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Identifying Modulators of the Development of Acute Functional Tolerance to Ethanol in Caenorhabditis elegans.

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Identifying Modulators of the Development of Acute Functional Tolerance to Ethanol in *Caenorhabditis elegans*.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

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List of Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
AFT	Acute Functional Tolerance
BEC	Blood (Brain) Ethanol Concentration
BK	Big calcium-activated voltage-gated potassium (K^+) channels; SLO-1 is a BK channel
CI	Chromosome One
CIII	Chromosome Three
COGA	Collaborative Studies on Genetics of Alcoholism
CNS	Central Nervous System
CREB	cAMP Responsive Element Binding Protein
ddH20	Double Deionized Water
DIC	Differential Interference Contrast microscopy
DNA	Deoxyribonucleic Acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders Fourth Edition
EMS	Ethyl methanesulfonate
EtOH	Ethanol
F_1	First filial generation after a cross
F_2	Second filial generation after a cross
FDA	Food and Drug Administration
ff	Full-functioning allele

$GABA_A$	Gamma-Aminobutyric Acid alpha
GK	Glycerol Kinase
GPO	Glycerol Phosphate Oxidase
HAFT	High Acute Functional Tolerance
hf	High-functioning allele
HHMPS	N-(3-sulfopropyl)-3-methoxy-5-methylaniline
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kfg	Kapo's favorite gene; gene of interest
L3	*C. elegans* Larval Stage Three
L4	*C. elegans* Larval Stage Four
LAFT	Low Acute Functional Tolerance
LB	Lysogeny Broth
lf	Low-functioning allele
LPL	Lipoprotein Lipase
LORR	Loss of Righting Reflex, a rodent assay that measures the hypnotic effect of a drug
NaAzide	Sodium Azide
NAD(H)	Nicotinamide adenine dinucleotide (hydride)
NGM	Nematode Growth Medium
NMDA	N-Methyl-D-aspartic acid
NPF	*Drosophila melanogaster* Neuropeptide F
NPR-1	*C. elegans Neuropeptide -Y- like Receptor 1*
NPY	*Mammalian Neuropeptide -Y*
PCR	*Polymerase Chain Reaction*
PKCε	*Protein Kinase C epsilon*
P_0	Parental Generation

POD	Peroxidase
RIL	Recombinant Inbred Line
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
SNP	Single Nucleotide Polymorphisms
SRE	Self-Rating of the Effects questionnaire on alcohol-related outcomes
TAG	Triacylglyceride/Triacyglycerol
VTA	Ventral Tegmental Area
YAC	Yeast Artificial Chromosome

Abstract

IDENTIFYING MODULATORS OF THE DEVELOPMENT OF ACUTE FUNCTIONAL TOLERANCE TO ETHANOL IN *CAENORHABDITIS ELEGANS*.

By Ka-Po Leung

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Major Director: Dr. Jill C. Bettinger, Ph.D., Pharmacology and Toxicology

Alcohol abuse is a problem in our society. There are few treatments available, in part due to the unclear molecular mechanisms of ethanol's effects. Human studies indicate that there is a genetic component influencing disease susceptibility, and that an individual's initial response to alcohol can predict their development of addiction. We have taken a forward genetics approach to study one component of initial response, acute functional tolerance (AFT), in *Caenorhabditis elegans*. We identified *bet11*, a mutation that causes animals to be defective in the development of AFT. Genetic analysis suggested that the gene that *bet11* disrupts participates in a synthetic genetic interaction with an unlinked natural allelic variant in another gene that alters ethanol

response. We also examined the role of lipid membrane composition in the response to ethanol. Identification of modulators that are responsible for alcohol-induced responses will provide a greater understanding of the mechanisms that cause alcohol-related diseases.

