers (Mello and Fire, 1995).

2.10. Growth and maintenance of bacteria and yeast strains

The *Escherichia coli* strains DH5α was routinely grown in LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were maintained on YPD medium and grown at 28-30°C. The yeast transformants were selected and maintained on SD medium with supplements as per the requirement.

2.11. Transformation in *E. coli*

E. coli competent cells were prepared using calcium chloride method in accordance with standard protocols (Ausubel et al., 1994). Transformation was carried out by adding plasmid or ligation mixture to the competent cells, incubation, followed by a thermal shock at 42 ° C for 1 minute. Transformed cells were incubated in LB at 37°C for 45 min, and plated on LB medium containing ampicillin.

2.12. Transformation in yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method (Ito *et al*., 1983). *S. cerevisiae* cultures were grown in YPD at 30^{o}C with shaking for 16-24 hours and then reinoculated in fresh YPD media to an initial OD_{600} of 0.1, cells were allowed to grow at 30^{o}C for 4-5 hours with shaking. Cells were harvested at 5000 rpm for 5 min, were washed with sterile water followed by subsequent wash with 0.1 M lithium acetate solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution. Cells were incubated at 30^{o}C for 30 min with shaking. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of 1×10^{9} cells/ml and divided into 100µl aliquots. Approximately 50 µg (5 µl of 10 mg/ml stock solution) of heat denatured, salmon sperm carrier DNA, followed by 0.3 µg- 0.7µg of plasmid/DNA fragment were added to each aliquot and whole cell suspension was incubated at 30^{o}C for 30 min. After the incubation, 0.3 ml of 50% PEG 3350 (prepared in 0.1 M lithium acetate, pH 7.5) was added to each tube, mixed well and again kept at 30^{o}C for 45 min. The cell suspensions were subjected to heat shock at 42^{o}C for 10 min. and the cells were allowed to cool at room temperature. The cells were pelleted down at 5000 rpm for 3 min. The cell pellet was resuspended in sterile water and appropriate volume of cell suspension was plated on selection plates.

2.13. Pharmacological assays

All the assays were performed with the experimenter blind to the genotypes. Each assay was performed at least three times as indicated at the base of each bar with > than 20 animals for each replicate.

2.13.1. Aldicarb assay: The Aldicarb assays were performed as described previously (Mahoney et al., 2006). Briefly, fresh Aldicarb plates were made

previous day by adding 100mM stock solution of Aldicarb to molten NGM at a final concentration of 1mM. Plates were then seeded with OP50 *E. coli* and stored in dark at room temperature overnight. For each assay,~ 20 young adult hermaphrodites were transferred on to Aldicarb plates and scored for paralysis every 15 minutes for more than 2.5 hours. Animals were considered paralyzed when they failed to show any body bends following prodding three times on the head.

For heat shock experiments, *C. elegans* were grown till young adult stage, heat shock was given at 34^{O}C for 1 hour, and following recovery for 3 hours at 20^{O}C. Aldicarb assay was performed after recovery period is over.

For optogenetics-based aldicarb assay, animals were exposed to a 1-minute pulse of low-intensity blue light before and after every 30 minutes during the aldicarb assay. For this assay, aldicarb plates were seeded with OP50 containing 0.8 mM all-trans Retinal (ATR).

For Histamine induced silencing of command interneurons, NGM-HA plates were prepared. For this, 1M histamine stock solution was added to NGM agar at a final concentration of 10 mM at ~65 °C immediately before pouring plates (Pokala et al., 2014). Histamine-free control plates were poured from the same NGM batch. Histamine-containing plates were used for Aldicarb within two days after pouring.

2.13.2. Levamisole assay: The Levamisole assay was performed by exposing *C. elegans* to Levamisole at a final concentration of 0.5mM. Plates were prepared as described previously for Aldicarb assay. Young adult hermaphrodites were transferred on to Levamisole plates and scored for paralysis every 15 minutes. Animals were deemed paralyzed when they do not show any body bends following three prodding's on the head (Mahoney et al., 2006).

2.13.3. Pentazylenetetrazole (PTZ) assay: PTZ assays were performed as described previously (Locke et al., 2008). Plates were made fresh on the day of experiment by spreading PTZ stock solution (100 mg/ml) on solidified NGM plates at a final concentration of 10 mg/ml. The plates were allowed to dry for about 2 hours and then seeded with concentrated OP50 *Escherichia coli.* For each assay,~ 10 young adult hermaphrodites were transferred to PTZ plates and then scored by visual inspection under a stereomicroscope for the presence of anterior head bends called

'head bobs' at 30 and 60 minute time points. *unc-25* mutant was used as positive control.

2.13.4. Muscimol assay: Muscimol assays were carried out with young adult hermaphrodites as previously described. Briefly,~ 10 *C. elegans* were placed on NGM plates containing 0.5mM Muscimol. The animals were then scored visually after 30 minutes by observing under a stereomicroscope for the presence of the "rubber band" phenotype (de la Cruz et al., 2003; Dabbish and Raizen, 2011).

2.14. Locomotion assays

Well-fed synchronized animals were picked onto new 90 mm NGM plates seeded with a uniform lawn of OP50 *E. coli*. These plates were seeded 12 hours before the start of the assay and the bacteria were allowed to grow at 20°C. The animals were allowed to equilibrate for 1- minute after transfer to the assay plate. For all analysis, a 3-minute video was made using the Zeiss Lumar V12 fluorescence Stereomicroscope. This video was then segmented into shorter videos of 20 seconds using custom code written in MATLAB (MATLAB Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States). The 20 seconds long video was then scored by eye for body bends. A body bend was defined as a change in the direction of propagation of the part of the animal corresponding to the posterior bulb of the pharynx along the y-axis, assuming the *C. elegans* was traveling along the x-axis (Tsalik and Hobert, 2003).

For monitoring locomotion in the absence of food, well-fed *C. elegans* were picked onto an unseeded 90 mm NGM plate and allowed to crawl free from any adherent food. After 1 minute, they were picked onto a second unseeded NGM plate. Further analysis was performed as described above.

2.15. Imaging experiments

Animals were immobilized with 30mg/ml 2, 3-butanedione monoxamine on 2% agarose pads. All quantitative imaging was done using Zeiss AxioImager microscope with a 40x or 63x 1.4 NA Plan APOCHROMAT objective equipped with a Zeiss AxioCam MRm CCD camera controlled by Axiovision software (Zeiss Micro-imaging). For comparing WT animals with *casy-1* mutants, >25 *C. elegans* were analyzed for each genotype (Babu et al., 2011).

For the morphological analysis of GABAergic neurons (*juIs76* [P*unc-25*::GFP]) and cholinergic neurons (*nuIs321* [P*unc-17*::mCherry]), z-stacks of the entire *C. elegans* (from overlapping fields of view) were taken using a 63x objective and a 10 µm optical slice (20 slices at 0.5 micron distance). Image J software was used to derive maximum intensity projection images from z-stacks. These images were then analyzed for gross morphological analysis of neurons.

All fluorescent SV marker imaging and quantitative imaging for GLR-1::GFP and NMR-1::GFP was done with the 63x objective. Animals were imaged for the dorsal nerve cord in the posterior portion of the *C. elegans* halfway between the vulva and the tail. For fluorescent analysis, image stacks were taken (approximately 10 µm) and the maximum intensity projections were obtained using Image J software. For all quantitative analysis, identical camera gain, exposure settings, and fluorescence filters were used for a particular transgenic line. For all the Figures, an average of the values for each *C. elegans* in the data set ± S.E.M. is plotted. Statistical difference between WT and mutant values was determined using the Student's t-test ($p \leq 0.05$) in Graph Pad Prism 7. Graphs of punctal and axonal fluorescence show data normalized to WT values.

Transcriptional reporter expression, *supercliptic pHluorin*, *iGluSnFR*, FLP-18::*sl2*::GFP and co-localization imaging was done using Leica HC PL APO 63x/ TCS SP8 confocal microscope (Leica Microsystems) with Multi-Ar (457, 488, and 515 nm), and He-Ne (543 and 633 nm) laser lines and HyD detectors. For iGluSnFR analysis, imaging was done with the 63x objective. Animals were imaged in the head region of *C. elegans* for the AVA neuron cell body.

For co-localization analysis, image stacks were taken (four times of average for 1024x1024 scan format at speed 400 Hz) and maximum intensity projections were obtained for each channel separately. Maximum intensity projections were then merged to obtain co-localization images using the Image J software.

2.16. Electrophysiology

Strains for electrophysiology were maintained on plates seeded with HB101 *Escherichia coli* at 20°C. Adult *C. elegans* were immobilized on Sylgard coated coverslips with cyanoacrylate glue. Dissections and whole-cell recordings were performed as described previously (Madison et al., 2005; Richmond, 2006; Richmond

and Jorgensen, 1999). Statistical significance was determined using the Student's t-test for comparison of mean frequency and amplitude for endogenous excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs).

2.17. Optogenetics

Optogenetic experiments were performed as described previously (Liewald et al., 2008). All trans-retinal (ATR) plates were prepared fresh and used within one week. ATR plates were made by spotting NGM plates with 50 µl of OP50 *E. coli* containing 0.8mM ATR and allowed to grow overnight at 37^{o} C in dark. Control plates were made by seeding NGM plates with 50µl of OP50 lacking ATR. Transgenic animals carrying GABAergic Channelrhodopsin *zxIs3* [P*unc-47*:: ChR2(H134R)::YFP + lin-15(+)] were synchronized and grown on ± ATR plates at 20^{O}C. For the assay,~ 10 young adult hermaphrodites were picked on fresh seeded NGM plates. For all analysis, a 20- second video was made using the Zeiss Lumar V12 fluorescence Stereomicroscope. Video recording was started as soon as the animals were seen crawling, blue light was turned on within 4 seconds and then left on for the duration of recording. The light intensity was approximately 57.5 mW/cm^2 from HXP 120V light source. Two frames were selected from all the videos for each animal, one before blue light was turned on and one after that with the maximum relaxation of the *C. elegans*. Analysis was performed as described previously (Fry et al., 2014). In brief, Image J on a Wacom Bamboo tablet and stylus were used to trace a freehand line from the nose tip down to the posterior-most point of the *C. elegans*. The length of the animal before and after exposure to blue light was measured and difference in these lengths divided by the starting *C. elegans* length was determined. This data was then plotted as percentage change in body length (relaxation).

2.18. Fluorescence Recovery after Photobleaching (FRAP)

FRAP was performed on transgenic WT and *casy-1 (tm718)* mutants carrying GABAergic *nuIs376* [P*unc-25*:: SNB-1::GFP] or cholinergic *nuIs152* [P*unc-129*::SNB-1::GFP]. For RNAi- based FRAP experiments, the F2 generation of *C. elegans* exposed to *unc-104/kif1a* RNAi were used. FRAP experiments were carried out using Leica TCS SP8 confocal microscope. For the FRAP experiments, *C. elegans* were immobilized on 10% agarose pads containing 0.2µl of 0.1µm polystyrene microspheres (Polysciences). To account for the focal drift, image stacks

were taken, and maximum intensity projections were obtained using Image J. For this experiment, three frames were taken for one representative puncta in the posterior half of the animal, followed by 100 iterations of photobleaching in the defined region (25% power of a 488 nm Argon laser with bleaching power of 2%). This was followed by monitoring 10 iterations of recovery every 25s for up to six minutes. The intensities were normalized against a non-bleached ROI within the same animal.

2.19. RNA interference (RNAi)

RNAi experiments were carried out as described previously (Timmons and Fire, 1998). All assays were performed in *eri-1(mg366); lin-15B(n744)* background which makes animals more sensitive to RNAi in the nervous system (Wang et al., 2005). Briefly, animals were raised on bacteria expressing double-stranded RNA containing *casy-1*, *unc-104/kif1a* or empty vector for two generations. F2 generation animals were analyzed on acute Aldicarb assays in duplicates.

2.20. Live imaging and analysis of SV transport dynamics

For in vivo time-lapse imaging of the motor neuron commissures, 1-day adult hermaphrodites with the GABAergic *nuIs376* [P*unc-25*::SNB-1::GFP] or cholinergic *nuIs152* [P*unc-129*::SNB-1::GFP] transgene were immobilized in 3 mM tetramisole in M9 and mounted on a 5% agarose pad. Imaging was performed in the posterior commissures. SNB-1::GFP time-lapse images were obtained with an Olympus IX83 microscope (Olympus, Tokyo, Japan) using a Plan Apochromat objective (100X, 1.4 NA) attached with a spinning disk confocal head (CSU22; Yokogawa, Tokyo, Japan) and equipped with an electron-multiplying charge-coupled device (EMCCD) camera (iXon 897; Andor Technology, South Windsor, CT). A 488 nm laser (25 mW) was used at 10% power for imaging (100X objective, 300 ms exposure time). Moving particles were defined as puncta that were displaced by more than 3 pixels in less than 5 consecutive time frames. The flux of particles was calculated as the total number of puncta moving in either direction in an entire kymograph, then normalized to a 10 μm region, further normalized to time (1 minute) {Unit of flux here is number of events/10μm/min}. Movies were acquired at a constant frame rate of 3 frames/s for a total of 700 (again average number- varies across movies) frames. Each frame was 512x512 pixels dimension. Kymographs were generated and analyzed using ImageJ software, version 1.41 (National Institutes of

Health, Bethesda, MD), and statistical significance was determined using a two-way ANOVA with Bonferroni's multiple comparison post-test.

2.21. Quantitative PCR (qPCR) experiments:

For Real time qPCR analysis, fresh RNA was isolated from mixed stage populations of WT and *casy-1(tm718)* mutants using the RNeasy Mini Kit (Qiagen). Total RNA was quantified with spectrophotometer by measurement at OD260/280nm (Nanodrop). The cDNA was synthesized with random hexamers using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Real time qPCR reactions were carried out in 20µl volumes using the SYBR *Premix Ex Taq* II (Tli RNase H Plus) (Clontech) in triplicates in an Eppendorf RealPlex Mastercycler. Thermal cycling was initiated at 94°C for 30 sec followed by 40 cycles of PCR (94°C for 5 s and 68°C for 30 s). *act-1* was used as an internal control and RNA levels of the gene of interest was normalized to *act-1* levels for comparison. The normalized expression of *casy-1* isoforms in *casy-1* mutants relative to WT animals was calculated using the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct = [(Ct\ G.O.I_{Control} - Ct\ H.K.G_{Control}) - (Ct\ G.O.I_{Experimental} - Ct\ H.K.G_{Experimental})$ and *Ct* represents threshold cycle number, G.O.I and H.K.G represents gene of interest and housekeeping gene (*act-1*) respectively A list of primers used for these experiments is listed in Table 2.5.

2.22. Yeast two-hybrid assay

All the yeast two-hybrid constructs used in this study are mentioned in Table 2.4. Briefly, pGBDUC1 vector was used as bait containing the yeast URA3 selectable marker and the C-terminal of CASY-1 is expressed as fusion to the Gal4p activation domain. While the pGADC1 vector used as prey carries the LEU2 selection marker and generates protein fusions with Gal4p DNA binding domain. pGAD-C1 and pGBDU-C1 clones containing genes of interest were co-transformed into PJ69-4α using the Lithium acetate method (Gietz and Schiestl, 2007) and were then plated onto SC-Ura-Leu plates and incubated at 30°C. Following this, the double transformants were inoculated into 5 mL SD-Ura-Leu and grown overnight in a shaking incubator (New Brunswick, Thermo Scientific) at 200 rpm at 30°C. Cell count was determined by monitoring O.D, followed by spotting serial dilutions of equal number of cells onto SC-Ura-Leu-His and SC-Ura-Leu+His plate and grown for 72 h at 30 °C. For CASY-1 interaction with UNC-104/KIF1A, serial dilutions of

equal number of cells were spotted on SC-Ura-Leu-His and SC-Ura-Leu+His plates containing 5 µM 3-Amino-1, 2, 4-triazole (AT).

For identifying the domain in UNC-104/KIF1A that might be interacting with CASY-1 C- terminal, a series of constructs were generated covering various domain of UNC-104/KIF1A as published previously (Wagner et al., 2009). These constructs cover several domains in UNC-104/KIF1A - Motor domain (aa 1-356), the forkhead domain FHA (aa 460-633), the SYD-2/Liprin binding domain STALK (aa 623-1105) and the PH domain (aa 1087-1628).

2.23. Protein expression and Glutathione S-transferase pull down assay

For GST- pull down experiment C-terminal of *casy-1* cDNA was subcloned into the pGEX-KB vector and expressed as N-terminal GST fusion proteins in *Escherichia coli* BL21 cells. Cells were grown at 37°C to an OD_{600} nm of 0.6, induced with 0.1 mM isopropylthiogalactoside (IPTG) and grown for further 16 h at 18 °C. Harvested cells were resuspended in a lysis buffer containing 50 mM Tris at pH 7.5, 300 mM NaCl, Protease inhibitor (Roche) and DNase I (Sigma) and disrupted by freeze–thawing followed by short sonication. The cell lysate was centrifuged at 14,000 rpm for 20 min at 4°C and the cleared supernatant was incubated with glutathione beads (Sigma) for 2 h at 4 °C. The beads were washed with lysis buffer three times. The beads were then packed into a syringe column. GST-tagged protein immobilized on glutathione–Sepharose beads was incubated with the supernatant from *E.coli* BL21 cells expressing UNC-104-HA for 2 hours at 4°C. The supernatant was prepared as mentioned above. The beads were then washed extensively five times. After extensive washing with lysis buffer, bound proteins were added directly into 5×SDS/PAGE sample buffer, boiled for 5 min, separated by SDS/PAGE and subjected to immunoblot analysis. Western blot using Anti-HA Antibody (1:1000) was then performed to identify interaction between CASY-1 and UNC-104.

2.24. Western Blot analysis

Protein content was estimated by the Bradford method. Equal amount of protein samples denatured in (100mM Tris-HCl, pH-8, 4% (w/v), 5mM EDTA, 40% (v/v) glycerol; 5% β-mercaptoethanol and 0.05% (w/v) bromophenol blue) at 95^{0}C for 10 min were resolved by reducing SDS-PAGE (10% acrylamide gel), electroblotted onto

nitrocellulose membrane and was probed with mouse monoclonal anti-HA primary antibody at the dilution of 1:1000. The proteins were finally probed with goat anti-mouse HRP conjugated secondary IgG and visualized using chemiluminescence detection reagent (GE Biosciences).

2.25. Statistical analysis

All statistical analysis was performed using GraphPad Prism V7. Experimental data are shown as mean ± S.E.M. Statistical comparisons were done using the Student's t-test, two-way ANOVA or one-way ANOVA with Bonferroni's multiple comparison post-test. A level of $P<0.05$ was considered significant.

SECTION C: PRINCIPLE FOR ASSAYS USED

2.26. Aldicarb assay: At the neuromuscular junction, acetylcholine released from cholinergic motor neurons binds to cholinergic receptors resulting in muscle contraction. The remaining acetylcholine is cleared from the synaptic cleft by a group of enzymes called acetylcholinesterases that breakdown acetylcholine to acetate and choline. The drug aldicarb constitutively binds acetylcholinesterase in the synaptic cleft and inhibits the degradation of acetylcholine; resulting in accumulation of acetylcholine at the NMJs. This persistent depolarization of muscle leads to hypercontraction, leading to complete paralysis of animal (Figure 2.1) (Mahoney et al., 2006).

Hypersensitivity to inhibitors of acetylcholine esterase could arise from two different variations at the NMJ. Firstly, a decrease in GABAergic signaling would cause less muscle relaxation, accelerating the paralysis of animals. Secondly, an increase in cholinergic signaling or overall synaptic release will result in more muscle excitation; thus increasing the paralysis rate of animals substantially compared to WT worms. On the other hand, an increased GABAergic signaling or decreased cholinergic signaling will results in a resistance to aldicarb. The aldicarb assay has been used in the identification of a number of genes involved in the regulation of Excitation: Inhibition balance (Sieburth et al., 2005; Vashlishan et al., 2008).

2.27. Levamisole assay: Levamisole is another drug that acts at the NMJ to cause spastic paralysis. Levamisole is a selective agonist for the major subtype of ligand gated-acetylcholine receptors present on the muscle and acts as major determinant of

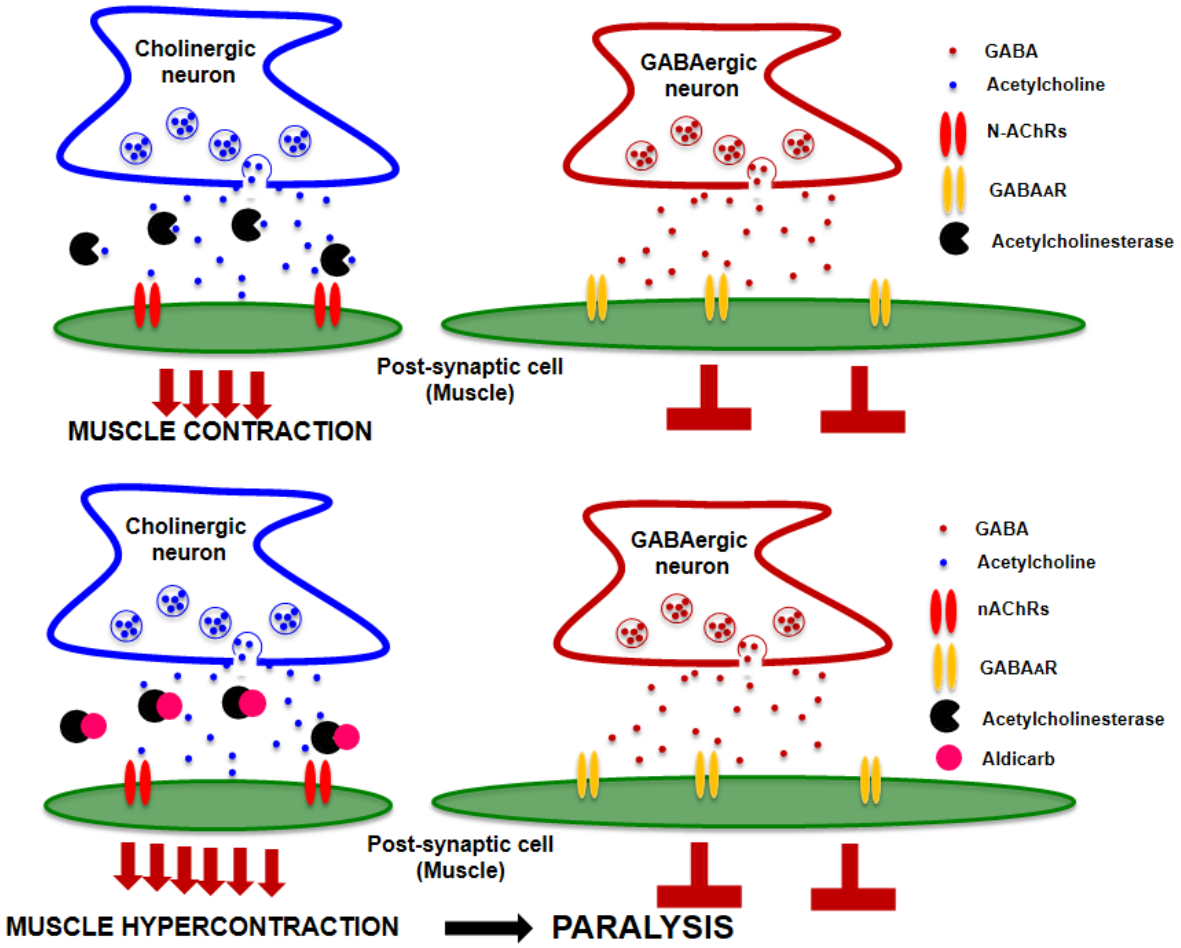


Figure 2.1: Mechanism of aldicarb-induced paralysis. In the absence of aldicarb, acetylcholinesterase degrades acetylcholine, stopping excess cholinergic signaling. When animals are treated with aldicarb, acetylcholinesterase is inhibited, excessive amounts of acetylcholine leads to muscle hypercontraction and eventually, paralysis of the animal. The aldicarb assay monitors the time-course of paralysis with respect to WT worms. Animals that paralyze faster, are considered hypersensitive to aldicarb and the animals that paralyze slower, are considered resistant to aldicarb.

synaptically evoked muscle contraction (Fleming et al., 1997; Richmond and Jorgensen, 1999). The action of levamisole is independent of presynaptic acetylcholine release and provides information about the muscle responsiveness or postsynaptic receptor levels. This assay also monitors the time-course induced paralysis compared to WT worms. If postsynaptic L-AChRs levels are unaffected, sensitivity to levamisole will remain unchanged (Figure 2.2).

2.28. Pentylenetetrazole (PTZ) assay: $GABA_A$ receptors in *C. elegans* are homopentameric ion channels. The receptor carries a GABA binding site (shown in red) and a neurosteroid (example PTZ) binding site (shown in yellow). PTZ is a pharmacological inhibitor of GABA signaling that acts as a competitive inhibitor of GABA. If the presynaptic GABA release is less, PTZ binds at the neurosteroid binding site thus interfering with GABA binding and further inhibitory GABA signaling. In the absence of GABA signaling, excitatory cholinergic signaling will increase resulting in enhanced muscle contraction. The most prominent phenotype shown by GABA signaling-deficient mutants is the 'head bobs or anterior convulsions' where worms rapidly convulse the anterior portion of their body (Locke et al., 2008) (Figure 2.3).

2.29. Muscimol assay: Muscimol is a GABA agonist. Muscimol assay is used to determine any change in the GABA receptor levels in the postsynaptic muscle cells. If there is less GABA receptors on the muscle worms will show resistance in Muscimol assay however, if GABA receptor levels have increased worms will show an increased sensitivity (de la Cruz et al., 2003) (Dabbish and Raizen, 2011) (Figure 2.4).

2.30. Optogenetics-based experiments: Optogenetic experiments are based on neuron-specific expression of light activated-cation channels called channelorhodopsin that allows non-specific flow of cations in to the neuron of interest resulting in neuron depolarization and its activation (Liewald et al., 2008). These channels uses all trans- Retinal (ATR) as a cofactor. In the presence of ATR, when blue light falls on channelorhodopsin, it results in neuronal activation while in the absence of ATR, blue light has little or no effect on channel activity. These channels can be expressed in specific subsets of neurons using neuron-specific promoters to

optogenetically stimulate those neurons. For example, optogenetic stimulation of GABAergic motor neurons results in muscle relaxation (Figure 2.5).

2.31. pHluorin experiment: In this experiment the luminal domain of synaptobrevin, a synaptic vesicle protein is tagged with superecliptic pHluorin, a pH-sensitive GFP reporter (Kavalali and Jorgensen, 2014). pHluorin is highly pH-sensitive and its fluorescence remains quenched in the acidic environment of the SV lumen, however, there is a dramatic increase in the fluorescence as soon as the vesicle fuses onto the membrane relieving the tag from the acidic environment of the SV. The surface fluorescence of this tag is then quantified to monitor the dynamics of synaptic vesicle fusion onto the membrane. If the surface fluorescence of pHluorin is reduced, this suggests that less number of synaptic vesicles are fusing onto the membrane (Figure 2.6).

2.32. Electrophysiology: In *C. elegans*, whole-muscle patch clamp recordings are performed to monitor the flow of ions across one type of channel (Richmond, 2006). For this, a single worm is glued onto the surface of a Sylgard coated-coverslip using WormGlu. An incision is made on one side of the worm, to expose the NMJ in an intact form. Once the muscle is clearly visible, a recording pipette is brought in close contact with the muscle membrane, a slight negative pressure is applied that ruptures the membrane, to attain a whole cell configuration (Figure 2.7). In this configuration, the solution inside the recording pipette comes in close contact with the muscle cytosol. Since, *C. elegans* muscles contain both excitatory cholinergic receptors (movement of positive ions) and inhibitory GABAergic receptors (movement of negative Cl^- ions) the current trace we get is a mixture of two ion flows. To distinguish between these two currents, either pharmacological inhibitors of one kind of channel is used, or the concentration of chloride ions is adjusted in the recording solution to differentiate between two kinds of ion flows.

Once this is done, we get individual excitatory and inhibitory post synaptic current (EPSCs and IPSCs) traces. Each spike in the current trace represents the fusion of one quanta (synaptic vesicle) on the presynaptic membrane. So the frequency of spikes gives information about the number of presynaptic vesicles fusing. If quantal content is same, the amplitude of each spike gives information about the number of postsynaptic receptors on the muscle.

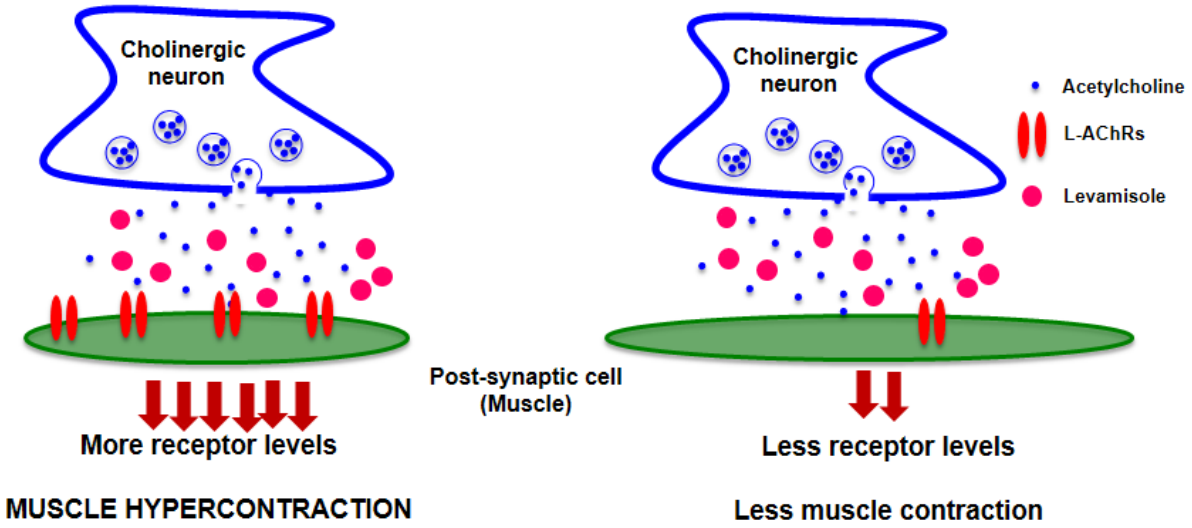


Figure 2.2: Mechanism of Levamisole-induced paralysis. Levamisole assay monitors the time course-induced paralysis upon application of Acetylcholine agonist Levamisole. If postsynaptic L-AChRs receptor levels are more, worms show hypersensitivity to Levamisole. If postsynaptic L-AChRs receptor levels are less, worms show resistance to Levamisole.

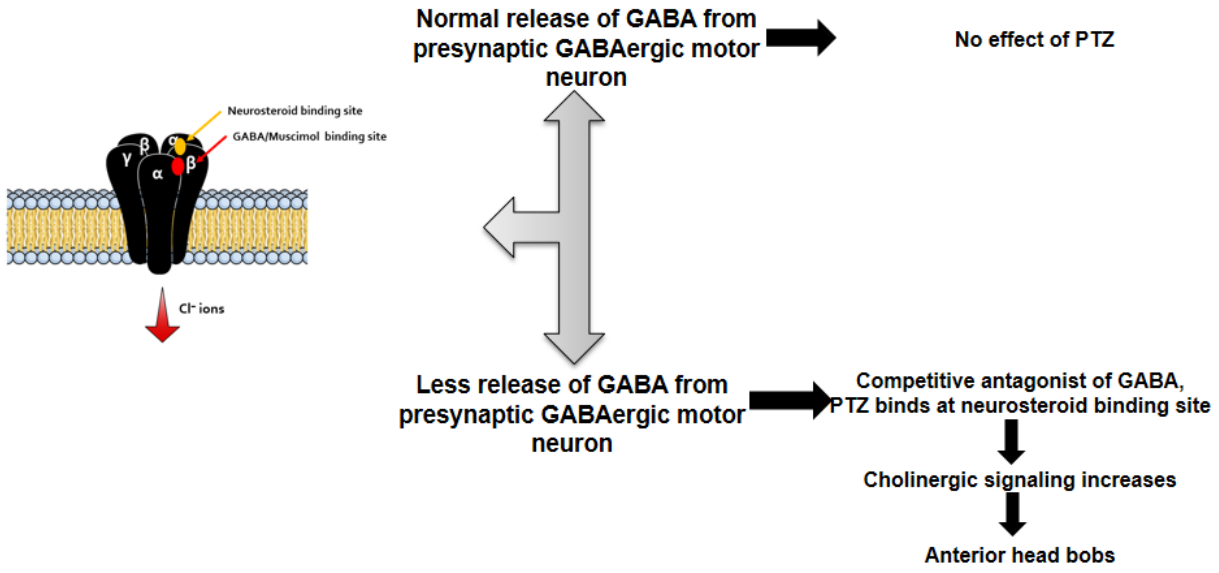


Figure 2.3: Mechanism of PTZ-induced anterior head bobs. PTZ is a competitive antagonist of GABA. If presynaptic GABA release is less, PTZ binds at neurosteroid binding site, thus hampering the binding of GABA at GABA- binding site. In the absence of GABA signaling, excitatory cholinergic signaling increases resulting in anterior convulsions called 'head-bobs'. Mutants showing anterior convulsions on PTZ plates suggests defective presynaptic GABA release.

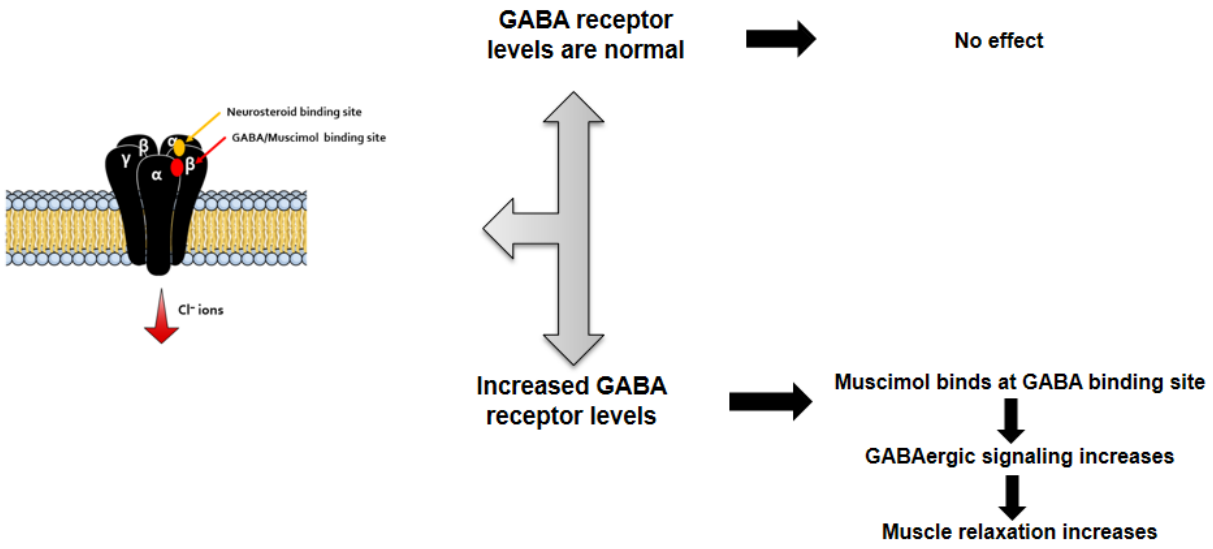


Figure 2.4: Mechanism of Muscimol induced body wall relaxation. Muscimol is a GABA agonist. If GABA receptor levels are increased on the post synaptic muscle cells, Muscimol binds at the GABA binding site on the GABA receptor, thus increasing the GABAergic signaling. Due to increased GABA signaling worm muscle relaxes, resulting in straightening of the worm on Muscimol-containing plate. Thus sensitivity to Muscimol points towards altered GABA receptor levels on the postsynaptic cell.

