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A GAIN OF FUNCTION MUTATION IN THE P/Q TYPE VOLTAGE GATED Ca^{2+} CHANNEL, UNC-2, STIMULATES ACETYLCHOLINE RELEASE IN CAENORHABDITIS ELEGANS

A Major Qualifying Project Report

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Samuel Raj Dhruv Sarin

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APPROVED:

Mark Alkema, Ph.D.
Neurobiology
Umass Medical Center
Major Advisor

David Adams, Ph.D.
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

zf35 is a mutation in the *unc-2* gene of *C. elegans* that causes a hyperactive phenotype. *unc-2* encodes the alpha-1a subunit of a P/Q type calcium channel involved in synaptic transmission in *C. elegans*. Double mutants were constructed of *zf35* and known synaptic transmission mutants *egl-30, unc-13, snt-1,* and *unc-31*, to determine how *zf35* affects neurotransmitter release. Also, a genetic screen was done to identify mutations that suppress the *zf35* phenotype, revealing novel genes in this pathway. We conclude that *zf35* mutants have defects in the presynaptic pathway.

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BACKGROUND

C. elegans as an Animal Model

The nematode *Caenorhabditis elegans* is a highly useful model for genetic research. This organism can conveniently be grown *in vitro* on OP50 *E. coli* spotted on agar plates. *C. elegans* grows rapidly, has its complete genome sequenced, has a short life cycle, has short clearly defined stages of development, is easily available, and its research is relevant to higher level organisms. Having only 6 total chromosomes, namely 1, 2, 3, 4, 5, and X, has helped biologists understand its genes in more detail.

C. *elegans* has two sexes: male or hermaphrodite. Hermaphrodites have two sex chromosomes (XX) while the males have one sex chromosome (X0). Hermaphrodites can self propagate and lay eggs, or they can mate with males. The life cycle of C. *elegans* is important in research especially when setting up crosses. C. *elegans* has 4 larval stages consisting of L1, L2, L3, and the L4 stage. The L4 stage is an important stage for distinguishing phenotypes, staging strains, mating, and is easily distinguished by a white crescent in the vulval region of the worm. After the larval stages, C. *elegans* develops into young adults and eventually adults. The entire life cycle is shown in figure 1 (Altun and Hall, 2006).

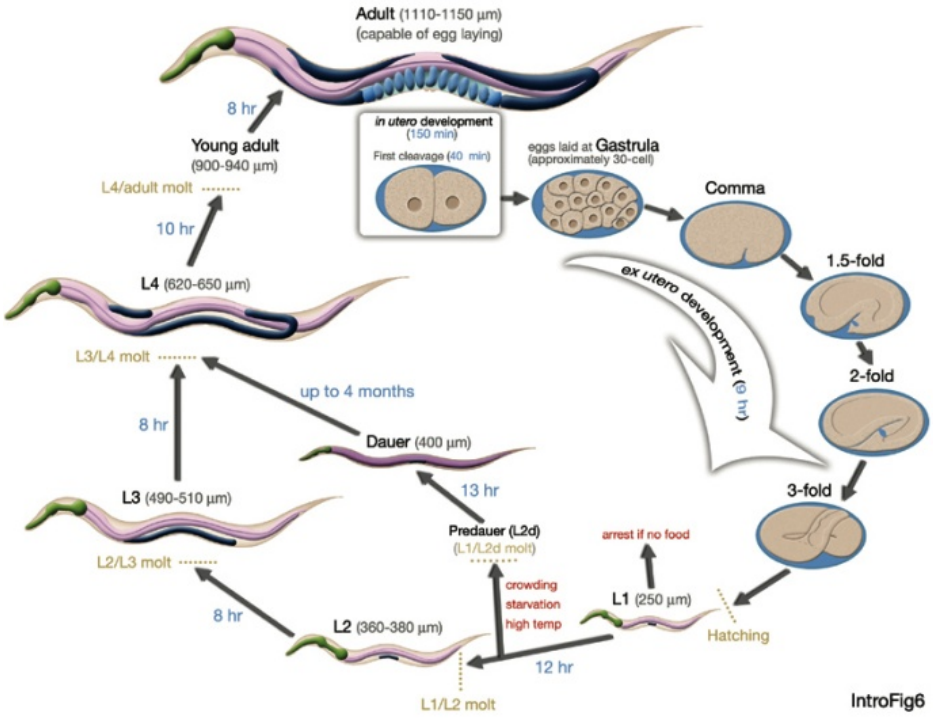


Figure 1: The *C. elegans* Life Cycle. Shows the complete life cycle of the C. *elegans* worm from egg to adult. Major stages include egg, L1, L2, L3, L4, young adult, and adult (Altun and Hall, 2006).

Research with *C. elegans* includes studying apoptosis, inter/intracellular signaling, parasitic interactions, and neuronal growth. The implications of C. *elegans* research in vertebrate neuronal growth and function is important because this work helps better understand human neuronal growth and function (Chou *et al.,* 2008). *C. elegans* has proven to be an important asset in neurobiology. For behavioral research, *C. elegans* shows a large variety of behaviors including locomotion, foraging, feeding, defecation, egg laying, dauer larvae formation, and responses to touch, smell, taste and temperature. More intricate behaviors include male mating, social behavior, learning and memory (Altun and Hall, 2006). Thus, the study of how neurons interact and the responses by the body can easily be studied using C. *elegans*.

Green fluorescent protein (GFP) is a widely used reporter protein that has been used as a genetic marker in *C. elegans.* The transparency of C. *elegans* makes it amenable for viewing GFP, as the worms can be viewed microscopically without dissection. Transgenic strains can be established using germ line transformation (Boulin *et al.*, 2006).

Another advantage of C. *elegans* in genetic analysis is reverse genetics can be used to characterize genes. Reverse genetics is initiated with the study of the genomic sequences. Using mutagenesis techniques such as EMS, gene sequences are randomly changed, and the resulting phenotype is analyzed. The advantages of reverse genetics include the ability to analyze all the genes in a gene family, analyze the function of homologous genes in other organisms, and study abundant genes that are still unaltered. Many of these goals cannot be achieved using forward genetics (Ahringer, 2006).

C. elegans Nervous System

The *C. elegans* nervous system is compromised of only 302 neurons (Nonet *et al.,* 2008). The organization of the C. *elegans* nervous system consists of ganglia in the head and the tail. There are also smaller ganglia and scattered neurons positioned throughout the body. The processes of all neurons project to the nerve ring inside the head as seen in figure 2 (Altun and Hall, 2006).

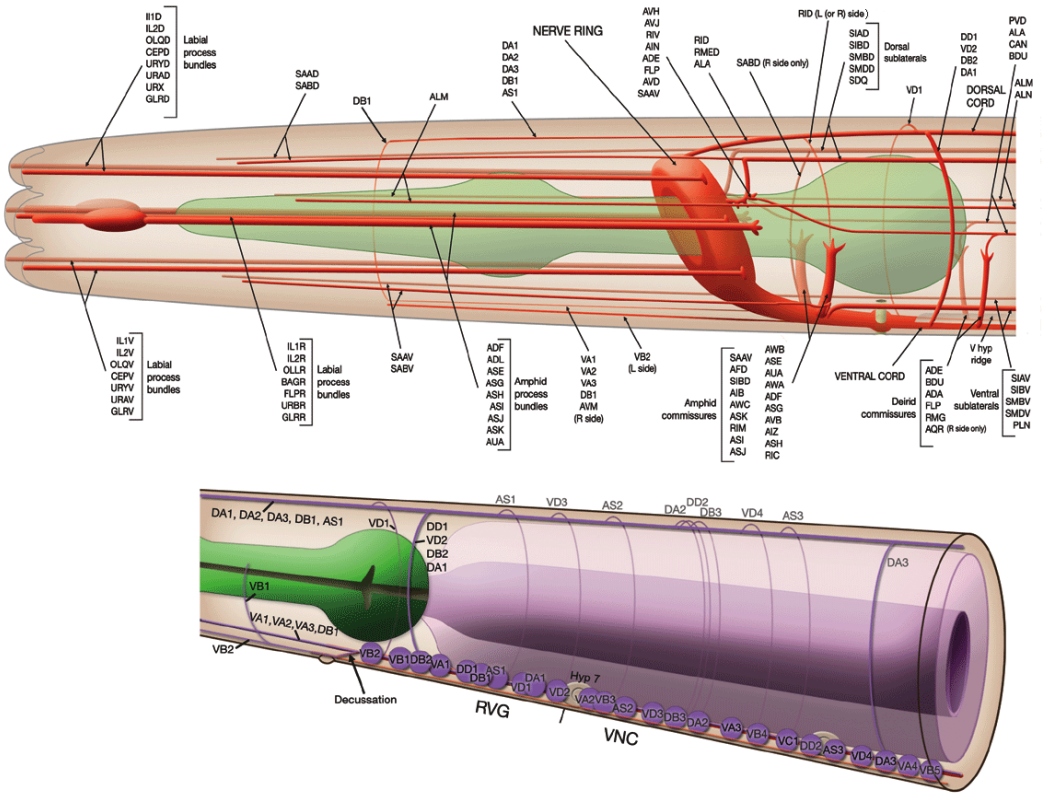


Figure 2: Key Features of the *C. elegans* Nervous System. Highlighted above are the major features of the C. *elegans* nervous system and its organization (Altun and Hall, 2006).

There are two main strains of wild-type *C. elegans* used in labs today: the N2 or Bristol strain, and the CB4856 or Hawaiian strain. These two strains were geographically separated for so long that millions of years of genetic drift caused polymorphisms in the DNA between the two strains. On average, these differences occur every 1000 base pairs, primarily in the non-coding regions of the genetic code, and cause no observed functional changes between the two strains (Jakubowski and Kornfeld, 1999; Koch *et al.*, 2000; Wicks *et al.*, 2001; Swan *et al.*, 2002). A few rare changes may affect protein activity and gene expression resulting in varying mating behavior as well as varying responses to interference RNA (Tijsternman *et al.*, 2002).

Synaptic Transmission

Synaptic transmission is a complex series of events that happen in sequence that result in relaying an electrical signal from the presynaptic neuron to the post-synaptic neuron. This process involves the conversion of an electrical impulse, the action potential, to the chemical signal of a neurotransmitter. The synapse can carry a vast array of signals. The specificity of these signals depends on where the signal originated, where the signal is going, the neurotransmitter used, and its mechanism of action.

There are some major steps required for synaptic transmission that should be mentioned (Finlay and Markha, 1997). Neurotransmitters are chemicals produced in a presynaptic axon terminal that act as the main messenger and signaling molecules of the nervous system. Once secreted from the presynaptic neuron into the synaptic space, they act upon the postsynaptic neuron by binding specific sets of receptors. Some examples of neurotransmitters include acetylcholine, serotonin, and dopamine. Neurotransmitters are stored inside vesicles in the axon terminal of the presynaptic neuron. Once an action potential comes down from an axon to the axon terminal, calcium channels open and let calcium in. This influx of calcium signals the presynaptic vesicles to release the neurotransmitter into the synaptic cleft. This process requires the presynaptic vesicles to dock at the axon terminal and to sense changes in calcium. During release, the neurotransmitter molecules travel by the process of diffusion towards the postsynaptic neuron where they bind to receptors to initiate ion flow across the post-synaptic membrane. In many cases another action potential is initiated by the influx of sodium ions (Finlay and Markha, 1997).

Once a neurotransmitter has bound its receptor, it must stop its signal or risk an undesired effect on the postsynaptic neuron. This inhibition is provided by the process of degradation of the neurotransmitter by an enzyme (in the case of acetylcholine this enzyme is acetylcholine esterase) or reuptake by the presynaptic neuron or glia. In the case of uptake, vesicles are reformed around the neurotransmitter. The vesicles then wait for the next action potential to induce another synaptic transmission (Finlay and Markha, 1997).

Acetylcholine Neurotransmitter

Acetylcholine is one of the major excitatory neurotransmitters of the nervous system. It was the first neurotransmitter ever discovered in 1955 by Helen Mellanby. Acetylcholine also acts as a major excitatory neurotransmitter in nematodes, over 1/3 of all cells in the *C. elegans* nervous system secrete it at the neuromuscular junction for a variety of purposes, including movement, feeding, male mating, and egg laying.

Relative to the topic of this MQP, it is important to examine cholinergic neurotransmission to better understand how acetylcholine works as a neurotransmitter. Acetylcholine is created by an enzyme called choline acetyltransferase (Rand, 2007). After being synthesized, the vesicular acetylcholine transporter loads acetylcholine into synaptic vesicles. As mentioned before, synaptic vesicles are used to store the acetylcholine until an action potential arrives at the axon terminal. This vesicular acetylcholine transporter is driven by a proton gradient that exists between the cytoplasm of the neuron and the synaptic vesicle. As seen in figure 3, the vesicular acetylcholine transporter is *unc-17* (uncoordinated) (Rand, 2007).

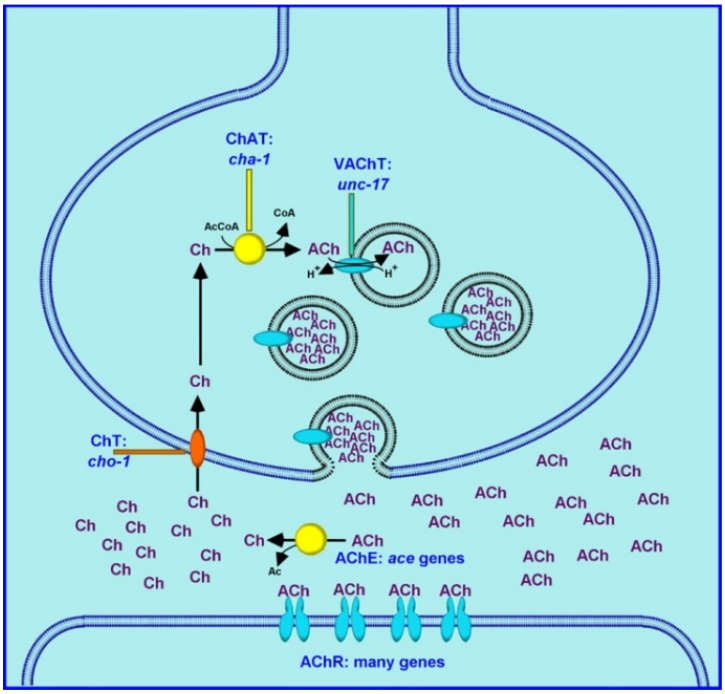


Figure 3: The Mechanism of Acetylcholine Formation and Breakdown. Shows the full cycle of acetylcholine from its formation, release, breakdown, reuptake, and reformation (Rand, 2007).

The vesicular acetylcholine transporter uses an anti-port system in which one proton is exported from the synaptic vesicle while one acetylcholine molecule is pumped into the synaptic vesicle. For this to be possible, the concentration of protons outside the synaptic vesicle has to be less than within the vesicle, or the internal pH of the vesicle has to be higher than the cytoplasm of the neuron (Rand, 2007).

Vesicle Docking to the Membrane

Once the acetylcholine has been inserted into the synaptic vesicles, a series of steps occur that result in the vesicle docking to the neuronal membrane and releasing acetylcholine into the synaptic cleft. The cycle that a synaptic vesicle goes through is diagramed in figure 4 (Rand, 2007).