Chapter 1: Introduction

Alcohol

Alcohol is a widely used and accepted drug in our society. Even with the associated negative consequences of decreased mental health, increased social problems, and increased risk of other diseases and disorders, the use of alcohol has lead to high incidences of abuse, dependence, and addiction. Alcohol abuse occurs when alcohol consumption leads to problems such as physical injury and failure to attend to important responsibilities at home, work, or school, but not physical addiction, which produces symptoms such as anxiety, nausea, and sweating (O'Connor 2007). Alcohol addiction is a compulsive disorder where continued use and abuse of the drug persists despite its negative consequences. Human alcohol dependence is a chronic relapsing disorder with characteristics of tolerance, drug withdrawal, excess consumption, and impaired social and work activities (DSM-IV 1994). Studies have shown that alcoholics have a higher risk for psychiatric disorders, which is likely to be due to intoxication and withdrawal (Robins, Helzer et al. 1984; Kessler, Crum et al. 1997; Schuckit, Tipp et al. 1997; Raimo and Schuckit 1998). A variety of independent outcomes can be linked to repeated heavy alcohol drinking. These include, but are not limited to changes in mood, anxiety, schizophrenia, bipolar manic-depressive disease, attention deficit hyperactivity disorder (ADHD), and other substance use

(Tsuang and Lohr 1994; Winokur, Coryell et al. 1996; Disney, Elkins et al. 1999). Alcohol abuse and the comorbidities associated with alcoholism have not only produced physical detrimental effects, but have also caused high economical expenditures. The overall effects of alcohol abuse and dependence are a serious societal problem.

Alcohol interactions

Alcohol abuse and dependence may involve many neural mechanisms. These mechanisms play a role in a person's initial sensitivity, development of tolerance, and continued desire or craving for the drug. The contribution and influences of these neural mechanisms to an individual's drinking behavior remains unclear. Currently, it is thought that alcohol can act directly and indirectly on ethanol targets to produce a response and has been commonly accepted to have a neurochemical effect (Lovinger and Crabbe 2005; Vengeliene, Bilbao et al. 2008). Due to ethanol's effects on a broad range of targets identified with *in vitro* studies, our understanding of how these molecular mechanisms are involved in the *in vivo* responses to ethanol are limited and have made development of new treatments less successful. These limitations are chiefly due to the simple molecular structure of ethanol. Ethanol has only two reactive sites present: a hydroxyl (-OH) group and a short carbon backbone (Lovinger and Crabbe 2005). In addition, the molecule does not form ionic or covalent bonds, which causes it to have poor reactivity. This poor reactivity leads to low potency of the drug allowing for a large range of ethanol effects from intoxication to anesthesia. Ethanol is also widely distributed to many cellular compartments and has weak interactions with many potential target molecules making it harder to pinpoint and specifically target and measure its affinity.

Ethanol has a promiscuous nature and studies have elucidated a myriad of neuronal substrates, proteins, and processes that are affected by the drug. Other drugs of abuse share some of the same targets and interactions. Ethanol is predicted to have an effect on neuronal plasticity and regulation. It has been shown to influence a wide range of areas including the brain glutamatergic system, CREB proteins, stimulation of VTA dopaminergic neurons, and involvement of mesocorticolimbic and amygdala circuitry (Lovinger and Crabbe 2005). Other potential proteins, genes, and enzymes that might also influence an alcoholic's vulnerability include adenylyl cyclase, G-proteins, protein kinase C, the neuropeptide Y pathway, the serotonin system, dopamine, GABA, noreprinephrine, monoamine oxidase, and the alcohol metabolizing enzymes, alcohol dehydrogenase and aldehyde dehydrogenase. Furthermore, many of these regulators interact with one another and when one is disrupted, other substrates are effected as well and it could lead to detrimental changes (Schuckit 2002).

Available treatments for alcohol dependence

Current treatments for alcohol abuse and dependence include drugs that hinder alcohol consumption and support programs that guide alcoholics to wean alcohol from their lives. Currently there are three FDA approved drugs to treat alcohol dependence: Disulfiram (Antabuse), Naltrexone (ReVia), and Acamprosate (Campral). Disulfiram targets the ethanol metabolic pathway. After alcohol consumption, ethanol is metabolized by alcohol dehydrogenase into acetaldehyde. Acetaldehyde is further broken down into acetic acid by aldehyde dehydrogenase, which is excreted by the body. Disulfiram blocks the enzyme acetaldehyde dehydrogenase. This inhibition results in an increase in acetaldehyde when alcohol is consumed. Acetaldehyde is