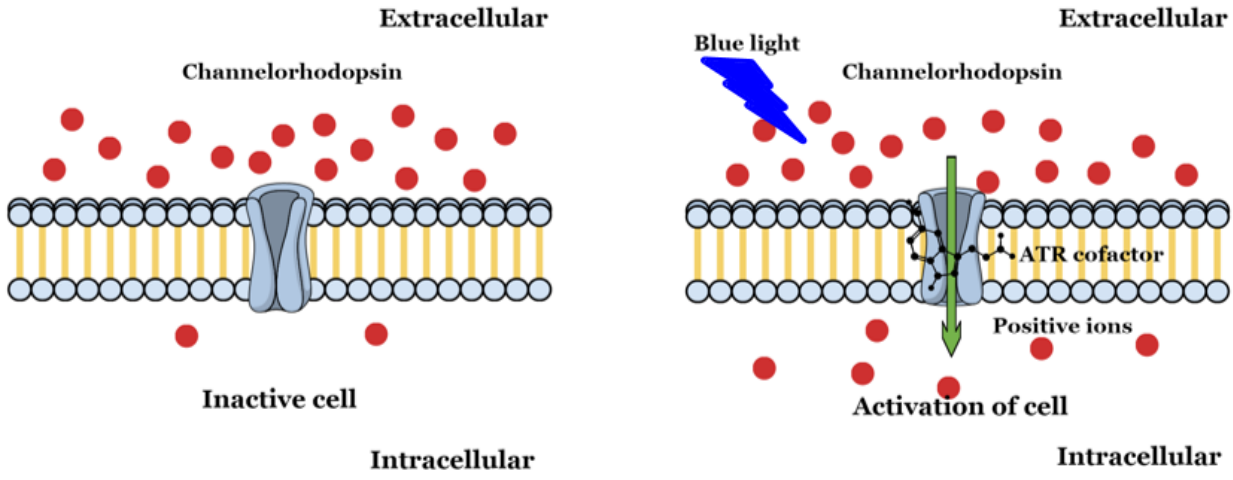


Figure 2.5: Mechanism of Optogenetics-based cell activation. Optogenetic experiments are based on neuron-specific expression of light activated-cation channels called channelorhodopsin that allows non-specific flow of cations in to the neuron of interest resulting in neuron depolarization and its activation. This channels uses all trans-Retinal (ATR) as a cofactor. In the presence of ATR, when blue light falls on channelorhodopsin, it results in neuronal activation.

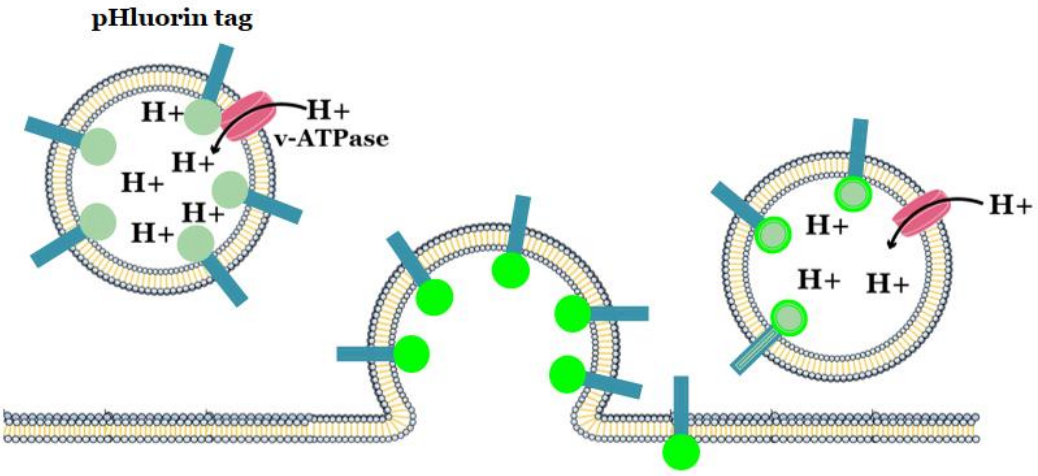


Figure 2.6: Mechanism of pHluorin experiment. pHluorin is highly pH-sensitive tag and its fluorescence remains quenched in the acidic environment of the SV lumen, however, there is a dramatic increase in the fluorescence as soon as the vesicle fuses onto the membrane relieving the tag from the acidic environment of the SV. This surface fluorescence is then quantified to monitor the dynamics of synaptic vesicle fusion in the neuron.

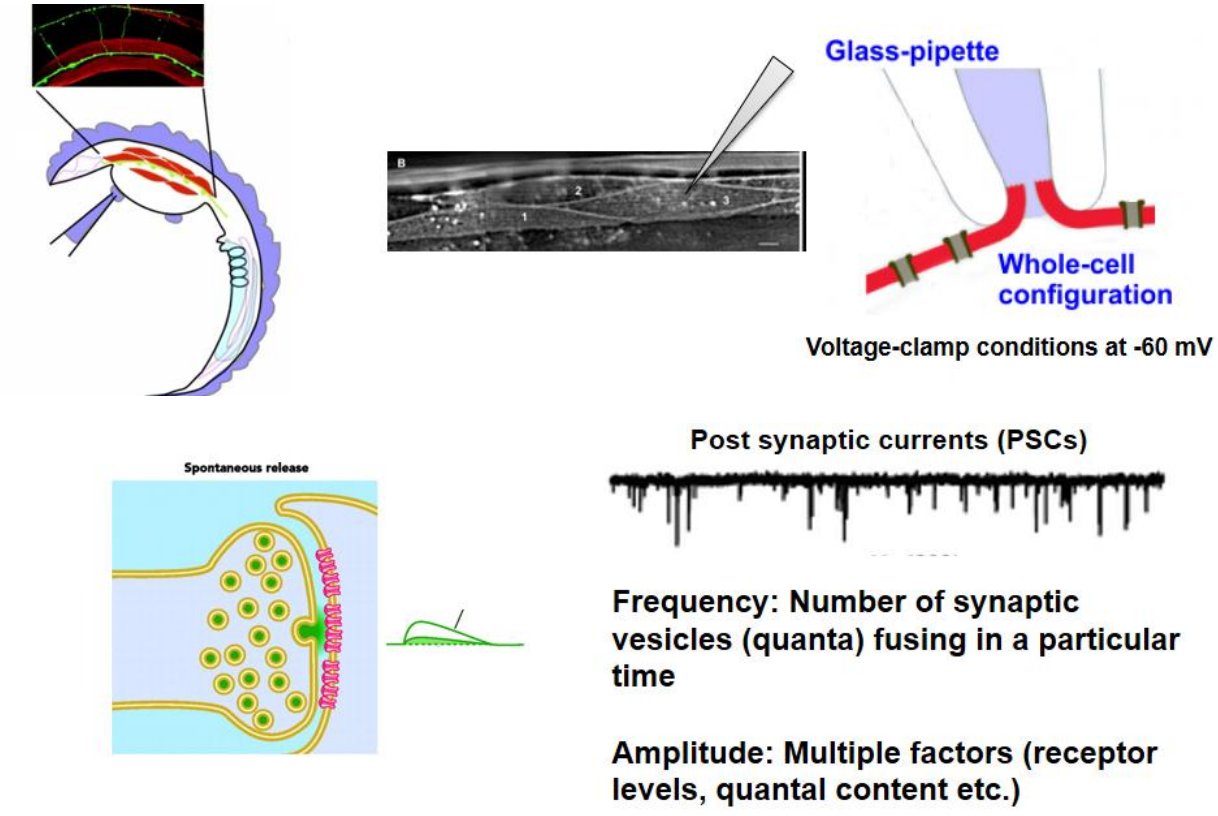


Figure 2.7: Method of whole muscle patch clamp recordings from *C. elegans* NMJ (Image adapted from Richmond. J.E., 2005).

CHAPTER 3:

Deciphering the role of CASY-1 at the C. elegans NMJ

Shruti Thapliyal designed, performed and analyzed all the experiments except the electrophysiological recordings and the synaptic trafficking experiments. Dr. Jihong Bai and Dr. Yongming Dong performed the electrophysiological recordings. Amruta Vasudevan performed the synaptic trafficking experiments under the supervision of Sandhya P Koushika.

Introduction:

A remarkable feature of the nervous system is the specific connections between neurons, which is responsible for orchestrated neural networks and circuitry of the brain. Many cell adhesion molecules (CAMs) are concentrated at synaptic sites in neuronal axons and dendrites, serving as dynamic regulators of synaptic function. Considering the complexity of the nervous system, tightly controlled spatial and temporal regulation of several different classes of CAMs is essential. Existing literature suggests that neuronal CAMs are not only important for adhesion but are also required for various aspects of synapse development and function (Biederer et al., 2002; Chan et al., 2003; Chih et al., 2005; Chubykin et al., 2007; Futai et al., 2007; Goda, 2002; Graf et al., 2004; Kurusu et al., 2008; Linhoff et al., 2009; Nam and Chen, 2005; Okuda et al., 2007; Rougon and Hobert, 2003; Scheiffele et al., 2000; Takeda et al., 2001; Varoqueaux et al., 2006; Walsh and Doherty, 1997).

The *C. elegans casy-1* is an ortholog of mammalian Calsyntenin genes. Calsyntenins are type-I transmembrane proteins characterized by the presence of two cadherin-like tandem repeats, an LG/LNS domain in the extracellular region and an intracellular region that carries two kinesin light-chain binding domains (Hintsch et al., 2002; Konecna et al., 2006). All these regions are conserved in the three human Calsyntenin genes; *clstn1*, *clstn2* and *clstn3* as well as in the sole *C. elegans* Calsyntenin ortholog, *casy-1*. Despite considerable studies that have allowed us to understand the functions of Calsyntenins, we are still far from elucidating the molecular and physiological underpinnings of this molecule.

In this study, we propose a role for CASY-1 in regulating GABAergic SV precursor transport and hence maintaining normal GABAergic neurotransmission at the *C. elegans* neuromuscular junction (NMJ). We show that the two shorter isoforms of CASY-1, that only have the conserved C-terminal region and lack the entire extracellular N-terminal region, function in the GABAergic motor neurons to regulate GABA release by mediating the trafficking of GABA synaptic vesicle (SV) precursors via their interaction with the UNC-104/KIF1A motor protein.

Results:

3.1. *casy-1* mutants are hypersensitive to Aldicarb

The *C. elegans* NMJ is widely used as a model to understand various aspects of synapse development and function. The NMJ consists of body-wall muscles that receive synaptic inputs from both excitatory cholinergic and inhibitory GABAergic pre-synaptic neurons. An intricate balance between the excitatory and inhibitory signaling is responsible for the sinusoidal locomotion in *C. elegans* and any defect in this balance could result in altered synaptic function (reviewed in (Richmond, 2005; Seifert et al., 2006)).

Previously an RNAi screen was performed using the acetylcholine esterase inhibitor, Aldicarb, to identify the function of cell adhesion molecules at the *C. elegans* NMJ ((Babu et al., 2011) and Babu K. and Kaplan J.M.; unpublished data). The presence of Aldicarb causes acute paralysis due to accumulation of acetylcholine at the NMJ. Loss of function of genes that are necessary for synaptic function could cause either increased resistance or hypersensitivity to Aldicarb(Mahoney et al., 2006; Miller et al., 1996; Sieburth et al., 2005; Vashlishan et al., 2008). RNAi against the *casy-1* gene showed hypersensitivity to Aldicarb in this screen. To validate the results of RNAi screening, two different mutant alleles of *casy-1*, *tm718* and *hd41*, were obtained. Both alleles are putative null alleles as they carry deletions that start in the N- terminal region and result in frame-shift mutations (Ikeda et al., 2008) (illustrated in Figure 3.1A). Both *casy-1(tm718)* and *casy-1(hd41)* showed significant hypersensitivity to Aldicarb suggesting neuromuscular signaling defects in these mutants (Figure 3.1B). All further experiments were performed using the *casy-1(tm718)* mutant allele.

The *casy-1* locus in *C. elegans* is predicted to encode three isoforms based on EST evidence (Reboul et al., 2001). CASY-1A, a 984 residue full-length protein contains all the conserved domains of mammalian Calsyntenins (illustrated in Figure 3.1A). CASY-1B and CASY-1C are truncated proteins encoding 167 and 160 residues respectively and lack most of the N- terminal of the Calsyntenin gene (illustrated in the top right of Figure 3.1D). Real-time qPCR experiments revealed that all three isoforms of *casy-1* are significantly reduced in the *casy-1(tm718)* mutant allele (Figure 3.1C).

Analysis of the *casy-1* genomic locus revealed that *casy-1b* and *casy-1c* could be expressed by their own internal promoters that lie in the intronic region of *casy-1a*.

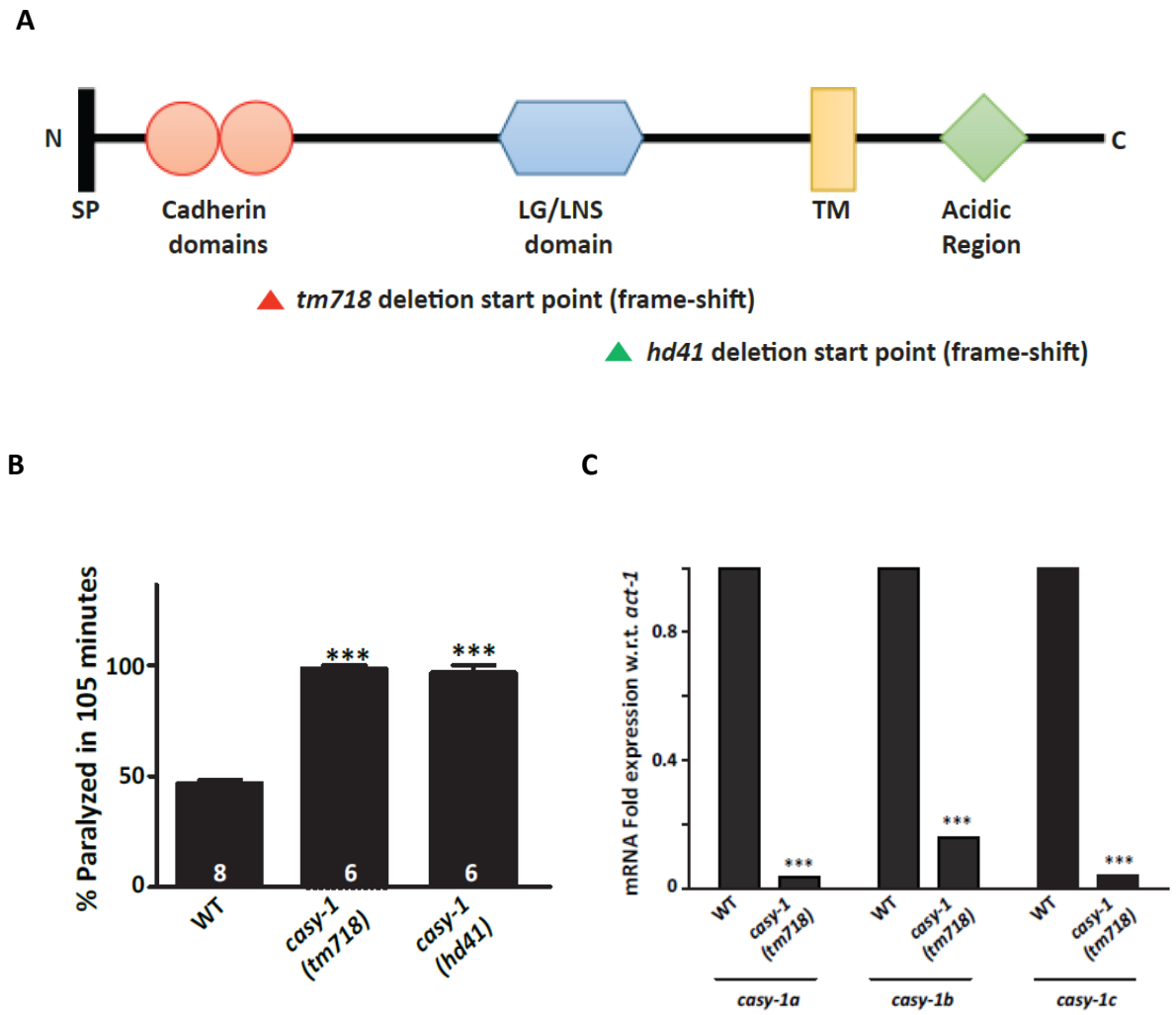


Figure 3.1a: Mutants in *casy-1* are hypersensitive to Aldicarb. (A) A schematic representation of the CASY-1 protein showing the N-terminal signal peptide (SP), two-tandem cadherin repeats, LG/LNS domain, transmembrane region (TM) and cytosolic acidic region. The domains deleted in *casy-1* mutants are indicated as triangles. The *tm718* and *hd41* alleles are putative null alleles as deletion starts in the N-terminal region and results in frame-shift. (B) Aldicarb-induced paralysis in *casy-1* mutants was compared to wild-type (WT) animals. Both *casy-1* mutant alleles (*tm718* and *hd41*) are hypersensitive in the Aldicarb assays. Assays were done at least 6 times (~20 *C. elegans*/assay) as indicated for each genotype. Data are represented as mean ± S.E.M. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (C) Expression levels of *casy-1* isoforms; *casy-1a*, *casy-1b* and *casy-1c* in wild-type (WT) and *casy-1(tm718)* mutant background in a mixed stage population of *C. elegans*. Fold change mRNA levels are indicated after normalization with *act-1* levels, which served as an internal control. Real time PCR was done twice from two independent samples and the data was analyzed using regular two-way ANOVA in GraphPad Prism V7 software.

Choi *et al.* have proposed two pre-requisites to function as an internal promoter both of which are fulfilled for the shorter isoforms of *casy-1* (Figure 3.1D). First, the introns acting as internal promoters should be significantly longer than the other introns. In the *casy-1a* locus, the 8th intron which could be acting as the internal promoter for *casy-1b* and *casy-1c* is significantly longer than the other introns (approximately 4 kb in size vs 1.5kb for other introns). And second, the internal SL1-coupled introns are more prone to function as independent promoters to produce protein isoforms (Choi and Newman, 2006). The 8th intron in *casy-1a* carries an internal SL1 acceptor leader sequence. To further support our hypothesis, we analyzed the sequence of the *casy-1* gene in closely related nematode, *Caenorhabditis briggsae*. *C. briggsae* has just one *casy-1* isoform reported, which is 100% identical in the coding region to the *casy-1a* isoform in *C. elegans* (www.wormbase.org). It carries a 7th intron, which is significantly longer than the other introns (~3700bp). We also did a pairwise sequence alignment for these introns in the two *Caenorhabditis* species using NBLAST (NCBI) and found close to 79% identity in the two sequences, whereas intronic sequences normally show less than 20% identity (Shabalina and Kondrashov, 1999). This strongly suggests conservation of elements in these intronic sequences. To validate the functionality of the *casy-1a*, *casy-1b* and *casy-1c* promoters we generated translational fusion lines in which *casy-1* isoforms were expressed under their own specific promoters (3 kb directly upstream of the isoform's initiator methionine) that were used in rescue experiments (Figure 3.1D).

An isoform-specific rescue of the *casy-1* mutant phenotype was then performed using each isoform with their native promoters (Figure 3.1D and E). Surprisingly, all three isoforms could fully rescue the Aldicarb hypersensitivity of the *casy-1* mutants (Figure 3.1D) suggesting that (1) the *casy-1* isoforms are each expressed by their own specific promoters and (2) all three isoforms could be functioning to regulate synaptic transmission at the NMJ.

The *casy-1a* transcriptional reporter has previously been shown to express throughout the nervous system in *C. elegans* (Ikeda et al., 2008). We also observed strong expression of *casy-1a* in a lot of head neurons including the amphid sensory neurons, the ventral nerve cord and some tail neurons. Expression was also observed in the somatic tissues like gonadal sheath and intestine (Figure 3.1F). To examine the expression pattern of *casy-1b* and *casy-1c,* transcriptional reporter lines expressing

NLS-GFP under isoform-specific promoters were generated. Compared to *casy-1a,* which is highly enriched in the head and tail neurons, *casy-1b* and *casy-1c* show a more limited expression in the head neurons. However, both *casy-1b* and *casy-1c* are strongly expressed in the ventral nerve cord motor neurons, a pattern not seen with *casy-1a*. No expression was observed in the gonadal sheath for the shorter isoforms (Figure 3.1F).

3.2. CASY-1 is not required for neuronal development

The mammalian orthologs of CASY-1 have been shown to regulate various aspects of neuronal development (Pettem et al., 2013; Ponomareva et al., 2014; Ster et al., 2014). Since defects in the synthesis or release of either excitatory (acetylcholine) or inhibitory (GABA) neurotransmitter could result in Aldicarb hypersensitivity (Vashlishan et al., 2008), we speculated that defects in cholinergic or GABAergic neuronal development could explain the Aldicarb hypersensitivity in *casy-1* mutants. To examine this, transgenic lines that express soluble markers driven by acetylcholine (ACh; *unc-17*) or GABA (*unc-25*) neuron-specific promoters were used. These transgenes were introduced in the *casy-1* mutant background. Analysis of the imaging data revealed no gross morphological defects in GABAergic or cholinergic motor neurons in *casy-1* mutants (Figure 3.2A).

To further validate that CASY-1 is not essential for neuronal developmental functions, a transgenic line that expresses the CASY-1A isoform conditionally under a heat shock promoter *hsp16.2* was used. Aldicarb hypersensitivity of *casy-1* mutants was completely rescued, and the *C. elegans* became resistant to Aldicarb when CASY-1A was expressed transiently in the mature nervous system (Figure 3.2B). These results further elaborate on the role of CASY-1 in regulating synaptic function in a mature nervous system rather than in neuronal development.

Since all three CASY-1 isoforms can completely rescue the Aldicarb hypersensitivity of *casy-1* mutants (Figure 3.1D), we decided to determine the region that is required to regulate synaptic transmission at the NMJ. Two transgenic lines were utilized in which either the entire extracellular N-terminal region [CASY-1A (ΔN)] or the entire intracellular C-terminal region [CASY-1A (ΔC)] were removed (Ikeda et al., 2008). Both constructs were expressed under the *ins-1* promoter in the ventral cord motor neurons. Transgenes lacking the N-terminal domains could rescue the Aldicarb hypersensitivity of *casy-1* mutants but the transgene lacking the C-

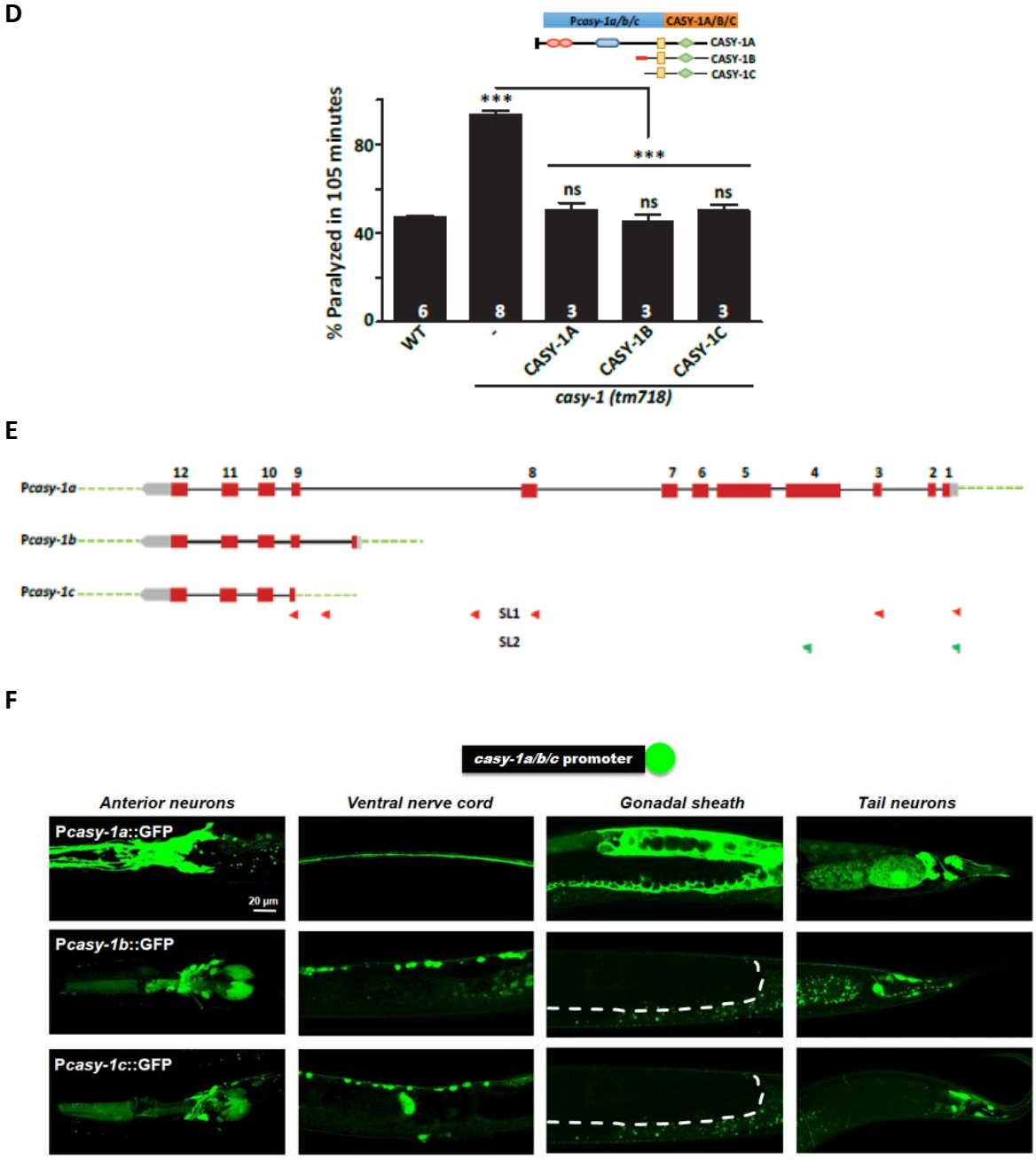


Figure 3.1b: Mutants in *casy-1* are hypersensitive to Aldicarb. (D) Expression of *casy-1* isoforms under their endogenous promoters completely rescues the Aldicarb hypersensitivity of *casy-1* mutant animals. Number of assays (~20 *C. elegans*/assay) is indicated for each genotype. Data are represented as mean ± S.E.M. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (E) Pictorial representation of the genomic locus of three isoforms. CASY-1B and CASY-1C are expressed by alternative promoters that exist in between the 8th and 9th intron of CASY-1A isoform, which is unusually long (~4000bp) and carries their own SL1 leader sequences. (F) Representative confocal images of transcriptional reporters of the three *casy-1* isoforms. Expression of GFP under isoform-specific promoters showed expression of *casy-1a* in most of the head neurons including amphid sensory neurons, in ventral nerve cord, some tail neurons, in the intestine as well as in the gonadal sheath. *casy-1b* and *casy-1c* also showed expression in some head neurons, in the ventral cord motor neurons and some tail neurons but no expression in the gonadal sheath. Dotted lines indicate the position of gonadal sheath.

terminal region could not rescue the Aldicarb defects (Figure 3.2C). These results indicate that the extracellular domains are dispensable for the synaptic function of CASY-1 in motor neurons. The requirement of just the cytoplasmic C-terminal region for regulating synaptic function at the NMJ further explains how all three CASY-1 isoforms, which are structurally different can rescue the Aldicarb defect of *casy-1* mutants. Since the entire C-terminal is conserved in all three CASY-1 isoforms (Figure 3.2D) it is conceivable that they utilize this region for their synaptic function when expressed in motor neurons.

3.3. CASY-1 regulates synaptic transmission in GABAergic motor neurons

To identify specific neurons where CASY-1 might be functioning to regulate synaptic transmission, all isoforms of CASY-1 were expressed under cholinergic (*unc-17*) or GABAergic (*unc-25*) neuron-specific promoters. All three isoforms completely rescued the Aldicarb hypersensitivity of *casy-1* mutants when expressed in GABAergic neurons but not in cholinergic neurons (Figure 3.3A). These results indicate that CASY-1 might be regulating synaptic transmission in GABA motor neurons at the NMJ. Although expression of CASY-1 isoforms was not observed in the body-wall muscle, however, to remove the possibility of any retrograde signaling from muscles effecting neuronal behavior at the NMJ, a transgenic line expressing CASY-1A under a body-wall muscle specific promoter (*myo-3*) was generated. Expression of CASY-1A in muscles failed to rescue the Aldicarb hypersensitivity of *casy-1* mutants (Figure 3.3A).

Previous experiments (Figure 3.2C) show that the C-terminal of CASY-1 is sufficient to rescue the Aldicarb hypersensitivity of *casy-1* mutants. To further strengthen our hypothesis, a transgenic line CASY-1A (ΔC) was generated, in which the entire C- terminal (880-984 aa) was removed from CASY-1A and expressed under the GABA-specific promoter (*unc-25*). This line failed to rescue the Aldicarb defects of *casy-1* mutants (Figure 3.3B), further signifying the importance of C-terminal of CASY-1 at the NMJ.

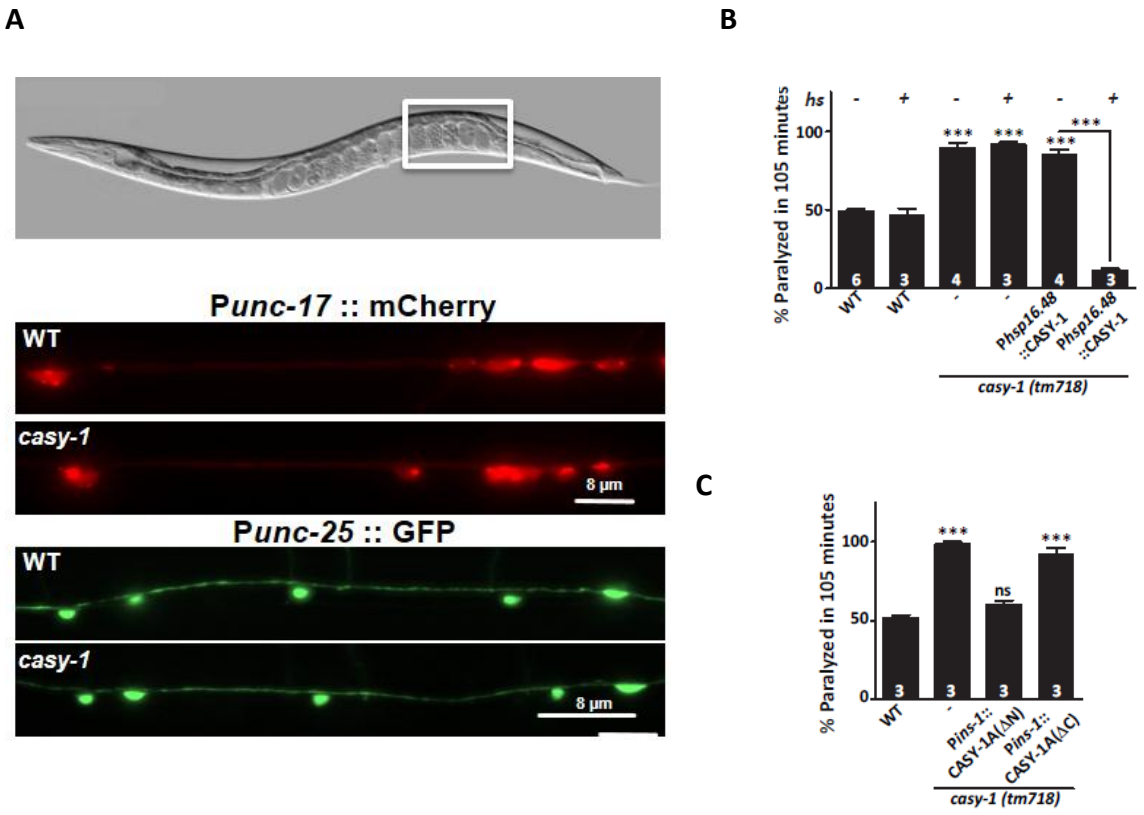
To further investigate the role of GABA signaling in *casy-1* mutants, an assay using the drug Pentylenetetrazole (PTZ), a potent antagonist of $GABA_A$ receptors (Rocha et al., 1996), was performed. PTZ has been shown to be effective in *C. elegans* for generating a convulsive phenotype called ‘head bobs’, which is used as an

indicator of reduced GABAergic synaptic-transmission (Williams et al., 2004). Mutants in *unc-25*, a GABA-synthesizing enzyme, were used as positive controls (Figure 3.3C). The *casy-1* mutant animals showed a strong convulsion phenotype after 30-minute exposure to 10mg/ml PTZ and by the end of 60 minutes, a significant fraction of *casy-1* mutants showed convulsions (Figure 3.3C). Additionally, *casy-1* mutants also showed a tail shrinking phenotype, a characteristic of GABA mutants in *C. elegans* (Schuske et al., 2004) (Movies 3.1-3.3). The convulsive phenotype of *casy-1* mutants was completely rescued by expressing the CASY-1 isoforms in GABAergic neurons but not in cholinergic neurons or muscle (Figure 3.3C) further implicating CASY-1 in GABA signaling.

Since *casy-1* mutants do not display any developmental defects, Aldicarb hypersensitivity could be due to several reasons (Figure 3.3D). Signaling defects at *casy-1* NMJ could be presynaptic or postsynaptic. Presynaptic defects could be because of increased acetylcholine release or decreased GABA release and postsynaptic defects could occur due to increased expression of cholinergic receptors or decreased expression of GABA receptors. In all cases, the excitation to inhibition ratio will increase, resulting in Aldicarb hypersensitivity. Each of these possibilities was investigated.

Reduced GABAergic synaptic transmission could be either due to reduced release of GABA from the GABAergic motor neurons (presynaptic) or reduced response of muscle to GABA due to lower expression of $GABA_A$ receptors on the muscle (postsynaptic). To differentiate between these two possibilities, presynaptic and postsynaptic markers were analyzed in the *casy-1* mutant background.

Initially the expression of postsynaptic markers under cholinergic and GABAergic neuron specific promoters was investigated. The *C. elegans* body muscles express two classes of Acetylcholine receptors (AChRs); nicotine-sensitive (N-AChRs) and levamisole-sensitive (L-AChRs) receptors as well as a single class of $GABA_A$ receptors. There was no significant change in the fluorescent intensity of the GFP-tagged N-AChRs subunit ACR-16 or the L-AChRs subunit, UNC-29 (Figure 3.3E) in the *casy-1* mutants. The $GABA_A$ receptor, UNC-49 also showed similar levels of expression and localization in *casy-1* mutants when compared to WT animals (Figure 3.3E).