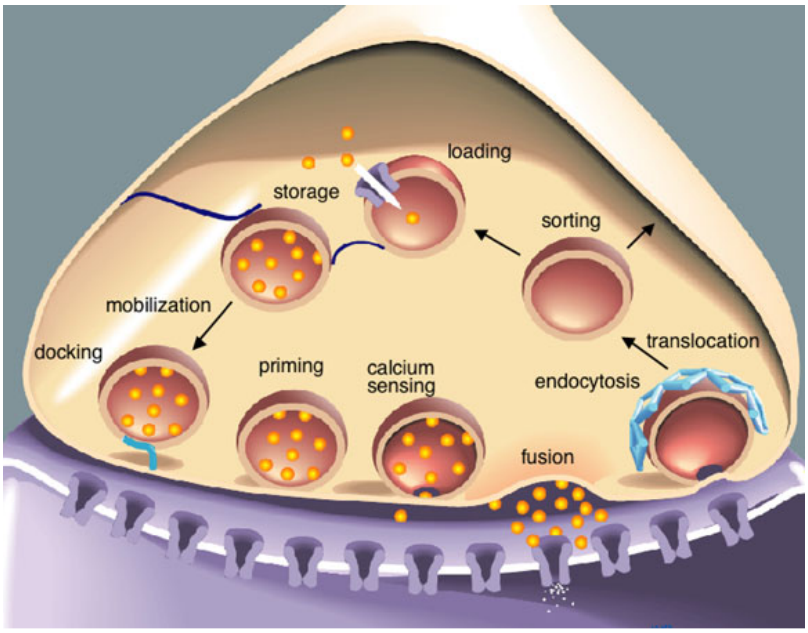


Figure 4: The Synaptic Vesicle Cycle. Diagramed are the important steps in the vesicle life cycle, including its function as transporter of acetylcholine neurotransmitter (Rand, 2007).

The mechanism of vesicle docking has been a hotly debated research topic. It has been shown that synaptic vesicles dock near active zones through molecular recognition. Various SNARE proteins and receptors are involved in docking. The SNARE synaptobrevin is encoded by the gene *snb-1* in *C. elegans.* This protein is located on the vesicle itself during docking. Syntaxin (encoded by *unc-64*) and SNAP-25 (encoded by *ric-4*) are on the plasma membrane. All of these together make the docking complex in *C. elegans*. Rab3 GTPase is also a co-factor to various proteins in the "active zone" on the plasma membrane. One of the proteins Rab3 acts with Rim, encoded by *unc-10*. *unc-10* is also known to play a role in priming (see below). A study of the *unc-18* mutant has revealed a protein family named SM and its importance in docking. SM proteins interact with syntaxins. However, the mechanism of action of SM's role in vesicle docking is still unknown (Rand, 2007).

Vesicle Priming

After vesicle docking to the membrane is complete, the vesicle now has to be directed and primed for fusion. SNAREs such as syntaxin and SNAP-25 located on the plasma membrane and synaptobrevin in the vesicle membrane start forming complexes that move the vesicle in proximity to the plasma membrane. The *unc-13* mutant has been implicated as an important priming factor. The protein Rim has been shown to play a complementary role in vesicle priming. Rim has been characterized by the *unc-10* mutant. Rim's exact purpose is still speculative (Rand, 2007).

Ca^{2+} Sensing

Once the vesicle has been primed for fusion, fusion to the membrane occurs only when calcium levels in the nerve terminal rises. The calcium sensing in the presynaptic terminal is centered on the voltage-gated calcium channels which contain the UNC-2 α_1 subunit. Other channels may also contribute to calcium sensing, such as *unc-36, egl-19* (egg laying deficient), and *unc-68*. Synaptotagmin, encoded by *snt-1*, is a vesicle associated protein that has been implicated as a calcium-sensor in exocytosis as well as an AP-2 binding partner in endocytosis. AP-2 is a clathrin adaptor that plays a major role in fission, uncoating, and sorting of vesicles (Rand, 2007).

Vesicle Fusion

Once calcium has entered the axon terminal, the vesicles that were primed and docked fuse with the plasma membrane. Neurotransmitter held in this vesicle is then released into the synaptic cleft. SNARE proteins, syntaxin, SNAP-25, and synaptobrevin all play a role in vesicle fusion. A SNARE complex forms, bringing the vesicle in close proximity to the plasma membrane where fusion occurs with Ca^{2+} influx (Rand, 2007).

Once acetylcholine has been released into the synaptic cleft, it diffuses across the cleft and activates acetylcholine receptors on the post-synaptic cell. Acetylcholine is cleared from the synapse by the enzyme acetylcholinesterase shown on figure 5 (Rand, 2007). Acetylcholinesterase hydrolyzes acetylcholine by breaking it down into acetic acid and choline.

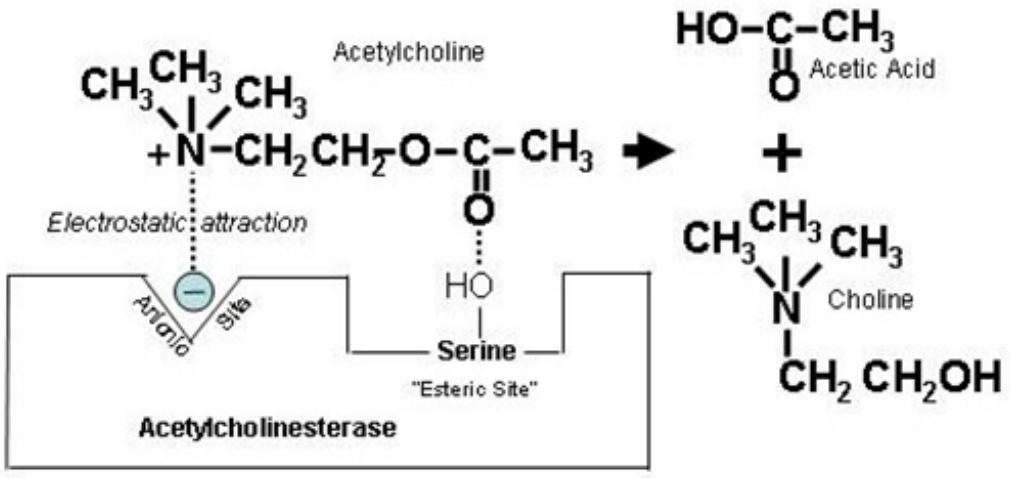


Figure 5: Acetylcholine Hydrolysis. The above reaction is mediated by the enzyme acetylcholinesterase that catalyzes the reaction in which acetylcholine is broken down to choline and acetic acid (Cholinesterase, 2008).

Once acetylcholine is broken down, the choline byproduct is taken back up into the presynaptic neuron by a high affinity choline transporter. Choline can then be reconverted into acetylcholine by choline acetyltransferase. After fusion, vesicle proteins

and membrane fragments are reorganized into synaptic vesicles for more exocytosis. It should be noted that the vesicles are also involved in transporting back the choline that is floating in the synaptic cleft so transmission can continue (Rand, 2007).

UNC-2

The Ca^{2+} channel alpha subunit encoded by the *unc-2* gene is involved in proper muscle contraction and speed in movement as shown in figure 6. UNC-2 is homologous to the human calcium channel, CACNA1A, which has been associated with Familial Hemiplegic Migraine. Calcium channels are composed of α_1, α_2, β, and δ subunits. The α_1 subunit is the most important functional component of the Ca^{2+} channel, as it forms the voltage sensor and the ion-conducting pore. The human gene responsible for the effects of FHM-1is CACNL1A4, which encodes the brain-specific P/Q type Ca^{2+} channel α_1 subunit (Ophoff *et al.*, 1996).

In *C. elegans*, the *unc-2* gene is expressed in sensory, motor, VC (Ventral Cord), and HSN (Hermaphrodite Specific Neuron) neurons. The HSN and VC neurons control the egg laying and other motor functions in *C. elegans* (WormBase, 2008).

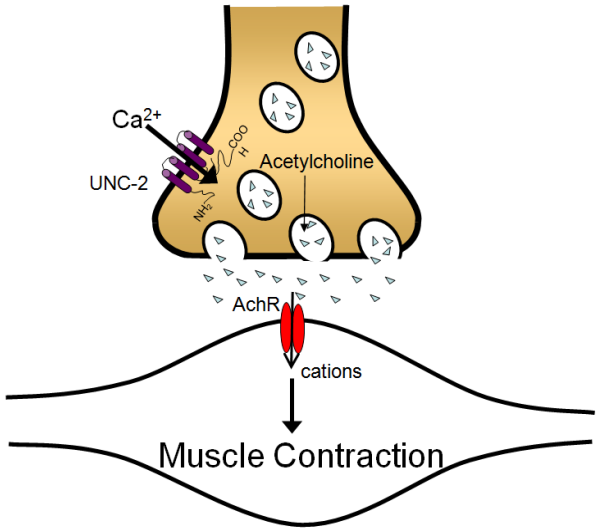


Figure 6: Role of *unc-2* and the Calcium Channel. Diagram shows the role of the calcium channel, one subunit is encoded by *unc-2* (Pirri & Alkema, Personal Communication).

The *zf35* Mutant

The *zf35* mutant was originally isolated in a genetic screen of *C. elegans* with previous defects in the *unc-2* gene. *unc-2* animals are uncoordinated, while the *zf35* mutant animals showed a higher reversal rate to a coordinated phenotype compared to wild-type. Also, *zf35* worms fail to suppress head oscillations in response to touch. Figure 7 shows the hyper-reversal and hyper-active egg laying behavior of the *zf35* mutant compared to wild-type (Pirri & Alkema, Personal Communication).

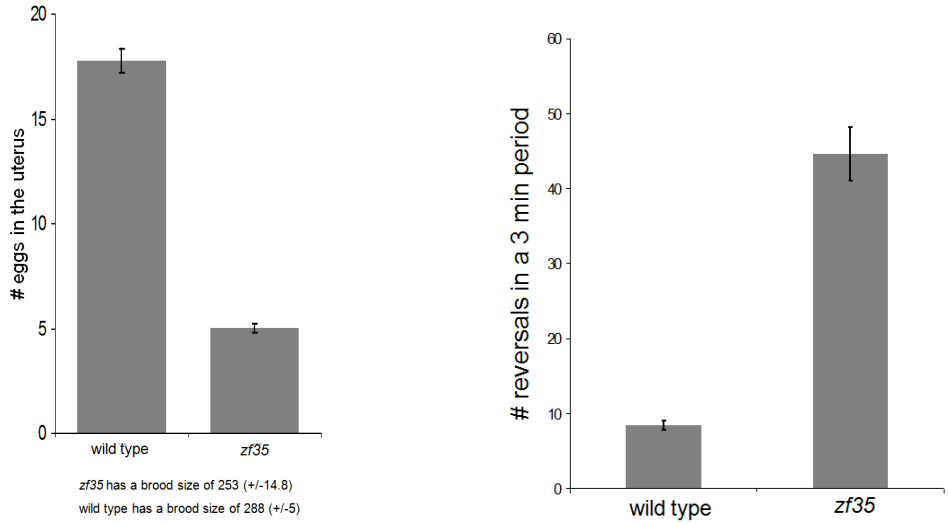


Figure 7: Effects of zf35 Mutant on Egg Laying and Phenotype Reversals. *zf35* is hyperactive in egg laying, showing a decreased number of eggs in the uterus (increased number laid), and has the phenotype hyper -reversal characteristic compared to the wild-type. In a phenotype reversal, a previously uncoordinated worm now coordinately moves backwards in response to a prodding (Pirri & Alkema, Personal Communication).

goa-1 and *zf35* worms are hyperactive in egg laying. The *goa-1* is a mutant hyperactive in acetylcholine release. This is a loss of function mutation involved in the Gα0 pathway. *zf35,* on the other hand, is a gain of function mutation in the *unc-2* gene. Figure 8 shows the hyperactive egg laying characteristic of *goa-1* mutant compared to the wild-type (Bastiani & Mendel, 2006).

Figure 8: The *goa-1* Mutant is Hyperactive for Egg Laying. Goa-1 (lower panel) is hyperactive in egg laying like *zf35* (fewer eggs present in the gonad), while wild-type animals (upper panel) retain fertilized eggs longer (for ~2.5 hours) before laying them (Bany *et al.*, 2003).

Aldicarb and Levamisole

Aldicarb and levamisole are drugs relevant to the cholinergic pathway. They can be used to determine pre vs. post-synaptic cholinergic signaling. Aldicarb is an acetylcholine esterase inhibitor (figure 9). The purpose of acetylcholinesterase is to prevent excessive and prolonged acetylcholine signaling. Without acetylcholinesterase there is a buildup of acetylcholine in the synaptic cleft resulting in hyper contraction, paralysis, etc. Defects in cholinergic signaling in *C. elegans* can be characterized with an aldicarb assay. Mutants that are resistant to aldicarb would move normally while wild-type worms have become paralyzed. Mutants that are hypersensitive to aldicarb would become paralyzed sooner than wild-type worms if placed on an aldicarb plate. A hypersensitive mutant to aldicarb would indicate that too much acetylcholine is being produced or not enough acetylcholine is being degraded. To facilitate the aldicarb assay, a levamisole assay further narrows down the biochemical cause of the phenotype (Mahoney et. al., 2006).

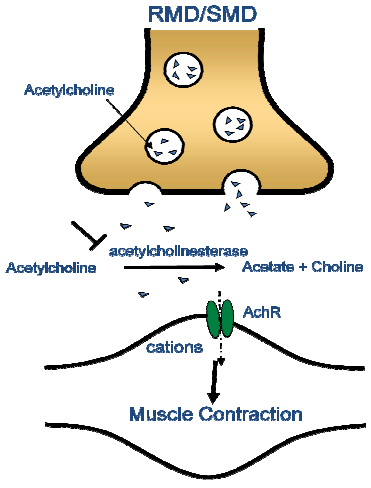


Figure 9: Aldicarb Mechanism. Aldicarb blocks the action of acetylcholine esterase and thus acetylcholine remains bound to the receptor longer eventually causing paralysis (Pirri & Alkema, Personal Communication).

Levamisole is a drug originally used as an antibiotic, pesticide, and cancer medication, and is a cholinergic agonist. Levamisole amplifies the effects of acetylcholine by activating acetylcholine receptors in a non-competitive allosteric manner. Assays using levamisole test whether there are too many receptors at the post synaptic level or too little. Levamisole assays also test for functional receptors. The amplified number or function of receptors of a *C. elegans* strain can be implied by sensitivity to levamisole relative to wild-type. Resistance to levamisole may suggest that the function or number of receptors in a *C. elegans* strain is less than wild-type (Lewis *et al.*, 1980). Hence, aldicarb assays combined with levamisole assays are an excellent method of predicting whether the biochemistry of the mutation is taking place postsynaptically or presynaptically.

PROJECT PURPOSE

The purpose of this project was to determine how the *zf35* mutation affects synaptic transmission. The *zf35* mutation was originally identified as an allele of the *unc-2* gene. We performed epistatic analysis by building double mutants of known synaptic transmission mutants, such as *egl-30, unc-13, snt-1,*and *unc-31*. The resulting phenotypes of the double mutants were studied and compared with both *zf35* and wild-type to dissect the genetic pathway and study gene interactions. The goal of these experiments was to determine how *unc-2 (zf35)* affects neurotransmitter release. Finally, a genetic screen was done to find the mutations that suppress the *unc-2 (zf35)* phenotype, hopefully revealing novel genes in this pathway.