extremely toxic and causes symptoms similar to a hangover such as nausea, vomiting, headache, and an increase in heart rate. These aversive effects discourage the consumption of alcohol. Another drug to treat alcohol dependence is naltrexone. While the mechanism of action of naltrexone is not fully known, it acts as a competitive antagonist at the μ- and κ-opioid receptors and minimally at the δ-opioid receptors (Shader 2003). It is proposed that an opioid receptor antagonist works within the dopaminergic mesolimbic pathway, which is linked to the rewarding effects of drugs. Naltrexone is prescribed to reduce alcohol craving, but will not relieve or stop withdrawal symptoms. Long-term use of alcohol causes an increase in the number of NMDA receptors. When a person is withdrawn from alcohol, there is a surge of excitatory neurotransmitters that activate these receptors at a higher than normal level (Tsai, Ragan et al. 1998). Acamprosate acts by targeting NMDA receptors and blocking the release of excitatory neurotransmitters that are produced after withdrawal from alcohol (De Witte, Littleton et al. 2005).

These drugs alone and in combination have been studied extensively to analyze their effectiveness. Disulfiram and naltrexone are FDA approved drugs, and are shown to be effective independently, but have room for improvement. These drugs in combination did not show any further advantage or improvement in treating alcohol dependence (Petrakis, Ralevski et al. 2007). Acamprosate, the newest and most promising of the drugs, is effective in reducing cravings and produces conditioned negative reinforcement and withdrawal (Littleton 1995; Kiefer and Mann 2010). Acamprosate has been shown to have fewer side effects and in combination with either naltrexone or dilsulfiram may be more effective than with treatment of acamprosate

alone (Hunter and Ochoa 2006). Due to the low success rate of current drug treatments, there are still areas that can be better understood to improve drug development.

More traditional methods of treatment based on behavioral therapies include Alcoholics Anonymous, the 12-Step program, and residential group therapies. These treatments show potential, but lack long-term effectiveness and have a high rate of relapse. Hospital or residential-based programs are more effective than outpatient programs in treating alcoholism (O'Malley, Jaffe et al. 1996; Fuller and Hiller-Sturmhofel 1999). However, due to health care costs outpatient programs are more desirable. Furthermore, it has been proposed that combination of psychotherapy and pharmacotherapy may be a promising supplemental approach.

Genetic factors influence susceptibility to alcoholism

There are two major factors that influence an individual's susceptibility to becoming an alcoholic, genetics and environment. First, it has been shown that genetic factors contribute to 40-60% of one's susceptibility to alcohol abuse and alcoholism (Kendler, Prescott et al. 1997; Schuckit 2002). The remainder of an individual's risk is attributed to non-genetic factors. Initial sensitivity differs among individuals and has been linked to the susceptibility to alcoholism later in life (Schuckit, Smith et al. 2004; Lovinger and Crabbe 2005). One study examined sons of alcoholics, between the ages of 18 and 20 years old, and evaluated their initial level of response to alcohol and then followed up 8.2 years later to assess their alcohol use and compared any known psychological disorders within their family. This study showed that family history plays a role in developing alcoholism, but prior psychological disorders were unrelated to

alcohol abuse and dependence (Schuckit and Smith 1996). Another study looked at subjects that were related to alcoholics and evaluated their level of response to alcohol challenges. Patients were tested and evaluated 15 years later for alcoholism using a 12-question Self-Rating of the Effects (SRE) of alcohol. From this study, it was shown that young adults that had a relatively low level of response to alcohol, when tested later in life, had a higher incidence of alcoholism (Schuckit, Smith et al. 1997). These studies confirm that an individual's genetic background plays a significant role in the predisposition to the future development of alcoholism.

The second non-genetic factor that plays a role in susceptibility to alcoholism is a person's environmental experiences (Kendler, Prescott et al. 1997; Schuckit 2002). Studies analyzing different populations have shown that there is a correlation between diagnosed alcohol abusers and their environmental influence. While there have been implications that environment plays a role in the development of alcoholism, population-based studies of twins have shown that it has only a minimal effect (Prescott and Kendler 1999). Twin studies from the Vietnam Era Twin Registry analyzed environmental factors that contributed to the decision to seek treatment and found there was a 40% variance between the shared environment and the influence to seek treatment (True, Heath et al. 1996). The decision to seek treatment is an inadequate measure for alcoholism, but is influenced by both genetic and environmental factors and may play a role in the continuation and degree of drinking. Understanding that environmental and more importantly that genetic factors play a large role in the development of alcoholism, it provides a gateway into identifying other mediators and the mechanisms of action that are involved in development alcoholism.