D

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CASY-1a         AQPDPIQFNFNSGETALDSLELIERHFEPAFDQLGSSRLQNILEMDLPRPKALLSHHGYD 840
CASY-1b         ------------------------------------MFVNILEMDLPRPKALLSHHGYD 23
CASY-1c         ----------------------------------------MDLPRPKALLSHHGYD 16
                                                        ****************

CASY-1a         VGQGAIAGGAVAVVVVVCVGFLLVLLVIGVLKMRDTPMPRRRRQKRQSDGGMHWDDSGMN 900
CASY-1b         VGQGAIAGGAVAVVVVVCVGFLLVLLVIGVLKMRDTPMPRRRRQKRQSDGGMHWDDSGMN 83
CASY-1c         VGQGAIAGGAVAVVVVVCVGFLLVLLVIGVLKMRDTPMPRRRRQKRQSDGGMHWDDSGMN 76
                ************************************************************

CASY-1a         ITVNPLDDVEKNGGAIDEFSDEEEEEETDGESECSYRDEEDDVSEDEEDQTEVLPHLDAN 960
CASY-1b         ITVNPLDDVEKNGGAIDEFSDEEEEEETDGESECSYRDEEDDVSEDEEDQTEVLPHLDAN 143
CASY-1c         ITVNPLDDVEKNGGAIDEFSDEEEEEETDGESECSYRDEEDDVSEDEEDQTEVLPHLDAN 136
                ************************************************************

CASY-1a         QRVVGGLEWDDEDAISTNARSYRV 984
CASY-1b         QRVVGGLEWDDEDAISTNARSYRV 167
CASY-1c         QRVVGGLEWDDEDAISTNARSYRV 160
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Transmembrane domain

Acidic region

Figure 3.2: Neuronal development is normal in *casy-1* mutants. (A) Representative fluorescent images of WT and *casy-1* mutant animals expressing GFP in all cholinergic neurons (*nuIs321* [P*unc-17*::mCherry]) and GABAergic neurons (*juIs76* [P*unc-25*::GFP]). The number of cell bodies and axonal commissures were largely normal in *casy-1* mutant animals (n-25) analyzed. Box in the DIC image displays the region imaged. (B) CASY-1 functions in the mature nervous system to regulate synaptic transmission at the NMJ. Expression of CASY-1A isoform just three hours before the assay using a heat- shock promoter completely rescues the Aldicarb hypersensitivity phenotype seen in *casy-1* mutant animals. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (C) The C-terminal of the CASY-1A isoforms is required to regulate synaptic transmission at NMJ. Transgenic lines expressing either the CASY-1A N-terminal (ΔC) or C-terminal (ΔN) alone expressed under *ins-1* promoter suggest that the CASY-1A C- terminal is sufficient to rescue the Aldicarb hypersensitivity in *casy-1* mutants. Assays were done 3 times as indicated in Figures (~20 *C. elegans*/ assay). Data are represented as mean ± S.E.M. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (D) Clustal omega alignment of the C-terminal of three *casy-1* isoforms. All the isoforms are identical in their C-terminal. Purple and green sequences denote the transmembrane domain and the acidic region in CASY-1 respectively. Red denotes the six amino acids that are present in CASY-1B but not in CASY-1C.

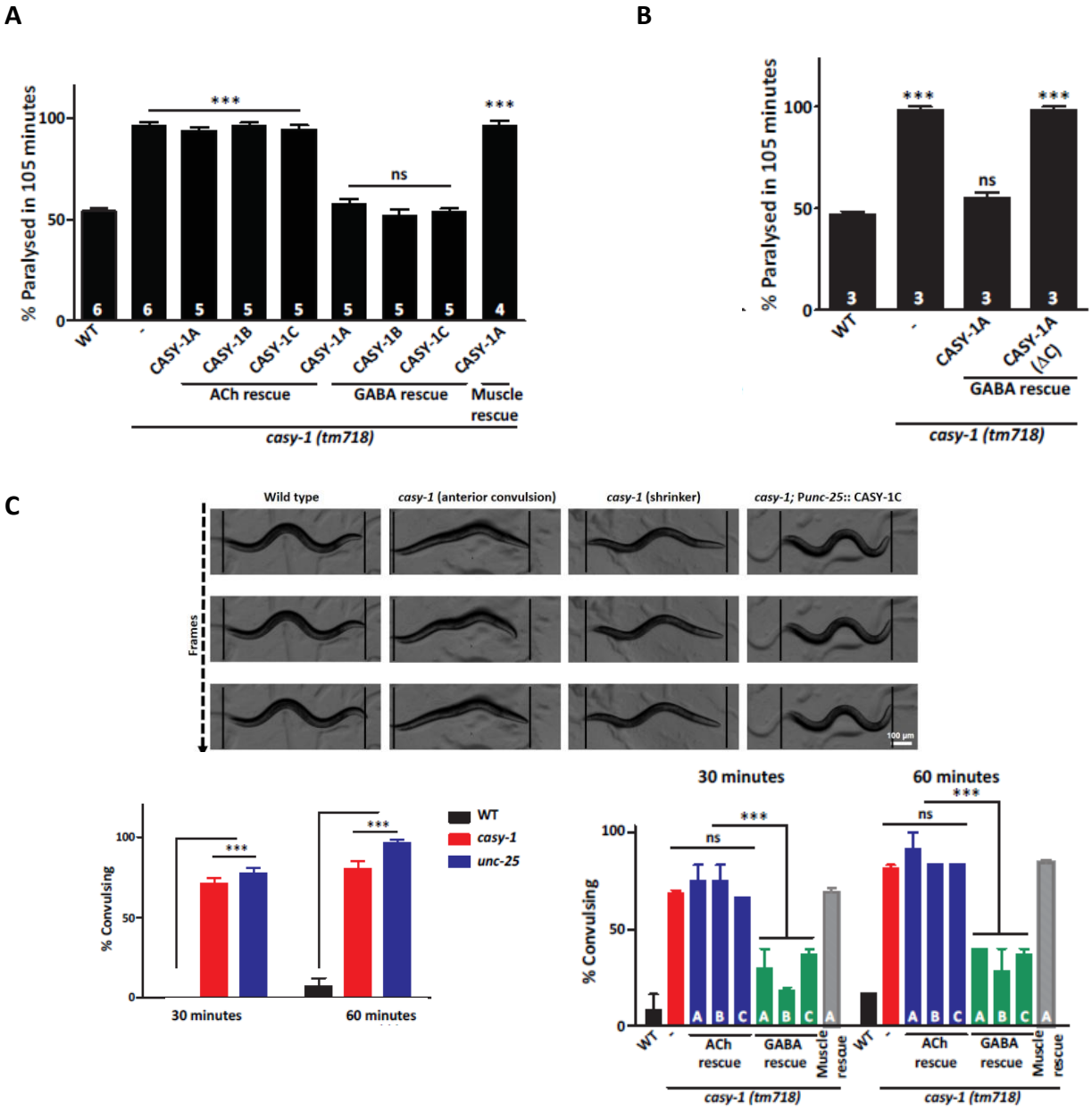


Figure 3.3a: The *casy-1* isoforms functions in GABAergic neurons to regulate synaptic transmission. (A) Expression of CASY-1 isoforms using tissue specific promoters; ACh neurons (P*unc-17*), GABA neurons (P*unc-25*) and muscle (Pmyo-3) establishes that expressing CASY-1 in GABAergic neurons completely rescues the Aldicarb hypersensitivity in *casy-1* mutants. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (B) The C-terminal of the CASY-1A isoform functions in GABAergic neurons to regulate synaptic function. The CASY-1A isoform expressed under GABAergic promoter completely rescues the Aldicarb hypersensitivity in *casy-1* mutants. However, removing the entire C-terminal from the CASY-1A [CASY-1A (ΔC)] isoform does not rescue the Aldicarb hypersensitivity in *casy-1* mutants. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (C) The *casy-1* mutants show higher sensitivity to GABA receptor antagonist PTZ than the WT animals. Representative still frame images demonstrating *casy-1* mutant *C. elegans* with anterior convulsions and tail shrinker phenotype. The still frame images are representative frames from movies (7 frames/second). Mutants in *unc-25*, the GABA synthesis enzyme were used as positive controls. The graph shows the fraction of animals showing anterior 'head bobs' after 30 minute and 60-minute exposure to 10 mg/ml PTZ. The sensitivity to PTZ could be fully rescued by expressing all *casy-1* isoforms in GABAergic neurons but not in cholinergic neurons or muscle. Assays were done (~10 *C. elegans*/assay) at least thrice. Values that differ significantly from WT animals are indicated (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). Data are represented as mean ± S.E.M.

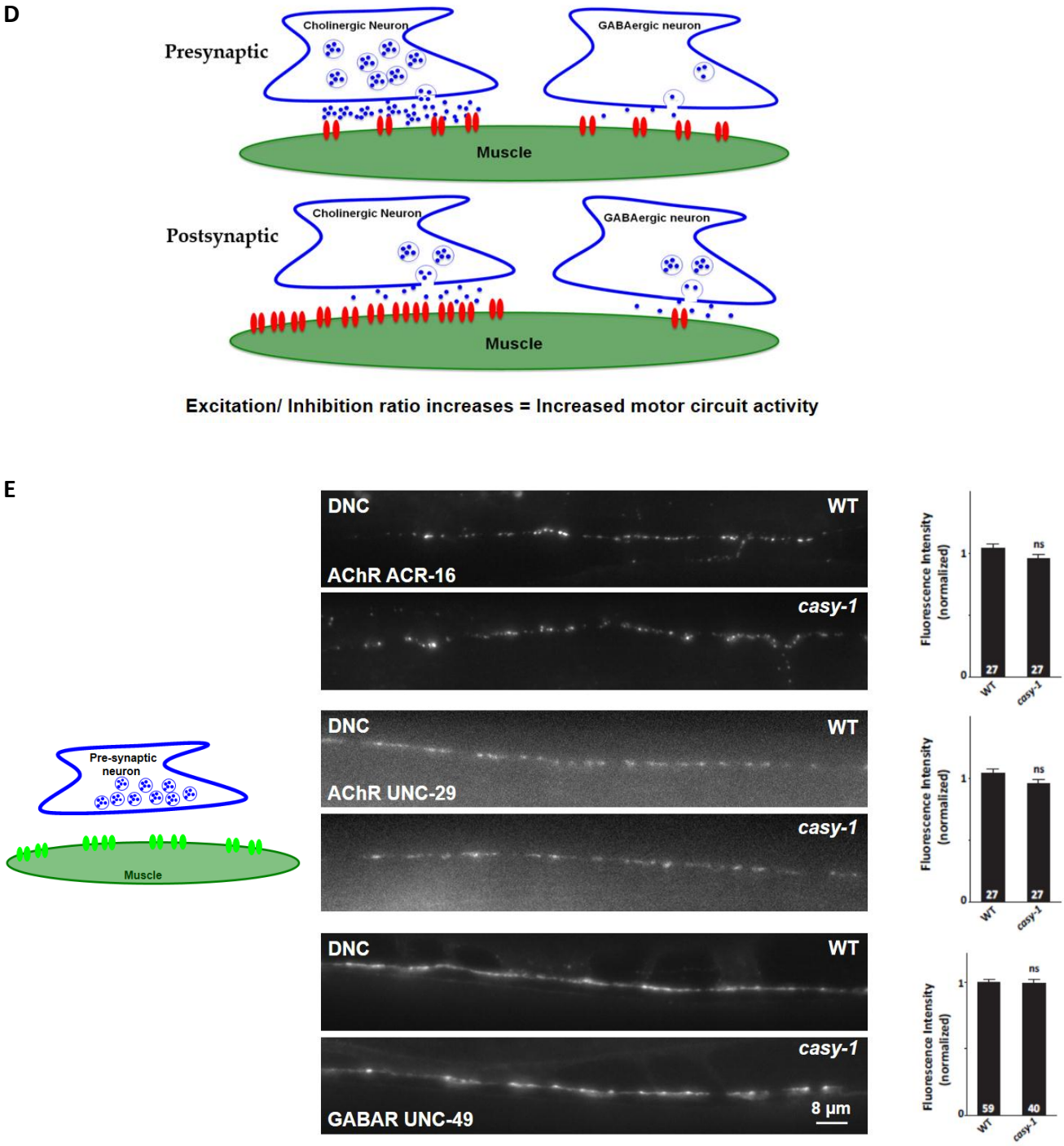


Figure 3.3b: The *casy-1* isoforms functions in GABAergic neurons to regulate synaptic transmission. (D) Schematic representing the possible reasons for hypersensitivity in Aldicarb assay. Hypersensitivity to Aldicarb could be due to pre-synaptic defects (increased acetylcholine release or decreased GABA release) or due to post-synaptic defects (increased expression of cholinergic receptors or decreased expression of GABA receptors). (E) Representative fluorescent images of cholinergic: N-type [*nuIs299* (P*myo-3*::ACR-16::GFP)] or L-type [*akIs38* (P*myo-3*::UNC-29::GFP)] and GABAergic [*nuIs283* (P*myo-3*::UNC-49::GFP)] receptors in the muscle of WT and *casy-1* mutants. The expression of receptors is unaltered in *casy-1* mutants. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test, "ns" indicates not significant in all figures.

Next, several different presynaptic markers were analyzed. The synapse density in the dorsal cord was examined by quantifying SYD-2 puncta, an ortholog of the mammalian active zone protein α-Liprin. The density of cholinergic and GABAergic synapses was largely normal, indicating that synapse development is unaffected in *casy-1* mutants (Figure 3.3F). The integrity of the actin cytoskeleton was subsequently determined by examining Gelsolin levels in the cholinergic and GABAergic motor neurons. Gelsolin is an actin-binding protein that sever, cap and nucleate actin filaments in a calcium dependent-manner (McGough et al., 2003). Analysis of Gelsolin levels revealed that the cholinergic or GABAergic actin cytoskeleton is not disturbed in the *casy-1* mutants (Figure 3.3G).

Next, GFP –tagged SNB-1, a *C. elegans* ortholog of the mammalian SV protein synaptobrevin was analyzed. Synaptobrevin localizes in a punctate pattern along the dorsal cord of *C. elegans*. Mutants in *casy-1* showed a subtle but significant decrease in GABAergic::SNB-1::GFP levels, suggesting less GABA vesicles at the synapse (Figure 3.3H). Fluorescence intensity of cholinergic synapses was indistinguishable in *casy-1* mutants from wild-type (WT) animals, suggesting normal cholinergic signaling (Figure 3.3H).

To further ensure that post-synaptic $GABA_A$ receptors functioned normally in the mutants, a GABA agonist Muscimol was utilized. *C. elegans* placed on Muscimol show various degrees of responses depending on their sensitivity to the drug. The most characteristic phenotype is the "rubber band phenotype", wherein the animals contract and relax without displacement following prodding on the head (de la Cruz et al., 2003). If reduced GABAergic signaling in *casy-1* mutants is due to lower release of GABA from the pre-synaptic terminal, no difference in the severity of the rubber band phenotype would be observed following incubation with Muscimol. When *casy-1* mutants were tested in the Muscimol assay they behaved like WT *C. elegans* (Figure 3.3I) again strengthening our hypothesis that CASY-1 is functioning pre-synaptically at NMJ. To further ensure that cholinergic transmission is normal in *casy-1* mutants, we performed a Levamisole assay. Levamisole is an agonist for L-type cholinergic receptors and exposure to Levamisole results in a time-course induced paralysis in WT animals. Mutants in *casy-1* showed a response similar to WT animals in the Levamisole assays suggesting normal L-AChR signaling at NMJ (Figure 3.3J).

3.4. Mutants in *casy-1* have reduced GABAergic neurotransmission at the NMJ

The *C. elegans* nervous system consists of 26 GABAergic neurons which include six DD and thirteen VD neurons that innervate the dorsal and ventral body-wall muscles respectively, four RME motor neurons that innervate head muscles, the AVL and DVB neuron that are pre-synaptic to the enteric muscles and RIS which is an interneuron (Schuske et al., 2004; White et al., 1986). To confirm that CASY-1 functions specifically in GABA motor neurons (D- type neurons), a transgenic line expressing the CASY-1C isoform specifically in DD and VD class of GABA motor neurons using the *unc-30* promoter that also expresses in some non-GABAergic neurons was generated. Expression of CASY-1C in just D-type motor neurons completely rescued the Aldicarb hypersensitivity of *casy-1* mutants, confirming that CASY-1 is functioning in motor neurons to regulate synaptic transmission at NMJ (Figure 3.4A).

To further explore the function of *casy-1* in GABA neurotransmission, we utilized an optogenetic tool, where channelrhodopsin (ChR2), a light-gated cation channel that allows non-specific flow of cations into the cell leading to electrical excitation, was used (Nagel et al., 2003). A transgenic line that expresses ChR2 specifically in GABAergic neurons was utilized. The light-based activation of GABA motor neurons leads to a measurable change in body length (relaxation). Activation of GABAergic ChR2 results in a reduced relaxation in *casy-1* mutants in comparison to WT controls. These results suggest a lower release of GABA from the pre-synaptic termini in *casy-1* mutants, which in turn results in less relaxation upon optogenetic stimulation. Expressing the CASY-1C isoform in GABAergic neurons completely rescues the decreased relaxation defect of *casy-1* mutants (Figure 3.4B).

To evaluate changes in endogenous synaptic transmission, whole-cell patch clamp recordings of the muscles under voltage-clamp conditions from dissected *C. elegans* were analyzed. Endogenous excitatory and inhibitory post-synaptic currents (EPSCs and IPSCs) that indicate the frequency and amplitude of neurotransmitter release from cholinergic and GABAergic motor neurons onto the body-wall muscle were measured in the WT and *casy-1* mutant animals. The *casy-1* mutants had a significantly lower endogenous IPSC rate compared to WT animals (Figure 3.4C). The amplitude of IPSCs was unaltered. Collectively, these results indicate that release of GABA is reduced in *casy-1* mutants while the muscle responsiveness to GABA remained

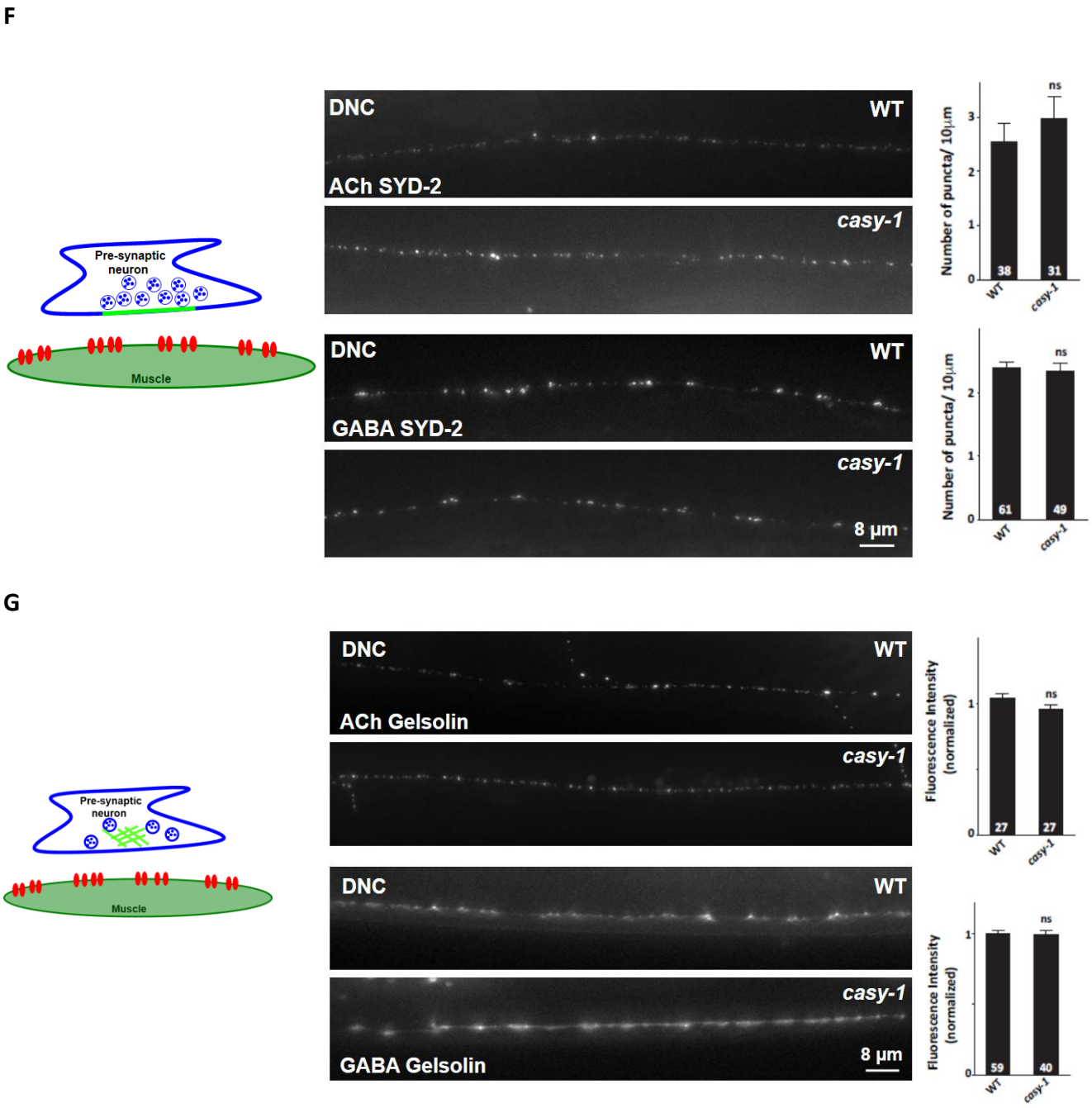


Figure 3.3c: The *casy-1* isoforms functions in GABAergic neurons to regulate synaptic transmission. (F) Representative fluorescent images of cholinergic [*nuIs160* (P*unc-129*::SYD-2::GFP)] or GABAergic [*hpIs3* (P*unc-25*::SYD-2::GFP)] synapses in the dorsal cord of WT and *casy-1* mutant *C. elegans*. The density of cholinergic and GABAergic synapses was largely normal in the mutants, indicating normal synapse development. (G) Representative fluorescent images of cholinergic [*nuIs169* (P*unc-129*::GELSOLIN::VENUS)] or (b) GABAergic [(P*unc-25*:: GELSOLIN::VENUS)] synapses in the dorsal cord of WT and *casy-1* mutant *C. elegans*. The actin cytoskeleton of cholinergic and GABAergic synapses was largely normal in the mutants. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test, "ns" indicates not significant in all figures.

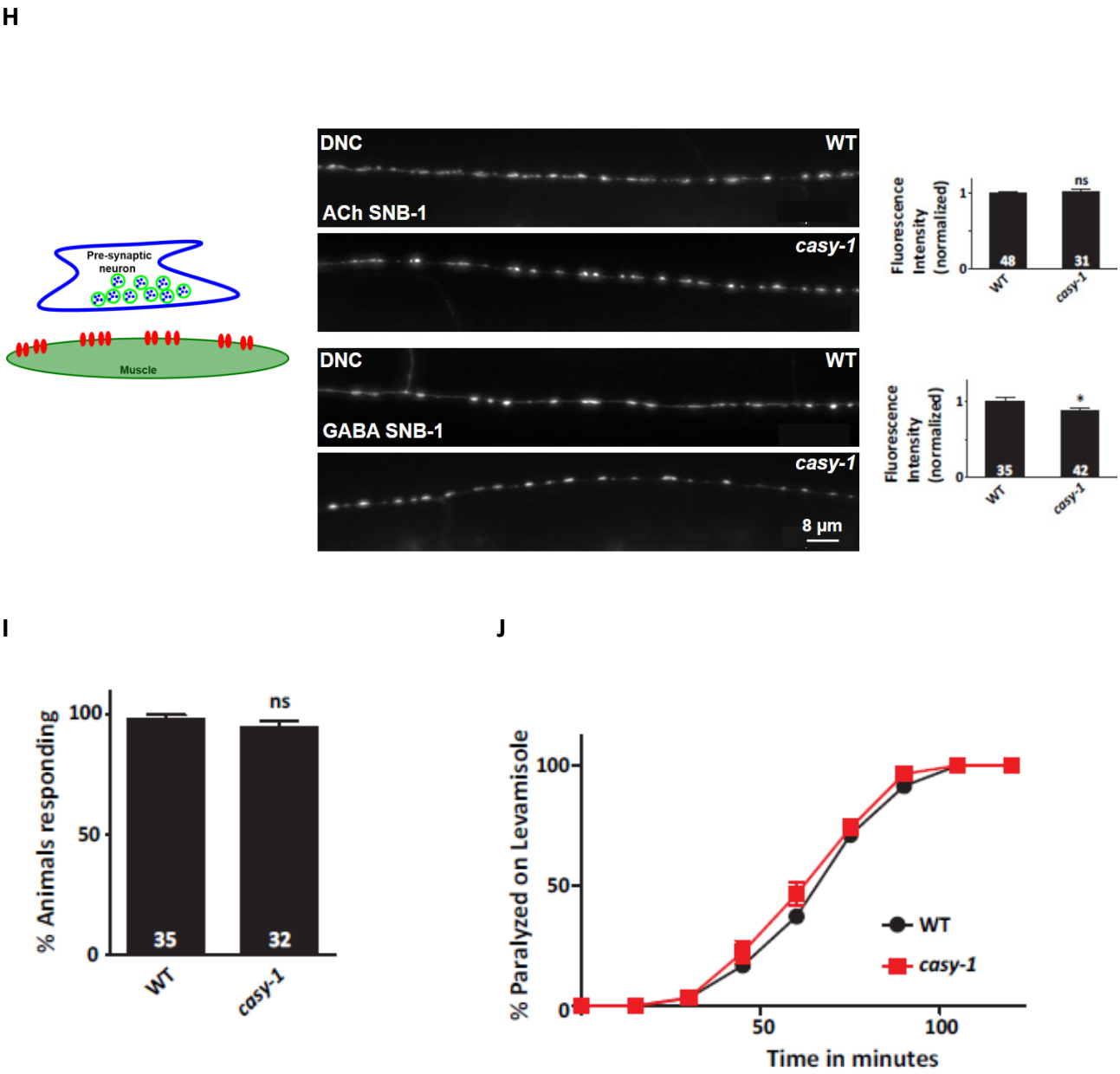


Figure 3.3d: The *casy-1* isoforms functions in GABAergic neurons to regulate synaptic transmission. (H) Representative fluorescent images of cholinergic [*nuIs152* (P*unc-129*::SNB-1::GFP)] or GABAergic [*nuIs376* (P*unc-25*::SNB-1::GFP)] synapses in the dorsal cord of WT or *casy-1* mutant. Cholinergic synapses are largely normal in *casy-1* mutants, while GABAergic synapses showed a subtle but significant decrease in fluorescent intensity when compared to WT animals. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test, *P<0.01 and "ns" indicates not significant in all Figures. (I) *casy-1* mutants show normal muscle response to 0.5 mM Muscimol, a GABA receptor agonist. Assays were done three times and total number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test. (J) *casy-1* mutants show normal muscle response to 0.5 mM Levamisole, a cholinergic receptor agonist. Assays were done three times (~20 *C. elegans*/assay).

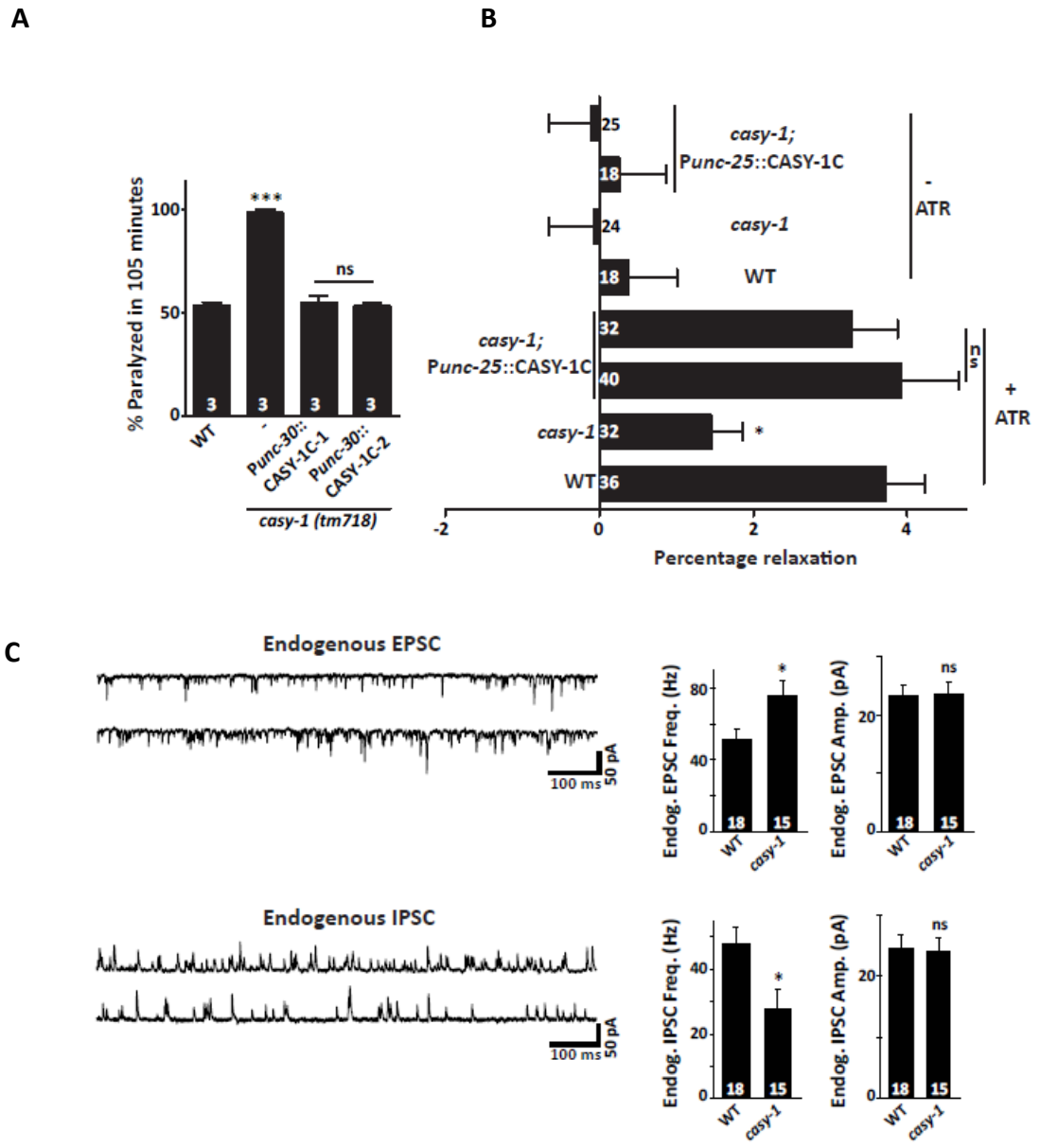


Figure 3.4: *casy-1* mutants show decreased GABAergic synaptic transmission at the NMJ. (A) Expression of the CASY-1C specifically in DD and VD neuronal subtypes using GABAergic motor neuron specific promoter (P*unc-30*) establish that CASY-1 is explicitly functioning in GABAergic motor neurons to regulate synaptic transmission. Assays were done 3 times as indicated in Figures (~20 *C. elegans*/ assay). Data are represented as mean ± S.E.M. (***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (B) Optogenetic stimulation of GABAergic neurons using Channel Rhodopsin (ChR2) [***zxIs3*** (P*unc-47*::ChRX2(H134R)::YFP)] showed that the *casy-1* mutant animals relax significantly less than WT *C. elegans* upon blue light exposure (percent change in body length before and after optogenetic stimulation). Expressing the CASY-1C isoform under a GABAergic neuron specific promoter (P*unc-25*) completely rescues the relaxation defect in *casy-1* mutant animals. Data is shown for two independent rescue lines. The graph shows the percentage change in body length for both +ATR and –ATR controls. The numbers of animals analyzed for each genotype are indicated. Data are represented as mean ± S.E.M. (*P<0.01 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (C) mEPSCs and mIPSCs were recorded from body wall muscles of adult animals for the indicated genotypes. Representative traces of mEPSCs and mIPSCs and summary data for frequency and amplitude are shown. The *casy-1* mutants showed a significant decrease in mIPSCs rate compared to WT *C. elegans*, suggesting a decreased GABAergic neurotransmission at the NMJ. The mIPSC amplitude, however, remains unaltered suggesting normal muscle responsiveness in the mutant. There is a subtle but significant increase in mEPSC frequency in *casy-1* mutants. The number of animals analyzed for each genotype is indicated. Data are represented as mean ± S.E.M. (*P<0.05 using two-tailed Student's t-test).

unaffected. Surprisingly, we found that the endogenous EPSC rate showed a significant increase in *casy-1* mutants when compared to WT animals (Figure 3.4C). The amplitude of EPSCs was again unaffected. Changes in acetylcholine release from cholinergic motor neurons is likely to be secondary, as functional rescue experiments showed that expressing *casy-1* in GABAergic, but not in cholinergic motor neurons, rescue the Aldicarb hypersensitivity of *casy-1* mutants. Thus, it is possible that the increased EPSC frequency in *casy-1* mutants is due to functionality of CASY-1 in some higher levels of neuronal circuits. This will be described in some detail in the next chapter.

3.5. *casy-1* isoforms express in different neuronal subtypes and are targeted to the pre-synaptic termini

Expression of all CASY-1 isoforms in GABAergic neurons completely rescues the Aldicarb hypersensitivity of *casy-1* mutants (Figure 3.4A). Also, *casy-1b* and *casy-1c* transcriptional reporters express in motor neurons (Figure 3.1F). To determine the specific class of motor neurons where *casy-1* isoforms are expressing, isoform specific- GFP transcriptional reporters were co-expressed with cholinergic or GABAergic specific mCherry-tagged reporter lines. The *casy-1a* transcriptional reporter showed no co-localization with either cholinergic or GABAergic motor neurons (Figure 3.5A, first column). However, *casy-1b* and *casy-1c* showed co-localization with both cholinergic and GABAergic neuronal markers (Figure 3.5A, 2nd two columns). This data suggests that the shorter isoforms of *casy-1* are probably functioning in GABA motor neurons to regulate GABA release and hence affect synaptic transmission at the NMJ, however, the role of these isoforms in cholinergic neurons is still unclear.