METHODS

Isolation of Plasmid DNA and Sequencing

Isolation of plasmid DNA was performed using the QIA prep Spin Miniprep Kit Protocol. Sequencing was performed by an offsite company (GeneWiz).

EMS Mutagenesis

Plates of *zf35* L4 larvae were washed with M9 (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, dH_2O) using sterile glass pipettes. The worms were transferred to a 15 ml sterile plastic centrifuge tube and centrifuged at 1000 rpm for 30 sec. The supernatant was removed and the worms were washed twice with M9 before being suspended in a final volume of 3 ml. Next, 20 µl of EMS (methanesulfonic acid, ethyl ester, Sigma #M-0880) was added to 1 ml of M9 in a separate 15 ml tube under the hood. The EMS suspension was mixed until it was no longer cloudy. Next, 3 ml of *zf35* worms were transferred into the tube of EMS. The top of the tube was parafilmed and placed on a spinning wheel at 20° for 4 hours. After 4 hours, the worms were centrifuged and the supernatant was removed. The worms were then washed twice with ~3 ml of M9, and transferred in a few drops of M9, using a sterile glass pipette, to a plate seeded with OP50 *E. coli*.

F2 Mutant Screen

After EMS treatment, the worms were left on the plates to recover for 15-20 minutes, and then healthy looking late L4 animals were picked off and placed on new plates, and allowed to recover for 24 hours. 3-4 gravid adult P_0 worms were put on 60 90

mm plates, and allowed to lay eggs for 4 hours. The 4 P_0 worms per plate laid an average of 70 eggs after 4 hours, resulting in ~70 F_1 progeny per plate. The F_1 animals were removed from the plate after they laid the F_2 eggs. The F_1 animals were washed off the plate by gently swirling the plate after adding M9, and then pouring the M9 off the plate. This removes most of the adults remaining, leaving the eggs behind. The remaining F_1 worms were sucked off the plate using a pasteur pipette hooked up to a vacuum line under the dissecting scope with a 12X objective. Once the EMS mutagenesis was complete, the worms from the F2 generation were placed on 9 cm NGM (NaCl, Bacto-Agar, Peptone, H_2O, 1M $CaCl_2$, 5 mg/ml cholesterol in EtOH, 1M $MgSO_4$, 1M KPO_4 buffer) agar aldicarb plates. After 1 hour, worms that showed movement were picked and singled to small NGM agar plates.

Aldicarb and Levamisole Assays

Aldicarb and levamisole assays were used to monitor the sensitivity of C. *elegans* to the paralyzing effects of these drugs. The worms were assayed on NGM agar containing 1mM aldicarb or 100 µM levamisole seeded with OP50 *E. coli*. About 16-24 hours before the assay was performed, L4 larval stage worms of each genotype were picked onto a fresh NGM plate containing OP50 *E. coli*. Twenty young adult worms were transferred to each aldicarb and levamisole plate at the start of the assay. Other *C. elegans* strains such as wild-type and *zf35* were also assayed as controls. The worms were checked every 15 minutes for paralysis. A worm was defined as paralyzed if there was no movement when prodded three times with a platinum wire on the head and tail. The worms were scored until all of the worms paralyzed or if the majority of the worms were not paralyzed within 3 hours.

The following information was used to analyze the results of the aldicarb and levamisole assays. Mutant worms with a block in acetylcholine release are generally aldicarb resistant, and worms with enhanced acetylcholine release are aldicarb hypersensitive. Aldicarb assays alone cannot determine if a mutant worm has pre or postsynaptic defects since a mutation in a postsynaptic cholinergic receptor can cause resistance to aldicarb. The resistance to aldicarb is caused by a decrease in the number of functional receptors. Levamisole activates acetylcholine receptors which act in postsynaptic cells. Worms with mutations in presynaptic genes generally show a similar rate of paralysis as wild-type worms. However, mutations in post-synaptic genes result in levamisole resistance. Aldicarb resistance and levamisole sensitivity suggests defects in synaptic release (presynapse). Aldicarb resistance and levamisole resistance suggests postsynaptic defects.

Preparation of C. *elegans* Culture Lysates

The worm lysates were prepared by first picking worms into 1X Lysis buffer placed 0.2 ml PCR tubes (1 µl of 1X Lysis buffer was used per worm). After the worms were picked into the lysis buffer, the PCR tubes with the worms in solution were frozen at –20°C overnight, or at -80°C for about an hour. The worms were then lysed at 65°C for 1 hour and at 95°C for 15 min in an Eppendorf Thermalcycler.

Polymerase Chain Reaction (PCR)

For the PCR, 10.0 µL master mix containing 1 µl of 10X PCR Buffer , 1 µl of 2 mM dNTPs, 1 µl of DNA (worm lysate), 0.1 µl of Taq (WoostaQ), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, and 5.9 µL of dH_2O were made for each reaction. The sample was then placed in the thermalcycler and heated at 94°C for 2 minutes. Next, 35 cycles of the following temperatures and times were conducted: 94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 1 minute. Next, a 5 minute 72°C step was performed. Following this reaction, 4 µl of 5X orange G loading buffer was added to each reaction. Finally, 14 µl of each reaction was run on a 1.0 % agarose gel at 150V for 40-45 minutes.

SNP Mapping

SNPs or small nuclear polymorphisms are single base changes. Recognition sites are created using SNPs for endonucleases, which cleave phosphodiester bonds internally within a sequence of DNA. Because of the divergent evolution of C. *elegans* resulting in the N2 and CB4856 strains, there are a number of differences in the genetic code occurring about every 1000 base pairs. These changes are DNA polymorphisms which are used in SNP mapping to locate mutations to specific chromosomes. Furthermore, SNPs that have the length of a restriction fragment (RFLPs) are preferred because restriction enzymes can then be used. The cleavage resulting from restriction enzymes can occur in either the N2 fragments or the CB4856 fragments. The differentiation of the isolates is more important than the specificity of the differentiation. The two major advantages of SNP mapping are cloning of a wide variety of mutations, and narrowing down the locus of mutations within 30,000 base pairs or 6-7 genes (Fay & Bender, 2008).

SNP mapping was used to determine the identity of the *zf35* mutation. SNP mapping uses DNA sequence polymorphisms between the N2-Bristol and CB4856-Hawaiian strain of C. *elegans* as genetic markers (Davis *et al.* 2005). The DNA was isolated from the mutants by setting up the following cross. The *zf35* hermaphrodites were mated with CB4856 males. Then 8-10 non-mutant (F_1 *zf35*/+ hermaphrodites) worms were picked to single plates and they were left to self-fertilize. Then ~50 (F_2 *zf35*/*zf35* hermaphrodites) worms showing the mutation were picked to single plates. The F_2 worms were then left to self-fertilize. The plates were then checked to see that each plate population is homozygous for the mutation. From each of the 50 plates, 50 worms were picked to make lysates. To determine the chromosome on which the mutation lies, 4 µl of lysate from each of the 50 tubes was pooled into a 1.5 ml tube. Figure 10 shows the total of 8 SNP primer pairs per chromosome that were used for each chromosome mapping.

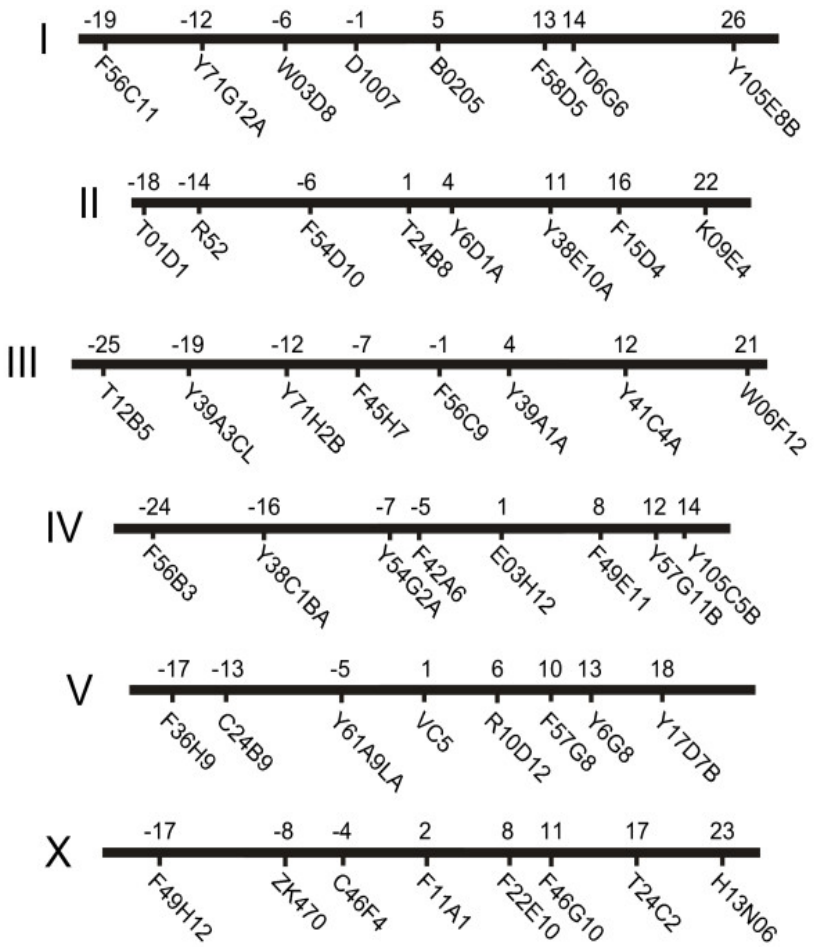


Figure 10: DraI SNPs in N2-Bristol and CB4856-Hawaiian (Davis *et al.,* 2005).

The reaction was set up in a 96-well plate consisting of the mutation mix and an N2/CB4856 control against all 8 pairs of SNPs on all 6 chromosomes (48 wells for each). Figure 11 shows the setup used.

		SNP 1 H	SNP 2 G	SNP 3 F	SNP 4 E	SNP 5 D	SNP 6 C	SNP 7 B	SNP 8 A	
50 mutation mix	1									Chromosome I
N2/CB control mix	2									
50 mutation mix	3									Chromosome
N2/CB control mix	4									II
50 mutation mix	5									Chromosome
N2/CB control mix	6									III
50 mutation mix	7									Chromosome
N2/CB control mix	8									IV
50 mutation mix	9									Chromosome V
N2/CB control mix	10									
50 mutation mix	11									Chromosome X
N2/CB control mix	12									

Figure 11a: Chromosome Mapping 96-well Plate (McPherson & Alkema, Personal Communication).

To set up the PCR reaction, two separate 50 reaction master mixes were made, one for the mutant lysates and one for the N2/CB4856 control. The master mix for 50 reactions consisted of 50 µl of 10X PCR Buffer, 50 µl of 2mM dNTPs, 50 µl of pooled lysate (25 µl N2 & 25µl CB), 5 µl of Taq (WoostaQ), and 299 µL of dH_2O. After both the master mixes were made, 9 µl of the master mix was added to each of the 48 mutant wells, and 9 µl of the control mix was added to each of the 48 control wells. Then 1 µl of the 10 µM primer pairs made from the clones shown on figure 10 were added with a pin replicator since the stock solutions of the 10 µM primer pairs were also stored in 96 well plates in the same orientation as the table above. The plate was sealed with a mat, and the PCR reaction was run under the conditions listed under the PCR protocol.

The digest for the chromosome mapping was then prepared. The restriction enzyme, DraI was used for chromosome mapping. The reagents for 1 reaction included 1.6 µl of 10X buffer, 0.1 µl of DraI, and 4.3 µl of dH_2O. A total of 100 reactions of the DraI cocktail were prepared, and 6 µl was added to each well using a multichannel pipet. The PCR products were digested overnight for 12-15 hrs at 37°C. After ~12 hours, 4 µl

of 5X orange G loading buffer was added to each well, and 15 µl from each well was loaded onto a 2.0% agarose gel as shown in figure 11b.

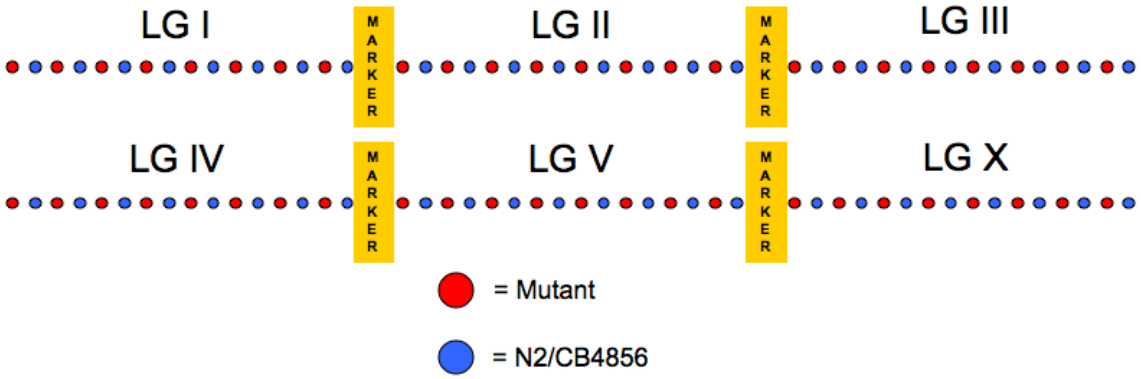


Figure 11b: Loading a SNP Gel (McPherson & Alkema, Personal Communication).

The samples were run at 150V for 40-45 minutes. The following criteria were used to interpret the results. If there is no linkage, then the lanes with the mutant DNA should look identical in band position and intensity as the 50:50 N2/CB4856 mix. However, if there is linkage to a chromosome then the N2 band should be enriched (much more intense) and CB4856 should be decreased in comparison to the N2/CB4856 mix. If the mutation is located closer to the SNP being tested, there is more chance for an enhancement of the N2 DNA to be seen in a homozygous mutant and a lesser chance of a recombination event taking place. Consequently, there is a lesser chance for the CB4856 allele to be observed in a homozygous mutant as the mutation gets closer to the SNP tested. Furthermore, if the SNP and the mutation are in very close proximity to each other, only the N2 band may appear for the homozygous mutants that are located at this SNP.

Complementation Test

Complementation tests are crosses where an unknown mutant strain is crossed with a strain carrying a known mutation. The progeny of this cross is then analyzed for a distribution of 50% wild type and 50 % mutant, or 100% wild type. If the former case occurs where one-half of the progeny shows the mutant phenotype and the other half shows the wild-type phenotype, then the unknown strain is considered the same as the known mutant. Half the progeny of the F1 generation should have the genotype $\frac{unknown}{mutant}$ while the other half should have the genotype $\frac{unknown}{wild\ type}$. If the unknown mutation is the same as the known mutant, the mutant phenotype should appear for the $\frac{unknown}{mutant}$ genotype. If the unknown strain is not the mutant, then a wild-type phenotype should appear for the $\frac{unknown}{mutant}$ genotype so the progeny are wild-type. Figure 12 shows the cross that was setup for the complementation tests.