Acute functional tolerance

A significant problem in many drugs of abuse is the development of tolerance, which leads to an increase in drug dose or use within an individual. In contrast, drug resistance is the requirement of a larger dose of a drug in order to experience the same effect compared to another subject. Tolerance can be due to both the break down or metabolic (pharmacokinetic) response to ethanol, as well as metabolism-independent changes. Tolerance is commonly a lower responsiveness to a drug in the nervous system and is known as pharmacodynamic tolerance or functional tolerance (Atkinson 2009). The pharmacodynamic effects of tolerance to ethanol can be found in three different forms: acute, rapid, and chronic (Mellanby 1919; Tabakoff, Cornell et al. 1986; Khanna, Morato et al. 2002). Acute functional tolerance (AFT) to ethanol is described as a pharmacodynamic adaptation within a short, single exposure to ethanol that counteracts the cellular disturbances. The degree of AFT is assumed to develop in a linear fashion over time and the slope of the line is a measure of the magnitude of AFT effect. Rapid tolerance is the diminished response to alcohol upon administration of a second dose within 24 hours after the first administration. Chronic tolerance is defined as the reduction in sensitivity to a drug that results from repeated administration of the drug over a period of time. Largely, the main difference between the different types of tolerance described here is that acute tolerance is the reduction in sensitivity to a drug that results from a single administration or within the duration of one continuous dose of a drug rather than following repeated doses (Kalant, LeBlanc et al. 1971).

The development of AFT has been linked to the predisposition of alcohol-related diseases later in life, which makes it a strong candidate to study. The development of

AFT is thought to play a role in neuronal plasticity and advance the development of alcohol dependence. Frequently AFT is measured in combination with initial sensitivity, the first detectable response to ethanol. In studying behavior and other neuronal processes, it is difficult to accurately measure a subject's true initial sensitivity. Since the response is measured immediately after drug administration, experimental error limits the accuracy of the results due to the fact that it is predicted that neurons start adapting almost immediately after ethanol exposure. Thus initial sensitivity and AFT are combined to give a more accurate measure of sensitivity and response to ethanol. Through these measures, it is thought that functional tolerance to alcohol is due to a neuronal resistance response and is involved in plasticity changes that are similar to those involved in learning and memory (Tabakoff, Cornell et al. 1986; Berger, Kong et al. 2008).

Neuropeptide Y (NPY) receptors have been shown in different studies to influence AFT. NPY receptors are a class of G-protein coupled receptors and NPY is the most abundant neuropeptide in the brain (Erickson, Clegg et al. 1996). In *C. elegans,* neuropeptide Y-like receptor 1 (NPR-1), which is homologous to the human NPY receptor was found to be a negative regulator of AFT. Natural variation in *npr-1* activity in *C. elegans* causes variation in ethanol response between wild strains. It has been shown that NPR-1 regulates AFT and has been predicted that increased function of this protein will decrease the rate of recovery from ethanol (Davies, Bettinger et al. 2004). Furthermore, Davies et al. has demonstrated that extended exposure to ethanol caused animals to down-regulate their NPR-1 activity. Other supporting genetic and pharmacological studies done on flies and mammals have shown that NPF and NPY

respectively, also regulate the response to ethanol. In mammals, NPY has also been shown to suppress anxiety and the responsiveness to stress and aversive stimuli, which may be common reasons why individuals are drawn to drinking (Palmiter, Erickson et al. 1998; Bannon, Seda et al. 2000; Heilig and Thorsell 2002). NPY, while a strong candidate in mediating ethanol tolerance, is not believed to be the sole contributor. Since ethanol is a very simple, widely distributed drug that has been shown to hit multiple targets, it is proposed that there are many mediators and interactions with targets that lead to ethanol sensitivity and tolerance.