To examine specific subcellular localization of the shorter *casy-1* isoforms, CASY-1C::mCherry was expressed under its own promoter. The CASY-1C::mCherry protein localizes all along the dorsal cord axons and shows co-localization with both cholinergic and GABAergic pre-synaptic SNB-1::GFP markers (Figure 3.5C). This suggests the presence of CASY-1C at the synapse. The CASY-1C::mCherry also co-localizes with the GABAergic active zone marker protein SYD-2 on the ventral nerve cord (Figure 3.5E). The CASY-1C::mCherry co-localizes with both cholinergic and GABAergic neuronal cell bodies in the ventral nerve cord tagged with SNB-1::GFP (Figure 3.5B). Expression of mCherry and GFP- tagged CASY-1C fully rescued the

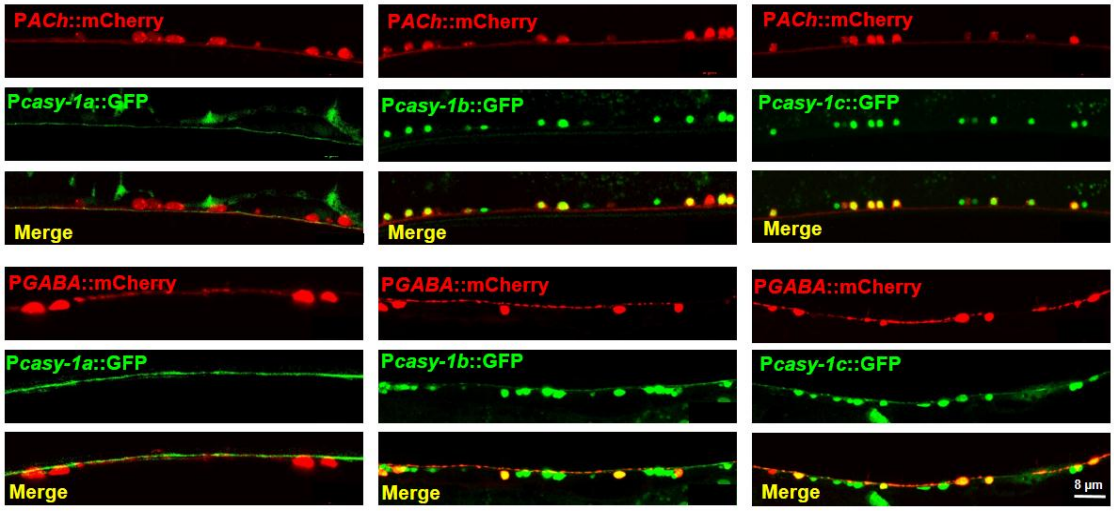
Aldicarb sensitivity of *casy-1* mutants suggesting that the tagged proteins are functional (Figure 3.5D).

The mammalian CLSTNs have been shown to be cleaved at the extracellular region (Araki et al., 2007; Vogt et al., 2001). Further, the CASY-1A isoform has previously been shown to be cleaved in its extracellular region juxtaposed to the membrane by synaptic cleft peptidases, resulting in the release of entire N- terminal into the synaptic cleft (Ikeda et al., 2008) (illustrated in Figure 3.5F, left panel). To figure out if CASY-1B and CASY-1C are also cleaved once they reach the synapse, N- terminal mCherry fusion transgenes of CASY-1B and CASY-1C were generated. The mCherry tagged N-terminal of the shorter CASY-1 isoforms was detected in the coelomocytes (Figure 3.5F; right panels), which are macrophage- like scavenger cells that take up any waste or secreted proteins from the body cavity (reviewed in (Grant and Sato, 2006)). These results allow us to conclude that all CASY-1 isoforms are cleaved at their N-terminal region by synaptic cleft peptidases, resulting in the release of the ectodomain into the body cavity.

3.6. Distribution of the SV protein SNB-1 is altered at GABAergic synapses in *casy-1 mutants*

Reduced SNB-1::GFP levels, less relaxation upon optogenetic stimulation of GABAergic neurons and decreased endogenous IPSC rate (Figure 3.3H and 3.4B and 3.4C) all highlight the role of CASY-1 in regulating GABA release from motor neurons. To further address this role of *casy-1*, a transgenic line in which the luminal domain of synaptobrevin is tagged with superecliptic pHluorin; a GFP reporter expressed specifically in GABAergic neurons (Ernstrom et al., 2012) was utilized. pHluorin is highly pH-sensitive and its fluorescence remains quenched in the acidic environment of the SV lumen, however, there is a dramatic increase in the fluorescence as soon as the vesicle fuses onto the membrane relieving the tag from the acidic environment of the SV (Miesenböck et al., 1998; Sankaranarayanan et al., 2000). To examine if *casy-1* mutants have less GABA vesicles at the synapse, and hence less release of GABA, we monitored the fluorescence intensity of pHluorin at the dorsal cord synapses. pHluorin intensity was significantly reduced in the dorsal cord synapses of *casy-1* mutants further confirming the role of CASY-1 in the release of GABA at NMJ (Figure 3.6A).

A



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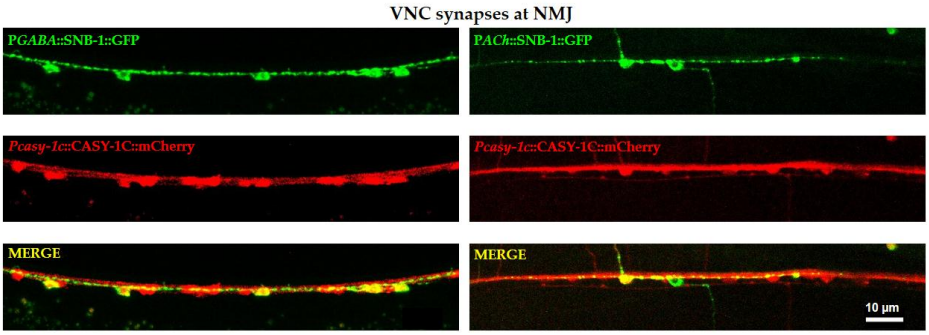

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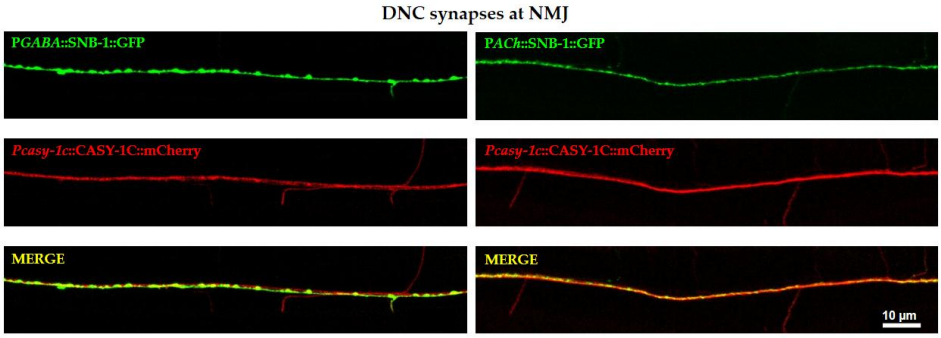

Figure 3.5a: CASY-1 isoforms shows differential spatial localization. (A) Expression of GFP under isoform-specific *casy-1* promoters. *casy-1a* transcriptional reporter does not co-localize with mCherry marked cholinergic or GABAergic motor neurons. *casy-1b* and *casy-1c* reporters show expression in both cholinergic and GABAergic motor neurons. Anterior is to the left in all panels. (B) and (C) Representative fluorescent images of P*casy-1c*::CASY-1C::mCherry translational reporter showing co-localization with the GABAergic *nuIs376* [P*unc-25*::SNB-1::GFP] and cholinergic [*nuIs152* (P*unc-129*:: SNB-1::GFP)] pre-synaptic markers in the ventral (B) or dorsal (C) nerve cords suggesting presence of CASY-1C in the GABAergic and cholinergic cell body as well as at the NMJ pre-synaptic terminal.

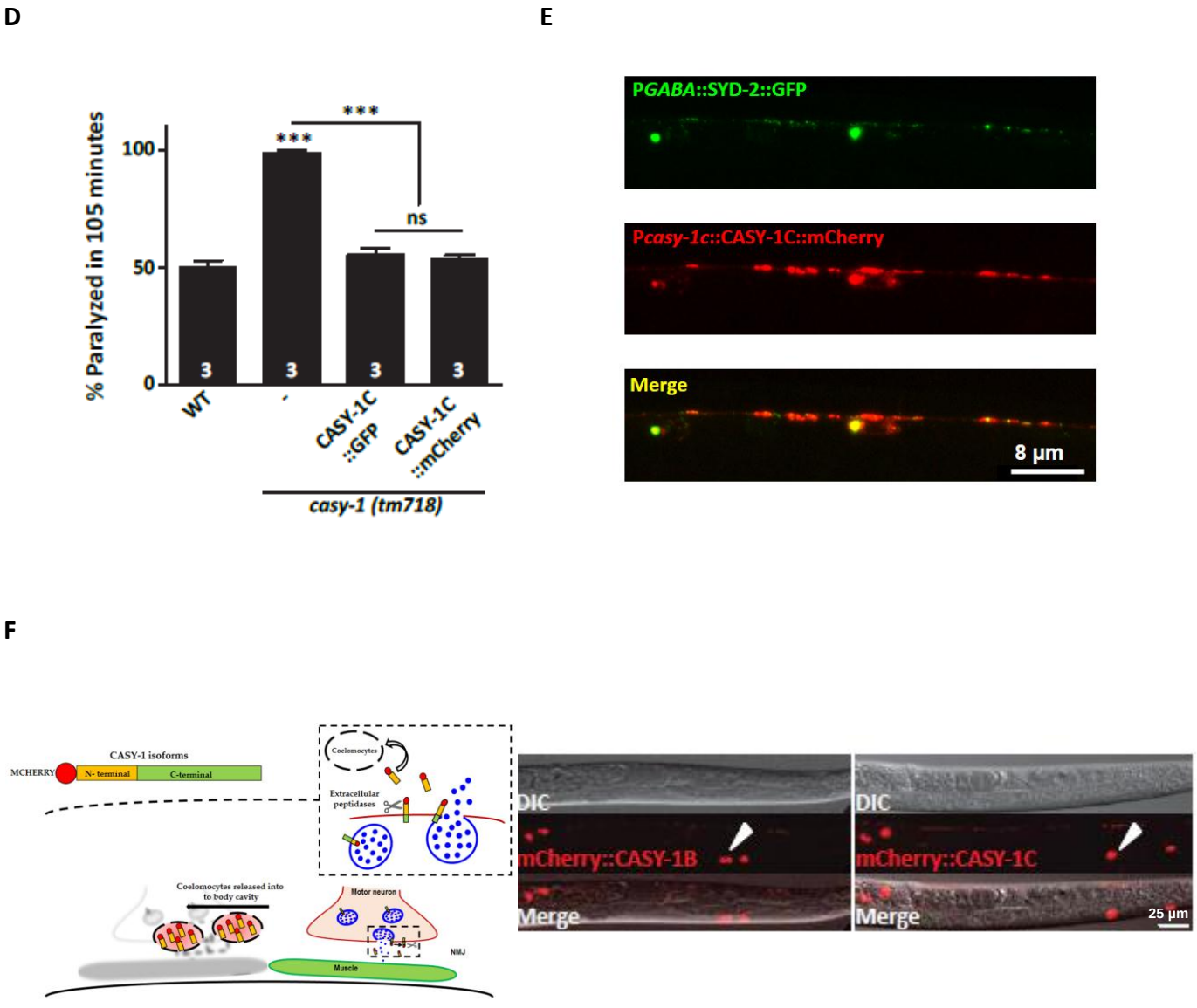

Figure 3.5b: CASY-1 isoforms shows differential spatial localization. (D) Aldicarb-induced paralysis in *casy-1* mutants is completely rescued by expressing GFP or mCherry-tagged CASY-1C expressed under their own promoter, suggesting that the tagged versions of CASY-1C are functional. Assays were done three times as indicated in the figures (~20 *C. elegans*/assay). Data are represented as mean ± S.E.M. "***" indicates P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test and "ns" indicates not significant in comparison to WT. (E) Representative fluorescent images of P*casy-1c*::CASY-1C::mCherry translational reporter showed co-localization with the GABAergic [*hpIs3* (P*unc-25*::SYD-2::GFP)] pre-synaptic markers suggesting presence of CASY-1C at the GABAergic pre-synaptic terminal. (F) Schematics showing the processing of *casy-1b* and *casy-1c* isoforms at the NMJ. *casy-1a* isoform has been shown to be cleaved at its N-terminal by extracellular peptidases resulting in the secretion of entire N- terminal domain (Ikeda et al. 2008). P*casy-1b/c*::mCherry::CASY-1B/C translational reporter shows that CASY-1B and CASY-1C isoforms, which lack a validated signal sequence also reach the synapse where their N- terminal is cleaved resulting in the release of entire N- terminal into the synaptic cleft. Representative fluorescent images showing the release of mCherry tagged N-terminal of CASY-1B and CASY-1C at the synapse followed by their uptake in the coelomocytes (solid arrowheads).

Further, we addressed the possibility of the involvement of CASY-1 in GABA vesicle trafficking by performing fluorescence recovery after photobleaching (FRAP) analysis of SNB-1::GFP levels. The recovery rate of SNB-1::GFP depends upon two factors; transport of new SV precursors at the synapse by motor-mediated trafficking or diffusion from neighboring synapses into the bleached area. The recovery rate of GABAergic SNB-1::GFP was significantly reduced in *casy-1* mutants suggesting that the mobility dynamics of SVs at the NMJ is compromised in the mutants. The recovery rate was completely restored by expressing the CASY-1C isoform specifically in GABAergic neurons (Figure 3.6B). We also monitored the mobility dynamics in cholinergic motor neurons but found no significant change in *casy-1 mutants* compared to WT animals (Figure 3.6B), suggesting that CASY-1 is specifically functioning to regulate SV release in GABA motor neurons.

3.7. CASY-1 regulates the trafficking of GABAergic synaptic precursors along the commissures

In *C. elegans*, the soma of motor neurons are present at the ventral nerve cord which sends out their axons as commissures antero-posteriorly and then dorsally to the dorsal nerve cord (DNC) where synapses are formed with the muscles. To address how *casy-1b/casy-1c*, monitor the trafficking of SV precursors from motor neuron cell bodies to the DNC synapses, the transport characteristics of SNB-1::GFP tagged vesicles along the commissures in live animals using time lapse imaging were examined. We first assayed SV transport in D- type GABAergic motor neurons. In wild type animals, SNB-1:: GFP labeled vesicles move in both anterograde and retrograde direction, but had a bias that was towards anterograde trafficking. However, in *casy-1* mutants, the SV anterograde transport was significantly reduced, although, the velocity of SNB-1::GFP was similar to WT animals. Decrease in anterograde vesicular flux was completely rescued by expressing the CASY-1C isoform in GABAergic motor neurons (Figure 3.7A-Cand movies 3.4-3.6). We next analyzed the transport characteristics of Cholinergic SNB-1::GFP vesicles. Vesicular flux for pCholinergic::SNB-1::GFP was higher than pGABAergic::SNB-1::GFP in WT *C. elegans*. Analysis of SNB-1::GFP transport in cholinergic motor neurons revealed wild-type-like vesicular flux and velocity in both anterograde and retrograde direction in *casy-1* mutant animals, suggesting normal cholinergic trafficking (Figure 3.7D-F and movies 3.7 and 3.8). This data suggests that the shorter CASY-1 isoforms

function specifically in GABAergic motor neurons to modulate the release kinetics of GABA by directly influencing the transport of SV precursors.

3.8. CASY-1 is a potential cargo adaptor for UNC-104/KIF1A mediated transport of SV precursors

casy-1 mutants have less GABA vesicles at the synapse as demonstrated by significant decrease in SNB-1::GFP levels at the NMJ. They also showed reduced recovery rates of SNB-1::GFP following FRAP analysis, suggesting the possibility that fewer SV precursors are trafficked to the NMJ. UNC-104/KIF1A is a kinesin-3 motor that has a conserved role in trafficking SV precursors from the cell body to the synapse (Hall and Hedgecock, 1991; Okada et al., 1995; Otsuka et al., 1991; Pack-Chung et al., 2007). To address if CASY-1 might be interacting with UNC-104/KIF1A to regulate the anterograde trafficking of SVs in GABAergic motor neurons, Aldicarb assays were performed after silencing the *casy-1* gene in the *unc-104/kif1a* mutant background using RNAi and vice versa. RNAi knockdown was performed as we were unable to make *unc-104/kif1a; casy-1* double mutants due to close proximity of the genes on Chromosome II (~1 cM apart). Both the knock-down and knock-out of *unc-104/kif1a*, results in resistance to Aldicarb. Further, RNAi knock-down of *unc-104/kif1a* in the *casy-1* mutant background, completely abolished the hypersensitivity of *casy-1* mutants and resulted in resistance to Aldicarb just like what was seen in *unc-104/kif1a* mutants, suggesting that UNC-104/KIF1A may genetically interact with CASY-1 (Figure 3.8A).

To further validate the genetic interaction, FRAP analysis of SNB-1::GFP in GABAergic motor neurons after RNAi knockdown of *unc-104/kif1a* in a *casy-1* mutant background was performed. The recovery rate of SNB-1::GFP was significantly reduced after knockdown of *unc-104/kif1a*. However, no significant difference between the recovery rates was observed after *unc-104/kif1a* knockdown in wild type and *casy-1* mutant background, again supporting the genetic interaction (Figure 3.8B).

The *C. elegans* CASY-1 has previously been shown to physically interact with the kinesin light chain-2 (KLC-2) in yeast two-hybrid assays (Ohno et al., 2014). These studies suggest CASY-1 to act as a broad regulator for transport of multiple neuronal cargo. To investigate the role of CASY-1 in general transport mechanisms, a possible

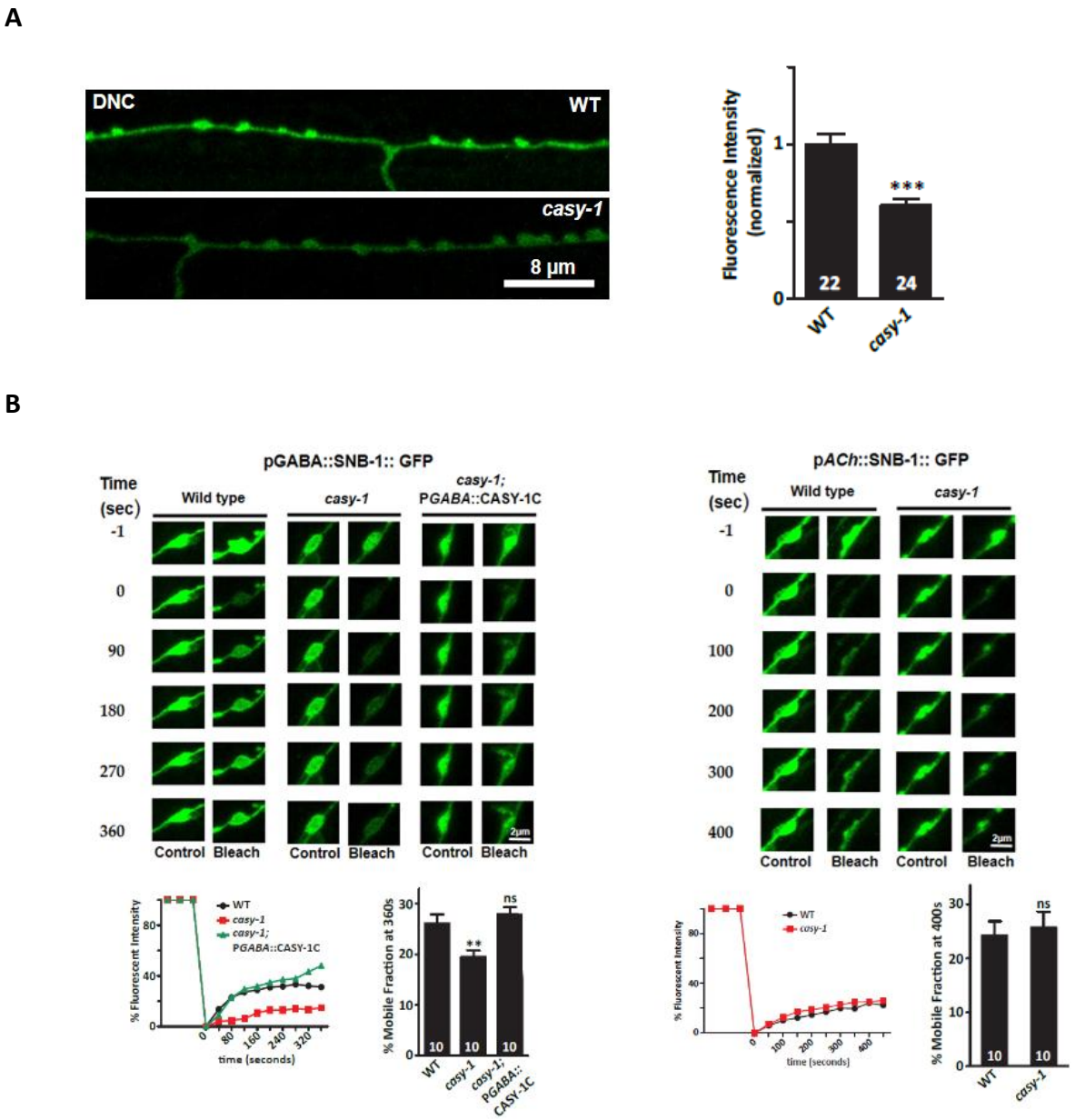

Figure 3.6: ***casy-1* isoforms are required for normal GABA release at NMJ.** (A) GABA SV release is compromised in *casy-1* mutants. Images show the dorsal nerve cord of adult hermaphrodites expressing pH-sensitive GFP reporter (*superecliptic pHluorin*) tagged to the luminal domain of synaptobrevin. pHluorin fluorescent intensity is significantly reduced in the *casy-1* mutants suggesting less number of GABA vesicles functional at the synapse. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test. (B) FRAP analysis of SNB-1::GFP levels in GABAergic motor neurons reveals that the dynamics of SV mobility is reduced in *casy-1* mutants. Representative confocal images of pGABAergic::SNB-1::GFP levels compared between WT, *casy-1* and *casy-1*; pGABAergic::CASY-1C rescue shows images before photo-bleaching *(pre-bleach)*, immediately after photo-bleaching *(post-bleach)* and 360 sec after photo-bleaching *(recovery)*. FRAP analysis of SNB-1::GFP levels in cholinergic motor neurons illustrated that mobility dynamics of SNB-1::GFP levels is normal in cholinergic motor neurons in *casy-1* mutant animals. At time 0, a single puncta of SNB-1::GFP was photo-bleached. Recovery of SNB-1::GFP levels were subsequently monitored at the photo-bleached and a neighboring control puncta. The fractional recovery of fluorescence 360 sec and 400 sec after photo-bleaching is shown. Recovery was measured with the pre-bleach fluorescence intensity being 100% and the post-bleach intensity at time 0 being 0%. The number of animals analyzed are indicated for each genotype. Data are represented as mean ± S.E.M. (**P<0.001 using one-way ANOVA and Bonferroni's Multiple Comparison Test).

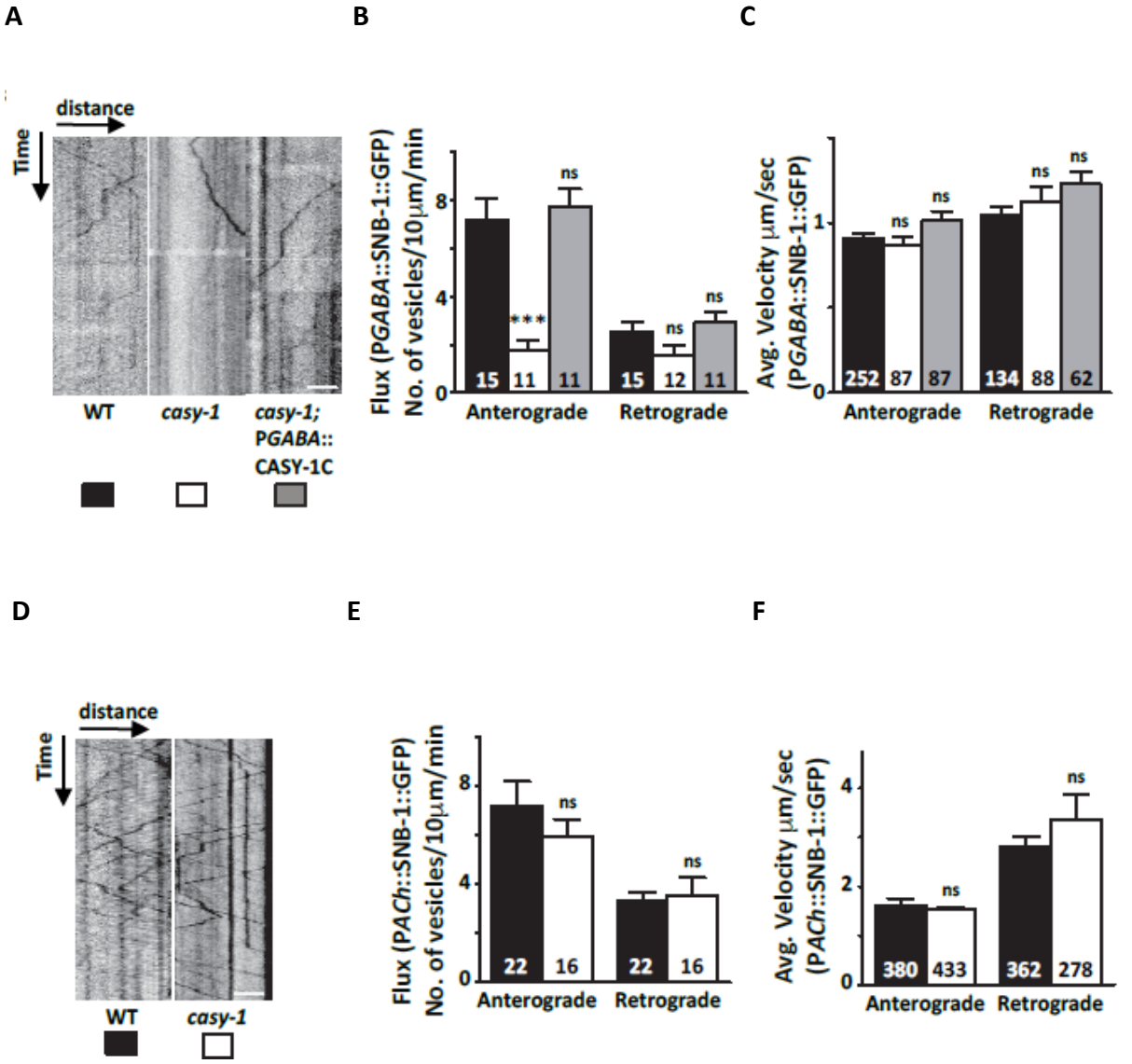

Figure 3.7: CASY-1 is required for trafficking of SV precursors in GABAergic motor neurons. (A) Representative GABAergic::SNB-1::GFP trafficking kymographs in WT, *casy-1* and *casy-1*; pGABAergic::CASY-1C rescue. For all kymographs, ventral cell body (anterograde) is to the right. All kymographs are clipped to 10 µm (distance) x 1 min (time) (77x180 pixels). Scale bar drawn is 3 µm. (B-C) Quantification of anterograde and retrograde SV flux in young adult animals (B) Comparison of mean anterograde and retrograde flux (normalized to a distance of 10 µm and a time of 1 min) between WT, *casy-1* mutant and *casy-1*; pGABAergic::CASY-1C rescue animals. (C) Comparison of mean anterograde and retrograde velocity between WT, *casy-1* mutant and *casy-1*; pGABAergic::CASY-1C rescue animals are shown. *casy-1* mutants show significantly reduced anterograde vesicular flux compared to WT animals. Reduced GABAergic anterograde flux was completely rescued by expressing CASY-1C specifically in GABAergic motor neurons. (D) Representative pCholinergic::SNB-1::GFP trafficking kymographs in WT and *casy-1* mutant animals. For all kymographs, ventral cell body (anterograde) is to the right. All kymographs are clipped to 10 µm (distance) x 1 min (time). Scale bar drawn is 3 µm. (E-F) Quantification of anterograde and retrograde SV transport in young adult animals (E) Average flux and (F) Average velocity are shown. Flux is defined as the number of moving particles per 10 µm per min. n represents number of particles analyzed for the analysis. Data are represented as mean ± S.E.M. (***P<0.0001 using two-way ANOVA and Bonferroni's Multiple Comparison Test, "ns" indicates not significant in all Figures).

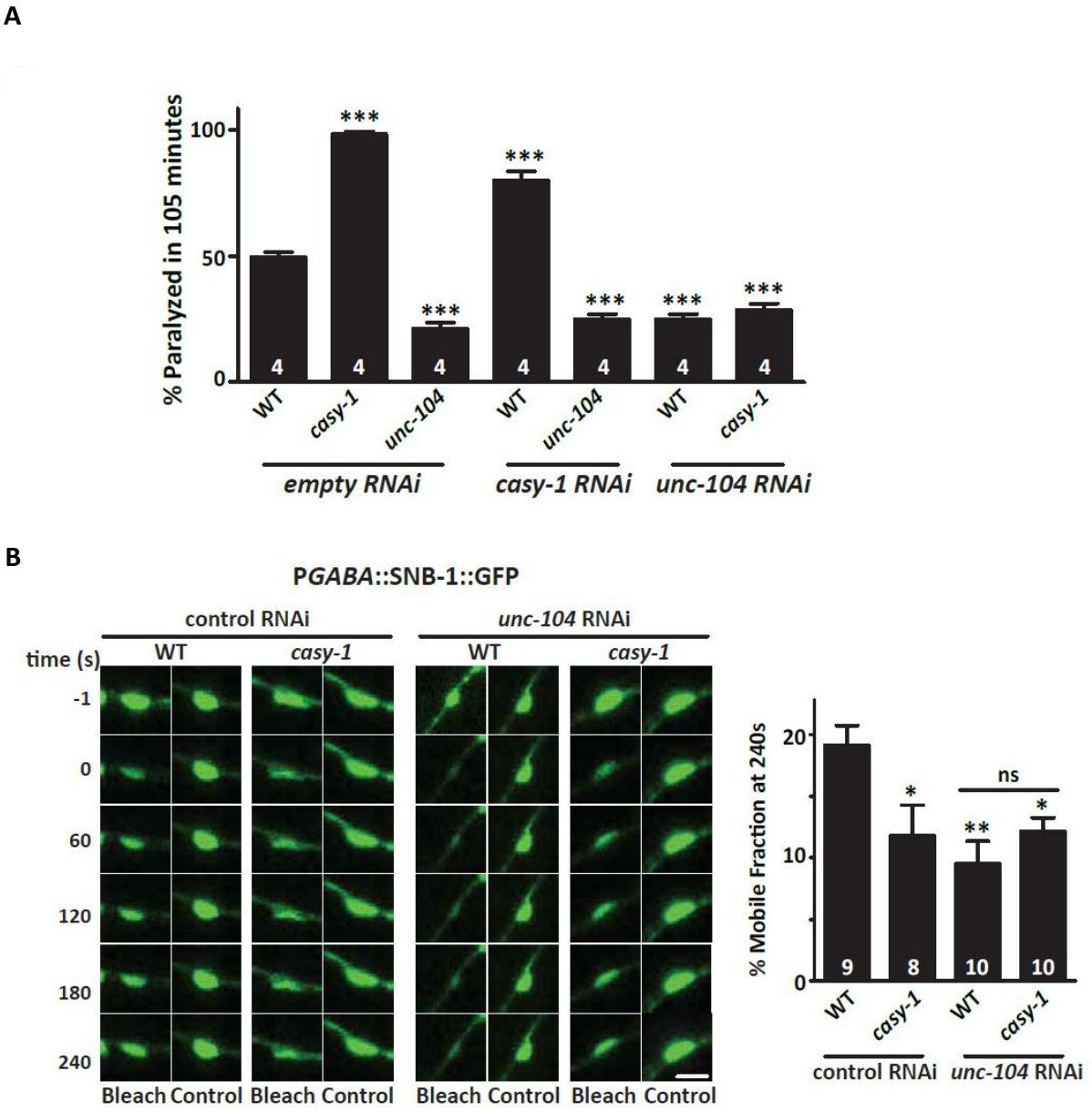

Figure 3.8a: CASY-1 interacts with the kinesin motor UNC-104/KIF1A to regulate the trafficking of GABA vesicles. (A) Aldicarb- induced paralysis was compared following RNAi treatments with control (empty vector), *unc-104/kif1a* or *casy-1* vectors as indicated in the graph. Knockdown of *unc-104/kif1a* results in a similar Aldicarb resistance phenotype in both the WT and *casy-1* mutants indicating that *casy-1* and *unc-104/kif1a* could be interacting genetically. Assays were done 4 times as indicated in Figure (~20 *C. elegans*/ assay). Data are represented as mean ± S.E.M. (***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (B) FRAP analysis of SNB-1::GFP levels in GABAergic motor neurons reveals that the dynamics of SV mobility is reduced after *unc-104/kif1a* RNAi. Representative confocal images of pGABAergic::SNB-1::GFP levels compared between WT, *casy-1* mutant, *unc-104/kif1a* RNAi and *casy-1*; *unc-104/kif1a* RNAi shows images before photo-bleaching *(pre-bleach)*, immediately after photo-bleaching *(post-bleach)* and 240 sec after photo-bleaching *(recovery)*. Scale bar, 2 µm. The fractional recovery of fluorescence 240 sec after photo-bleaching is shown.

physical interaction between CASY-1 and UNC-104/KIF1A in a yeast two-hybrid assay was examined. The interaction of the CASY-1 C- terminal with several UNC-104/KIF1A domain constructs was examined. Our data suggests that a weak interaction occurs between the cytoplasmic tail of CASY-1 and the C-terminal of UNC-104/KIF1A that largely includes the stalk region and the Pleckstrin Homology (PH) domain, a domain responsible for cargo vesicle binding to UNC-104/KIF1A (Klopfenstein and Vale, 2004). Subtle interactions were also observed with UNC-104/KIF1A motor domain and FHA domain. This data suggests that the C-terminal of CASY-1 could directly interact with UNC-104/KIF1A. (Figure 3.8D). However, since the interaction between UNC-104/KIF1A and CASY-1 appeared weak in the yeast-two-hybrid assay, we cannot rule out the possibility that CASY-1 interacts indirectly with UNC-104/KIF1A, through other adaptor molecule/s. This could also allow for an explanation on the specificity of this interaction occurring only in GABAergic motor neurons. A GST- pull down experiment was additionally performed to validate the physical interaction of UNC-104 C- terminal with CASY-1. A significant pull down of UNC-104-HA was obtained with GST-CASY-1 bound on beads, giving us further evidence of a possible physical interaction between the two proteins (Figure 3.8E).

To further understand the role of UNC-104/KIF1A in axonal trafficking of CASY-1, we determined the localization of CASY-1C::GFP in *unc-104/kif1a* mutant animals. In WT *C. elegans*, CASY-1C::GFP shows significantly lower expression in the VNC cell bodies owing to its trafficking to the DNC synapses. However, in *unc-104/kif1a* mutants we observed significant accumulation of CASY-1C::GFP in some neuronal cell bodies (Figure 3.8C), suggesting that *unc-104/kif1a* is a potential candidate for trafficking of CASY-1C::GFP to the synapse in these neurons.