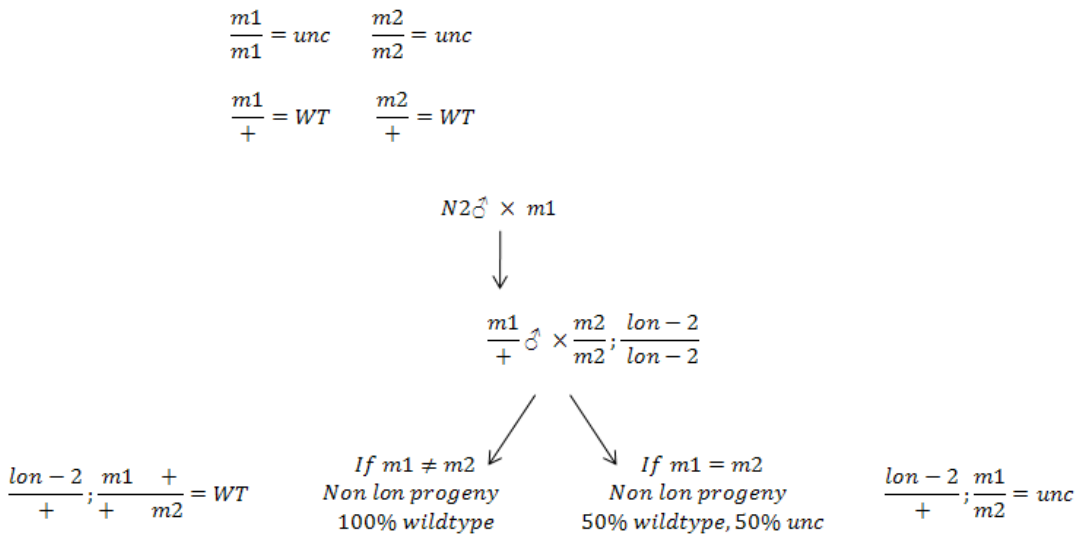


Figure 12: The Complementation Test. Note that the *lon-2* gene is marker for the long phenotype.

RESULTS

The main goal of this project was to characterize the *zf35* mutant by identifying the gene causing the hyperactive phenotype and performing epistatic analysis using double mutants in order to support the hypothesis that the *zf35* mutant had defects in the presynaptic pathway. Furthermore, an EMS suppressor screen with a *zf35*;*lon-2* background was done to provide novel mutations for further analysis. The characterization of *zf35* began by performing aldicarb and levamisole assays on the wild-type and *zf35*.

Aldicarb & Levamisole Assays

The N2 (WT) and *zf35* strains were shown to have different sensitivities to 1mM aldicarb. Aldicarb is an acetylcholine esterase inhibitor, which causes an increase of acetylcholine. This leads to eventual hyper-contraction at the synapse and paralysis. Figure 13 shows the time course aldicarb sensitivity assay of the N2 and *zf35* strains.

The *zf35* mutant paralyzes earlier than N2 WT, suggesting that the mutation could act presynaptically by causing the release of more acetylcholine into the synaptic cleft than the wild-type strain. Alternatively, there could be a problem with the choline reuptake mechanism, as well as the reassembly of choline and acetyl CoA into acetylcholine by the enzyme choline acetyltransferase. Another possible scenario could be that the zf35 sensitivity to aldicarb may be caused by an increase in the number of functional receptors. Hence, to fully understand where in synaptic transmission the *zf35* mutation acts, we performed levamisole assays to complement the aldicarb assays.

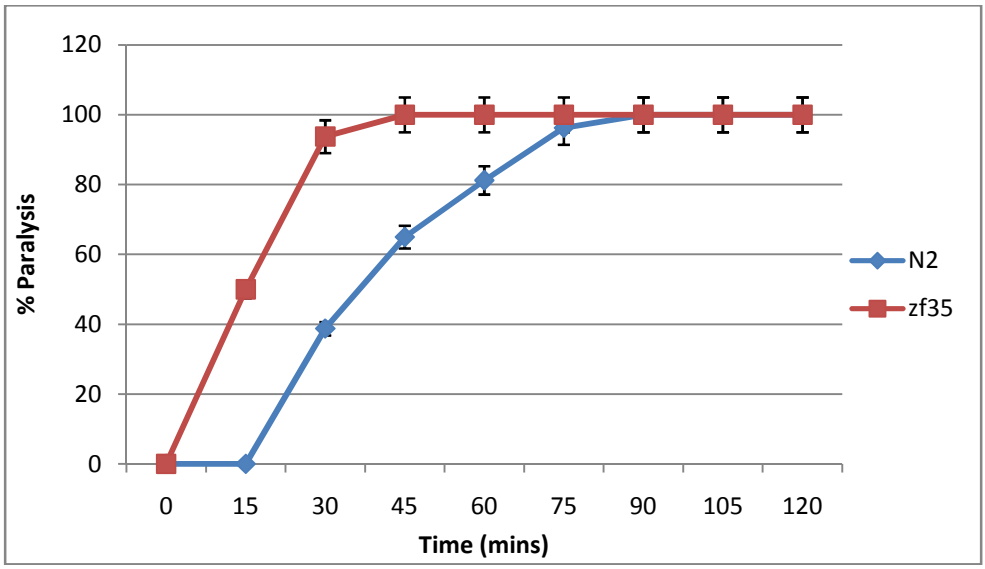


Figure 13: N2 and *zf35* Aldicarb Assay. *zf35* (red curve) is more sensitive to aldicarb than N2 WT (blue curve) as indicated by early paralysis.

Levamisole assays were performed on the N2 and *zf35* strains to determine if there were postsynaptic defects in the *zf35* mutants. Figure 14 shows the time course of the levamisole sensitivity assay. Based on this data, it was determined that the N2 wild type and the *zf35* mutant showed a similar sensitivity to levamisole. This indicates that *zf35* did not alter the post-synaptic function of acetylcholine release. Thus, analysis of *zf35* using aldicarb and levamisole suggests that the gene containing the *zf35* mutation functions presynaptically.

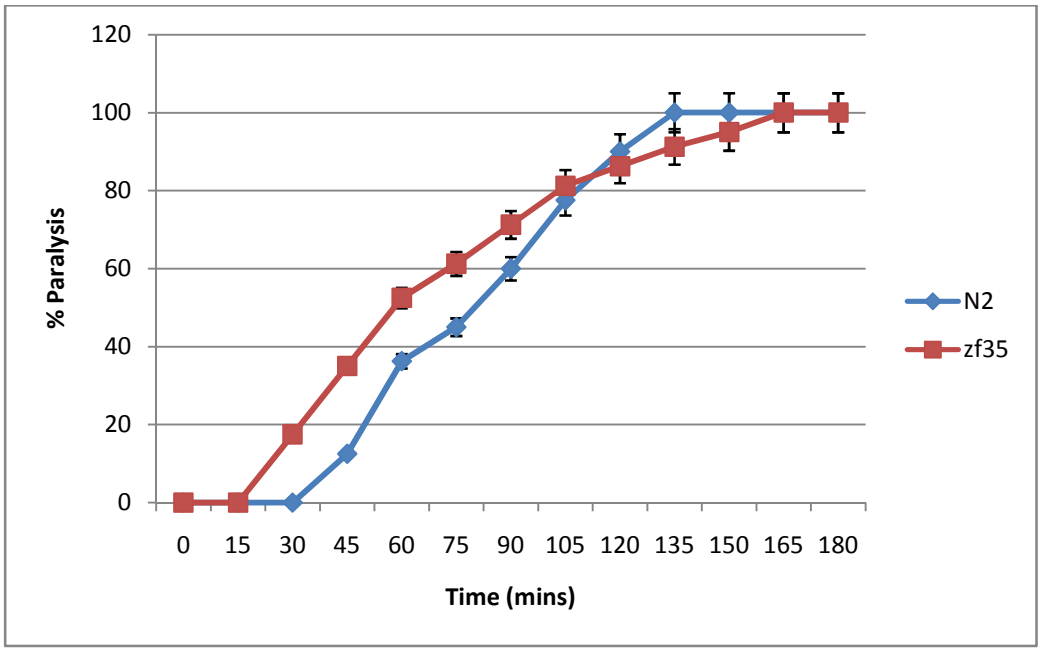


Figure 14: N2 & *zf35* Levamisole Assay. *zf35* and N2 (WT) have a similar rate of paralysis in the presence of Levamisole.

Chromosome SNP mapping was used to determine the locus of *zf35*. This work was initiated by Pirri and Alkema, and extended in this project. As shown in figure 15, evidence of the strongest linkage was observed in chromosome X (Pirri & Alkema, Personal Communication). This area of linkage is highlighted by the dotted red box in figure 15.

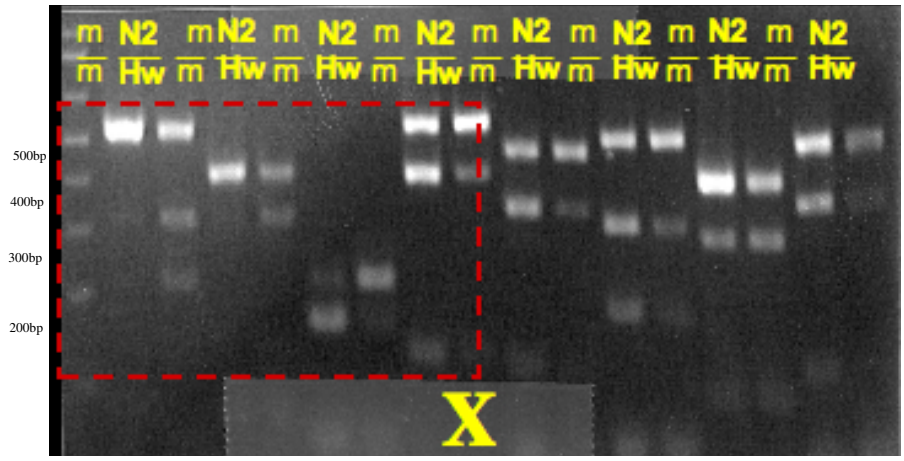


Figure 15: SNP mapping of *zf35*. The red box (chromosome X) indicates the locus where strongest linkage is observed (Pirri & Alkema, Personal Communication).

Fine mapping was performed to further narrow down the interval on the X chromosome where *zf35* might be located. In order to do this, recombinants were made by crossing the *zf35* strain with *dpy-3; lon-2*. Figure 16 shows the results from the fine mapping using the recombinants. The mutation was mapped to a small interval of 1.18 centimorgans, -14.02 to -13.2 on chromosome X. Recombinants are used in fine mapping to facilitate recombination events. The closer one gets to the mutation, the lesser the chance of recombination exists (Pirri & Alkema, Personal Communication).

Mutant	X, -15.58	X, -14.3	X,-14.04	X, C09B7	X,T02C5	X,-13.7	X, -13.2	X, -12.5
2	het	N2	N2			N2	N2	N2
4	het	het	het			het	N2	N2
19	het	het	het			het	het	N2
29	het	het	het	CB	N2	N2	N2	N2
33	het	N2	N2			N2	N2	N2
41	het	N2	N2			N2	N2	N2
47	het	het	het	N2	N2	N2	N2	N2
55	N2	het	het			het	het	het
57	N2	het	het			N2	het	het
61	N2	N2	het			het	het	het
70	N2	N2	N2			N2	N2	het
71	N2	het	het			het	het	het
73	N2	N2	N2			N2	het	het
75	N2	N2	N2			N2	het	het
81	het	N2	N2			N2	N2	N2
84	N2	N2	het			het	het	het
91	het	het	het			het	N2	N2
95	N2	N2	N2			N2	N2	het

Figure 16: *Unc-2* Fine Mapping. *unc-2* was fine mapped to the interval of -14.02 and -13.02 (Pirri & Alkema, Personal Communication).

Figure 17 shows the 8 genes known to exist in this region. Researching each of these genes revealed that *unc-2* was the only plausible gene that fit the presynaptic characterization determined by previous experiments. The α_1 subunit that *unc-2* (uncoordinated) encodes in the calcium channel is involved in the proper muscle contraction and speed in movement. This channel is homologous to the mammalian calcium channel, CACNA1A. This neuronal calcium channel is used for regular release

of neurotransmitters from the axon terminal. Therefore, disruption of this voltage gated calcium channel may cause abnormal release of acetylcholine into the synaptic cleft.

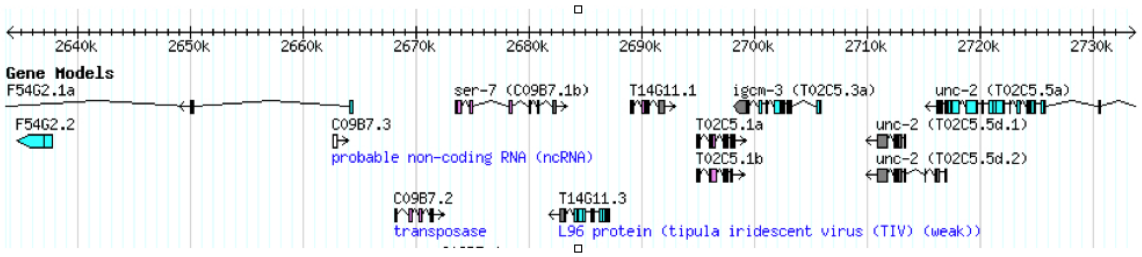


Figure 17: The 8 Known Genes Located on Chromosome I Between -14.02 and -13.2.

Double Mutants

Epistatic analysis of the double mutants was used to determine where in the synaptic release pathway *zf35* acts. Double mutants of *zf35* with various known presynaptic mutants were created and assayed. The cross used to construct these double mutants can be seen in figure 18. The double mutants *egl-30*; *zf35*, *unc-13*; *zf35*, *snt-1*; *zf35*, and *unc-31*; *zf35* were constructed to look for interactions between genes. Possible suppression was observed in the *egl-30*; *zf35*, *unc-13*; *zf35*, and *snt-1*; *zf35* double mutants based on aldicarb assays and the resulting visible phenotypes.