SLO-1, an ethanol regulator

SLO-1, an important regulator of neurotransmitter release, controls the synaptic strength and plasticity for higher brain function. A variety of potassium channels are located in the presynaptic nerve terminal along with SLO-1 channels. SLO-1 channels are activated by both calcium and membrane depolarization. Calcium activated BK channels were first discovered in *Drosophila* through slowpoke. Slowpoke, is homologous to *slo-1* in *C. elegans* and interestingly, *slo-1* was selectively isolated in a forward genetic screen as the only ion channel mutant that plays a role in neurotransmitter release (Wang, Saifee et al. 2001). This suggested that *slo-1* is an important and unique regulator of neurotransmitter release. The identification of the action of ethanol on SLO-1 channels has provided a gateway and tool to further studies on the role of ethanol. Importantly, SLO-1 has high homology between *C. elegans*, *Drosophila*, rodents, and humans where the structural components are similar. SLO-1 in *C. elegans* is expressed in neurons and body-wall muscle. SLO-1 is critical for muscle

function and mutant *slo-1* worms have muscle degeneration similar to those seen in dystrophin mutants (Carre-Pierrat, Grisoni et al. 2006).

slo-1, in *C. elegans* is a voltage-gated potassium channel, which is activated by ethanol . In ethanol target screens, which are used to identify mechanisms that are responsible for ethanol intoxication, numerous loss-of-function *slo-1* alleles were isolated (Davies, Pierce-Shimomura et al. 2003). It was shown that *slo-1* loss-of-function mutants have a strong resistance to ethanol in speed and egg-laying behavior. In contrast, *C. elegans* with hyperactivated SLO-1 channel showed behaviors similar to ethanol intoxication in the absence of ethanol. Additionally, it has been found that over expression of *slo-1* also restored ethanol intoxication to *slo-1* mutant animals. Through electrophysiological experiments, it has been shown that pharmacologically relevant concentration of ethanol activates the SLO-1 channel. Studies on mammalian BK channels have also suggested that ethanol directly affects these channels at relevant concentrations (Dopico, Lemos et al. 1996; Dopico, Anantharam et al. 1998).

Membrane lipids influence ethanol sensitivity and tolerance

Most studies on ethanol sensitivity and tolerance focus on protein targets, however recent studies have shown that membrane lipids may play a larger role. BK and other voltage-gated potassium channels are sequestered mostly in lipid microdomains of lipid membranes and cholesterol content modulates the role of ethanol activation of BK channels (Marty 1981; Dopico, Lemos et al. 1996; Crowley, Treistman et al. 2003; Yuan, O'Connell et al. 2008). Acute ethanol tolerance has been observed in stable (20:1) phosphatidylcholine-dioleoylphosphatidylethanolamine (PC-DOPE) and the activity of the BK channel is influenced by the ratio of cholesterol to phospholipids in

the membrane in which the channel is located. Changes in the lipid bilayer composition may also cause changes in ethanol sensitivity by influencing the BK channels (Yuan, O'Connell et al. 2008). Ethanol modifies the gating and conductance properties of the BK channel and increases channel activity (Dopico, Lemos et al. 1996). When ethanol is presented into the system for a period of time and leads to the development of tolerance, there is a decrease in ethanol activation on the channel and a decrease of BK current density due to the possible increase in cholesterol that plays a role in the overall lipid composition and sequestering of BK channels (Pietrzykowski, Martin et al. 2004).

Triacylglycerides (TAG), a type of lipid is modulated by lipases. The lipase, LIPS-7 negatively regulates the level of TAGs in *C. elegans* and ultimately may alter the ethanol response through CTBP-1 (Figure 1). CTBP-1 is a NAD(H)-dependent transcriptional co-repressor that is homologous to humans and has been linked to increased lifespan in *C. elegans* (Chen, Whetstine et al. 2009). When CTBP-1 is inhibited, *lips-7* is over expressed, which leads to a decrease of TAG. On 400mM ethanol, *lips-7(ok3110)* develops AFT faster and has higher resistance to ethanol than N2, while *ctbp-1(eg613)* is more sensitive to ethanol and has a slower development of AFT (Bettinger, Leung et al. 2011, unpublished). By altering levels of *lips-7, ctbp-1* inversely influences the lipid composition and the levels of TAG, and thus producing opposing effects to ethanol sensitivity and AFT. Mammalian BK channels have been found within lipid microdomains and their activation and potentiation may be modified in response to changes in the lipid membrane composition (Bravo-Zehnder, Orio et al. 2000; Treistman and Martin 2009). Together these findings suggested that TAGs might

play a role in the sensitivity and development of tolerance in response to ethanol.