A previously reported interaction of mammalian calsyntenins with kinesin light chain motor proteins was also confirmed for *C. elegans* homologs as a part of this study. First, to address if CASY-1 might be interacting with kinesin light chain motor proteins *klc-1* and *klc-2*, Aldicarb assays were performed. The knock-down of *klc-1* and *klc-2*, results in resistance to Aldicarb. Further, removing *klc-1* and *klc-2* in the *casy-1* mutant background, completely abolished the hypersensitivity of *casy-1* mutants and resulted in resistance to Aldicarb just like what was seen in *klc-1* and *klc-2* mutants, suggesting that KLC-1 and KLC-2 might interact genetically with CASY-1 (Figure 3.8F). Further, to investigate a possible physical interaction of CASY-1 with

KLC-1 and KLC-2, a yeast two-hybrid assay was performed. The interaction of the CASY-1 C- terminal with full-length KLC-1 and KLC-2 constructs was examined. Our data suggests that a strong interaction occurs between the *C. elegans* CASY-1 and kinesin light chain motor proteins just like its mammalian homologs (Figure 3.8G).

Finally, we also determined the localization of CASY-1C::GFP in *klc-1* mutant animals. As explained above, in WT *C. elegans*, CASY-1C::GFP shows significantly lower expression in the VNC cell bodies. However, in *klc-1* mutants a significant accumulation of CASY-1C::GFP in several neuronal cell bodies was observed (Figure 3.8H), validating that *klc-1* is a potential candidate for trafficking of CASY-1C::GFP to the synapse in these neurons.

3.9. Discussion

GABA is a major inhibitory neurotransmitter functioning in both vertebrate and invertebrate nervous systems. In vertebrates, nearly 30-40% of the CNS synapses are thought to be GABAergic (Docherty et al., 1985) and alterations in GABA neurotransmission have been associated with several different neurological disorders (reviewed in (Chiapponi et al., 2016; Contestabile et al., 2017; Schür et al., 2016)). In *C. elegans*, very few reports have documented the role of genes regulating GABAergic synaptic transmission at the NMJ (Kowalski et al., 2014; Sun et al., 2013). In this study, we are proposing a novel role of CASY-1, in regulating GABA signaling at the NMJ in an isoform-specific manner. Despite considerable reports emphasizing the involvement of Calsyntenins in GABA synapse development and function, it has been very difficult to deduce the cellular and molecular mechanisms and implications of these proteins on animal behavior and function. Our results indicate that the short isoforms of CASY-1, CASY-1B and CASY-1C, are involved in modulating GABA synaptic release from motor neurons (Figure 3.9).

Mutants in *casy-1* show significant hypersensitivity to Aldicarb, suggesting an increased synaptic transmission at the NMJ. Neuron-specific rescue experiments suggested that the shorter CASY-1 isoforms function specifically in GABAergic neurons to regulate synaptic signaling. Domain mapping experiments suggested that the conserved C-terminal of CASY-1, present in *casy-1b/casy-1c* is functioning to rescue the hypersensitive phenotype of *casy-1* mutants. The expression analysis of

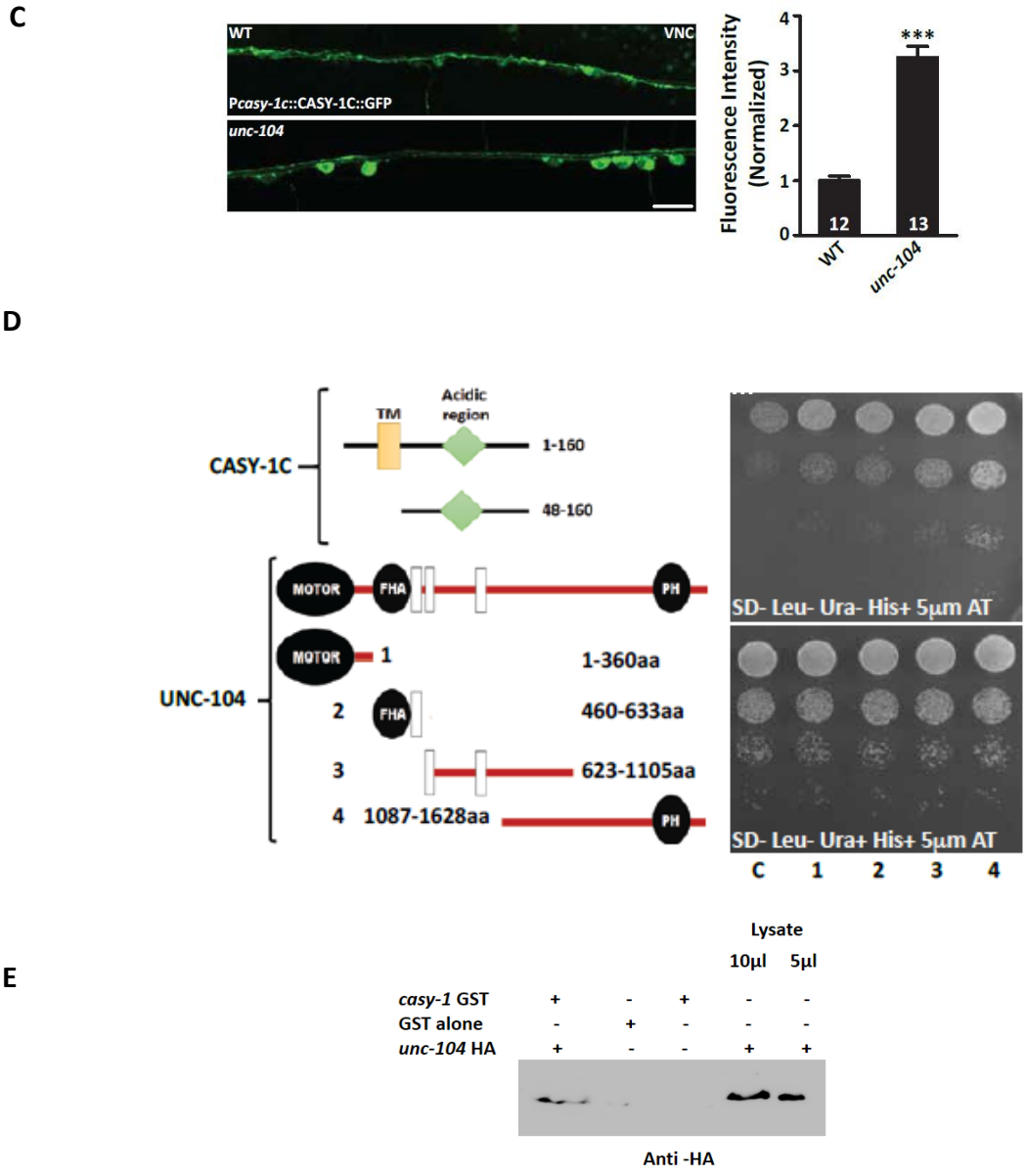

Figure 3.8b: CASY-1 interacts with the kinesin motor UNC-104/KIF1A to regulate the trafficking of GABA vesicles. (C) Intracellular localization of P*casy-1c*::CASY-1C::GFP in VNC cell bodies in *unc-104* mutant background. In this mutant background, CASY-1C::GFP is significantly sequestered in cell bodies compared to WT control animals. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test. (D) The left panel indicates a schematic representation of CASY-1C C-terminal and UNC-104/KIF1A constructs utilized in this study. UNC-104/KIF1A constructs cover several domains; the UNC-104/KIF1A - Motor domain (aa 1-356), the fork head domain FHA (aa 460-633), the SYD-2/liprin binding domain STALK (aa 623-1105) and the PH domain (aa 1087-1628). The lower panel on the right indicates the yeast two hybrid data showing the control spotting in the presence of a selection component while the upper panel shows significant interaction of CASY-1 C terminal with several UNC-104/KIF1A domains. The most prominent interaction occurs with the tail region of UNC-104/KIF1A spanning the stalk and PH domain. (E) *In vitro* binding assay between GST-CASY-1 C- terminal (aa 48-160) and UNC-104 (aa 1087-1628)- HA. Glutathione beads loaded with recombinant GST and GST-CASY-1 were incubated with *in-vitro* expressed UNC-104-HA and then washed to remove unbound proteins. Bound proteins were eluted and analyzed by Western blot using anti-HA. A GST pull-down assay showed that GST-CASY-1 interacts directly with UNC-104-HA. Input lanes contain in vitro-expressed UNC-104-HA used in the binding assays added at 10 µl and 5 µl volumes.

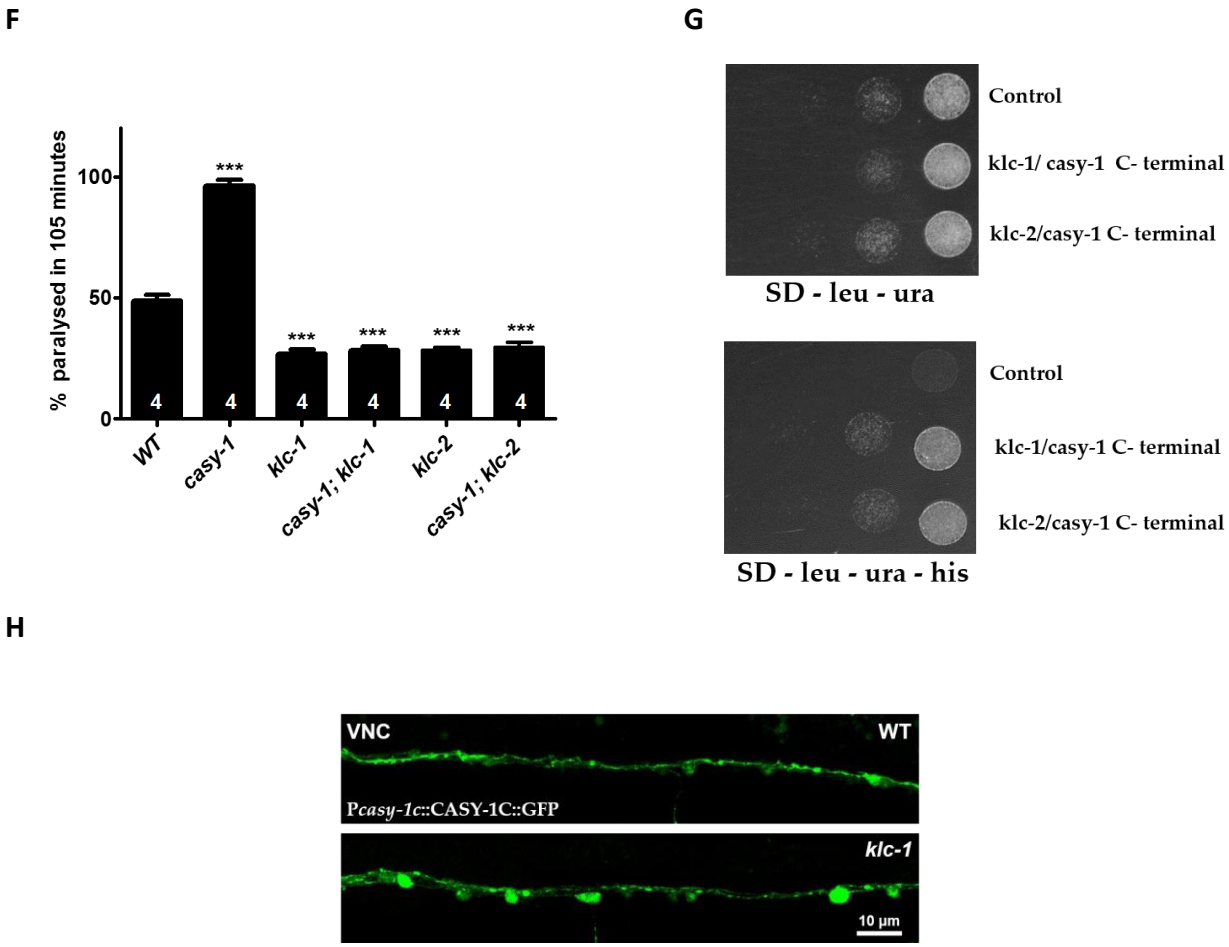

Figure 3.8c: CASY-1 interacts with the kinesin motor UNC-104/KIF1A to regulate the trafficking of GABA vesicles. (F) Aldicarb-induced paralysis at 105 minutes is shown for the indicated genotypes. The *casy-1* Aldicarb hypersensitivity was suppressed by mutations inactivating kinesin light chain motor proteins *klc-1* and *klc-2*. Mutations in *klc-1* and *klc-2* results in an Aldicarb resistance phenotype in the *casy-1* mutants indicating that *casy-1* and *klc-1* and *klc-2* could be interacting genetically. Assays were done 4 times as indicated in Figure (~20 *C. elegans*/ assay). Data are represented as mean ± S.E.M. (***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (G) Yeast- two hybrid assay revealed significant physical interaction between CASY-1 C- terminal and full length KLC-1 and KLC-2 proteins. The lower panel indicates the yeast two hybrid data showing the control spotting in the presence of a selection component while the upper panel shows significant interaction of CASY-1 C terminal with KLC-1 and KLC-2. (H) Intracellular localization of P*casy-1c*::CASY-1C::GFP in VNC cell bodies in *klc-1* mutant background. In this mutant background, CASY-1C::GFP is significantly sequestered in cell bodies compared to WT control animals. This suggests that CASY-1 C-terminal also interacts with kinesin light chain motor proteins just like its mammalian homologs.

CASY-1 isoforms further showed that only CASY-1B and CASY-1C express in GABAergic motor neurons, while CASY-1A expression is restricted mainly to head neurons and is not seen in the ventral cord motor neurons. Interestingly, *C. elegans* has devised a fascinating strategy wherein isoform expression and function is spatially regulated using alternative promoters. The shorter isoforms of CASY-1, which are essentially just the C-terminal region of mammalian Calsyntenins and required for this function, are expressed in GABAergic motor neurons to regulate GABA release at NMJ.

As discussed before, several studies of mammalian synapses using primary hippocampal neuronal cultures and knockout mice have established that Calsyntenins are involved in the development and functioning of inhibitory GABA synapses (Pettem et al., 2013). CLSTN knockout mice also show reduced GABAergic neurotransmission (Lipina et al., 2016) but the molecular basis of this regulation is unknown. In our study, we are showing, how the C-terminal of mammalian Calsyntenins which is conserved in CASY-1B/CASY-1C, can regulate GABAergic neurotransmission pre-synaptically. Using pharmacological, behavioral, optogenetic and electrophysiology approaches, we established defects in GABA signaling in *casy-1* mutants at NMJ. All these mutant phenotypes could be completely rescued by expressing CASY-1B/CASY-1C specifically in GABAergic motor neurons. Although, mammalian calsyntenins are reported to be post-synaptic membrane proteins, here we are demonstrating a pre-synaptic role of the *C. elegans* orthologs of mammalian Calsyntenins. Our study opens up the possibility of exploring the potential existence of similar mechanisms regulating GABAergic neurotransmission in the mammalian nervous system.

We are also proposing a mechanistic insight into how the CASY-1 C-terminal can regulate GABA release at NMJ. Mammalian CLSTN1 has been well documented for its role in regulating trafficking of various axo-dendritic synaptic components via its interaction with kinesin light chain motor protein (*klc-1*). *C. elegans* CASY-1 has also recently been shown to interact physically with KLC-2 (Ohno et al., 2014), suggesting a role for CASY-1 in trafficking of synaptic components as well. In this study, we have determined a novel interaction where the C-terminal of CASY-1 interacts with the tail region of the motor protein UNC-104/KIF1A that harbors the stalk region as well as PH domain. UNC-104/KIF1A is established as an evolutionarily conserved

motor protein required for trafficking of SV precursors from the soma to the synapse. Our results unlock the possible existence of similar interaction in mammalian system. However, we cannot refute the possibility that CASY-1 might also affect SV precursor trafficking via its interaction with kinesin light chain (*klc-1* and *klc-2*) as multiple studies also showed the involvement of *klc-1* motor in SV precursor trafficking (Byrd et al., 2001; Kurup et al., 2015; Mimori-Kiyosue et al., 2000).

Despite proposing a convincing mechanistic role of *casy-1* isoforms in modulating GABA signaling at NMJ, some major questions remain unanswered. First, *casy-1b/casy-1c* specifically act in GABAergic neurons although they are also expressed in cholinergic neurons. Several previous reports have highlighted the role of molecules that expresses in both cholinergic and GABAergic motor neurons but specifically functions in just one system to affect NMJ signaling (Hao et al., 2012; Opperman et al., 2017). Here we establish that *casy-1* isoforms function in GABAergic motor neurons to regulate Aldicarb responsiveness. However, we cannot rule out additional roles of CASY-1 in cholinergic motor neurons. Furthermore, electrophysiological recordings from the *casy-1* NMJ showed an increased EPSC frequency suggesting another mechanism for Aldicarb hypersensitivity, but expressing CASY-1 isoforms in cholinergic motor neurons could not rescue the Aldicarb phenotype. This implies that increased EPSC frequency is not an outcome of *casy-1* function in cholinergic motor neurons. To address how CASY-1 could affect EPSC frequency we propose a function for the CASY-1A isoform in higher levels of locomotion circuit (sensory neurons) to regulate cholinergic transmission extra-synaptically via increased glutamatergic and neuropeptidergic signaling (addressed in Chapter 4).

Despite the simplicity of *C. elegans* having just one gene coding for CASY-1, compared to multiple mammalian Calsyntenin genes, it emerges as an excellent regulator of diverse functions such as vesicular trafficking, functioning of GABAergic synapses and synaptic plasticity, functions that are performed by individual CLSTNs in the mammalian nervous system. This establishes *C. elegans* as an ideal model system to explore other functions of mammalian Calsyntenins. Further studies in this system could enhance our understanding about pathophysiological mechanisms that trigger calsyntenin-related brain disorders.

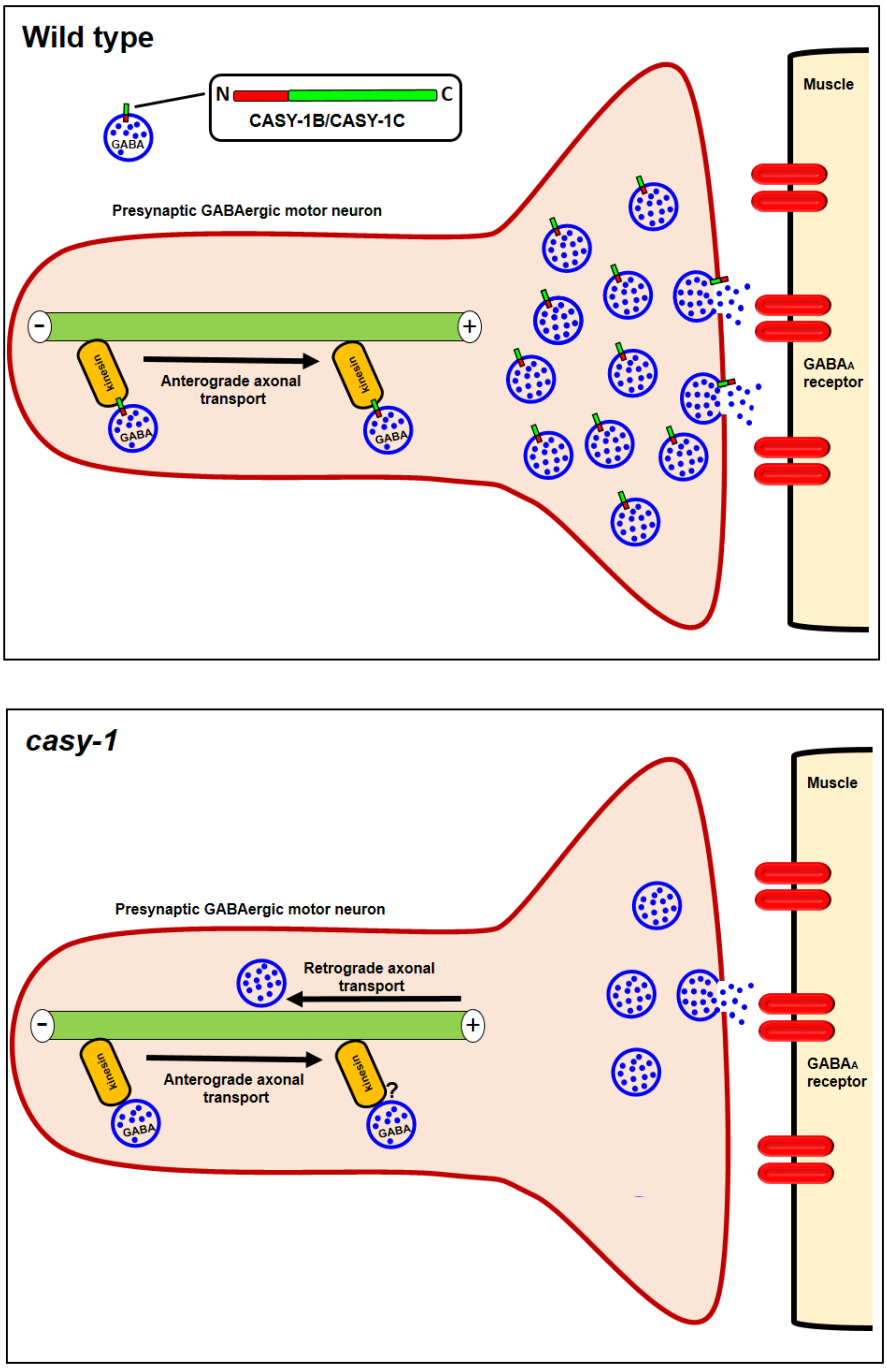

Figure 3.9: Possible model for CASY-1 functioning at the NMJ. CASY-1B and CASY-1C, the shorter isoforms are present on the SV precursors. The conserved C-terminal of CASY-1 act as an adaptor molecule to mediate interaction of GABA-specific SVs with the tail region of UNC-104/KIF1A motor protein to mediate fast anterograde axonal transport of synaptic cargo. Mobility dynamics of SV precursors in turn regulates the release kinetics of GABA at NMJ. However in the absence of *casy-1*, this cargo-adaptor-motor bridge is lost resulting in aberrant anterograde flux of the GABA-specific SV cargo along the axonal pathway. This function of CASY-1B/C shows some similarity to how the mammalian CLSTN1 is thought to function. CLSTN1 has been shown to be required for fast anterograde axonal transport and loss of interaction with kinesin motor results in decreased anterograde trafficking and an increase in retrograde transport of vesicular cargo (Konecna et al., 2006).

CHAPTER 4:

CASY-1 mediated regulation of motor circuit dynamics and behavior stemming from Sensory neurons

Shruti Thapliyal designed, performed and analyzed all the experiments except the movement experiments. Shruthi Ravindranath performed the *C. elegans* movement experiments under supervision of Shruti Thapliyal and Kavita Babu. Dr. Jihong Bai and Dr. Yongming Dong performed the electrophysiological recordings.

Introduction

Behavioral output of an organism depends on the combined activity of multiple neural networks in which individual circuits are either activated or inhibited by the action of neurotransmitters or modified through neuropeptides. Locomotion is a very basic yet complex behavior in most organisms. An in-depth understanding of the *Caenorhabditis elegans* neural connectome provides an excellent model to understand the complex molecular and cellular mechanisms operating in locomotory circuits. Neural circuits that produce coordinated dorso-ventral sinusoidal bends allow for normal locomotion in *C. elegans*. Locomotory behavior is synchronized at multiple levels and involves the integration of diverse sensory cues that are processed by the interneurons and ultimately cause changes at the neuromuscular junctions (NMJ) (Bargmann, 2012; de Bono and Maricq, 2005). At the *C. elegans* NMJ, cholinergic motor neurons stimulate muscle contraction as well as activate GABAergic motor neurons that inhibit contraction of the contralateral muscles (Alfonso et al., 1993; Duerr et al., 2008; McIntire et al., 1993a, 1993b; White et al., 1986). Despite an extensive understanding of the development and functioning of the ventral cord motor neurons, the mechanisms controlling motor coordination are still elusive. In mammals, disruption in this motor activity synchronization results in an excitation-inhibition imbalance that has been implicated in several neurological disorders like autism and epilepsy (Eichler and Meier, 2008; Fetissov et al., 2003; Lerner et al., 2010; Nelson and Valakh, 2015; Wu et al., 2011; Yizhar et al., 2011). Using *C. elegans* NMJ provides an excellent model to understand the genetic factors that coordinates this balance, thus providing deeper understanding of the pathogenesis of these disorders.

In the previous chapter, I have shown that the two shorter isoforms of CASY-1, CASY-1B and CASY-1C function in the GABAergic motor neurons to regulate GABA release by mediating the trafficking of GABA synaptic vesicle (SV) precursors through their interaction with the UNC-104/KIF1A motor protein. This investigation highlighted the role of CASY-1B/C in maintaining inhibitory GABAergic signaling (Chapter 3).

In this chapter, I propose a novel neuromodulatory role of CASY-1-dependent signaling in the regulation of motor circuit dynamics and locomotion. I show that the

CASY-1A isoform functions in the sensory neurons to inhibit the activity of command interneurons which in turn decreases the motor circuit activity and hence locomotion. Also, CASY-1A regulates the neuropeptide receptor NPR-1- mediated signaling at the level of sensory neurons. The reduced NPR-1 signaling in *casy-1* mutants represents one possible mechanism that increases glutamate and neuropeptide release from the sensory neurons resulting in enhanced motor circuit activity.

Results

4.1. Mutants in *casy-1* have elevated Aldicarb sensitivity and locomotion rates

We have recently shown that *casy-1* mutants are hypersensitive to Aldicarb when compared to wild-type (WT) *C. elegans* (Figure 4.1C and (Chapter 3)). Aldicarb is a pharmacological inhibitor of the enzyme acetylcholine esterase that prevents breakdown of the excitatory neurotransmitter acetylcholine at the NMJ. WT *C. elegans* show a time-course induced paralysis upon exposure to Aldicarb. It has been shown that mutations that increase synaptic transmission results in Aldicarb hypersensitivity, while mutations that decrease synaptic transmission results in Aldicarb resistance (Mahoney et al., 2006; Miller et al., 1996; Sieburth et al., 2005; Vashlishan et al., 2008). Significant Aldicarb hypersensitivity in *casy-1* mutants suggests an increased motor circuit activity (Fry et al., 2014). As locomotion directly reflects the coordination of motor circuit activity, the locomotory behavior of *casy-1* mutants on food was analyzed. To quantify the locomotory behavior, body bends of *C. elegans* on NGM agar seeded with bacteria were quantified. Although *casy-1* mutants maintain normal sinusoidal locomotion, they showed a significantly increased number of body bends in comparison to WT control animals (Figure 4.1D).

Our previous work has revealed that whole-muscle patch clamp recordings from *casy-1* mutants show a significant increase in baseline excitatory post synaptic currents (EPSCs) (Fugure 4.1A and Chapter 3), suggesting increased acetylcholine release at the NMJ. However, expressing CASY-1 isoforms in cholinergic motor neurons could not rescue the Aldicarb hypersensitivity in these mutants (Chapter 3). This suggests that the increased cholinergic transmission in *casy-1* mutants is not due to CASY-1 function in cholinergic motor neurons. CASY-1A expression is not seen in cholinergic or GABAergic ventral cord motor neurons. However, it is strongly expressed in several sensory neurons and interneurons located in the anterior region of

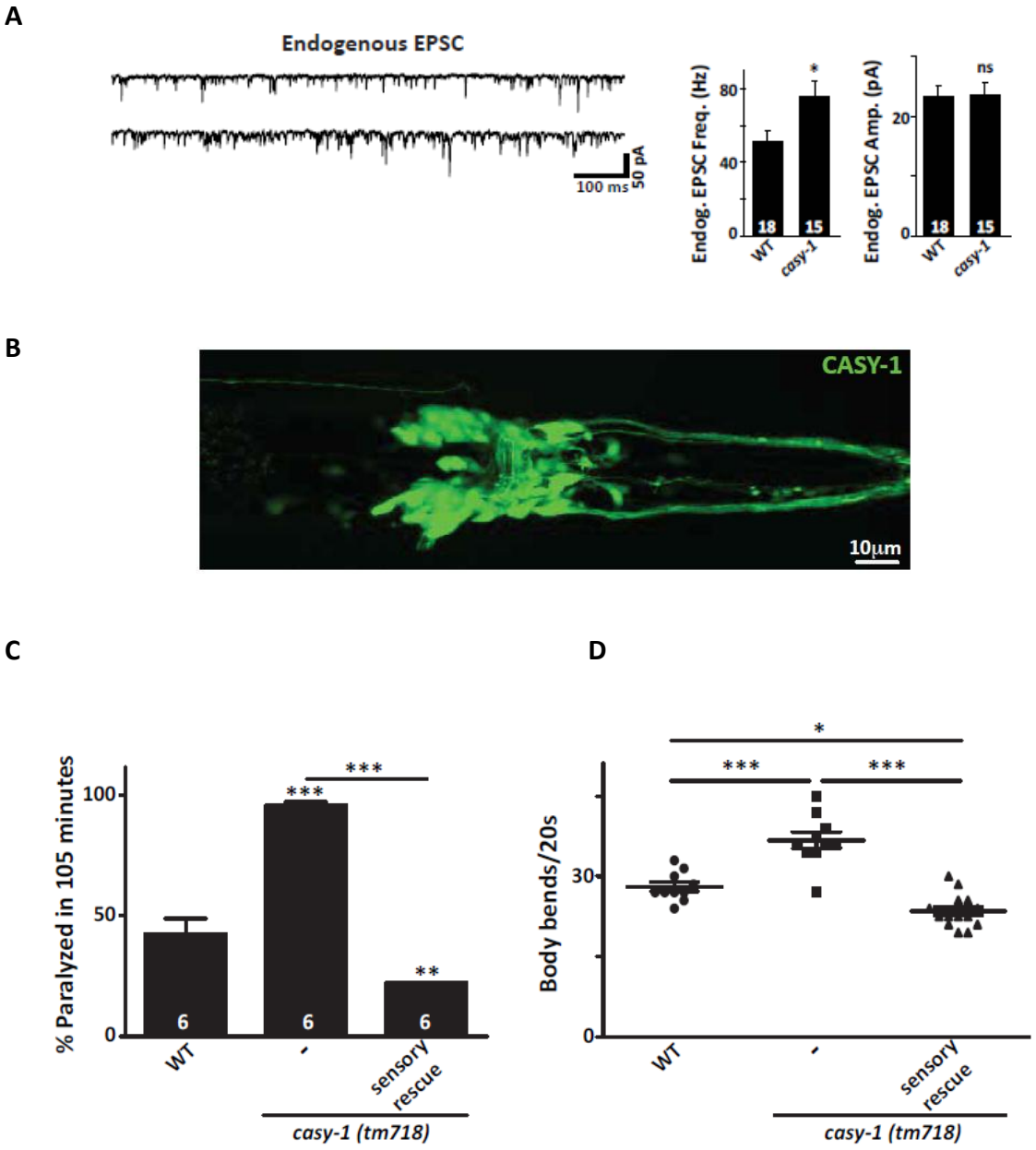

Figure 4.1: ***casy-1*** **mutant animals have increased motor circuit activity.** (A) mEPSCs were recorded from body wall muscles of adult animals for the indicated genotypes. Representative traces of mEPSCs and summary data for frequency and amplitude are shown. There is a subtle but significant increase in mEPSC frequency in *casy-1* mutants however the amplitude remains unchanged. The number of animals analyzed for each genotype is indicated. Data are represented as mean ± S.E.M. (*P<0.05 using two-tailed Student's t-test). (B) Representative fluorescent micrograph showing P*casy-1a*::GFP transcriptional reporter expression in *C. elegans* head neurons. (C) Aldicarb-induced paralysis in *casy-1* mutants was compared to wild-type (WT) animals. *casy-1 (tm718)* mutants are hypersensitive to Aldicarb, which is completely rescued by expressing CASY-1A in sensory neurons using the *odr-4* promoter. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (D) *casy-1* mutants crawl faster than WT animals on agar surface seeded with OP50 (Ravindranath S., 2016). Body bends/20sec are shown for WT, *casy-1 (tm718)* and rescue lines expressing CASY-1A in sensory neurons. n=10-15 animals. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test).

the animal (Figure 4.1B and (Ikeda et al., 2008 and Chapter 3)). Recent publications have nicely highlighted how genes expressed in higher levels of locomotory circuits i.e. in sensory and interneurons could affect synaptic signaling at the NMJ (Choi et al., 2013, 2015; Fry et al., 2014). Based on these previous publications, we hypothesized that the increased sensitivity to Aldicarb and the elevated locomotory rates in *casy-1* mutants could be due to increased signaling from the sensory neurons and/or interneurons. We were specifically interested in understanding the role of CASY-1A in sensory neurons, since previous reports have shown that learning deficits in *casy-1* mutants can be completely rescued by expressing CASY-1A in a single sensory neuron (Ikeda et al., 2008). To test whether CASY-1A could function at the level of sensory neurons to regulate NMJ signaling, a transgenic line expressing CASY-1A in several sensory neurons using the *odr-4* promoter was obtained. Expressing CASY-1A in sensory neurons completely rescued the Aldicarb phenotype of *casy-1* mutants, infact the animals became resistant to Aldicarb (Figure 4.1C). CASY-1A expression in sensory neurons also rescued the increased locomotion rate of *casy-1* mutants (Figure 4.1D). These results demonstrate that the CASY-1A isoform can regulate NMJ signaling from the sensory neurons. It also indicates that CASY-1A functions in the *C. elegans* nervous system to reduce the motor circuit activity.

4.2. CASY-1A regulates glutamate release from sensory neurons

CASY-1A expression in sensory neurons using the *odr-4* promoter rescued the Aldicarb hypersensitivity and increased locomotion rates of *casy-1* mutants. The *odr-4* promoter drives gene expression in 12 pairs of sensory neurons (ADF, ADL, ASG, ASH, ASI, ASJ, ASK, AWA, ASB, AWC, PHA, PHB) (Dwyer et al., 1998). The neurotransmitter identity of these 12 neurons was next examined, and it appeared that about 75% of these neurons are glutamatergic and release glutamate as the neurotransmitter (Gendrel et al., 2016). This analysis raised the possibility that increased synaptic signaling and increased locomotion rates in *casy-1* mutants could be due to increased glutamatergic signaling at the sensory level. To test this hypothesis, several experiments were performed.

To determine if increased glutamate signaling from sensory neurons mediates enhanced cholinergic transmission at the NMJ of *casy-1* mutants, *eat-4*/*vGLUT* (vesicular glutamate transporter) mutants, in which glutamatergic signaling is

disrupted were examined (Lee et al., 1999). The Aldicarb hypersensitivity in *casy-1* mutants was suppressed by inactivating *eat-4/vGLUT*, indicating that the increased glutamate signaling facilitates sensory-evoked increase in motor circuit activity in *casy-1* mutants (Figure 4.2A). To address the reason for increased glutamatergic signaling, the Aldicarb sensitivity of *casy-1* mutants was analyzed by introducing mutations that inactivate ion channels required for sensory transduction; *ocr-2/trpv* (transient receptor potential channel), *tax-4/cng* (a cyclic nucleotide-gated channel), and *mec-4/degenerin* (amiloride-sensitive Na+ channel protein) (Driscoll and Chalfie, 1991; Komatsu et al., 1996; Tobin et al., 2002). Mutations in these ion channels completely abolished the Aldicarb hypersensitivity seen in *casy-1* mutants (Figure 4.2B, 4.2C and 4.2D). Collectively, these results suggest that the Aldicarb hypersensitivity and increased locomotion in *casy-1* mutants is due to increased glutamatergic signaling triggered by heightened activity in the sensory circuit.