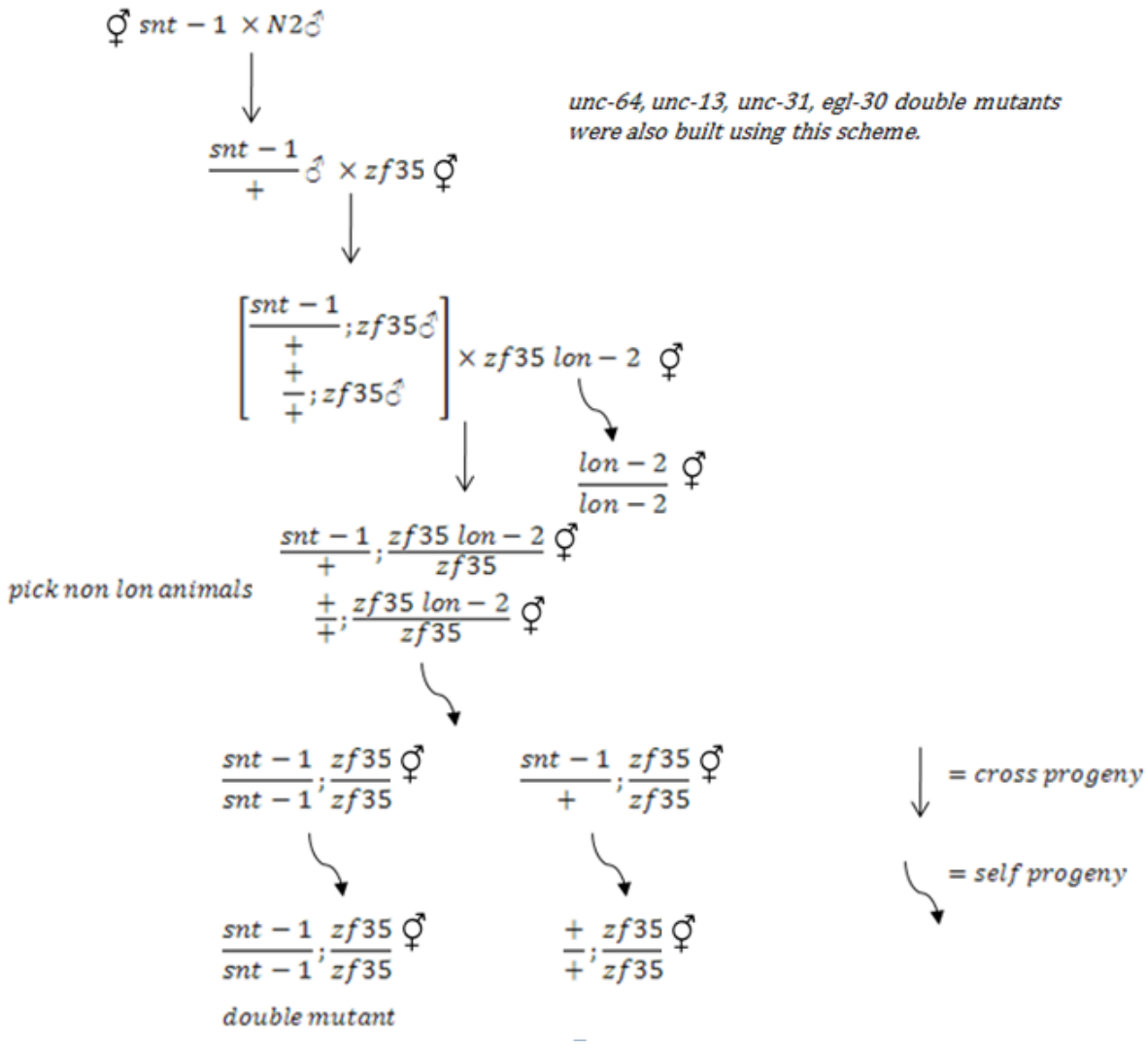


Figure 18: Cross Scheme for Formation of Double Mutants With *zf35.* *unc-64*, *unc-13*, *unc-31*, *snt-1* & *egl-30* were chosen because of their known role in presynaptic function.

Aldicarb assays were performed on each of these double mutants (Figure 19). These assays are displayed in graph form with N2, and the *zf35* single mutant serving as controls. *snt-1* worms show abnormal pharyngeal pumping as well as jerky backward movements. *snt-1* is also a partial loss of function, a type of mutant that contains less than the normal amount of gene product. In the aldicarb assay, the *snt-1* single mutant showed a slight resistance to aldicarb. The *snt-1*; *zf35* double mutant and the wild-type responded

similarly to aldicarb. Based on the aldicarb data, *zf35* restores *snt-1* to N2 like sensitivity to aldicarb. This meant that there was some interaction between the genes. This suggests that there is an interaction between *zf35* and *snt-1*, possible function in the same pathway.

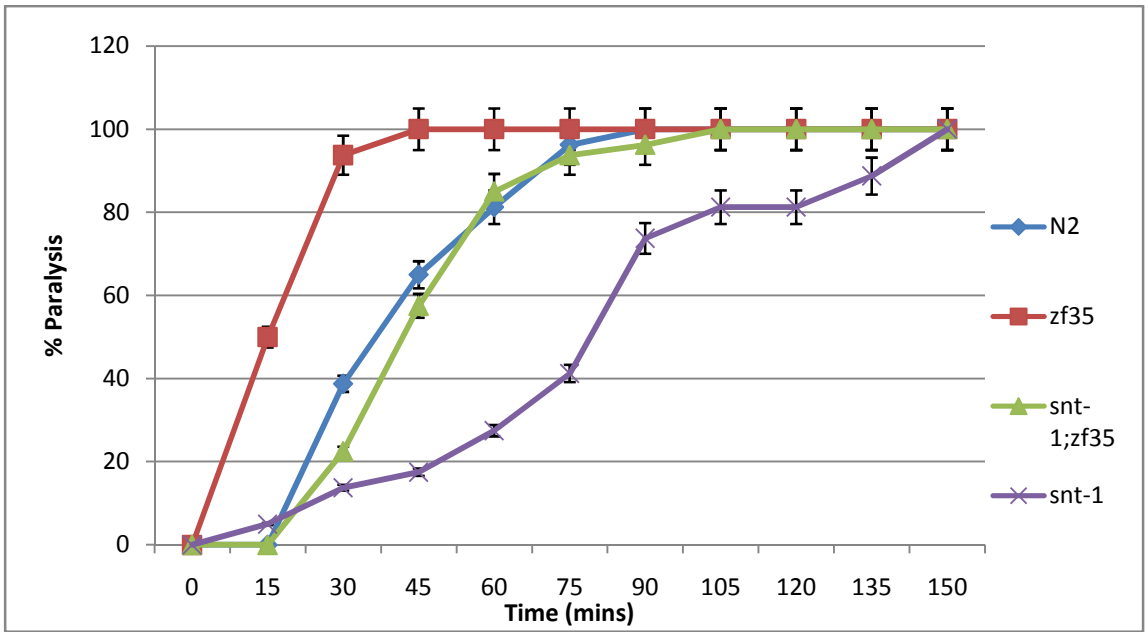


Figure 19: *snt-1*; *zf35* Double Mutant Aldicarb Assay. Aldicarb assays were performed on 1 mM NGM Aldicarb plates using 20 worms. Worms were assayed until 100% paralysis was seen. Paralysis is defined as lack of movement in response to an anterior and posterior touch with a platinum pick.

unc-13 worms are paralyzed. *unc-13* worms have abnormalities in pharyngeal pumping, axon morphology, protein sub cellular localization, and ectopic axon outgrowth (WormBase, 2008). *unc-13* is part of the pathway that uses synaptic vesicles for neurotransmission. In the aldicarb assay (Figure 20) the *unc-13* single mutant showed hyper-sensitivity to aldicarb similar to *zf35*. The *unc-13; zf35* double mutant and the N2 wild-type strain responded similarly to aldicarb. The formation of the double mutant with *unc-13* causes resistance towards aldicarb to increase and correspond with the N2 sensitivity. This means that *unc-13* did interact with *zf35,* and a possible mutant rescue might have taken place. Such an event was possible because the sensitivity of *zf35* was

not evident in the double mutant (WormBase, 2008). *unc-13* worms appeared hypersensitive as a result of their general locomotion defects. *unc-13* appeared to be paralyzed even in the absence of aldicarb. However, according to Richmond *et al.*, *unc-13* worms are resistant to aldicarb.

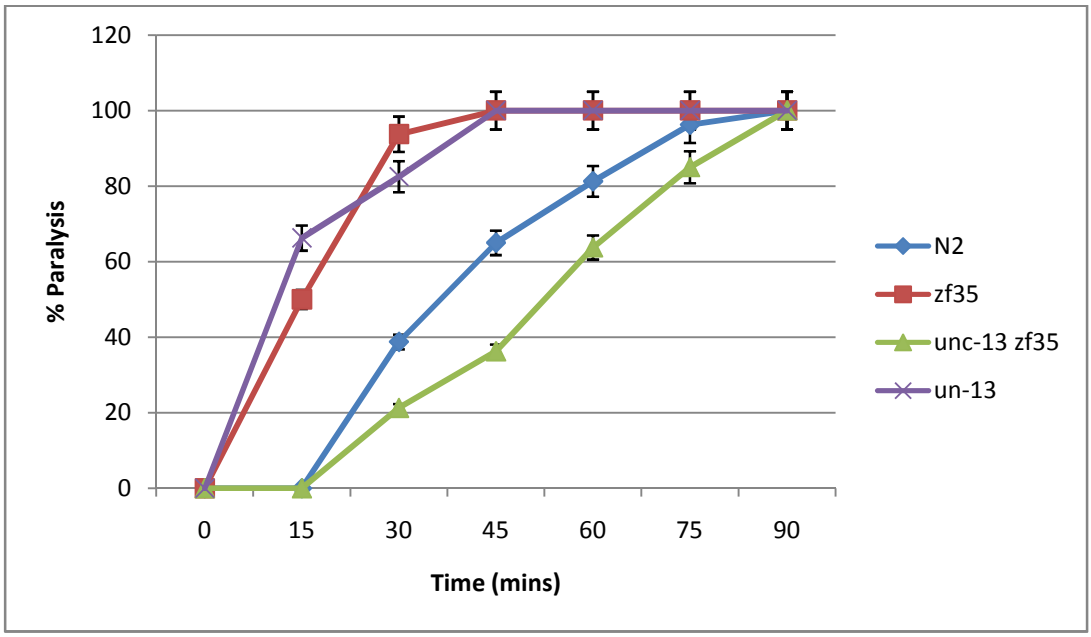


Figure 20: *unc-13*; *zf35* Double Mutant Aldicarb Assay. Aldicarb assays were performed on 1mM NGM Aldicarb plates using 20 worms. Worms were assayed until 100% paralysis was seen. Paralysis is defined as lack of movement in response to an anterior and posterior touch with a platinum pick.

unc-31 worms are slow moving, recessive, and mate poorly. The aldicarb assay done on the *unc-31* single mutant showed sensitivity to aldicarb similar to the wild-type N2 (Figure 21). The *unc-31*; *zf35* double mutant and the *zf35* strain responded similarly to aldicarb. No evidence of suppression or interaction was observed since the double mutant looked the same as the single mutant. The *unc-31* mutation acts in the pathway that uses dense core vesicles for synaptic transmission. *unc-31* looks wild-type on aldicarb because acetylcholine is released by synaptic vesicles and not released by dense core vesicles.

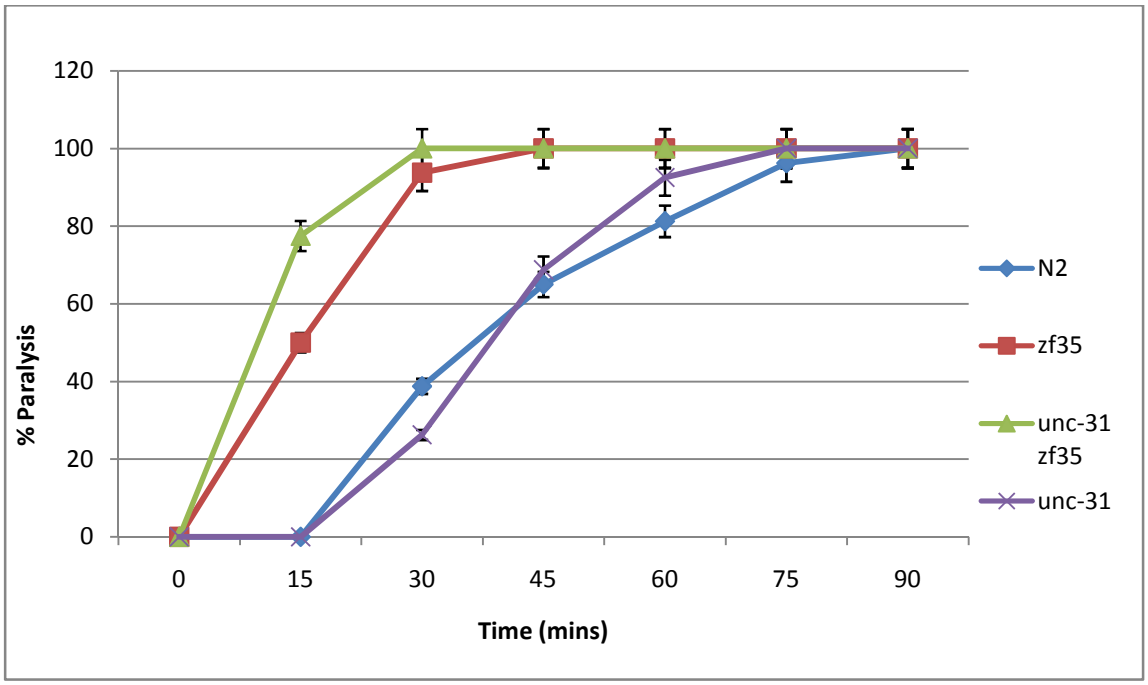


Figure 21: *unc-31*; *zf35* Double Mutant Aldicarb Assay. Aldicarb assays were performed on 1mM NGM Aldicarb plates using 20 worms. Worms were assayed until 100% paralysis was seen. Paralysis is defined as lack of movement in response to an anterior and posterior touch with a platinum pick.

egl-30 is an egg laying deficient mutant. It is a loss of function mutation that results in the excessive production of DAG and thus more acetylcholine is released than necessary. *egl-30* loss of function mutation up regulates the GαQ pathway resulting in an overproduction of DAG. DAG leads to the activation of the UNC-13 (priming factor). This results in an increase in acetylcholine release (Reynolds *et al.,* 2005). In the aldicarb assay (Figure 22), the *egl-30* single mutant showed sensitivity to aldicarb similar to the wild-type. Evidence of suppression in *egl-30;zf35* mutant worms was observed through phenotype assessment but not through the analysis of the aldicarb assay data. However, we were not sure if the *egl-30;zf35* carries the egl-30 allele. *egl-30;zf35* mutant worms showed locomotion similar to wild-type worms. These double mutants were unlike the paralyzed *egl-30* worms or the hyperactive *zf35* worms. Suppression would mean that there is some form of interaction between *egl-30* and *zf35*. Similar to *unc-13*, *egl-30*

worms also indicated hypersensitivity to aldicarb as a result of their general locomotion defects. Like *unc-13*, *egl-30* worms also appeared to paralyzed. However, according to Reynolds *et al.*, *egl-30* worms are resistant to aldicarb.

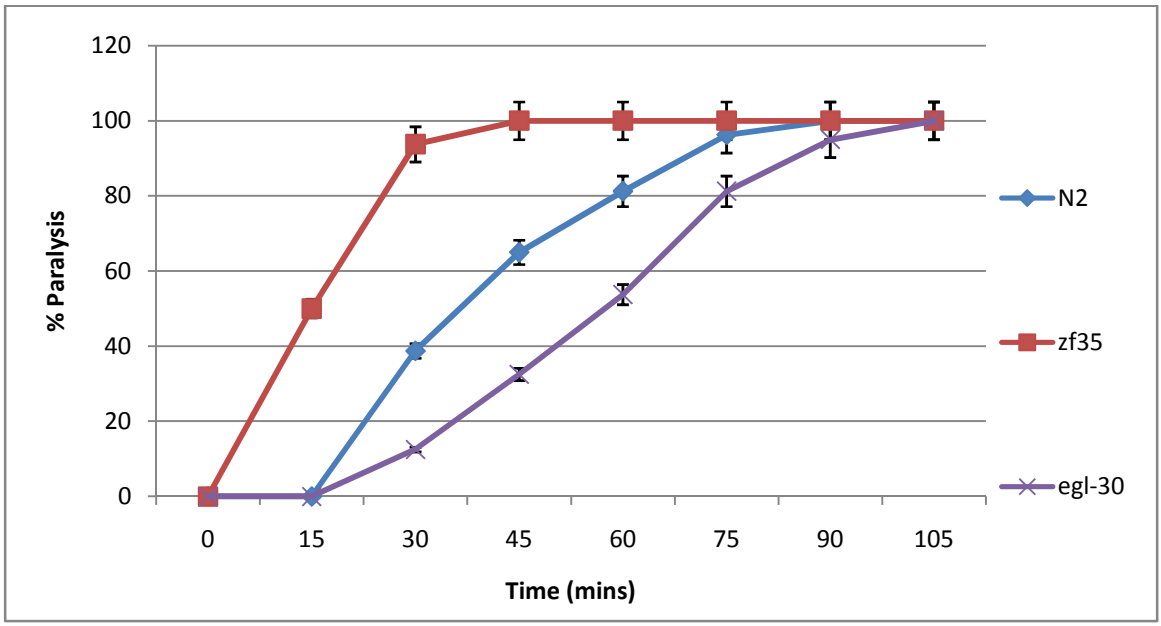


Figure 22: *egl-30*; *zf35* Double Mutant Aldicarb Assay. Aldicarb assays were performed on 1mM NGM Aldicarb plates using 20 worms. Worms were assayed until 100% paralysis was seen. Paralysis is defined as lack of movement in response to an anterior and posterior touch with a platinum pick.