Using model organisms to study alcoholism

Different model organisms have been used to research alcohol use, abuse, dependence, and comorbidities with alcoholism. Animal models allow researchers to conduct experiments that cannot be done on human subjects. Manipulation of genetic background, environment, route of administration, and diet are some advantages when studying ethanol. Also, the ability to test and analyze cells and tissues provides another asset in studying alcohol-related diseases. Due to the high homology between humans, rodents, and *C. elegans,* the ability to use different model organisms provides a powerful tool in identifying and understanding the role of alcohol in humans.

Ethanol studies in rodents

In rodents, the loss of righting reflex (LORR) and the blood (brain) ethanol concentration (BEC) are measured in tandem in order to analyze the acute effects of alcohol. The LORR assesses the level of sensitivity to the effects or sedation to alcohol. It is measured behaviorally by the ability of the animal to right itself after being turned on its back. AFT can be estimated by taking the BEC levels at two time points during ethanol impairment at the rising and falling arms of the BEC curve (Ponomarev and Crabbe 2004). This will allow comparison of the BEC for initial sensitivity to its recovery. AFT can be influenced by many factors including the genetic background of a subject and the experimental treatment. AFT can only be estimated due to the constraints of the

ability to accurately measure the true initial ethanol response once alcohol has been administered.

Alcohol tolerance has been seen in many observable effects of ethanol including motor impairment, sedation, and hypothermia. Studies on adolescent and adult rats have shown that alterations in the NMDA receptor system may decrease ethanol sensitivity in response to the enhancement of acute tolerance (Ramirez, Varlinskaya et al. 2011). Ethanol sensitivity has also shown to be regulated by Fyn, a non-receptor type tyrosine kinase. Animals lacking Fyn were hypersensitive to the effects of ethanol and found that there was no tyrosine phosphorylation in the NMDA receptor in the hippocampus as seen in control mice (Miyakawa, Yagi et al. 1997). Another study showed that a decrease of protein kinase C epsilon (PKCε) contributes to AFT. Knockout mice with a loss of PKCε showed signs of increased ethanol intoxication and reduced ethanol self-administration. In wild-type mice *in vivo* ethanol exposure produced AFT and yielded an increase in phosphorylation of PKCε (Wallace, Newton et al. 2007).

As mentioned previously, NPY receptors are an ethanol regulator among a number of homologous species. NPY deficient mice show an increase in tolerance to ethanol and an increase in alcohol consumption of different concentrations of ethanol (Thiele, Marsh et al. 1998; Thiele, Koh et al. 2002). NPY knockout mice were also less sensitive to the sedative and hypnotic effects of ethanol. Similarly, the plasma ethanol concentrations were unchanged compared to wild type controls. NPY receptor subtypes Y1 and Y5 in mice have been shown to regulate food intake and alteration in food consumption, anxiety, and ethanol consumption. An increase in subtype Y1 resulted in

a decrease in ethanol consumption, where knockout Y1 mice also showed a decreased sensitivity.

A method of bidirectional breeding allows for a more direct approach of analyzing ethanol sensitivity. Variations in genes that promote AFT and reduce the behavioral uncoordinated effects of ethanol can be identified using and comparing mice that have a high acute functional tolerance (HAFT) and mice that have a low acute functional tolerance (LAFT). The concept is that HAFT mice will have more genetic variants that contribute to AFT compared to LAFT mice and important mediators can be identified (Tabakoff, Bhave et al. 2003). This study identified candidate genes for the complex phenotype of ethanol tolerance. Specifically they indicated the importance of a signal transduction cascade that involved the glutamate receptor δ2 protein, the Ephrin B3 ligand, and the NMDA receptor. These studies confirm that there are multiple variations that may alone or in combination mediate the development of tolerance and addiction to the incoordinating effects of alcohol.