One possible reason for increased glutamatergic signaling in *casy-1* mutants could be an increase in glutamate release from CASY-1 expressing sensory neurons. In order to investigate if *casy-1* mutants have increased glutamate release from the sensory neurons, a single wavelength glutamate sensor, iGluSnFR was used. iGluSnFR responds to the glutamate *in situ* and has been used for robust long-term imaging of glutamatergic transmission (Marvin et al., 2013). A transgenic line, expressing iGluSnFR specifically in the AVA interneurons using the *rig-3* promoter was utilized for this experiment (Marvin et al., 2013). AVA is post-synaptic to 40 other neurons many of which are glutamatergic (White et al., 1986). Glutamate levels were monitored by measuring iGluSnFR fluorescence along on the AVA cell body. The iGluSnFR fluorescence was significantly increased in the *casy-1* mutants indicating the increased release of glutamate from glutamatergic sensory neurons seen at basal levels (Figure 4.2E). This increased iGluSnFR fluorescence was completely rescued by expressing CASY-1A specifically in sensory neurons using the *odr-4* promoter, thus validating the role of CASY-1A in regulating glutamate release from sensory neurons (Figure 4.2E).

4.3. Increased synaptic activity and glutamate release from sensory neurons is responsible for the *casy-1* locomotory and Aldicarb defects

To gain insight into the role of CASY-1A in sensory neurons, an optogenetic approach was utilized wherein sensory neurons were photo activated using a slow

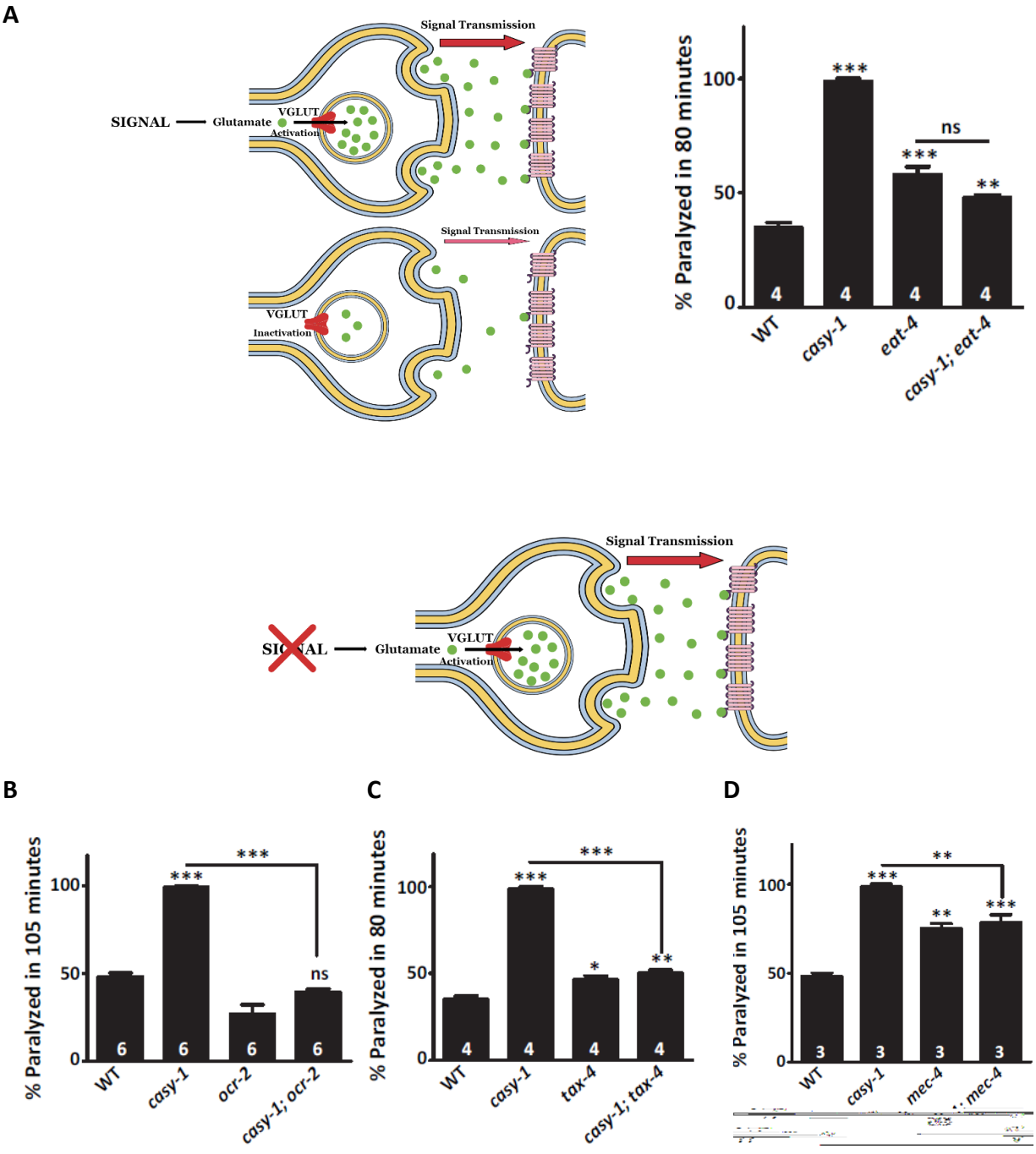

Figure 4.2a: Motor circuit activity is enhanced due to increased sensory activity in *casy-1* mutants (A-D) Aldicarb-induced paralysis at 80 or 105 minutes is shown for the indicated genotypes. Schematic of the principle of each assay is shown with the figures. The *casy-1* Aldicarb hypersensitivity was suppressed by mutations inactivating *eat-4/vglut*, *tax-4/cng* channels, *ocr-2/trpv* channels and *mec-4/degenerin* amiloride-sensitive Na+ channels. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). "ns" indicates not significant in all figures.

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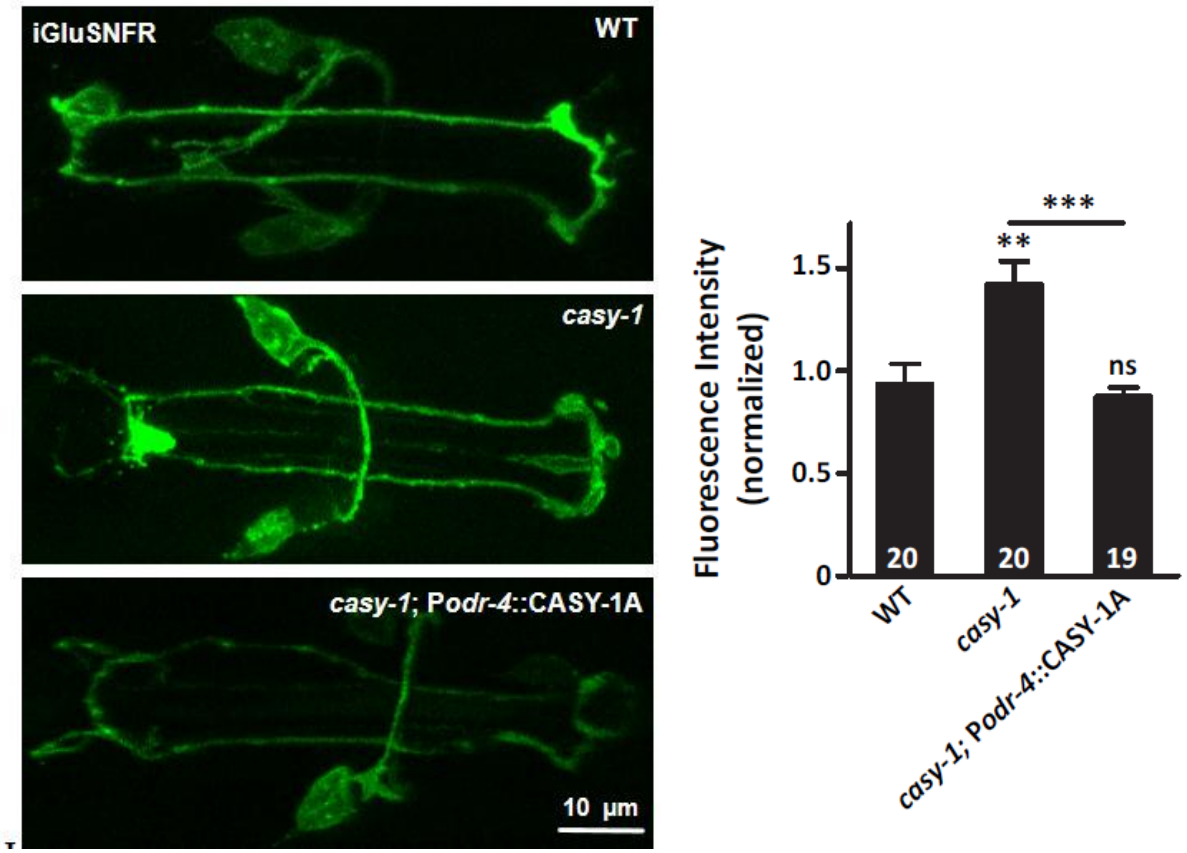

Figure 4.2b: Motor circuit activity is enhanced due to increased sensory activity in *casy-1* mutants. (E) Representative fluorescence micrographs of iGluSnFR expressed in AVA interneurons. iGluSnFR fluorescence intensity was significantly increased in *casy-1* mutants. iGluSnFR fluorescence intensity was completely rescued by expressing CASY-1A in sensory neurons using *odr-4* promoter. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by one-way ANOVA and Bonferroni's Multiple Comparison Test (**P<0.001; ***P<0.0001). "ns" indicates not significant in all figures.

inactivating variant of ChR2* (C128S) (Schultheis et al., 2011). The expression of ChR2* (C128S) was driven specifically in the sensory neurons using the *odr-4* promoter. To achieve this, a cell-specific, inverted Cre-Lox expression system was utilized as previously reported (Flavell et al., 2013; Fry et al., 2014). Briefly, in this approach, one promoter drives the expression of floxed ChR2* and the GFP construct (using a pan-neuronal *rab-3* promoter) and a second neuron-specific promoter drive the expression of Cre (the recombinase). Thus, by promoter overlap the expression of ChR2* will occur specifically in the neuron of interest (*odr-4* promoter driving expression in sensory neurons) (Flavell et al., 2013; Fry et al., 2014). This slow inactivating variant of ChR2* allowed us to monitor progressive paralysis in Aldicarb assays with intermittent blue light exposure (1-minute pulse of blue light after every 30 minutes). Interestingly, in WT animals photoactivation of sensory neurons results in Aldicarb hypersensitivity, a phenotype very similar to *casy-1* mutants. This experiment showed that increased activity of sensory neurons could result in Aldicarb hypersensitivity (Figure 4.3A).

This was followed by a complementary experiment, where the possibility of vesicle release from the sensory neurons being responsible for increased Aldicarb hypersensitivity in *casy-1* mutants was addressed. The same promoter-intersectional, inverted Cre-Lox strategy was utilized to express Tetanus toxin light chain (TeTx) specifically in the sensory neurons. TeTx blocks the release of vesicles containing both neurotransmitter and neuropeptides by cleaving the synaptic vesicle protein synaptobrevin (Fry et al., 2014; Schiavo et al., 1992; Sweeney et al., 1995). Blocking vesicle release from sensory neurons results in Aldicarb resistance in WT *C. elegans* (Figure 4.3B). Next, rescue of *casy-1* hypersensitivity by blocking synaptic release from sensory neurons using P*odr-4*::TeTx was examined. Indeed, the *casy-1* mutant Aldicarb response was restored to WT levels upon blocking synaptic release in *casy-1* mutants (Figure 4.3B). These results suggest that in *casy-1* mutants increased activity of sensory neurons trigger an increased release of glutamate and/or neuropeptide/s that facilitates an overall increase in the motor circuit activity. Collectively, these results suggest that in WT *C. elegans*, CASY-1A functions to regulate the activity of sensory neurons, which in turn allows for normal functioning of the *C. elegans* motor circuit.

4.4. CASY-1A modulates motor circuit activity by inhibiting command interneuron signaling

Our results so far suggest that in *casy-1* mutants, increased glutamatergic signaling from sensory neurons could affect motor neuron function. One possible way this could happen is through command interneurons that directly synapse onto the cholinergic motor neurons and hence could allow for modulating the motor circuitry (White et al., 1986). In *C. elegans*, the neurotransmitter glutamate mediates synaptic transmission from sensory neurons to downstream interneurons and the command interneurons (Brockie et al., 2001; Kaplan and Horvitz, 1993; Mellem et al., 2002).

To determine if the increased motor circuit activity in *casy-1* mutants is due to increased glutamate release from sensory neurons that in turn causes increased command interneuron signaling several experiments were performed. The command interneurons AVA, AVB, AVD, AVE and PVC are post-synaptic to sensory neurons as well as to several other interneurons (White et al., 1976, 1986). All these command interneurons express glutamate activated cation channels GLR-1/AMPA-type and NMR-1/ NMDA-type receptors that mediate excitatory neurotransmission (Hart et al., 1995; Maricq et al., 1995). If the hypersensitivity in *casy-1* mutants is due to increased glutamatergic signaling via command interneurons, then removing glutamate receptors should completely abolish the *casy-1* hypersensitivity. To discern this, *nmr-1; glr-1* double mutants and *nmr-1 casy-1; glr-1* triple mutants were generated. The *nmr-1; glr-1* double mutants showed a WT response to Aldicarb. However, mutations inactivating *nmr-1* and *glr-1* in the *casy-1* mutant background completely abolished the Aldicarb hypersensitivity of *casy-1* mutants (Figure 4.4A).

Next, we silenced the command interneuron signaling using Histamine-gated chloride channels, expressed specifically in command interneurons using the *nmr-1* promoter. Histamine-HisCl1 system has been well established to quickly and robustly silence the *C. elegans* neurons and is often used as a useful complement to optogenetic approaches (Pokala et al., 2014). Silencing command interneurons in WT *C. elegans* results in a WT response to Aldicarb (Figure 4.4B). This is not surprising as previously it has been shown that ablating command interneurons using cell-specific miniSOG (mini singlet oxygen generator)- based light-inducible cell ablation does not alter the Aldicarb hypersensitivity of the animals (Fry et al., 2014). However, silencing command interneuron signaling in *casy-1* mutants restored

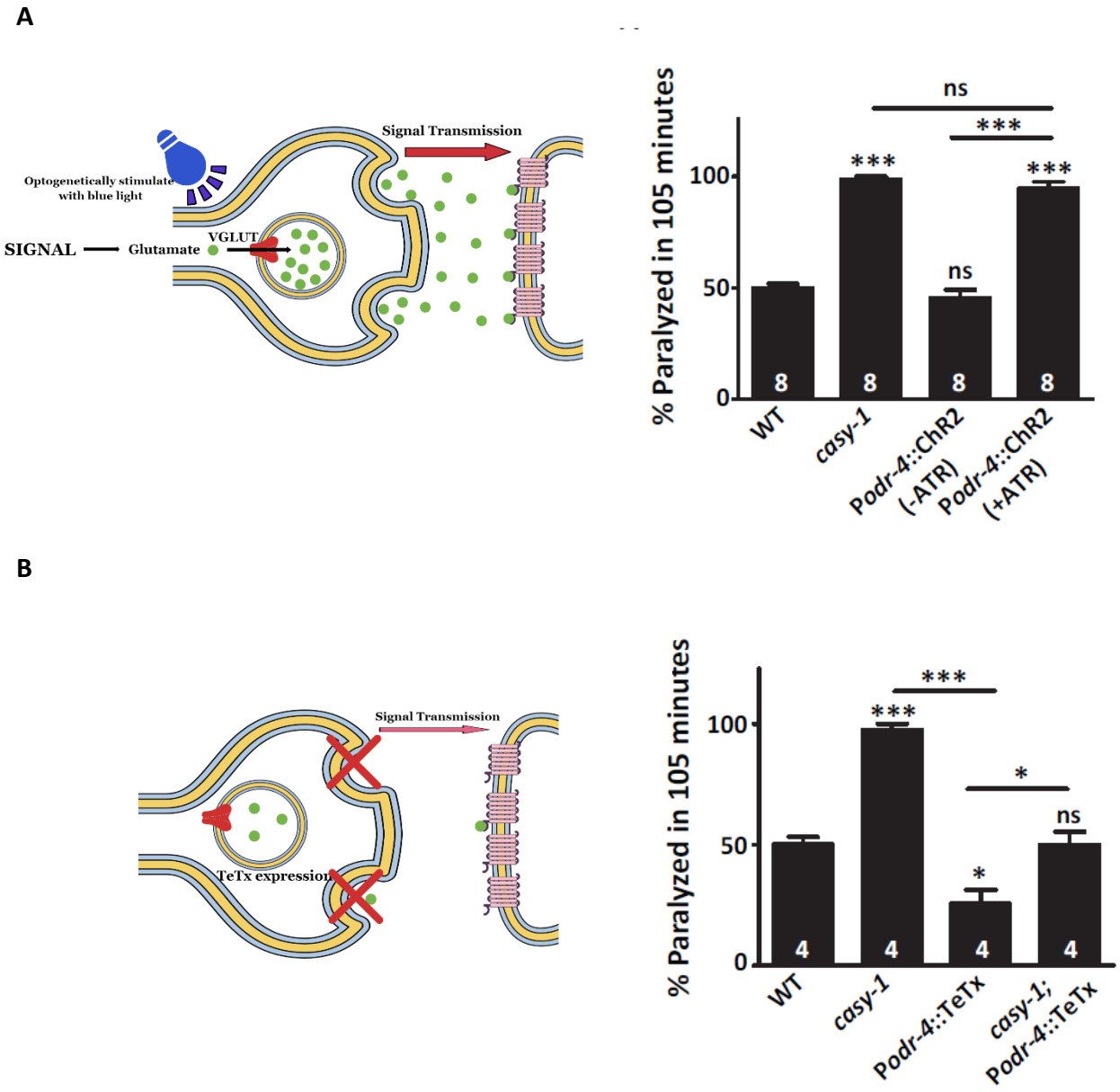

Figure 4.3: CASY-1A is required for the activity of sensory neurons, which promotes motor circuit activity. (A) Activation of sensory neurons using ChR2* (C128S) induces Aldicarb hypersensitivity in WT animals just like *casy-1* mutants. (B) Blocking vesicle release using sensory neuron-specific tetanus toxin (TeTx) induces Aldicarb resistance in WT animals. Blocking synaptic release from sensory neurons using TeTx, in the *casy-1* mutant background, causes the Aldicarb hypersensitivity of the *casy-1* mutants to reach WT levels. Promoter- intersectional, inverted Cre-Lox system is used to drive the expression of ChR2* (C128S) and TeTx in sensory neurons using *odr-4* promoter. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (*P<0.01, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test. "ns" indicates not significant in all figures).

the Aldicarb response of *casy-1* mutants to WT levels, indicating that the command interneuron serves as a link downstream of CASY-1A in sensory neurons to modulate the motor circuit activity (Figure 4.4B).

To further confirm that increased glutamatergic signaling is due to increased glutamate release from the sensory neurons and not due to increased expression of glutamate receptors on the post-synaptic interneurons, we imaged GLR-1::GFP and NMR-1::GFP in the *casy-1* mutant background. We found no significant change in the fluorescence intensity for both receptors suggesting that the post-synaptic glutamate receptor levels are normal in the *casy-1* mutants (Figure 4.4C and 4.4D).

4.5. Enhanced cholinergic transmission in *casy-1* mutants is due to reduced *npr-1* signaling

casy-1 mutants are hypersensitive to Aldicarb as previously discussed. Electrophysiological recordings from the *casy-1* NMJ revealed significantly increased endogenous EPSCs, suggesting enhanced endogenous cholinergic transmission in *casy-1* mutants (Chapter 3). The density of cholinergic α-liprin (SYD-2) puncta was unaltered in *casy-1* mutants, implying that increased cholinergic transmission is not due to an increase in the number of cholinergic NMJs. Further, the expression of the CASY-1 isoforms in cholinergic motor neurons could not rescue the Aldicarb hypersensitivity of *casy-1* mutants (Chapter 3).

CASY-1A expression in sensory neurons completely restored the Aldicarb sensitivity of *casy-1* mutants. *casy-1* mutants also show heightened sensory response to environmental cues resulting in enhanced glutamate release. This increased sensory acuity is also responsible for increased locomotion on food. Previous reports have highlighted behavioral outputs in *npr-1* mutants that are similar to those seen in *casy-1* mutants. The *npr-1* gene codes for a predicted G- protein coupled receptor homologous to mammalian NPY receptors (de Bono and Bargmann, 1998). NPR-1 expresses mainly in the nervous system where its expression is largely concentrated in neurons of the sensory circuit (de Bono and Bargmann, 1998; Coates and de Bono, 2002). Further, mutants in *npr-1* have been reported to show enhanced cholinergic transmission at NMJ. They have significantly increased EPSC frequency as well as significant Aldicarb hypersensitivity (Choi et al., 2013, 2015; Vashlishan et al., 2008). Adult *npr-1* mutants also exhibited enhanced locomotion rates. Moreover, the

cholinergic transmission defects of *npr-1* mutants were completely rescued by expressing NPR-1 in the RMG sensory neurons. In the head sensory circuit, RMG interneurons form gap junctions with several classes of sensory neurons. NPR-1 is expressed in some of these sensory neurons including the RMG interneuron to form an RMG circuit. Enhanced cholinergic transmission of *npr-1* mutants has been attributed to increased sensory acuity resulting in increased glutamatergic transmission and neuropeptide signaling (Choi et al., 2013, 2015).

Based on the above observations, we tested the idea that CASY-1 and NPR-1 could function in the same pathway at the sensory level. To verify this, *casy-1; npr-1* double mutants were generated and tested in Aldicarb assays. The Aldicarb hypersensitivity of *casy-1; npr-1* double mutant was similar to the single mutants (not additive) supporting their involvement in the same pathway (Figure 4.5A and 4.5B). We previously showed that *casy-1* mutants have accelerated locomotion on food (Figure 4.1D). In a complementary experiment we observed that locomotion rate was significantly reduced when placed on NGM plates with no food (Ravindranath S, 2016) (Figure 4.5C). Previous literature has reported a similar phenotype for *npr-1* mutants (de Bono and Bargmann, 1998). Although the mechanism behind this behavioral phenotype has not been addressed, our results indicate that CASY-1 and NPR-1 could be functioning in the same sensory circuit.

To further understand this interaction, we analyzed the sensory neurons that express the *odr-4* promoter utilized for sensory rescue. As mentioned before, the *odr-4* promoter expresses CASY-1A in 12 pair of sensory neurons (ADF, ADL, ASG, ASH, ASI, ASJ, ASK, AWA, ASB, AWC, PHA, PHB) (Dwyer et al., 1998). Our analysis revealed that four of these neurons belong to the hub and spoke RMG sensory circuit (ASH, ADL, ASK and ASI) (Coates and de Bono, 2002; Macosko et al., 2009). Further, CASY-1A expression specifically in the RMG neurons using the *flp-21* promoter completely restored the Aldicarb hypersensitivity in *casy-1* mutants to WT levels (Figure 4.5D). Prior studies have shown that several behavioral phenotypes exhibited by *npr-1* mutants are caused by enhanced secretion of a neuropeptide pigment dispersing factor (PDF-1) from touch neurons. In *C. elegans*, PDF peptides (PDF-1 and PDF-2) and their receptor (PDFR-1) have been previously identified (Janssen et al., 2008, 2009). PDF-1 overexpression rescued the Aldicarb resistance phenotype of *pdf-1* mutants (Figure 4.5E). Moreover, *pdf-1* and *pdfr-1*

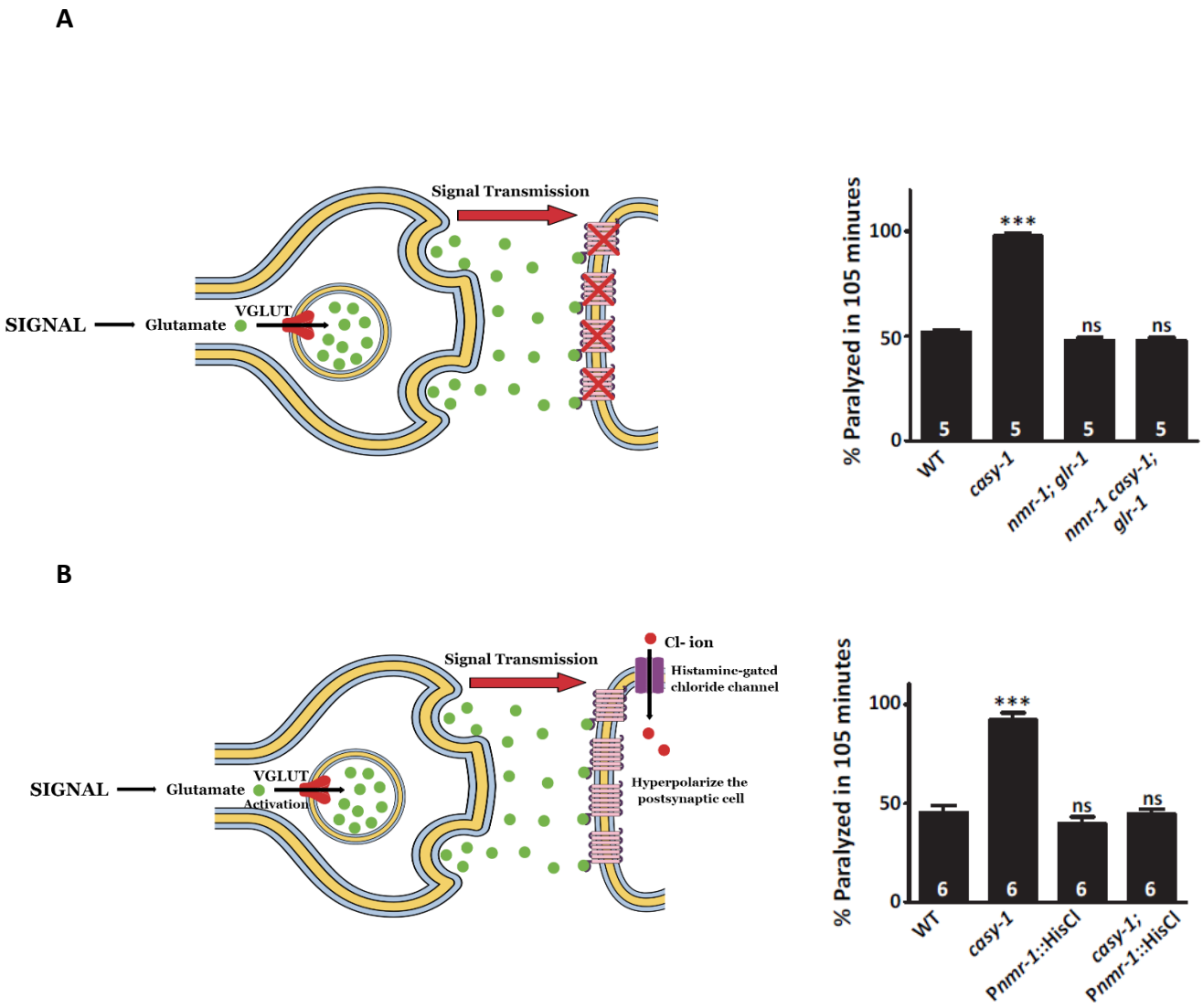

Figure 4.4a: CASY-1A functions in sensory neurons to inhibit the command interneurons of the locomotion circuit to balance the motor circuit activity. Schematic illustrating the mechanism of experiment is shown along with the graph. (A) Reduction of glutamate signaling in command interneurons using glutamate receptor (GluR) mutants suppresses the Aldicarb hypersensitivity of *casy-1* mutants. *nmr-1*; *glr-1* double mutant shows WT- Aldicarb response, and *nmr-1*; *glr-1* mutation in *casy-1* mutant background abolishes hypersensitivity in *casy-1* mutants. (B) Silencing command interneuron signaling using Histamine-gated chloride channels under *nmr-1* promoter does not affect Aldicarb sensitivity in WT *C. elegans*, but significantly suppresses hypersensitivity in *casy-1* mutants. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). "ns" indicates not significant in all figures.

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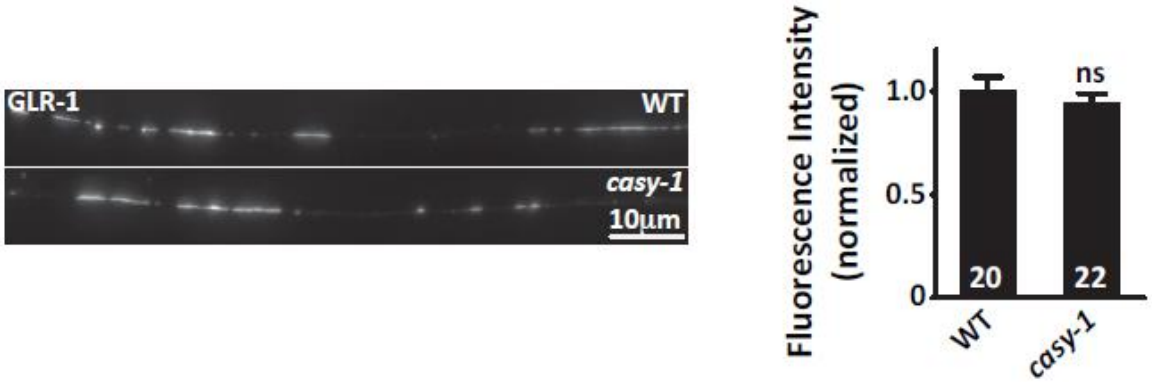

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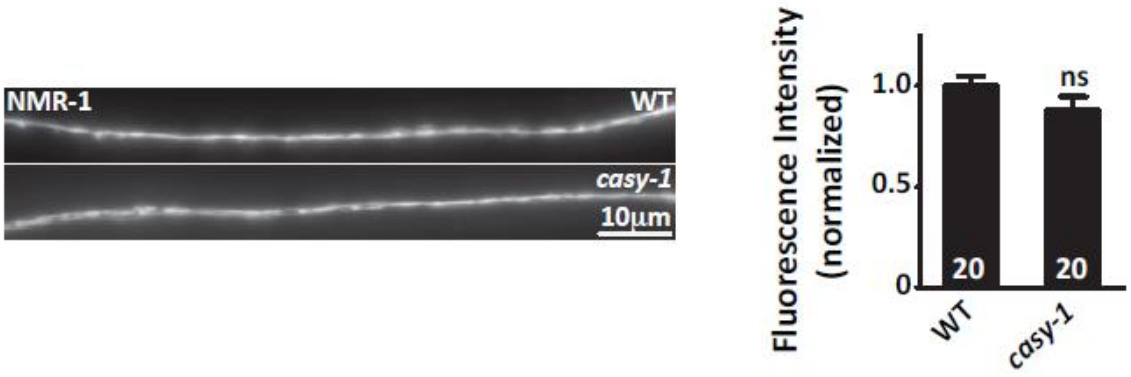

Figure 4.4b: CASY-1A functions in sensory neurons to inhibit the command interneurons of the locomotion circuit to balance the motor circuit activity. (C-D) Representative fluorescent images showing GLR-1::GFP and NMR-1::GFP in posterior ventral nerve cord of WT and *casy-1* mutant animals. No significant difference was observed in fluorescence intensity compared to WT *C. elegans*. The number of animals analyzed for each genotype is indicated at the base of the bar graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test. "ns" indicates not significant in all figures

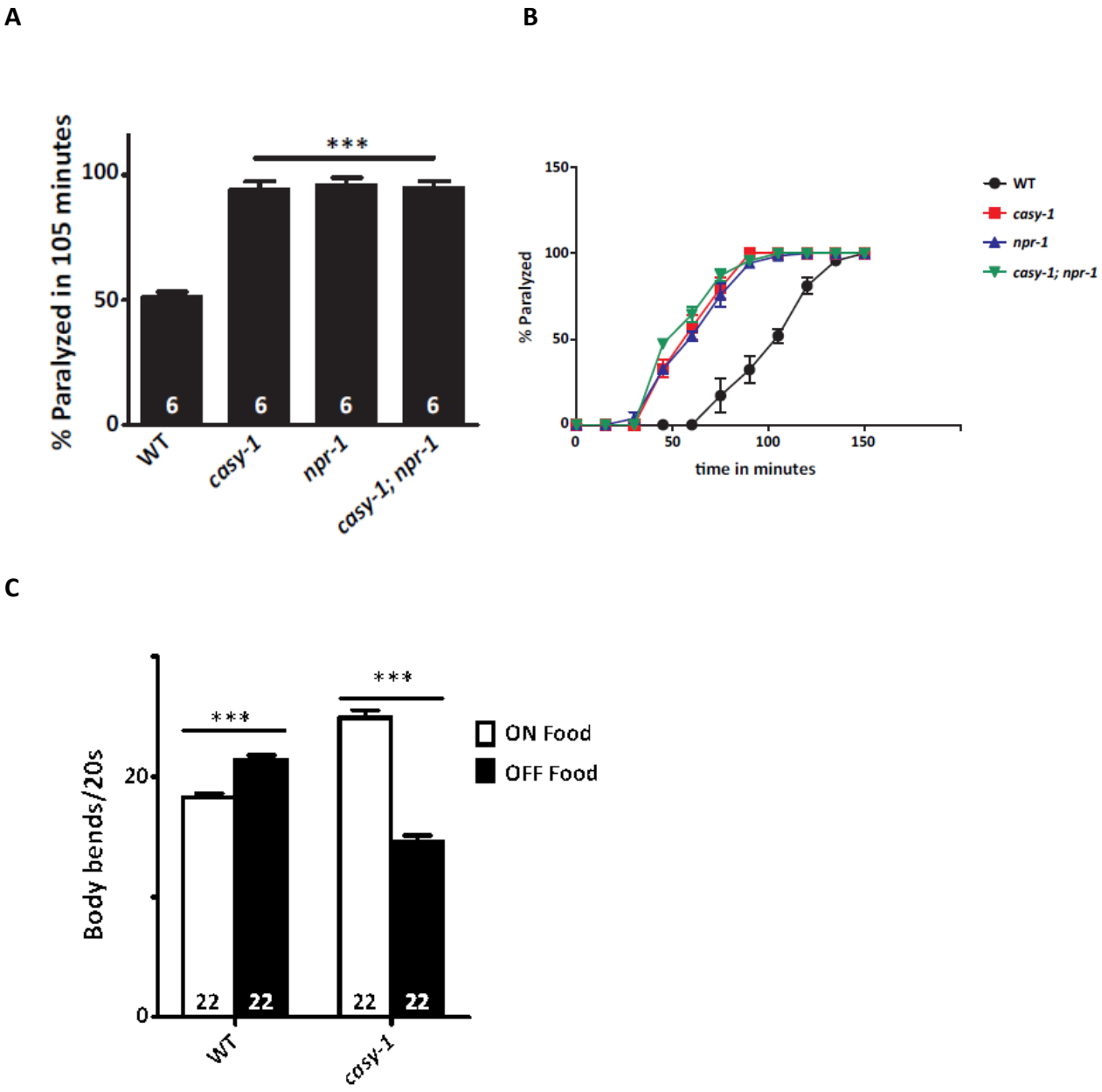

Figure 4.5a: CASY-1A affects NPR-1 signaling in the sensory-motor circuit. (A) Aldicarb-induced paralysis at 105 minutes is shown for the indicated genotypes. Mutants in *casy-1* and *npr-1* are hypersensitive in Aldicarb assays. Hypersensitivity of *casy-1; npr-1* double mutant is comparable to single mutants suggesting involvement in the same pathway. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). (B) The time-course of Aldicarb- induced paralysis (up to 150 minutes) plotted for the indicated genotypes. Mutants in *casy-1* and *npr-1* are hypersensitive on 1 mM Aldicarb. The hypersensitivity of *casy-1; npr-1* double mutants is comparable to the single mutants. (C) Body bends/20sec are shown for WT and casy-*1 (tm718)* mutant on and off food. Mutants in *casy-1* crawl faster than WT animals on food, however, off food speed is significantly reduced compared to WT controls (Ravindranath S., 2016). Similar on and off food behavior has previously been reported for *npr-1* gene. Isolated *npr-1* mutants are hyperactive on food and lethargic in the absence of food (de Bono and Bargmann, 1998). The number of animals analyzed is indicated at the base of the graph. (***P<0.0001 using two-way ANOVA and Bonferroni's Multiple Comparison Test).