Aldicarb and Levamisole assays were performed on the mutants derived from the EMS mutagenesis (Table I). *zf88*, *zf62*, and *zf65* were selected for complementation tests because of the uncoordinated locomotion observed. Three distinct mutants were selected from the genetic screen based on their sensitivity to aldicarb and levamisole. *zf88* was selected for being presynaptic because it was observed to be resistant to aldicarb and showed wild-type response to levamisole. This meant that either not enough acetylcholine was produced in *zf88* worms or there were other problems within the

presynaptic pathway. *zf62* and *zf65* were chosen because they were both resistant to aldicarb and levamisole.

Mutant Stains	Aldicarb Assay (% of worms moving after 1 hr)	Levamisole Assay (% of worms moving after 3 hrs)
zf57	90%	100%
zf58	60%	100%
zf59	80%	100%
zf60	95%	95%
zf61	100%	100%
zf62	100%	100%
zf63	100%	100%
zf64	100%	100%
zf65	100%	100%
zf66	100%	100%
zf67	100%	100%
zf68	100%	80%
zf69	100%	100%
zf70	100%	100%
zf71	100%	95%
zf72	100%	100%
zf73	100%	100%
zf74	100%	100%
zf75	100%	100%
zf76	100%	100%
zf77	50%	100%
zf78	85%	90%
zf79	100%	100%
zf80	85%	100%
zf81	95%	100%
zf82	100%	100%
zf83	100%	100%
zf84	100%	100%
zf85	100%	100%
zf86	100%	100%
zf87	20%	20%
zf88	100%	0%
zf89	20%	10%
zf90	20%	0%
zf91	35%	85%
zf92	0%	90%
zf93	0%	100%
zf94	0%	85%
zf95	0%	95%
zf96	25%	100%

Aldicard resistant, Levamisole resistant

Aldicard sensitive, Levamisole sensitive

Aldicard sensitive, Levamisole resistant

Aldicard resistant, Levamisole sensitive

Table 1: Summary of EMS Mutant Screening. The legends shows the how the mutants were characterized relative to their sensitivity to aldicarb and levamisole. Mutants from this procedure had aldicarb and levamisole assays performed on them to determine whether presynaptic or post-synaptic acetylcholine function was altered. The data from these assays is listed in table 1. For each mutant, 20 worms were placed on 1mM aldicarb NGM agar plates for 1 hour. In addition, 20 worms were also placed on 100uM levamisole NGM agar plates for 3 hours. At the end of the assays, the percentage of worms moving was recorded for each assay.

The mutagenesis was done in the *zf35 lon-2* background. To further characterize these mutations it was necessary to separate them from the *zf35 lon-2* background. To do

this (*zf62*, *zf65*, and *zf88*); *zf35 lon-2* animals were crossed with wild-type ♂. The cross used to achieve this goal is shown in figure 23. The wild type males were crossed with (*zf62*, *zf65*, and *zf88*); *zf35 lon-2* hermaphrodites. In the F1 generation, cross progeny were picked and selfed. In the F2 generation, non lon worms exhibiting the unc phenotype were picked and selfed. In the F3 generation, non lon worms were picked.

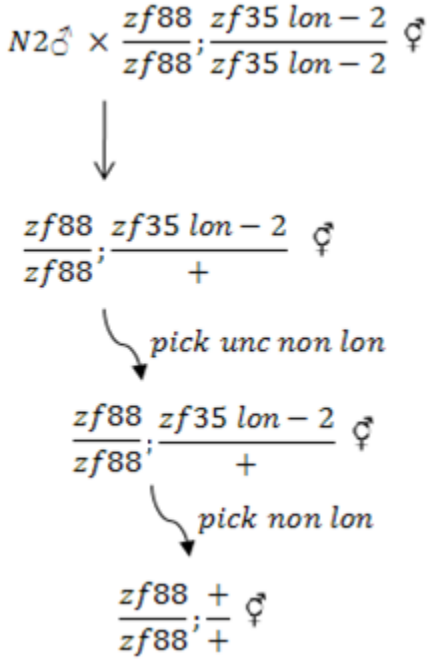


Figure 23: Scheme to Phase Out *zf35*-lon2. The cross was designed to remove the *zf35* lon-2 background from the EMS mutants selected for complementation .

SNP mapping was used to map the EMS mutants to the chromosome they were linked to. Figure 24a shows the chromosome I SNP gel for *zf62* using the listed primer pairs in figure 24b. The mutant DNA should look identical in band position and intensity to the 50:50 N2/CB4856 mix if there is no linkage to the chromosome. However, if there is linkage, the N2 band is enriched, and the intensity of the CB4856 band is decreased when compared to N2/CB4856 mix.

For the *zf62* mutant, linkage was predominantly found between the SNPs at -6 to +5 on chromosome I, suggesting that *zf62* is linked to chromosome I.

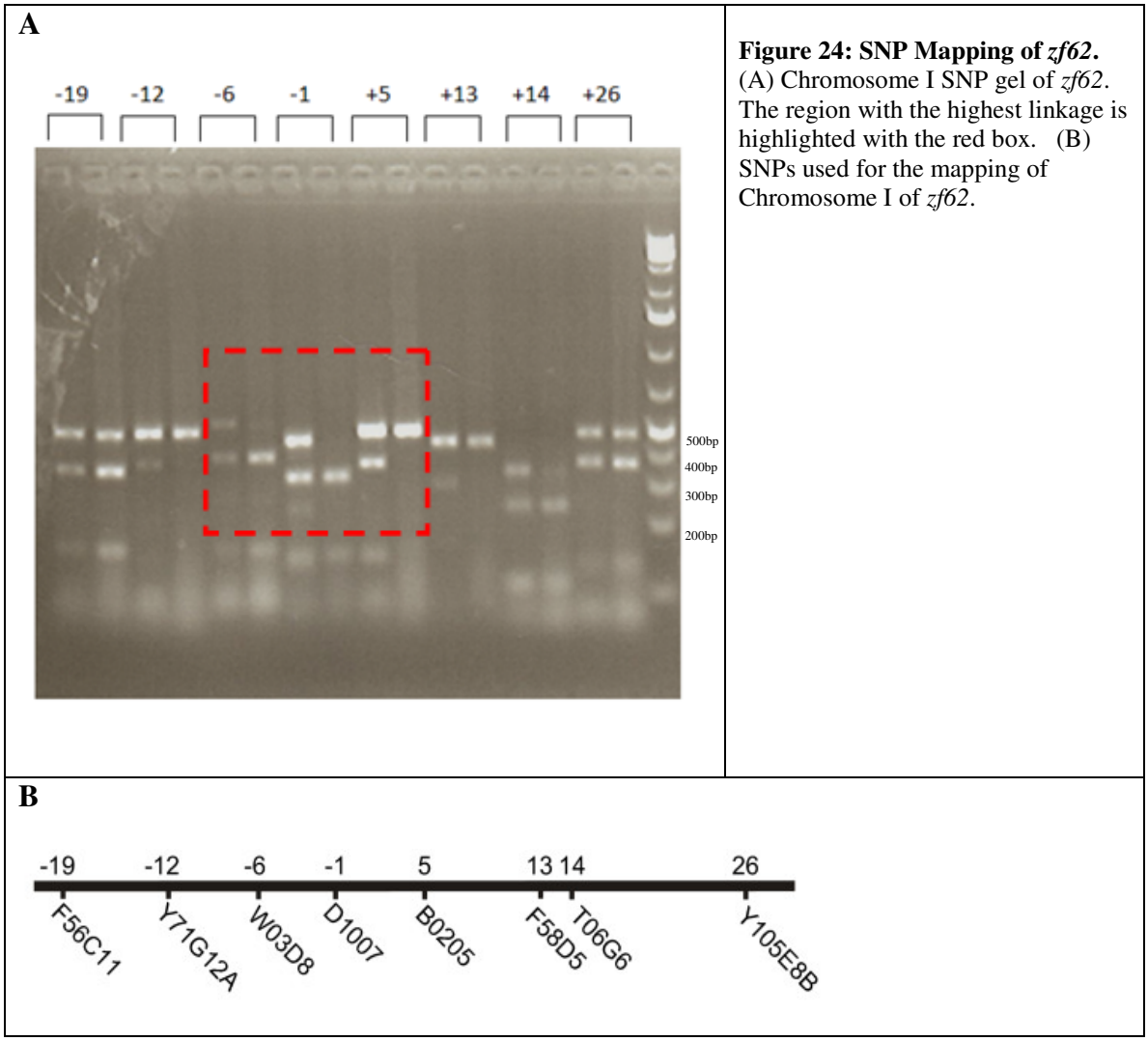


Figure 24: SNP Mapping of *zf62*. (A) Chromosome I SNP gel of *zf62*. The region with the highest linkage is highlighted with the red box. (B) SNPs used for the mapping of Chromosome I of *zf62*.

A similar method was used to interpret the *zf65* and *zf88* SNP gels. Figure 25a shows the chromosome I SNP gel of *zf65*. The strongest linkage can be seen at the interval -6 to +13 on chromosome I.

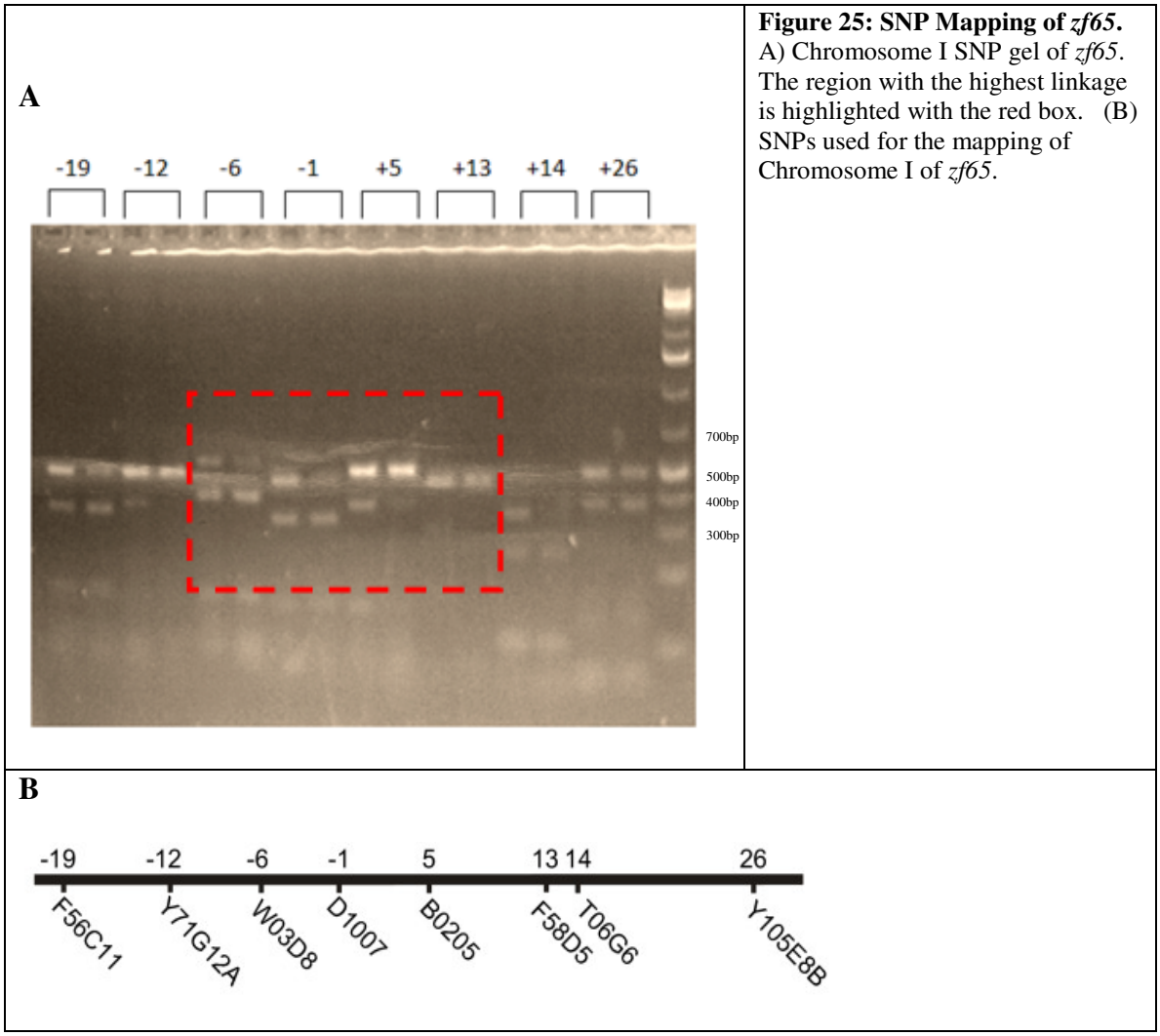


Figure 25: SNP Mapping of *zf65*. A) Chromosome I SNP gel of *zf65*. The region with the highest linkage is highlighted with the red box. (B) SNPs used for the mapping of Chromosome I of *zf65*.

As seen in figure 26a, the strongest linkage for *zf88* can be seen at the interval +1 to +18 on chromosome V.

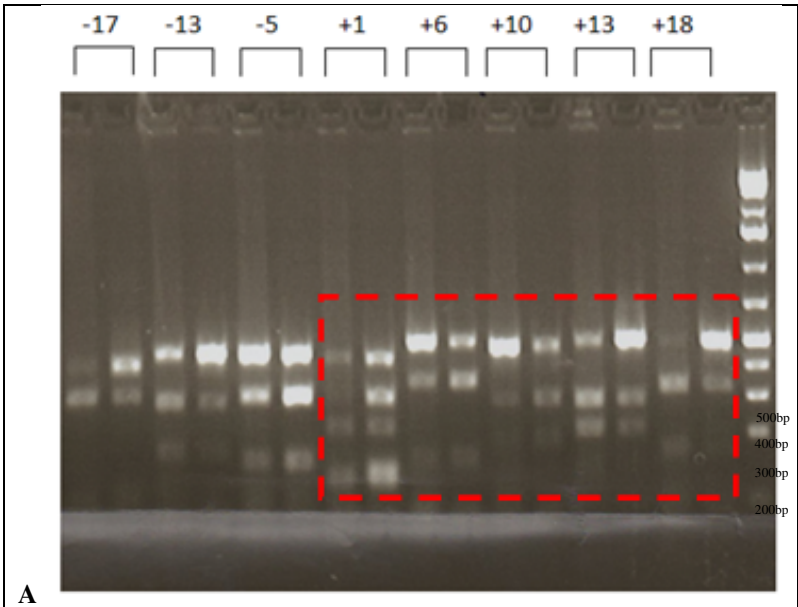


Figure 26: SNP Mapping of *zf88*. A) Chromosome I SNP gel of *zf88*. The region with the highest linkage is highlighted with the red box. (B) SNPs used for the mapping of Chromosome I of *zf88*.

Once the three mutants were mapped to their respective chromosomes, the interval was further narrowed down narrow using fine mapping. The concept behind this specific version of fine mapping is that the closer one gets to the mutation, the lesser the chance for recombination to occur. Thus seeing an enrichment of N2 would indicate that one is in close proximity to the mutation. The further away one is from the mutation, the more the chance for a recombination event to occur.

The fine mapping gel for *zf62* can be seen in Figure 27. Table 2 shows the complete fine mapping data for *zf62*. Figure 27a shows the left boundary at -3.14 and figure 27b shows the right boundary at +2.94.

Mutant	I, -6	I, -3.14	I, -1.67	I, -1	I, +0.06	I, +0.80	I, +2.94	I, +5
51	N2			N2				N2
52	het	N2		N2				N2
53	N2			N2				N2
54	N2			N2				N2
55	N2			N2				N2
56	het	N2	N2	N2	N2	N2	N2	het
57	N2			N2				N2
58	het	het	N2	N2				N2
59	N2			N2				N2
60	N2			N2				N2
61	N2			N2				N2
62	N2			N2				N2
63	N2			N2				N2
64	N2			N2				N2
65	N2			N2				N2
66	N2			N2				N2
67	N2			N2				N2
68	N2			N2				N2
69	N2			N2				N2
70	N2			N2				N2
71	N2			N2				N2
72	N2			N2				N2
73	N2			N2				N2
74	N2			N2				N2
76	N2			N2				N2
77	N2			N2				N2
78	N2			N2				N2
79	N2			N2	N2	N2	het	het
80	N2			N2				N2
81	N2			N2				N2
82	N2			N2				N2
83	N2			N2				N2
84	N2			N2				N2
85	N2			N2				N2
86	N2			N2				N2
87	N2			N2				N2
88	N2			N2				N2
89	het	N2		N2				N2
90	N2			N2				N2

Table 2: Fine Mapping Data for *zf62*. As one gets closer to the mutation, N2 enrichment is observed and the number of heterozygotes decreases. The left and right boundaries are determined by the transition from heterozygous (het) to N2. The boundaries are established where a minimal number of heterozygous (het) are seen at the point of transition.

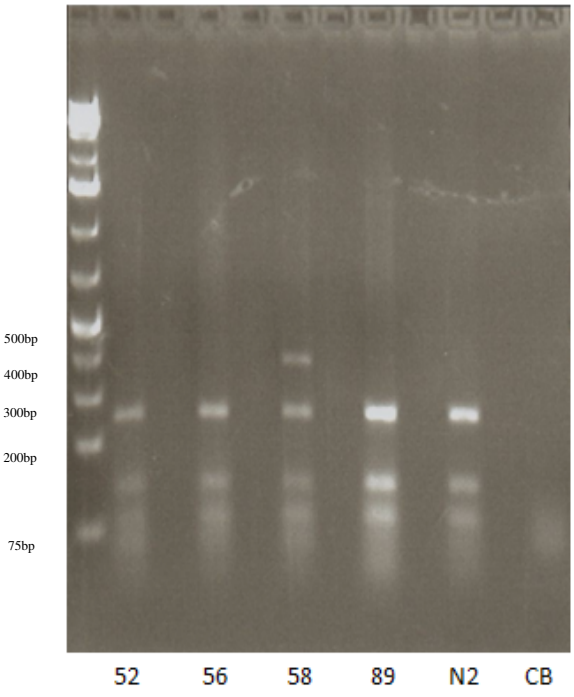


Figure 27a: Left Boundary Fine Mapping Gel of *zf62*. The locus was -3.14 and HinfI is used as the restriction enzyme The primers used were 5' primer = TTTTGGCTCCAAGGGTCACTC and 3' primer = CGAATGTTTGTTGAAAAAGCGG.

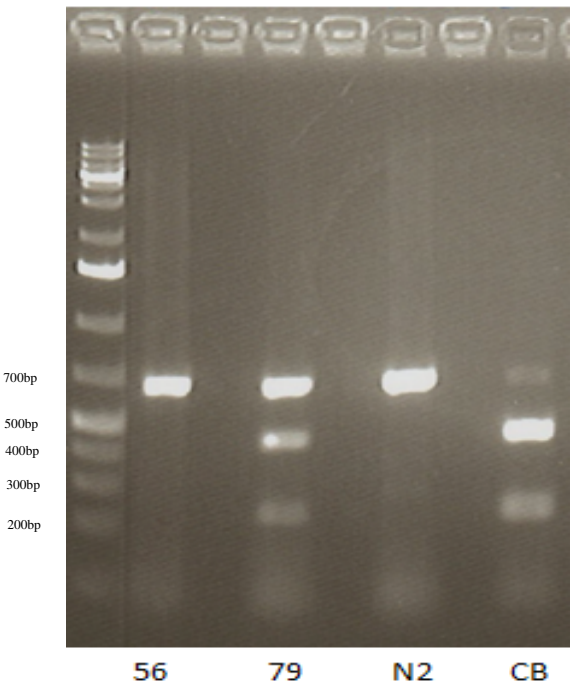


Figure 27b: Right Boundary Fine Mapping Gel of *zf62*. The locus was +2.94 and DraI is used as the restriction enzyme The primers used were 5' primer = TTCAGGCTCCACTTTATGCC and 3' primer = CATCTGGGACGTTCTTTCAC.

The fine mapping gel for *zf65* can be seen in figure 28. Table 3 shows the complete fine mapping data for *zf65*. Figure 28a shows the left boundary at -1.67 and figure 28b shows the right boundary at +0.06.

Mutant	I, -6	I, -3.14	I, -1.67	I, -1.17	I, -1	I, -0.70	I, +0.06	I, +5	I, +13
1		N2			N2	N2	het	het	CB
2	N2				N2			N2	N2
3	N2				N2			N2	het
4		N2			N2	N2	N2	het	het
5	N2				N2			N2	N2
6	N2				N2			N2	N2
7	N2				N2			N2	N2
8	N2				N2			N2	het
9	N2				N2			N2	N2
10	N2				N2			N2	N2
11	N2				N2		N2	het	het
12	N2				N2			N2	N2
13	N2				N2			N2	N2
14	N2				N2			N2	N2
15	N2				N2			N2	N2
16	N2				N2			N2	N2
18	N2				N2			N2	N2
19	N2				N2			N2	N2
20	N2				N2			N2	N2
21	N2				N2			N2	N2
23	N2				N2			N2	N2
24	N2				N2			N2	N2
25	N2				N2			N2	N2
26	N2				N2		N2	het	het
27	N2				N2			N2	N2
28	N2				N2			N2	N2
29	het	N2			N2			N2	N2
30	N2				N2			N2	N2
31	N2				N2			N2	N2
32	het	het	het	N2	N2			N2	N2
33	het	N2		N2	N2			N2	N2
34	N2				N2			N2	N2
35	N2				N2			N2	N2
36	N2				N2			N2	N2
37	N2				N2			N2	N2
38	het	het	N2		N2			N2	N2
39	N2				N2			N2	N2
40	N2				N2			N2	N2
41	het	het	het	N2	N2			N2	N2
42	N2				N2			N2	N2
43	het	het	N2		N2			N2	N2
44	N2				N2		N2	het	het
45	N2				N2			N2	CB

Table 3: Fine Mapping Data for *zf65*. As one gets closer to the mutation, N2 enrichment is observed and the number of heterozygotes decreases. The left and right boundaries are determined by the transition from heterozygous (het) to N2. The boundaries are established where a minimal number of heterozygous (het) are seen at the point of transition.

A

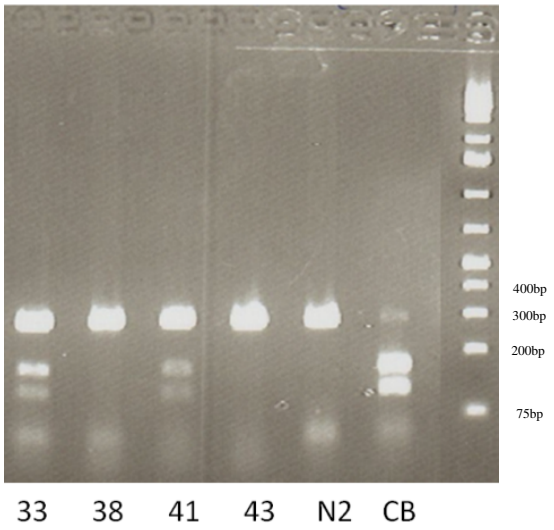


Figure 28a: Left Boundary Fine Mapping Gel of *zf65*. The locus was -1.67 and ClaI is used as the restriction enzyme The primers used were 5' primer = GTGAACGTCGTGATGAGGAG and 3' primer = TGAAGTGGTCCCGAATATATG.

B

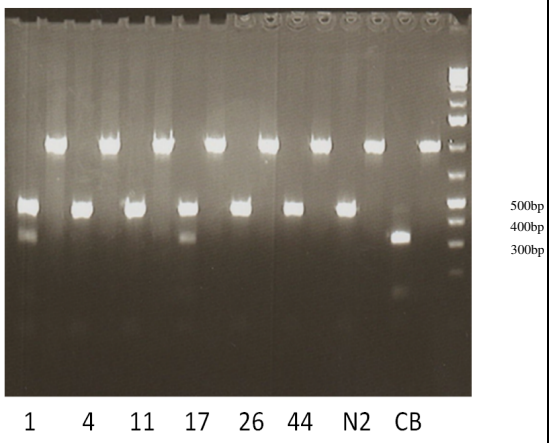
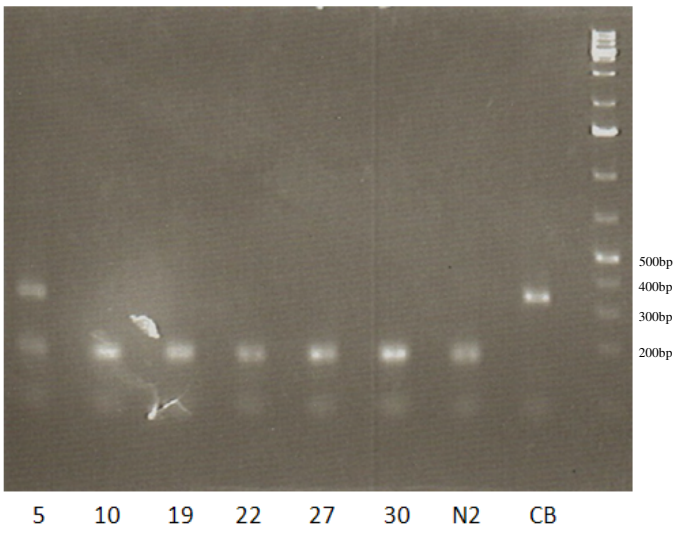


Figure 28b: Right Boundary Fine Mapping Gel of *zf65*. The locus was +0.06 and AvaI is used as the restriction enzyme The primers used were 5' primer = AATTCCGATGATGGAGACGG and 3' primer = GGTTACCATTCCAAATAGTGC.

The fine mapping gel for *zf88* can be seen in figure 29. Table 4 shows the complete fine mapping data for *zf88*. Figure 29a shows the left boundary at +23.25 and figure 29b shows the right boundary at +24.99.

Mutant	V, +1	V, +10	V, +18	V, +23.25	V, +24.46	V, +24.99	V, +25.21
1	het	het	N2			N2	N2
2	het	het	N2			N2	het
3	N2	N2	N2			N2	N2
4		N2	N2			N2	het
5	het	het	het	het	N2	N2	het
6	N2	N2	N2			N2	N2
7		N2	N2			N2	het
8	het	het	N2			N2	N2
9	N2	N2	N2			N2	N2
10	het	het	het	N2	N2	N2	N2
11	N2	N2	N2			N2	N2
12	N2	N2	N2			N2	N2
13	N2	N2	N2			N2	het
14		N2	N2		N2	het	het
15		N2	N2			N2	het
16	N2	N2	N2			N2	N2
17	N2	N2	N2			N2	N2
18	N2	N2	N2			N2	N2
19	het	het	het	N2	N2	N2	N2
20	het	N2	N2			N2	N2
21	N2	N2	N2			N2	N2
22	het	het	het	N2	N2	N2	N2
23	N2	N2	N2			N2	N2
24	N2	N2	N2			N2	N2
25	N2	N2	N2			N2	N2
26	N2	N2	N2			N2	N2
27	CB	het	het	N2	N2	N2	N2
28	N2	N2	N2			N2	N2
29	het	het	N2			N2	N2
30	het	het	het	N2	N2	N2	N2
31	N2	N2	N2			N2	N2
32	N2	N2	N2			N2	N2
33	N2	N2	N2			N2	het
34	het	het	N2		N2	het	het
35	N2	N2	N2			N2	het
36		N2	N2			N2	het
37	het	N2	N2			N2	het
38		N2	N2			N2	het
39	het	het	N2		N2	het	het
40		het	N2			N2	het

Table 4: Fine Mapping Data for *zf88*. As one gets closer to the mutation, N2 enrichment is observed and the number of heterozygotes decreases. The left and right boundaries are determined by the transition from heterozygous (het) to N2. The boundaries are established where a minimal number of heterozygous (het) are seen at the point of transition.



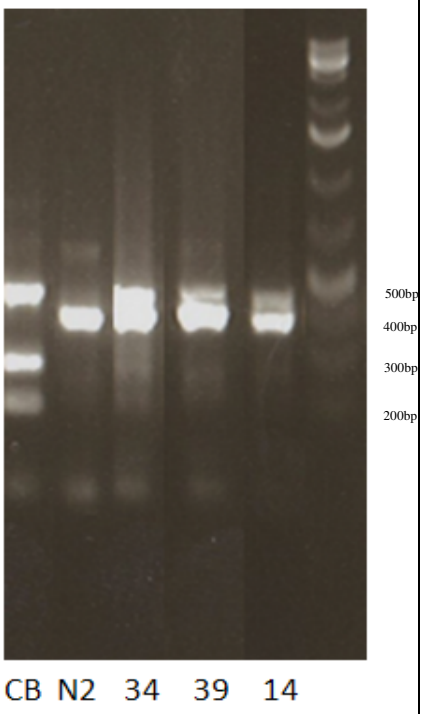


Figure 29a: Left Boundary Fine Mapping Gel of *zf88*. The locus was +23.25 and Tsp509I is used as the restriction enzyme The primers used were 5' primer = GACTTTTCGGATTGTCCATCAG and 3' primer = GACACTTACCGATATGCCGC.