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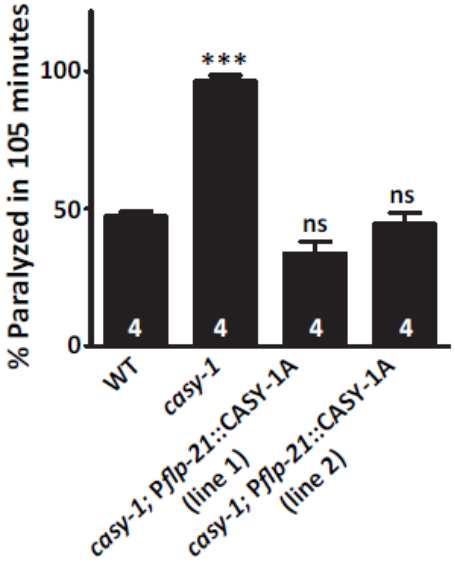

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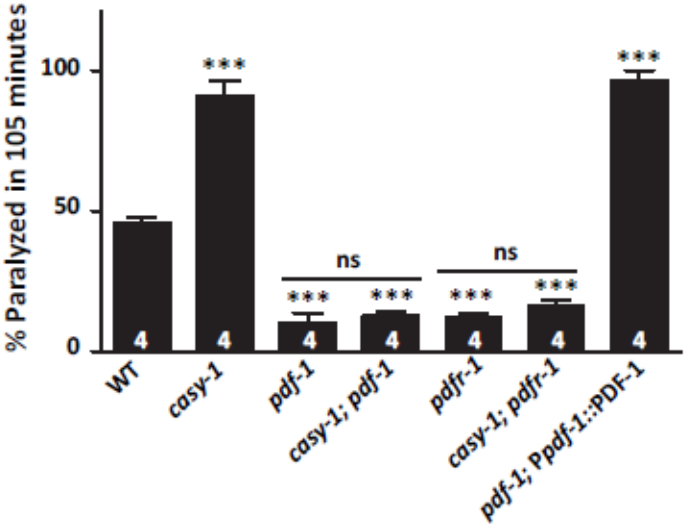


Figure 4.5b: CASY-1A affects NPR-1 signaling in the sensory-motor circuit. (D) Aldicarb hypersensitivity of *casy-1* mutants was completely rescued by expressing CASY-1A in the RMG sensory circuit using *flp-21* promoter. Rescue data for two independent transgenic lines is shown. (E) Both *pdf-1* and *pdfr-1* are resistant to Aldicarb and suppress the Aldicarb hypersensitivity seen in *casy-1* mutants. Overexpression of the PDF-1 neuropeptide rescues the Aldicarb resistance phenotype seen in the *pdf-1* mutants and causes the animals to become hypersensitive to Aldicarb. The percentage of animals paralyzed on 1 mM Aldicarb at 105 minutes was plotted for the indicated genotypes. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype at the base of the bar-graph. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test, "ns" indicates not significant in figure).

mutations completely suppressed the Aldicarb hypersensitivity of *casy-1* mutants (Figure 4.5E), supporting the contribution of PDF-1 signaling in Aldicarb hypersensitivity of *casy-1* mutants. These results give a strong indication that both CASY-1 and NPR-1 could be functioning in the same signaling pathway to modulate motor circuit activity via sensory evoked enhancement of cholinergic signaling at the *C. elegans* NMJ.

4.6. Discussion

Locomotion is one of the most studied behavioral output in *C. elegans*, it is mediated by a neuronal circuit that generates coordinated sinusoidal movement. The locomotor circuit in *C. elegans* works through a "cross-inhibition model" where the excitatory cholinergic motor neurons form dyadic synapses onto both the ipsilateral muscle as well as onto inhibitory GABAergic motor neurons that then synapse onto the contralateral muscle (White et al., 1976, 1986). An important principle that maintains locomotion is a coordinated balance between the excitation (E) and inhibition (I) (Isaacson and Scanziani, 2011; Stawicki et al., 2013). Despite considerable reports on the involvement of E/I imbalance in the pathogenesis of several neurological disorders, genetic factors that control and modulate this balance have not all been identified. Previously, *acr-2(gf)* has been reported to alter this E/I balance resulting in spontaneous convulsions (Stawicki et al., 2013). More recently, the neuronal calcium sensor protein NCS-2 has been reported to regulate asynchronous release mediated E/I imbalance (Zhou et al., 2017). Also, an endoplasmic-reticulum resident chaperone, RIC-3 phosphorylation status has been shown to dictate the E/I balance at the muscle (Safdie et al., 2016). In our study, we identified CASY-1 as an active regulator of both excitatory and inhibitory synaptic transmission, thus emerging as a valuable novel controller of excitation-inhibition balance.

Previously, we have reported the role of CASY-1 in regulating GABA release and hence maintaining normal GABAergic neurotransmission at the *C. elegans* NMJ. We showed that the two shorter isoforms of CASY-1, CASY-1B and CASY-1C, express and function specifically in the GABAergic motor neurons to regulate GABA release dynamics by mediating the trafficking of GABA synaptic vesicle (SV) precursors via their interaction with the KIF1A/UNC-104 motor protein (Chapter 3).

In this study, we report different mechanisms for CASY-1A mediated regulation of excitatory cholinergic transmission. Mutants in *casy-1* showed enhanced

locomotion on food suggesting heightened motor circuit activity. Our results demonstrate that CASY-1A functions in multiple classes of sensory neurons to decrease the motor circuit activity. In this study, we propose one such mechanism that might explain the role of CASY-1A in this function. Mutants in *casy-1* have heightened sensory responsiveness resulting in increased glutamate and neuropeptide release, which in turn affects interneuron signaling and finally results in enhanced motor circuit activity (Figure 4.6). We also show that CASY-1A functions in sensory neurons by promoting NPR-1 signaling. NPR-1 has previously been shown to promote behavioral quiescence during lethargus and in adult *C. elegans*. NPR-1 signaling functions in a local RMG sensory circuit by diminishing the sensitivity of many sensory modalities (Choi et al., 2013). The NPR-1 receptor is coupled to Gi/Go proteins and hyperpolarizes membrane potential through G protein-activated inwardly rectifying K+ channels (GIRKs) resulting in diminished activity of RMG circuit (Rogers et al., 2003). However, stimulating NPR-1 signaling could just be one mechanism for CASY-1A functioning. CASY-1A expresses in other non- NPR-1 expressing sensory neurons, thus we cannot rule out additional mechanisms that might be contributing to CASY-1A sensory functions.

Our preliminary results also implicate CASY-1A function in interneurons (discussed in Chapter 5) to regulate the motor circuit activity. Specifically, we report that CASY-1A expression in RIM or AVE interneurons can completely restore the motor circuit activity in *casy-1* mutants. The neural connectivity of RIM/AVE with the command interneurons along with our genetic, pharmacological and optogenetic data support a model wherein CASY-1A functions in RIM/AVE to specifically regulate the command interneuron signaling to inhibit the motor circuit activity.

Mammalian calsyntenins have been reported to be required for the development and functioning of both excitatory glutamatergic and inhibitory GABAergic synapses (Pettem et al., 2013). Our study provides some useful insights into possible roles for mammalian calsyntenins at glutamatergic synapses. Mammalian calsyntenins have not been addressed to interact with the components of neuropeptidergic signaling. Substantial conservation of behavioral and genetic mechanisms between mammalian Calsyntenins and *C. elegans* CASY-1A strengthens the likely existence of similar mechanisms in other systems.

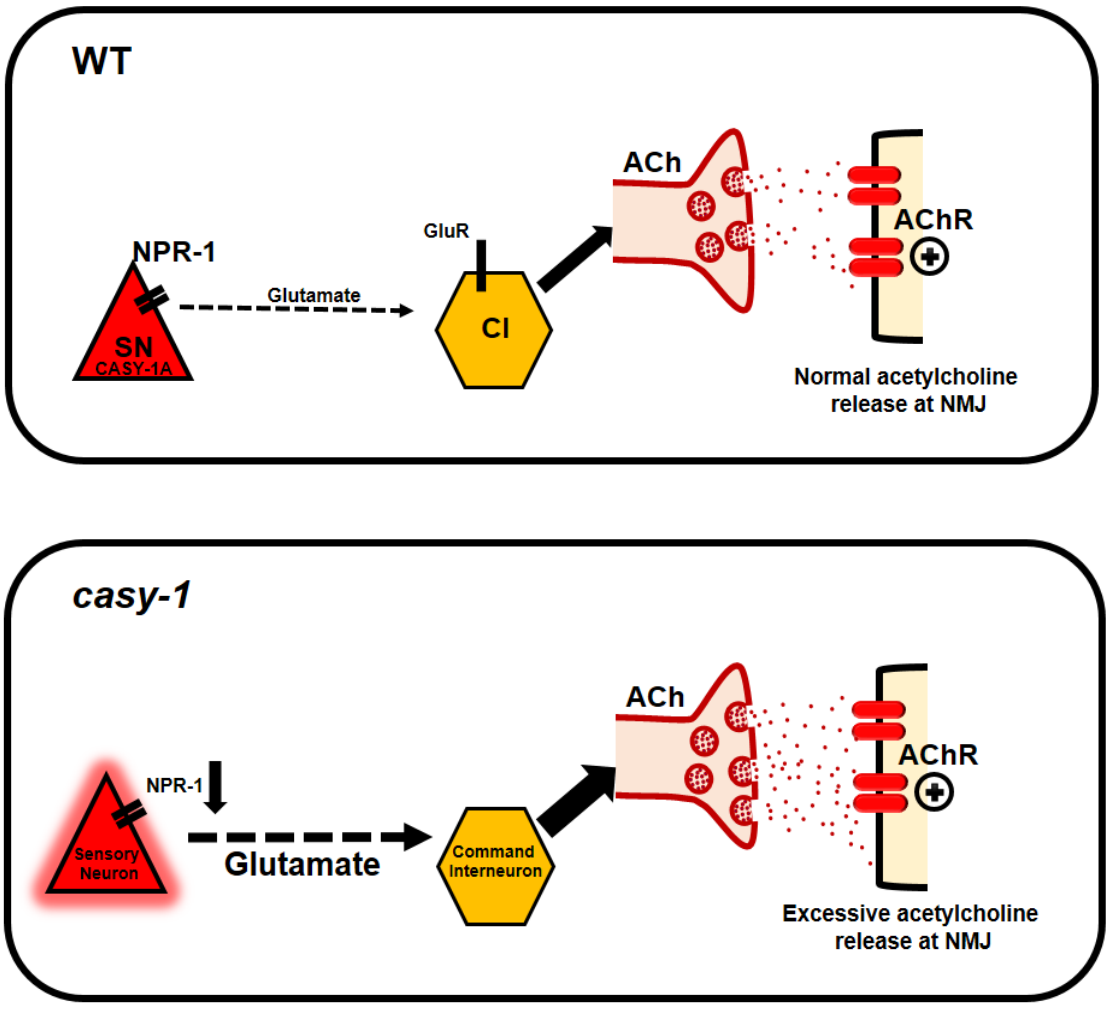


Figure 4.6: Proposed model for CASY-1A function in sensory neurons. Mutants in *casy-1* have an increased acetylcholine release at the NMJ. This Chapter addresses the role of CASY-1A in maintaining the excitatory cholinergic transmission at NMJ by employing multiple mechanisms of regulatory control from higher levels of locomotion circuit. In WT *C. elegans*, CASY-1A is expressed in sensory neurons to inhibit the motor circuit activity. In sensory neurons, it functions partly by activating NPR-1 signaling in the RMG hub-and spoke circuit. Activation of NPR-1 signaling results in a regulated release of the neurotransmitter glutamate or/and neuropeptide/s. Loss of *casy-1* could cause a disruption in NPR-1 signaling resulting in enhanced glutamate and neuropeptide release that exaggerates the motor circuit activity via increased signaling from command interneurons. Increased signaling from the command interneurons can in turn result in increased acetylcholine release at the NMJ thus increasing the excitatory signals ensuing enhanced motor circuit activity and Aldicarb hypersensitivity.

CHAPTER 5:

Conclusions and Future directions

The experiments discussed in this chapter are preliminary data that require additional experimentation to validate the results.

5.1. Implications of role of CASY-1 in regulating synaptic function

Diverse families of cell adhesion molecules present at the synapse highlight the pivotal role of these molecules in regulating various aspects of nervous system development and function including axon-guidance, synapse formation and maintenance, synaptic transmission and plasticity. Our study has focused on the functional role of one such cell adhesion molecule, CASY-1 that belongs to the cadherin superfamily. Our results demonstrate that CASY-1 functions to maintain the balance between excitation and inhibition at the *C. elegans* neuromuscular junction, thus coordinating locomotory behavior. This study provides new insights on isoform-specific CASY-1- mediated regulation of inhibitory GABAergic signaling by directly affecting GABA vesicle transport as well as CASY-1- mediated regulation of excitatory glutamatergic signaling that in turn could modulate cholinergic release from motor neurons.

Our study features an interesting mechanism where three isoforms of the gene, *casy-1,* function in different subsets of neurons to regulate entirely different signaling processes. The shorter isoforms function in GABAergic motor neurons to maintain balanced GABA release at the NMJ thus maintaining inhibitory signals. While the longer isoform function in higher levels of the locomotor circuits, in sensory neurons to regulate glutamatergic and neuropeptidergic signaling that ultimately functions to maintain excitatory acetylcholine release at the NMJ, thus maintaining excitatory signals. A precise regulation of these excitatory and inhibitory signals maintained by CASY-1 coordinates movement in *C. elegans* (Figure 5.1).

5.1.1. Regulation of inhibitory synaptic transmission at the *C. elegans* NMJ

GABA acts as a major inhibitory neurotransmitter in both vertebrate and invertebrate nervous system (Docherty et al., 1985). Despite considerable reports on the role of GABA signaling in proper maintenance of nervous system function (reviewed in (Chiapponi et al., 2016; Contestabile et al., 2017; Schür et al., 2016)), a comprehensive understanding of key genetic factors that regulate and maintain GABA synapse structure and function is still at its infancy.

In this thesis study, we have identified CASY-1 as a novel regulator of GABAergic synaptic transmission at the *C. elegans* neuromuscular junction. The shorter isoforms of CASY-1 expressed by alternative promoters: CASY-1B and

CASY-1C express in GABAergic motor neurons to explicitly regulate GABA release at the NMJ. CASY-1B/CASY-1C that are essentially just the conserved C- terminal of mammalian calsyntenin homologs and lack the entire extracellular N- terminal domains functions to regulate the trafficking of GABA synaptic vesicle precursors. Our study identifies a novel interaction of CASY-1 C- terminal with a conserved motor protein specifically required for transport of synaptic vesicle precursors UNC-104, a *C. elegans* homolog of mammalian KIF-1A. Our study will contribute to a better understanding of the role of mammalian calsyntenins in GABAergic synaptic transmission as well as provide a promising base to investigate the possible interaction of mammalian calsyntenins with other motor proteins.

5.1.2. CASY-1 mediated sensory regulation of motor circuit and behavior

Sensory inputs from diverse environmental stimuli are integrated and processed in several layers of neuronal circuits to modulate the behavioral responses of an organism. Most studies have focused on either the physiology of the sensory organs or on the motor output of organisms. However, the genetic factors that coordinate this information flow from sensory to motor circuit and the neuronal circuits that maintain sensory -regulated motor behaviors are largely unknown.

Our study highlights the role of longer CASY-1A isoform in the sensory neurons to regulate the motor circuit activity and dynamics. Our results demonstrate that CASY-1A functions to monitor the activity of sensory neurons thus maintaining the coordinated release of glutamate from these neurons. In a subset of sensory neurons, CASY-1A regulation of glutamate release could be mediated by positive regulation of NPR-1 signaling that has previously been reported to inhibit glutamate and neuropeptide release from a central sensory circuit that eventually reduces cholinergic transmission at the NMJ. Thus, CASY-1A functions in the RMG-sensory circuit to regulate NPR-1 signaling hence maintaining balanced acetylcholine release at the NMJ.

Our study contributes to a better understanding of genetic factors and neural circuits that coordinate sensory regulated motor behaviors.

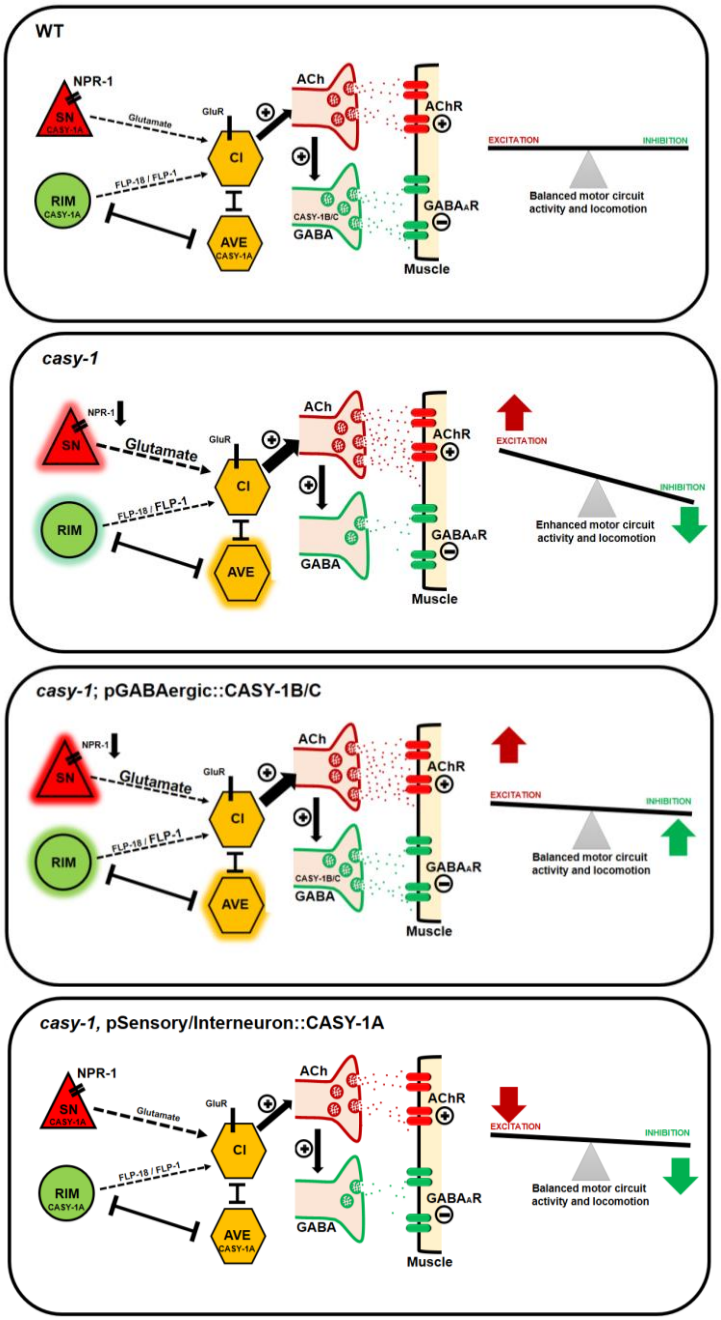


Figure 5.1: Proposed model for CASY-1 function in regulating excitation-inhibition balance at *C. elegans* NMJ. Mutants in *casy-1* have an excitation-inhibition imbalance at the NMJ. Cholinergic transmission is enhanced while the GABAergic transmission is reduced resulting in an overall increase in the motor circuit activity. CASY-1A isoform functions in sensory and interneurons to maintain excitatory cholinergic transmission while shorter isoforms CASY-1B/1C function in GABAergic motor neurons to maintain normal GABA release. Restoring this imbalance either via expression of shorter CASY-1 isoforms in GABAergic motor neurons or the expression of CASY-1A in sensory/interneuron restores the locomotion dynamics in *casy-1* mutants, thus establishing CASY-1 as a novel regulator to maintain excitation-inhibition balance at *C. elegans* NMJ. CI represents Command interneuron-AVE neuron.

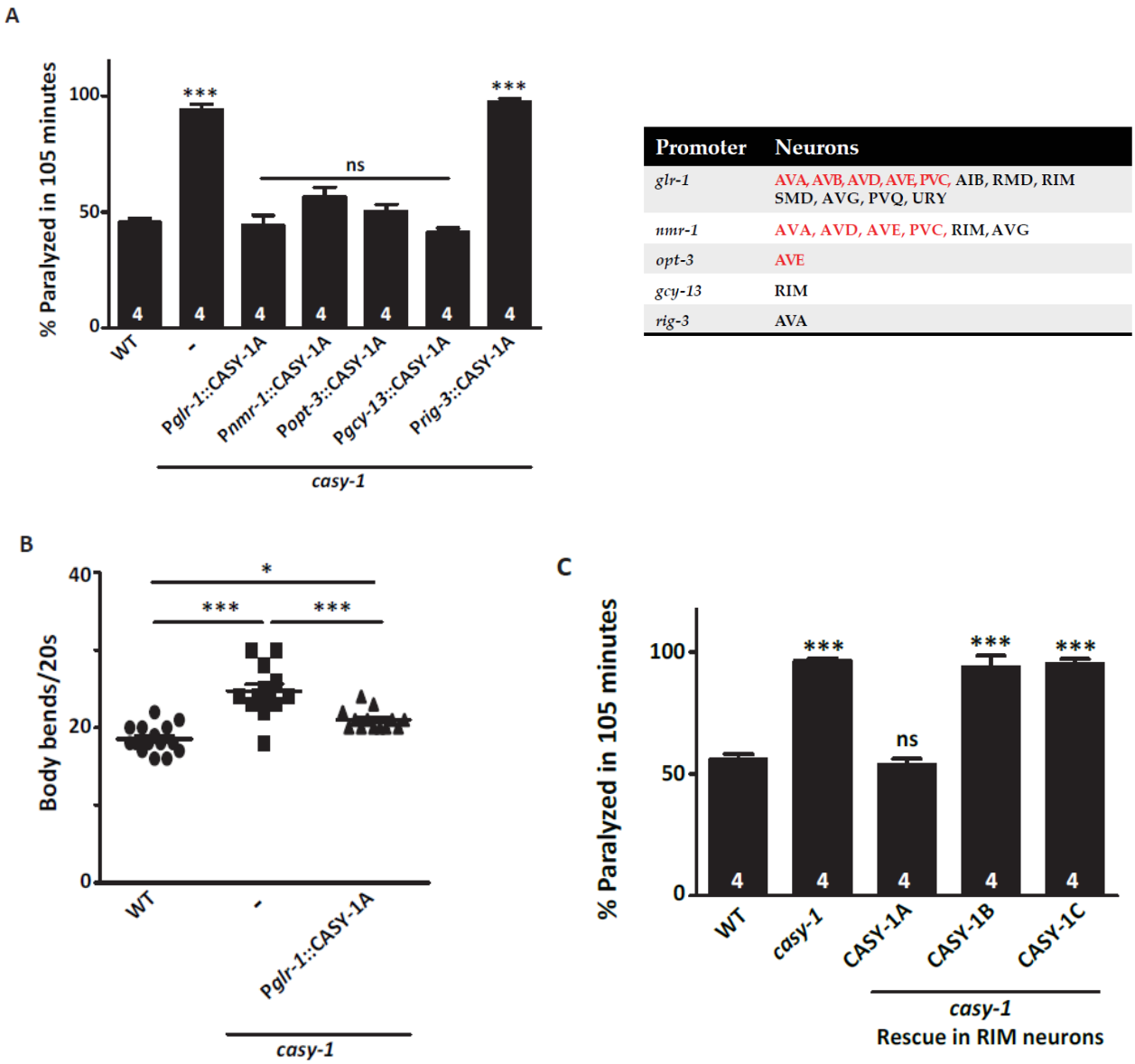
5.2. Areas for future investigations

5.2.1. Role of CASY-1 in interneurons

CASY-1A expression in several head sensory neurons ((Ikeda et al., 2008) and Figure 4.1A) indicates its possible role in transmitting the sensory signal that gives rise to motor output. Besides sensory neurons, CASY-1A expression was also observed in some interneurons (Ikeda et al., 2008). To address the possible role of CASY-1A in monitoring the motor circuit activity via functioning at the level of interneurons, a transgenic line was used that expresses CASY-1A under the *glr-1* promoter. This promoter is expressed in 12 pair of interneurons including the five command interneurons (Figure 5.2A). Interestingly, CASY-1A expression under *glr-1* promoter completely rescued the Aldicarb hypersensitivity seen in *casy-1* mutants, supporting our hypothesis that CASY-1A could also function in interneurons to modulate the motor output (Figure 5.2A). CASY-1A expression under *glr-1* promoter also partially rescued the increased body bends phenotype of *casy-1* mutants (Figure 5.2B). To narrow down the neurons where CASY-1A might function, a transgenic line was generated utilizing the *nmr-1* promoter that expresses CASY-1A in six interneurons (Figure 5.2A). CASY-1A expression under *nmr-1* promoter also rescued the Aldicarb hypersensitivity of *casy-1* mutants (Figure 5.2A). To specify the interneurons where CASY-1A might be functioning to regulate motor circuit activity, co-localization of P*nmr-1*::mCherry with P*casy-1a*::GFP was analyzed. Co-localization analysis revealed that CASY-1A does not express in all NMR-1 expressing cells. Based on previously published literature and utilizing the database of behavioral and structural anatomy of *C. elegans* (Hoerndli et al., 2009; Ikeda et al., 2008) and *Wormatlas*), CASY-1A was identified to co-localize with two interneurons, RIM (a motor cum- interneuron) and AVE (a command interneuron) (Movie 5.1). To validate the expression of CASY-1A in these two neurons transgenic lines P*gcy-13*::mCherry (expressed in RIM) and P*opt-3*::mCherry (expressed in AVE) were generated. CASY-1A shows expression and co-localization in both RIM and AVE (Figure 5.2D). As a control, P*rig-3*::mCherry (expressed in AVA) transgene was also generated, and no expression of CASY-1A was observed in AVA interneurons (Figure 5.2D, 3rd panel). Next, we investigated if CASY-1A expression in either RIM or AVE could specifically rescue the Aldicarb hypersensitivity in *casy-1* mutants. Transgenic lines expressing CASY-1A under *gcy-13* and *opt-3* promoters were

generated, and these lines completely restored the Aldicarb response of *casy-1* mutants to WT level (Figure 5.2A). Expressing CASY-1A in AVA interneurons using the *rig-3* promoter, did not rescue the Aldicarb hypersensitivity of *casy-1* mutants (Figure 5.2A). RIM is an interneuron which also functions as a motor neuron for head muscles (Gray et al., 2005; White et al., 1986), while AVE is a command interneuron that regulates reversal along with AVA and AVD (Alkema et al., 2005; Chalfie et al., 1985; Gray et al., 2005; Hart et al., 1995; Kaplan and Horvitz, 1993; Maricq et al., 1995; Zheng et al., 1999). Expression of CASY-1A in AVE rescues the Aldicarb phenotype of *casy-1* mutants but expression in AVA did not. This highlights additional functions of the AVE interneurons that are independent of AVA. Analysis of synaptic connectivity between RIM and AVE revealed that the two interneurons do not share chemical synapses but are connected by 10 gap junctions that could synchronize the activity of these two neurons and hence could explain the rescue specifically in the two neurons. For all further experiments the RIM interneuron promoter was utilized.

To determine whether CASY-1A plays a role in regulating the activation state of RIM (/AVE), the previously discussed promoter-intersectional, inverted Cre-Lox strategy was used. Here, ChR2* (C128S) and TeTx were expressed specifically in the RIM interneuron using the *gcy-13* promoter. The expression in RIM was confirmed by analyzing GFP expression using fluorescence microscopy. The photoactivation of RIM interneuron using ChR2* resulted in Aldicarb hypersensitivity in WT animals. This raises the possibility that in *casy-1* mutants RIM interneuron might show increased activation (Figure 5.2E). To further elucidate if the RIM interneuron is activated in *casy-1* mutants, a complimentary experiment was done where the release of neurotransmitter and neuropeptide/s from the RIM interneuron was blocked using TeTx. If the hypersensitivity in *casy-1* mutants is due to increased activity and hence increased synaptic release from RIM interneuron, then using this transgenic line, should abolish the Aldicarb hypersensitivity of *casy-1* mutants. Blocking basal synaptic release from RIM interneuron results in Aldicarb resistance in WT animals (Figure 5.2F), suggesting that RIM releases neurotransmitter and/or neuropeptide/s that promotes motor circuit activity. Further, blocking synaptic release in *casy-1* mutants using TeTx restores the hypersensitivity of *casy-1* mutants to WT levels. Collectively, these



Promoter	Neurons
glr-1	AVA, AVB, AVD, AVE, PVC, AIB, RMD, RIM SMD, AVG, PVQ, URY
nmr-1	AVA, AVD, AVE, PVC, RIM, AVG
opt-3	AVE
gcy-13	RIM
rig-3	AVA

Figure 5.2a: CASY-1A modulates motor circuit activity through the RIM and AVE interneurons (A) Aldicarb- induced paralysis at 105 minutes plotted for indicated genotypes. Hypersensitivity of *casy-1* mutants was completely rescued by expressing CASY-1A in interneurons using *glr-1* and *nmr-1* promoters. Expression in the motor and interneuron RIM and command interneuron AVE specifically, also restores Aldicarb hypersensitivity in *casy-1* mutants to WT levels. Expression of CASY-1A in command interneuron AVA does not rescue hypersensitivity of *casy-1* mutants. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. Table is highlighting the rescue promoters used in this study and the specific neurons where these promoters express. (B) Body bends/20sec are shown for WT, *casy-1 (tm718)* and rescue lines expressing CASY-1A in interneurons using *glr-1* promoter. CASY-1A expression in interneurons partially rescued the body bend defect in *casy-1* mutants. n=15-20 animals. (C) Aldicarb- induced paralysis at 105 minutes, plotted for the indicated genotypes. The hypersensitivity of *casy-1* mutants was completely rescued by expressing CASY-1A in RIM interneurons using the *gcy-13* promoter. However, expressing CASY-1B or CASY-1C under the *gcy-13* promoter could not rescue the Aldicarb hypersensitivity of the *casy-1* mutants. (*P<0.01, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test, "ns" indicates not significant in all figures).

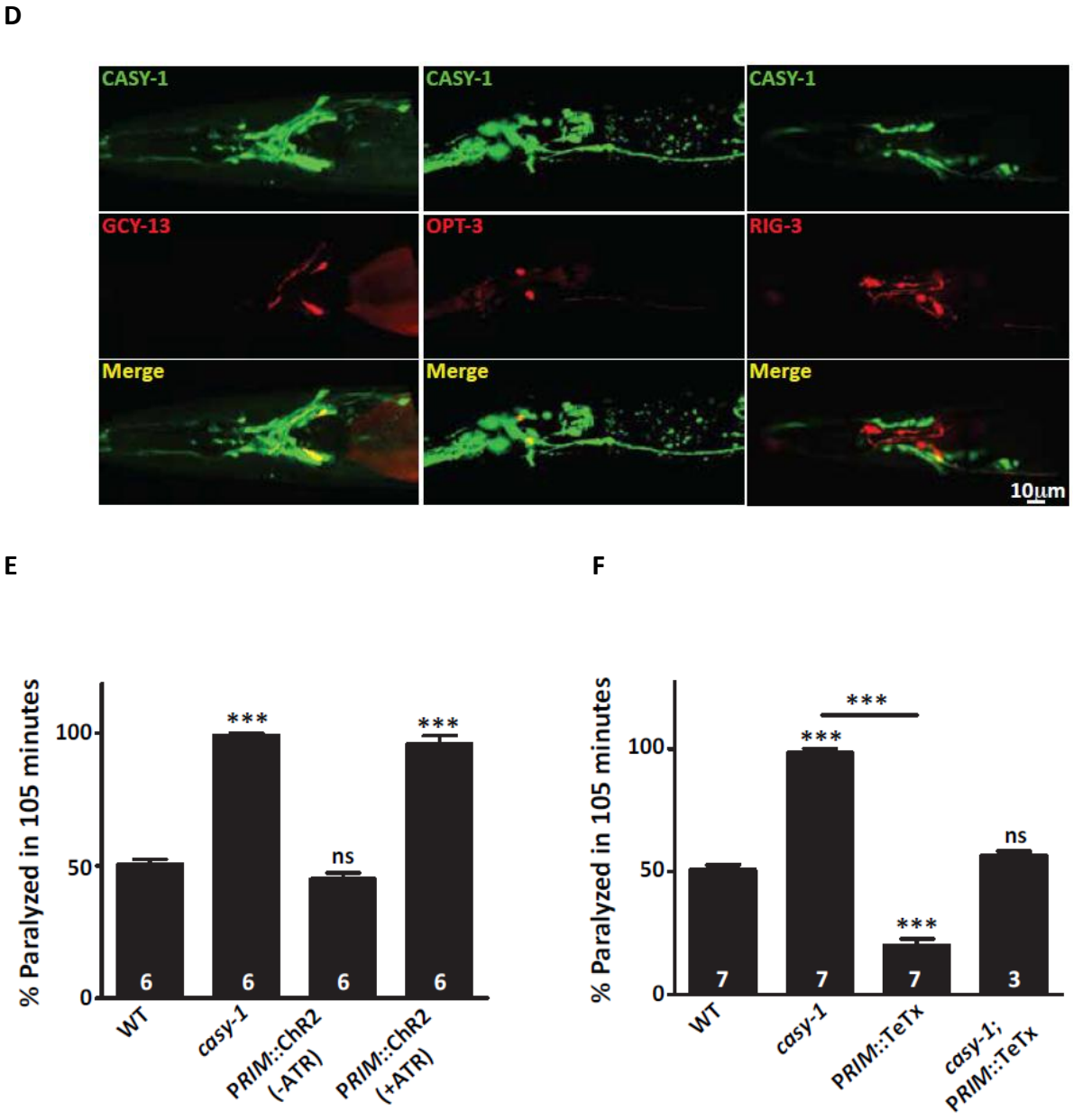


Figure 5.2b: CASY-1A modulates motor circuit activity through the RIM and AVE interneurons. (D) Representative fluorescent images showing colocalization of P*casy-1a*::GFP reporter with RIM- (*gcy-13* promoter) and AVE- (*opt-3* promoter) specific mCherry –tagged transcriptional reporters. P*casy-1a*::GFP reporter showed no colocalization with AVA (*rig-3* promoter) reporter line. (E) Activation of RIM using ChR2* (C128S) induces Aldicarb hypersensitivity in WT animals just like *casy-1* mutants. (F) Blocking vesicle release using RIM neuron-specific tetanus toxin (TeTx) induces Aldicarb resistance in WT animals. Blocking synaptic release from RIM using TeTx, in the *casy-1* mutant background, rescues Aldicarb hypersensitivity to WT levels. Promoter- intersectional, inverted Cre-Lox system is used to drive the expression of ChR2* (C128S) and TeTx in RIM neurons using *gcy-13* promoter. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test. “ns” indicates not significant in all figures).

results indicate that synaptic release from RIM (/AVE) interneuron contributes to the enhanced activity at the NMJ in *casy-1* mutants (Figure 5.2F).