Figure 29b: Right Boundary Fine Mapping Gel of *zf88*: The locus was +24.99 and AccI is used as the restriction enzyme The primers used were 5' primer =CCCTCAATCTTGCCCCATTC and 3' primer =AATGACGGGCAACTTTCAGG.

The fine mapping data was used to narrow down the genetic intervals in which the mutations lie. Once the intervals were narrowed down, we looked for candidate genes that acted presynaptically or might influence a calcium channel. For the intervals -1.67 to 0.06 and -3.14 to 2.94, *unc-63* was a likely mutant that fit these characteristics that *zf62* and *zf65* exhibits. *zf62* and *zf65* are postsynaptic according to the aldicarb assays done on them. *unc-63* is also postsynaptic.

The alpha subunit of the nicotinic acetylcholine receptor (nAChR) is encoded by *unc-63*. *unc-63* is expressed in many neurons, vulval muscles, and body wall muscles of *C. elegans*. *unc-63* is required for regular egg laying behavior and locomotion. The alpha subunit of a nicotinic acetylcholine receptor is encoded by *unc-63.* Functionally, *unc-63* acts as a ligand-gated ion channel that controls acetylcholine release at neuromuscular junctions (WormBase, 2008). Figures 30 and 31 show the fine mapped intervals of *zf62* and *zf65* respectively. In both cases, *unc-63* was selected for a complementation test.

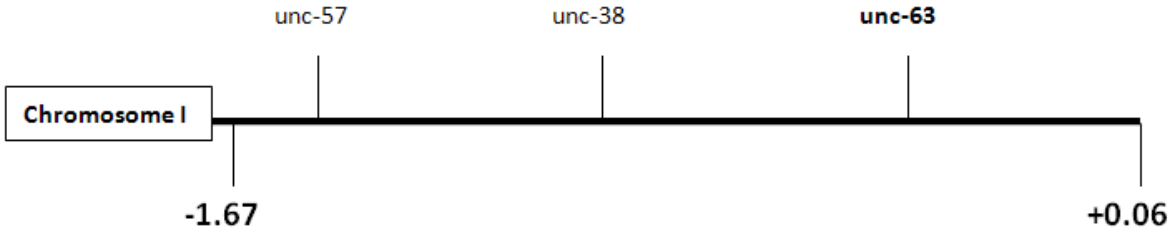


Figure 30: Known uncs Between the Loci -1.67 and +0.06 on Chromosome I.

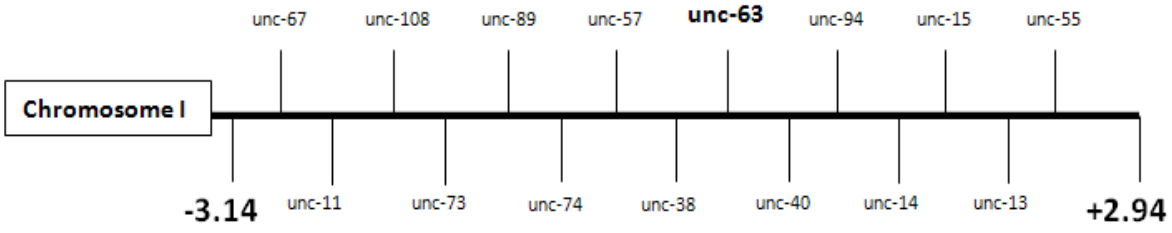


Figure 31: Known uncs Between the Loci -3.14 and +2.94 on Chromosome I.

unc-51 is a presynaptic gene that encodes a serine/threonine protein kinase. The axons that are located along the anterior-posterior axis require this protein kinase for regular outgrowth. *unc-51* is expressed in all neurons, pharyngeal muscles, and body wall muscles of *C. elegans* (WormBase, 2008). Figure 32 shows the fine mapped intervals of *zf88*. *unc-51* was selected for complementation since it was a uncoordinated presynaptic mutant in the interval.

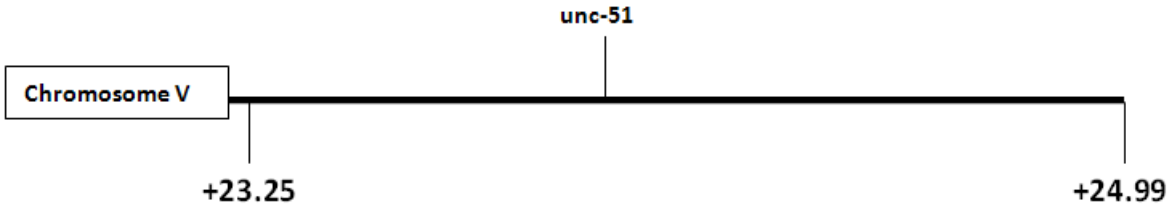


Figure 32: Known uncs Between the Loci +23.25 and +24.99 on Chromosome V.

The complementation test for *zf62* and *zf65* was performed using the cross in figure 33. For the mapped intervals of *zf62* and *zf65*, *unc-63* was chosen as a likely candidate based on the similarity of the phenotypes. Based on the results from the complementation test, both *zf62* and *zf65* failed to complement *unc-63*. Therefore, *zf62* and *zf65* were identified as *unc-63*.

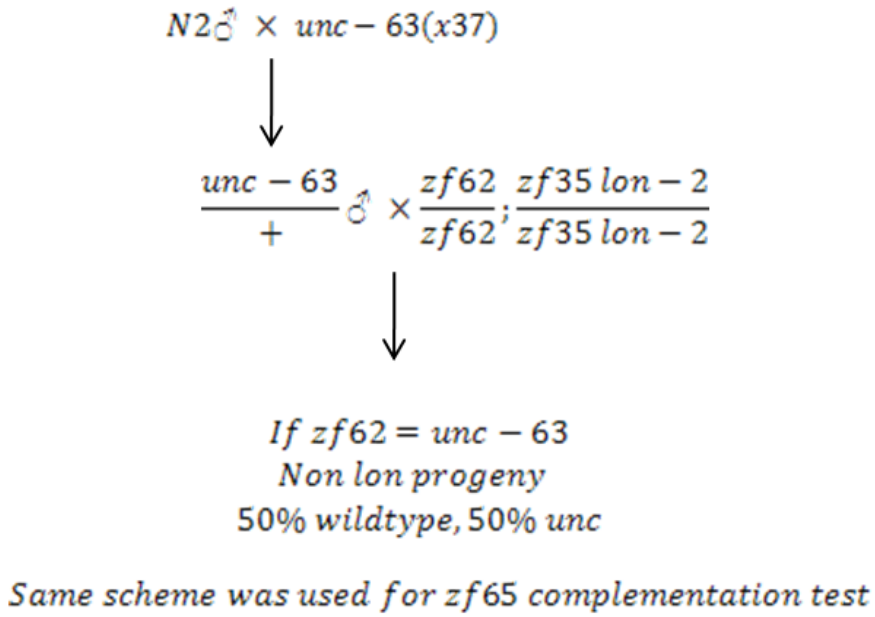


Figure 33: *zf62* & *zf65* Complementation Test With *unc-63*. The *zf62* & *zf65* mutants failed to complement with *unc-63*. Hence, *zf62*&*zf65* were identified as *unc-63*.

The complementation test for *zf88* was performed using the cross in figure 34. For the mapped intervals of *zf88*, *unc-51* was chosen as the most likely candidate based on the similarity of the phenotypes. Based on the results from the complementation test, *zf88* complemented *unc-51*. Hence, *zf88* was not unc-51. Note that rol-9 was a genetic marker that did not influence the complementation test results.

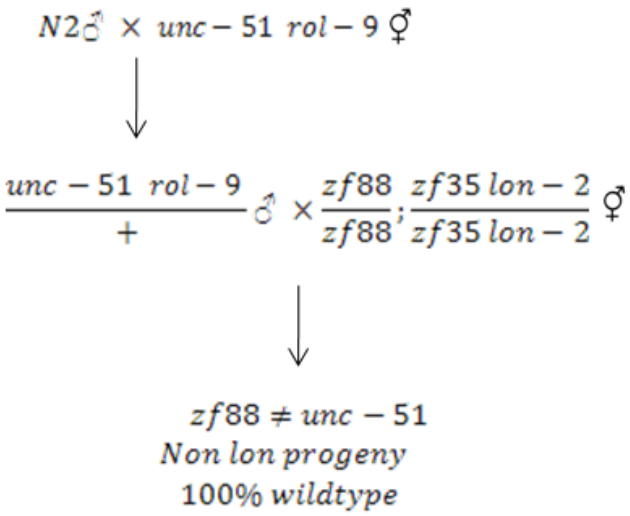


Figure 34: *zf88* Complementation Test With unc-51. The *zf88* mutant successfully complemented with unc-51. Hence, *zf88* was not identified as unc-51.

DISCUSSION

zf35 is a presynaptic gain of function mutant that displays genetic dominance and hyperactivity. These unique sets of characteristics make it a rare mutation. The sensitivity to aldicarb and the wild-type response to levamisole implied that *zf35* was a presynaptic hyperactive mutation with a defect causing an increase in acetylcholine at the synapse. Through fine mapping using SNPs, *zf35* was identified as an allele of the *unc-2* gene.

unc-2 encodes the alpha-1 subunit of a P/Q type calcium channel, similar to the mammalian CACNA1A. The P/Q type calcium channels are important for successful synaptic transmission. Previous research has implicated mutations of Familial hemiplegic migraines (FHM) and autism.

C. elegans UNC-2 is homologous to the CACNA1H channel in humans, a T-type CaV3.2 calcium channel. The electrophysiological, electrochemical, and biochemical data presented by Splawski *et al.* (2006) suggests that various combination of mutations present in the CACNA1H channel may play a role in Autism spectrum disorders. Autism is a neurological disease that affects brain development and neuronal function in humans. Symptoms include problems with communication, social interaction and restrictive and repetitive behavior. Splawski *et al.* identified 6 missense mutations in a conserved region of CACNA1H in patients that exhibited symptoms of autism. Splawski *et al.* concluded that these mutations reduced calcium channel activity and suggested that this could impact proper brain development and neuron function leading to Autism spectrum disorder (Splawski *et al.*, 2006).

Familial Hemiplegic Migraine is an autosomal dominant disorder that is associated with mutations in the CACNA1A calcium channel. Many of the symptoms of

FHM such as intense headaches, intolerance of loud sounds, and intolerance of intense light, are similar to those of a classic migraine. FHM is unique relative to classic migraines because FHMs are the associated with auras or other transient neurologic symptoms. These neurological symptoms may include cerebellar atrophy, the degeneration of the cerebellum, and ictal hemiparesis, the paralysis and weakness in one side of the body. The importance of studying FHM in detail is highlighted because of the implications of better understanding the complex factors influencing common migraines. (Hans *et al*., 1999). Further research on the CACNA1A calcium channel in *C. elegans* may prove fruitful to fully understand these debilitating diseases, and possibly suggest curative measures. As *unc-2* encodes the alpha-1 subunit of the CACNA1A of *C. elegans*, further analysis on this mutation may provide better understanding of these diseases.

unc-2 null animals are uncoordinated, aldicarb resistant and similar to wild-type on levamisole and nicotine. GFP fusion, unc-2 promoter bound to GFP, has also indicated that *unc-2* is expressed in the nervous system but not in the body wall, vulval or utrine muscles of *C. elegans*. Behavioral and expression data implies *unc-2* affects presynaptic neurotransmission in *C. elegans* nervous system (Mathews *et al.*, 2003). To date, many of the mutations in *C. elegans unc-2* have been loss of function mutations. z*f35* is one of the first gain of function *unc-2* alleles identified.

To further define the pathway through which *zf35* acts, epistatic analyses of double mutants were performed to search for suppressers. The mutants that *zf35* was crossed with were selected because they act in synaptic release. *snt-1* showed an interaction with *zf35* based on the aldicarb assay data. *snt-1* is resistant to aldicarb, and

according to Nguyen *et al., snt-1* mutants are defective in exocytosis and accumulate acetylcholine in their tissues. *snt-1*, a partial loss of function mutant, is capable of coordinated motor movements demonstrating that it does not have a complete block in acetylcholine release (Nguyen *et al.,*1995).

These observations suggest that *snt-1* restores the *zf35* mutant to wild-type because there is a decreased rate of neurotransmitter release caused by *snt-1*. This compensates for excessive release of acetylcholine by *zf35*. According to Richmond *et al.* (1999)*, unc-13* worms are also resistant to aldicarb. *unc-13* is involved in the priming of vesicles and is required for synaptic vesicle exocytosis (Richmond *et al.*, 1999). A problem in priming or exocytosis should cause less acetylcholine to be released even if a substantial increase in calcium occurs. In the case of the double mutant *unc-13*; *zf35*, the aldicarb data suggests that there is no significant change acetylcholine release even with the increased calcium influx caused by the *zf35* gain of function. The aldicarb sensitivity of *unc-31* worms is similar to wild-type. Acetylcholine is released by synaptic vesicles and not released by dense core vesicles. Because dense core vesicles are important in neurotransmitter release in the synaptic pathway in which unc-31 acts in, acetylcholine release is unaffected in the unc-31 mutants. Therefore, unc-31 worms show wild-type sensitivity to aldicarb (Charlie *et al.*, 2006).

egl-30 loss of function mutants are paralyzed in the absence of aldicarb. The g-alpha-q pathway is required for synaptic transmission and does not function properly in *egl-30* mutants. As a result, *egl-30* worms only release a small amount of neurotransmitter. Aldicarb does not drastically alter *egl-30's* paralyzed state; however, it causes a slight rescue of paralysis. Since aldicarb causes a buildup of acetylcholine in the

synaptic cleft, *egl-30* was observed as a phenotypic suppressor of *zf35*. However, it was necessary to confirm whether animals isolated from the *egl-30* and *zf35* cross contained mutant alleles of both genes. Furthermore, a balancer is necessary to confirm whether the egl-30 allele exists in the resulting double mutant (Reynolds *et al.*, 2005).

A genetic suppressor screen was performed by mutagenizing *zf35* which resulted in the isolation of 40 mutants. These 40 mutants were further characterized on aldicarb and levamisole plates, and 3 distinct mutants were picked for further study: *zf62*, *zf65*, and *zf88*. The *zf88* mutant was determined to function presynaptically due to the results of the aldicarb and levamisole assays. *zf88* was mapped between +23.25 and +24.99 on chromosome V, and *unc-51* was chosen as a candidate gene because *unc-51* is an uncoordinated presynaptic mutant (WormBase, 2008). A complementation test with *unc-51* resulted in a successful complementation; therefore zf88 is not an allele of *unc-51*. There are however, other candidate genes within the mapped interval on chromosome V. *ncx-1* was identified as a gene of interest because it is thought to play a critical role in neuronal Ca^{2+} homeostasis and function (Zamponi, 2005). *ncx-1* is a Na^{+}/ Ca^{2+} exchanger that is highly expressed in areas of high plasmalemmal Ca^{2+} flux, such as presynaptic terminals (Lytton, 2007) . The function of this exchanger in neurons is to extrude Ca^{2+} following its entry, thus terminating signaling events and restoring homeostasis for the next round of signaling.

Future experiments could include performing patch-clamp recordings on *Xenopus* oocytes, balancer confirmation of the egl-30 double mutant, fully characterizing every mutant derived from the EMS screen, creating double mutants with additional genes

involved in synaptic transmission, and sequencing the *zf88* mutant to determine if *zf88* is *ncx-1*.

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