To further validate the role of CASY-1A isoform in regulating synaptic release from interneurons and hence modulating the motor activity, transgenic lines expressing the shorter isoforms of CASY-1; CASY-1B and CASY-1C specifically in the RIM interneuron using the *gcy-13* promoter were generated. These transgenic lines failed to rescue the Aldicarb hypersensitivity of *casy-1* mutants, thus confirming the role of full-length CASY-1A isoform in this function (Figure 5.2C).

Further experiments are needed to elucidate the exact function of CASY-1A isoform in these two interneurons. How CASY-1A is modulating the activity of these neurons? Are there other interacting proteins that contributes to this role of CASY-1A isoform in these neurons?

5.2.2. Role of Neuropeptides in mediating enhanced motor circuit activity at the NMJ in *casy-1* mutants

Increased secretion of the neuropeptide PDF-1 from the RMG sensory circuit contributes to the enhanced motor activity at the NMJ in *casy-1* mutants (Figure 4.5E). This raises the possibility of the involvement of other neuropeptides in this regulation. *C. elegans* expresses more than 250 different neuropeptides (Li and Kim, 2008) that have been documented to modulate neural circuits involved in locomotion, feeding, reproduction and synaptic plasticity (Barrios et al., 2012; Beets et al., 2012; Chalasani et al., 2010; Garrison et al., 2012; Hu et al., 2011). Most of the sensory neurons and interneurons release one or more neuropeptides. Increased activity of sensory and interneurons in *casy-1* mutants might modify the release of neuropeptides from these neurons which can then extrasynaptically modulate acetylcholine release at the NMJ. To specifically test the role of neuropeptides on the increased Aldicarb sensitivity seen in *casy-1* mutants, mutants that are known to disrupt neuropeptide processing were first examined. The best- characterized enzymes reported for pro-neuropeptide processing are the proprotein convertase (PC2), EGL-3 and the carboxypeptidase E (CPE), EGL-21 (Jacob and Kaplan, 2003; Kass et al., 2001; Li and Kim, 2008). Both *egl-3* and *egl-21* mutants show resistance to Aldicarb as shown previously ((Jacob and Kaplan, 2003) and Figure 5.3A). Further, removing *egl-3* and *egl-21* in the *casy-1* mutant background completely suppressed the Aldicarb

hypersensitivity of *casy-1* mutants (Figure 5.3A) supporting the possible role of neuropeptides in modulating the motor circuit activity in *casy-1* mutants.

Previously the FMRFamide- family of neuropeptides FLP-1 and FLP-18 have been shown to act synergistically to reduce the excitation- inhibition imbalance at the NMJ in *acr-2 (gf)* mutants (Stawicki et al., 2013). Additionally, FLP-1 peptides have also been reported to alter the locomotory behavior (Nelson et al., 1998). FLP-1 and FLP-18 expression has been reported in several anteriorly positioned interneurons (Kim and Li, 2004; Rogers et al., 2003). Interestingly, FLP-1 is secreted by the interneuron AVE and FLP-18 expresses in RIM (Kim and Li, 2004; Nelson et al., 1998; Rogers et al., 2003). CASY-1A expression in RIM and AVE completely rescued the Aldicarb hypersensitivity in *casy-1* mutants (Figure 5.1A) thus raising the possibility that these two neuropeptides might play some role in the increased Aldicarb sensitivity seen in *casy-1* mutants.

Mutants in *flp-1* showed resistance to Aldicarb as shown previously ((Sieburth et al., 2005; Stawicki et al., 2013) and Figure 5.2B). Removing *flp-1* from the *casy-1* mutant background completely abolished the Aldicarb hypersensitivity of *casy-1* mutants (Fig. 7B). These results propose that *casy-1* mutants probably have increased secretion of the FLP-1 neuropeptide that might be responsible for the increased activity at the NMJ. Supporting our assumption, FLP-1 overexpression results in Aldicarb hypersensitivity in WT animals. Also, expressing FLP-1 under the *flp-1* promoter reinstated the Aldicarb hypersensitivity in *casy-1; flp-1* double mutants (Figure 5.3B).

Compared to *flp-1* mutants, *flp-18* mutants showed Aldicarb hypersensitivity (Figure 5.3C). This was surprising, as *flp-18* mutants have previously been reported to behave like WT animals upon Aldicarb treatment (Choi et al., 2013; Stawicki et al., 2013). One possible explanation for this discrepancy could be that both Choi S. *et al.* and Stawicki T.M. *et al.* utilized the *flp-18 (tm2179)* mutant allele, which removes the N- terminal of *flp-18* that largely includes the first intron. However, in our study we utilized the *flp-18 (gk3063)* mutant allele that removes the entire coding region of *flp-18* except for the first exon (*Wormbase*).

A double mutant of *casy-1* and *flp-18* resulted in hypersensitivity comparable to each of the single mutants (Figure 5.3C). These results demonstrate that FLP-18 functions to reduce the motor circuit activity in WT *C. elegans* just like CASY-1A. If this were the case, FLP-18 overexpression should result in Aldicarb resistance. We

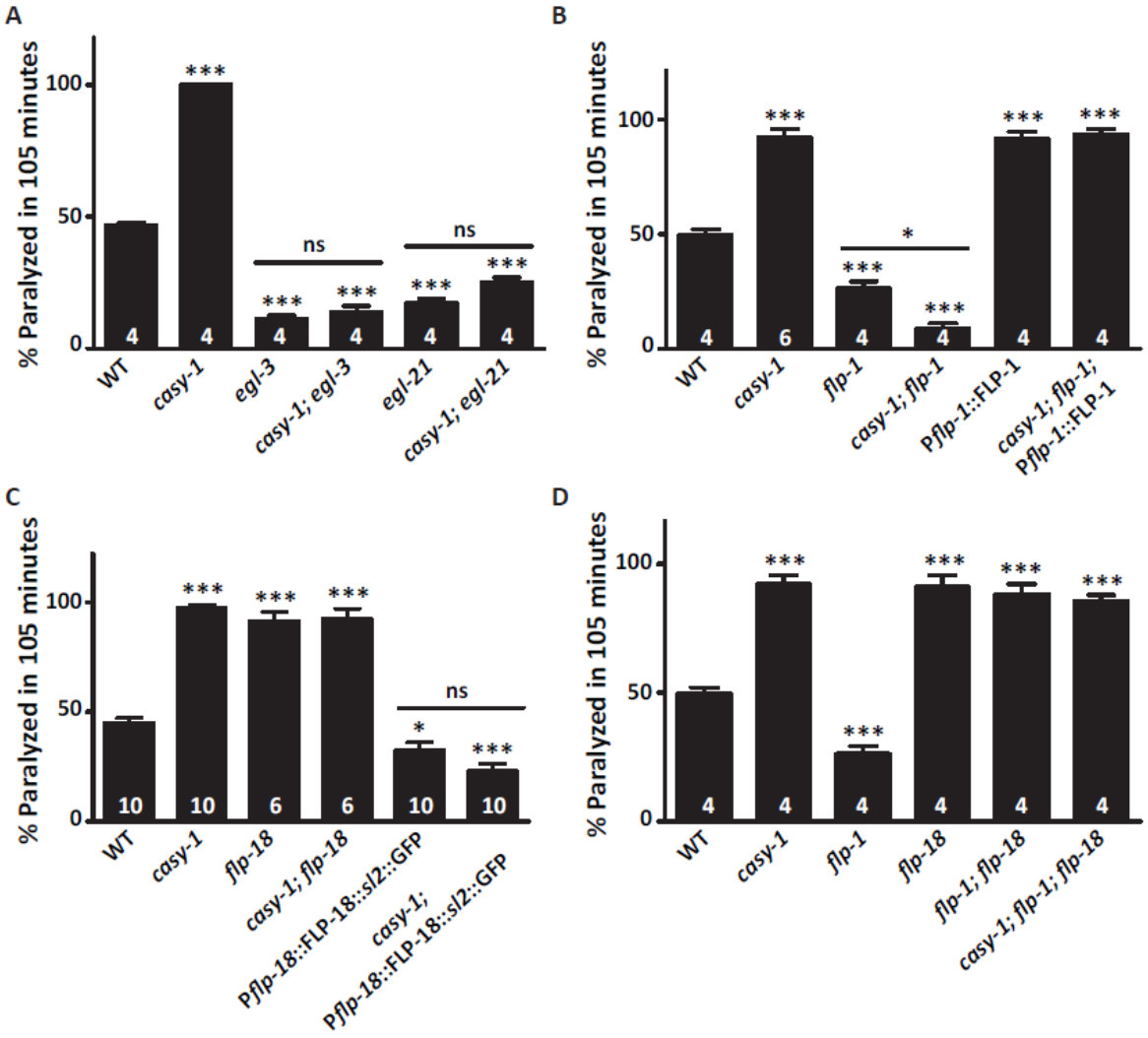


Figure 5.3a: CASY-1A modulates motor circuit activity through neuropeptides (A-D) Aldicarb- induced paralysis at 105 minutes plotted for indicated genotypes. (A) Mutations in neuropeptide processing enzymes *egl-3* and *egl-21* suppressed the Aldicarb hypersensitivity of *casy-1* mutants. (B) Mutants in *flp-1* are resistant in Aldicarb assay and also abolishes the hypersensitivity of *casy-1* mutants. FLP-1 overexpression in WT *C. elegans* results in Aldicarb hypersensitivity and expression in *casy-1*; *flp-1* double mutant reinstates the Aldicarb hypersensitivity of *casy-1* mutants. (C) *flp-18* mutants are hypersensitive in Aldicarb assay. Also, FLP-18 overexpression results in Aldicarb resistance and also suppresses the Aldicarb hypersensitivity of *casy-1* background. (D) The Aldicarb phenotype of *flp-18* is dominant over *flp-1*, as *flp-1; flp-18* double mutant are hypersensitive. Also, a triple mutant with *casy-1* showed hypersensitivity comparable to the single mutants. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test).

tested for FLP-18 overexpression utilizing a transgenic reporter P*flp-18*::FLP-18::*sl2*::GFP (Cohen et al., 2009). As expected, FLP-18 overexpression resulted in Aldicarb resistance in WT animals. Also, FLP-18 overexpression completely suppressed the Aldicarb hypersensitivity of *casy-1* mutants (Figure 5.3C). Collectively, these results allowed us to hypothesize that in *casy-1* mutants, there is lower expression/release of FLP-18 that might contribute to enhanced motor circuit activity. To confirm this, the fluorescence intensity of the neuropeptide using the above mentioned bicistronic FLP-18::GFP reporter was next monitored. In WT *C. elegans*, FLP-18::GFP was strongly expressed in several head neurons and a weak but detectable fluorescence was observed in the ventral nerve cord (Cohen et al., 2009). In *casy-1* mutant, no significant change in fluorescent intensity was observed in the ventral nerve cord (Figure 5.3F). However, FLP-18::GFP expression in the head neurons was significantly reduced (Figure 5.3E). These results substantiate that alterations in FLP-18 expression/release functions to modulate the motor circuit activity in *casy-1* mutants.

A double mutant of *flp-1; flp-18* exhibited hypersensitivity to Aldicarb, suggesting that the *flp-18* mutant phenotype is dominant over the *flp-1* mutation. Further, the *casy-1; flp-1; flp-18* triple mutant also showed hypersensitivity to Aldicarb just like the *flp-1; flp-18* double mutant (Figure 5.3D). Collectively, our data suggest that FLP-1 and FLP-18 functions antagonistically in *casy-1* mutants to promote motor circuit activity. Overall, our analysis supports the role of CASY-1A in regulating the activity of interneurons RIM and AVE that in turn modulate the motor circuit at the NMJ through antagonistic functions of *flp-1* and *flp-18* neuropeptides.

Both sensory neurons and interneurons are peptidergic and release neuropeptides along with classical neurotransmitters and hence can act as diverse modulator of neural circuit function. Enhanced PDF-1 neuropeptide secretion from the RMG sensory neurons suggest one possible mechanism operating in *casy-1* mutants to promote motor circuit activity. Previously, FMRFamide-family neuropeptides *flp-1* and *flp-18* have been reported to maintain excitation-inhibition balance at NMJ (Stawicki et al., 2013). We have found that the neuropeptides FLP-1 and FLP-18 act antagonistically to inhibit motor circuit activity. Our results demonstrate that FLP-18 functions to decrease the motor circuit activity in WT animals. Overexpression of FLP-18 results in resistance to Aldicarb suggesting diminished motor circuit activity. On the other hand, FLP-1 neuropeptides enhance motor circuit activity and are

responsible for increased locomotion in *casy-1* mutants. Taken together, our results support the antagonistic role of FLP-1 and FLP-18 neuropeptides in modulating the locomotor circuit in the *casy-1* background. Previously, *flp-1* and *flp-18* genes have been reported to modulate E/I balance in *acr-2 (gf)* mutants by primarily influencing GABAergic neurotransmission. Double mutants of *flp-1* and *flp-18* showed reduced GABA release at the NMJ (STAWICKI *et al.* 2013). Although we show that CASY-1A appears to function at the level of sensory and interneurons to modulate cholinergic transmission, however we cannot rule out the fact that *casy-1* mutants may also regulate GABAergic neurotransmission indirectly via *flp* neuropeptides.

Despite presenting a convincing mechanism for sensory and interneuron-evoked enhancement for cholinergic transmission at NMJ, several questions in this study remain to be determined. For example, expressing CASY-1A in either sensory neurons or interneurons fully restores the motor activity in *casy-1* mutants. How is it possible that expression in two different levels of locomotory circuit can compensate for CASY-1A function in the other level? One possible explanation could be the existence of negative feedback loops that could synchronize the activity of neurons with the dynamics of behavioral output. Existence of such feedback loops that can regulate sensory-> sensory, sensory->interneuron or interneuron->sensory modalities have been previously documented (Chalasani et al., 2010; Gómez et al., 2005; Gordus et al., 2015; Ignell et al., 2009; Kato et al., 2015; Stein et al., 2007; Tsunozaki et al., 2008; Wachowiak et al., 2009). Identification of such feedback loops will provide better understanding for CASY-1 role in these neurons. Another missing component of the study is how increased activity of the RIM interneuron relates to less release of FLP-18 neuropeptide. The RIM interneuron occupies a unique position in the command circuitry and may have a role in both forward and backward locomotion (Alkema et al., 2005; Husson et al., 2013; Piggott et al., 2011; Pirri et al., 2009; Zheng et al., 1999). Tyramine release from activated RIM can also bind to its receptor SER-2 on VD GABAergic motor neurons thus inhibiting GABA input onto the muscles (Donnelly et al., 2013). Hence, our understanding of how the activity and function of RIM interneurons affect the locomotion circuit is still not fully clear. Future investigations concentrating on RIM activity might explain some of the results obtained in this study.

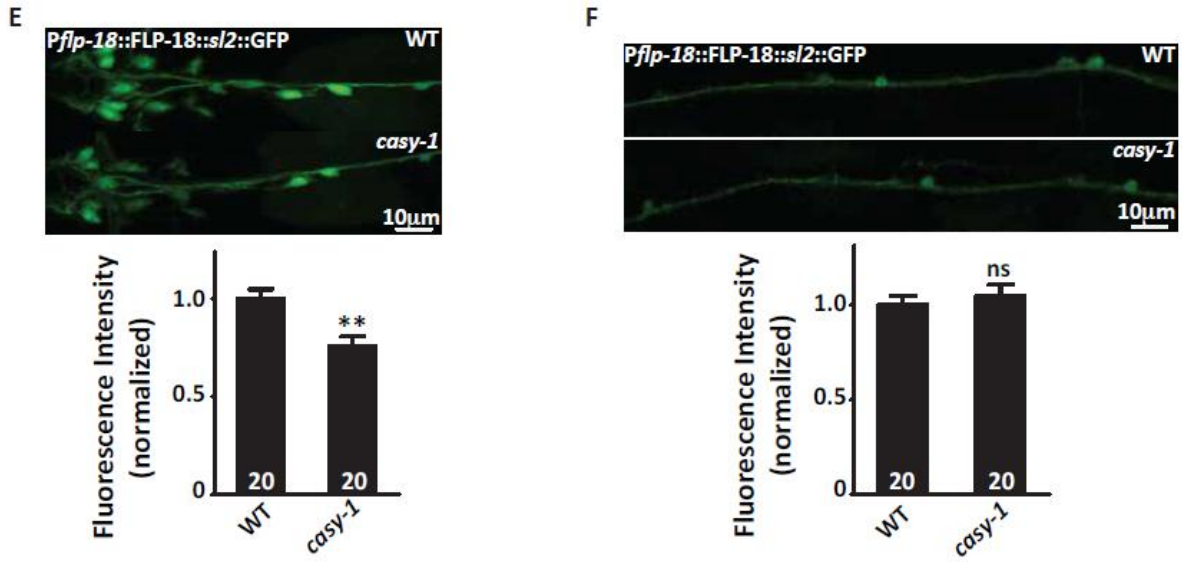


Figure 5.3b: CASY-1A modulates motor circuit activity through neuropeptides. (E) Representative fluorescence micrograph showing expression of bicistronic FLP-18::*sl2*::GFP reporter in the head neurons. Fluorescence intensity of FLP-18::GFP was significantly reduced in the *casy-1* head neurons. (F) Representative fluorescence micrograph showing expression of the bicistronic FLP-18::*sl2*::GFP reporter in the ventral cord motor neurons in the WT and *casy-1* mutants. No significant difference was observed in fluorescence intensity in *casy-1* mutants when compared to WT animals. Quantification of fluorescent intensity is normalized to WT values. The number of animals analyzed for each genotype is indicated at the base of the bar-graph. Quantified data are displayed as mean ± S.E.M. and were analyzed by two-tailed Student's t-test (**P<0.001). "ns" indicates not significant.

Further experiments are needed to validate how FLP-1 and FLP-18 are functioning antagonistically to regulate synaptic signaling at NMJ? How the increased activity of interneurons is affecting the levels of these neuropeptides *in vivo*? Are their additional mechanisms that might explain the role of these neuropeptides in NMJ signaling? A comprehensive understanding of these questions will provide essential insights into CASY-1's role in regulating neuropeptidergic signaling. Mammalian calsyntenins have not been addressed to interact with the components of neuropeptidergic signaling. Future investigations might unravel existence of similar interactions in the mammalian nervous system.

5.2.3. Role of CASY-1 in cholinergic motor neurons

Transcriptional reporter expression analysis revealed that both CASY-1B and CASY-1C expresses in cholinergic motor neurons. Translational CASY-1 reporters also reveal the presence of CASY-1 shorter isoforms at the cholinergic dorsal cord synapses. Functional rescue experiments suggested that CASY-1 isoforms are not functioning in cholinergic motor neurons to regulate acetylcholine release at the NMJ. This opens up the possibility to explore additional roles of CASY-1 isoforms in cholinergic motor neurons.

Further, several questions regarding the specificity of CASY-1 function in GABAergic motor neurons remain unanswered. Our results demonstrate that in GABA motor neurons CASY-1 isoforms interact with UNC-104 motor protein to regulate GABA synaptic vesicle trafficking. However, CASY-1 isoforms also express in cholinergic motor neurons. Also, UNC-104 is expressed ubiquitously in all *C. elegans* neurons. So what additional genetic factors renders specificity of CASY-1 function specifically in GABA release and does not affect ACh release. Utilizing a candidate gene approach or performing a genetic screen to identify additional adaptor molecules that function specifically in GABA motor neurons may provide deeper understanding of this phenomenon.

5.2.4. Presynaptic functions of CASY-1 isoforms

In this thesis we are reporting the role of shorter CASY-1 isoforms in regulating GABA release by directly monitoring the trafficking of GABA synaptic vesicle precursors to the synapse. The shorter CASY-1 isoforms are essentially the conserved C-terminal of mammalian Calsyntenins that lack the entire extracellular N- terminal

domains. The intracellular region of mammalian Calsyntenins has been reported to contains clusters of acidic amino acids that can bind calcium ions with high affinity and hence can regulate intracellular calcium signaling (Vogt et al., 2001). Calcium signaling plays an absolute essential role at the presynaptic termini where they directly monitor synaptic vesicle fusion and release machinery (Chan and Sieburth, 2012; Jackman et al., 2016; Michaelsen and Lohmann, 2010; Myoga and Regehr, 2011; Smith et al., 2012). These clusters of acidic glutamic acid residues are highly conserved in the *C. elegans* CASY-1 raising the possibility of existence of additional mechanisms that contributes to CASY-1 role in presynaptic neurotransmitter release. Our preliminary results suggest a possibility that *C. elegans* CASY-1 regulation of NMJ signaling might be Calcium-dependent. In the absence of calcium in the NGM media the Aldicarb sensitivity of WT worms remained unaffected. But, the Aldicarb hypersensitivity of *casy-1* mutants was completely abolished (Figure 5.4) suggesting that increased motor circuit activity of *casy-1* mutants could be calcium-dependent. Further experiments investigating the role of CASY-1 in regulating calcium signaling and/or its interaction with synaptic calcium channels might reveal additional mechanisms for CASY-1 functioning at the synapse.

5.2.5. CASY-1 mediated regulation of sensory and interneuron activity

Our results in Chapter 4 and 5 highlight the role of the CASY-1A isoform in regulating the activity of sensory neurons and interneurons. The mechanistic insight into how CASY-1 might be controlling the activity of these neurons has not been addressed. Mammalian calsyntenins have been reported to interact physically with Neurexins to promote excitatory and inhibitory synapse development (Lu et al., 2014; Um et al., 2014; Pettem et al., 2013). In *C. elegans*, neurexins and neuroligins are highly enriched in head neurons but their functions have been investigated mostly at the NMJ where they have been reported to mediate retrograde synaptic inhibition (Hu et al., 2012; Tong et al., 2017) and postsynaptic clustering of $GABA_A$ receptors (Maro et al., 2015). Recently, *C. elegans casy-1* has been shown to interact with neurexin-related protein *bam-2* promoting fasciculation between sensory neurons required in mating circuits of the adult male (Kim and Emmons, 2017). This raises the possibility that CASY-1, NRX-1 and NLG-1 might interact physically at the sensory and interneuron levels to mediate additional functions that might involve and explain the mechanisms for CASY-1 mediated regulation of sensory/interneuron activity. Our

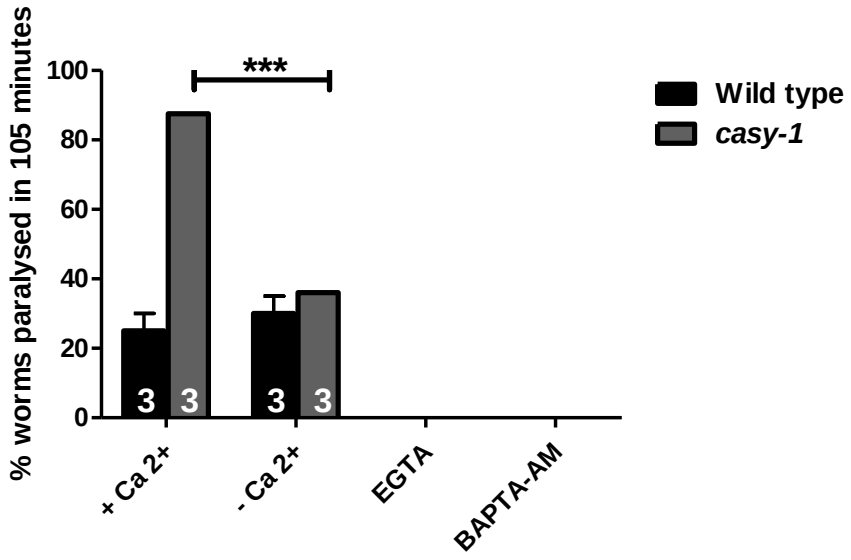


Figure 5.4: CASY-1 regulation of Aldicarb hypersensitivity is Calcium dependent (A-D) Aldicarb-induced paralysis at 105 minutes plotted for indicated genotypes in the presence or absence of calcium in the NMG media. Aldicarb sensitivity of WT worms remained unaffected however, the Aldicarb hypersensitivity of casy-1 mutants was completely abolished. Addition of calcium chelators EGTA and BAPTA- AM makes both genotypes extremely resistant to Aldicarb. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (***P<0.0001 using two-way ANOVA and Bonferroni's Multiple Comparison Test).

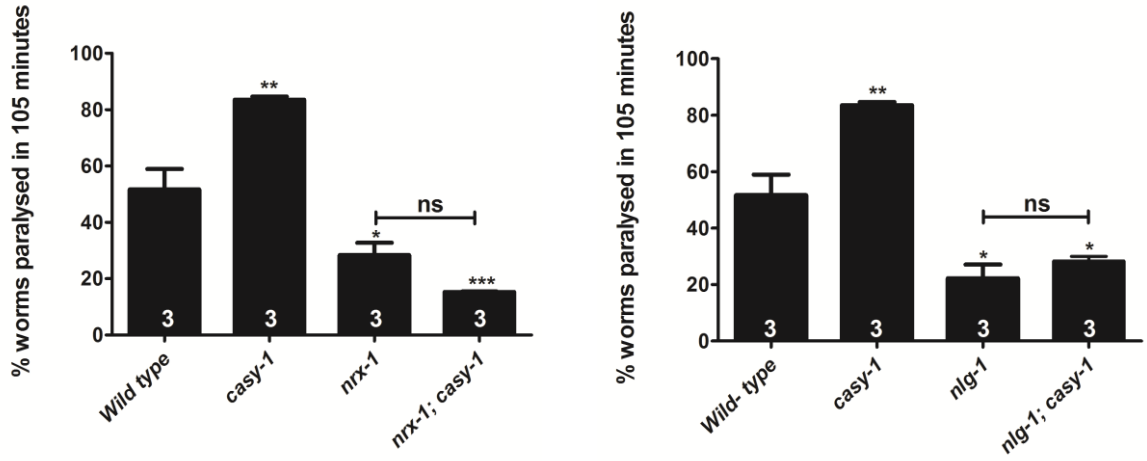


Figure 5.5: CASY-1 might interact with neurexins and neuroligins to monitor motor activity. Aldicarb-induced paralysis at 105 minutes plotted for indicated genotypes. *nrx-1* and *nlg-1* mutants show resistance to Aldicarb. Mutations in *nrx-1* and *nlg-1* suppresses the Aldicarb hypersensitivity of *casy-1* mutants. Data are represented as mean ± S.E.M. The number of assays (~20 *C. elegans*/assay) is indicated for each genotype. (*P<0.01, **P<0.001, ***P<0.0001 using one-way ANOVA and Bonferroni's Multiple Comparison Test). "ns" indicates not significant.

preliminary Aldicarb data supports the hypothesis that CASY-1 interacts genetically with NRX-1 and NLG-1 to regulate motor circuit. *nrx-1* and *nlg-1* mutants are resistant in the Aldicarb assay. The double mutant of *casy-1* with both *nrx-1* and *nlg-1* completely abolishes the Aldicarb hypersensitivity of *casy-1* mutants (Figure 5.5). Future investigations to understand the role of neurexins and neuroligins at higher levels of locomotion circuits may provide better understanding of this interaction.

5.2.6. Role of CASY-1 in regulating synaptic plasticity

C. elegans CASY-1 has been reported to function in sensory neurons to regulate synaptic plasticity, however, the mechanism is still unknown (Hoerndli et al., 2009; Ikeda et al., 2008). Mammalian CLSTN2 knock-out mice have been shown to be hyperactive with cognitive deficits that could be due to reduced GABAergic neurotransmission (Lipina et al., 2016; Ranneva et al., 2017). Our results provide a mechanistic insight into how CASY-1 might regulate GABA signaling at the NMJ. Further investigations involving the role of GABA signaling in higher levels of neuronal circuits in *casy-1* mutant learning defects might provide deeper insights into the role of CASY-1 in memory performance.

5.3. Significance of the study

Our study with *C. elegans* CASY-1, an ortholog of mammalian Calsyntenins dissects out multiple mechanisms for how CASY-1 could regulate synaptic signaling at the NMJ via functioning in distinct subsets of neurons. On one hand, our study provides mechanistic insights into how *casy-1* could regulate GABAergic neurotransmission. On the other hand, we report multiple mechanisms by which CASY-1 could regulate excitatory cholinergic neurotransmission at NMJ.

Further, all the key players in this proposed study are highly conserved, strengthening the possibility that a similar regulatory control could exist in other systems. Considering the conservation involved in the process of synaptic transmission, the implications of this work goes far beyond *C. elegans* and could provide a major impetus to study the roles and functions of Calsyntenins and their regulation in multiple systems and disease conditions. An inequity in excitation-inhibition balance forms the basis for multiple neuropsychiatric disorders like epilepsy, autism etc. Deregulation of mammalian Calsyntenins is also thought to be coupled with age-related- conditions like Alzheimer’s and Parkinson’s disease, hence

future investigations in this area based on our work could provide a deeper understanding of these disorders.

APPENDIX TABLE 1: Figure legends for the video files

Movie 3.1: WT *C. elegans* move normally on brief exposure to PTZ. This media file indicates a 6s clips of WT animals (7 frames/second) exposed to 10 mM PTZ for 30 minutes. As can be seen from the movie file the WT *C. elegans* shows normal sinusoidal locomotion after 30 minutes exposure to PTZ.

Movie 3.2: *casy-1* mutants show anterior convulsions on exposure to PTZ. This media file indicates a 6s clips of *casy-1* mutant animals (7 frames/second) exposed to 10 mM PTZ for 30 minutes. As can be seen from the media file, one *casy-1* mutant shows anterior convulsions or 'head-bobs' while another animal shows the tail shrinker phenotype characteristic of GABA mutants in *C. elegans*.

Movie 3.3: *casy-1* mutants, with GABAergic CASY-1C expression show normal locomotion on exposure to PTZ. The media file indicate a 6s clips of *casy-1*; P*unc-25*::CASY-1C *C. elegans* (7 frames/second) exposed to 10 mM PTZ for 30 minutes. Expressing the CASY-1C isoform in GABAergic neurons completely rescues the PTZ defects seen in the *casy-1* mutant animals.

Movie 3.4: Movie of the synaptic vesicle marker SNB-1::GFP trafficking in GABAergic neurons in WT *C. elegans.* Representative time-lapse recording of SNB-1::GFP in axons of wild-type GABAergic commissure. The media file represents a 14s clips at 7 frames/second. VNC is on the top.

Movie 3.5: Movie of the synaptic vesicle marker SNB-1::GFP trafficking in GABAergic neurons in the *casy-1* mutant line. Representative time-lapse recording of SNB-1::GFP in axons of *casy-1* mutant GABAergic commissure. The media file represents a 14s clips at 7 frames/second. VNC is on the right.

Movie 3.6: Movie of the synaptic vesicle marker SNB-1::GFP trafficking in GABAergic neurons in *casy-1* mutants that express CASY-1C in GABAergic neurons (GABA rescue of *casy-1*). Representative time-lapse recording of SNB-1::GFP in axons of rescue line GABAergic commissure. The media file represents a 15s clips at 7 frames/second. VNC is on the left.

Movie 3.7: Movie of the synaptic vesicle marker SNB-1::GFP trafficking in cholinergic neurons in WT animals. Representative time-lapse recording of SNB-

1::GFP in axons of wild-type cholinergic commissure. The media file represents a 14s clips at 7 frames/second. VNC is on the right.

Movie 3.8: Movie of the synaptic vesicle marker SNB-1::GFP trafficking in cholinergic neurons in the *casy-1* mutant line. Representative time-lapse recording of SNB-1::GFP in axons of *casy-1* mutant cholinergic commissure. The media file represents a 14s clips at 7 frames/second. VNC is on the down-left.

Movie 5.1: CASY-1A expresses in RIM and AVE interneuron. Movie is showing a 180^0 rotation of a transgenic line expressing P*nmr-1*::mCherry and P*casy-1a*::GFP. The co-localization between the two markers identifies CASY-1A as being expressed in the RIM and AVE interneurons (*Wormatlas*).

APPENDIX TABLE 2: Reagents used in the thesis

Lab	Reagent Strain/Plasmid	Citation
Yuichi Iino Lab	*casy-1 (tm718)*, *casy-1(hd41)*, JN442, JN1440, JN1442, JN485, JN484	Ikeda et al., 2008
Josh Kaplan Lab	*nuIs321*, *nuIs152*, *nuIs376*, *nuIs160*, *nuIs299*, *nuIs283*, *nuIs169*, KP5926, *nuIs24*	Babu et al., 2011 Sieburth et al., 2005 Rongo et al., 1998
Kenneth Norman Lab	Plasmids pKN22, pKN24 and pKN27	Fry et al., 2014
Erik Jorgensen Lab	*oxIs155*	Ernstrom et al., 2012
Cori Bargmann Lab	*kyEx4787* and plasmid pAG11 and pNP471	Marvin et al., 2013 Pokala et al., 2014
Sandhya Koushika Lab	*klc-2 (km11) and* tBIs147	Yandell et al., 1994 Kumar et al., 2010
Erik A. Lundquist Lab	*IhIs6*	Norris and Lundquist, 2011
Mario de Bono Lab	AX1444	Cohen et al., 2009
Yishi Jin Lab	*juIs76*	Baran et al., 2010
Andres Villu Maricq Lab	*akIs38*	Francis et al., 2005

APPENDIX TABLE 3: Collaborators and contributions

Collaborators	Experiments performed	Displayed in Section
Dr. Sandhya P. Koushika and Amruta Vasudevan	Live imaging of synaptic vesicle trafficking	Chapter 3- Figure 3.6
Dr. Jihong Bai and Dr. Yongming Dong	Electrophysiological recording from *C. elegans* NMJ	Chapter 3- Figure 3.4C Chapter 4- Figure 4.1A
Shruthi Ravindranath	Movement experiments	Chapter 4- Figure 4.1D and Figure 4.5C

APPENDIX TABLE 4: Pre-published images utilized with permission in thesis

Permission from	Image utilized	Citation
Dr. Masatoshi Takeichi	FIGURE 1.3	Hirano and Takeichi, 2012
Dr. Jonathan Pettitt	FIGURE 1.11	Pettitt, 2005
Dr. Alan M. Zahler	FIGURE 1.15	Zahler, 2005
Dr. Janet E. Richmond	FIGURE 2.7	Richmond, 2005

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