Ethanol studies in *Caenorhabditis elegans*

Natural wild strains, N2 and CB4856 have been shown to have different rates of development of AFT when analyzed for ethanol sensitivity and tolerance. N2 has a slower development of AFT, where CB4856 has a faster development of AFT. N2 and CB4856 are wild isolates that were found in two separate locations and evolved many millions of years apart. This promotes a low level of molecular diversity between the two strains and results in many single nucleotide polymorphisms (SNP) on average, one every 800 base pairs across the genome. The major genetic difference between the two

wild strains that confers ethanol sensitivity and tolerance was mapped to a single gene, *npr-1 (Davies, Bettinger et al. 2004)*. Mutations in *npr-1* cause behavioral changes in response to ethanol and it has been shown that a decrease in NPR-1 signaling occurs during extended ethanol exposure (~20 hours). By analyzing multiple alleles of *npr-1, npr-1* complete loss-of-function mutants developed tolerance more rapidly than weaker alleles. Unchanged internal ethanol concentrations confirmed that a decrease in *npr-1* modulates the increase in ethanol tolerance through a mechanism that does not involve ethanol metabolism. Varying the levels of the gene *npr-1* modifies the rate of development of acute functional tolerance to ethanol.

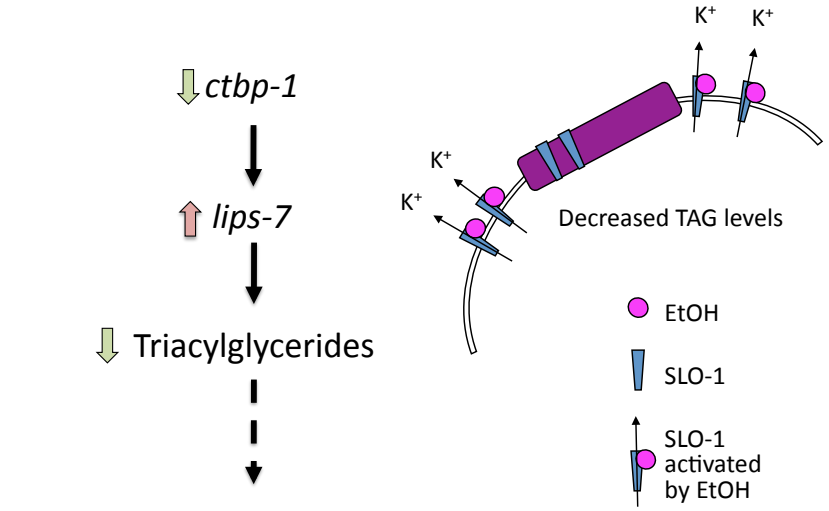
Two alleles of NPR-1 have been identified and are present in many wild isolates. A variation at position 215 in NPR-1 caused changes in social and ethanol behavior. N2 contains the NPR-1 isoform 215V, a solitary feeding strain, while CB4856 has the lower functioning *npr-1* allele isoform, 215F (de Bono and Bargmann 1998). NPR-1 expression was located by using a green fluorescent protein and was expressed in sensory neurons, interneurons, motor neurons, and pharyngeal neurons. Further analysis of neurons AUA, AQR, PQR, and URX, where their activity was suppressed through the activation of a K^+ channel, depicted a decrease in social feeding (Coates and de Bono 2002). These neurons are predicted to play a role in the integration of antagonistic signals that control the choice between social and solitary behavior and ultimately suggesting the importance of these neurons for this behavior. The two *npr-1* alleles, 215V or 215F can be found in many wild strains, but no other isolate has been identified (de Bono and Bargmann 1998).

In *C. elegans*, studies have shown that ethanol tolerance and food-dependent behaviors can be separable (Davies, Bettinger et al. 2004). CB4856, *npr-1*(215F), showed an increased speed on food and an increase in bordering, a preference for thicker parts of the lawn and a low O_2 environment (hyperoxia avoidance) (Chang, Chronis et al. 2006). The ocr-2(ak47) mutation suppressed the clumping and faster speeds on food phenotype of a loss-of-function *npr-1* mutation (de Bono, Tobin et al. 2002). When tested in a *npr-1(lf)* background, the *ocr-2(ak47)* mutation was not able to significantly suppress the *npr-1(lf)* rapid development of AFT phenotype (Davies, Bettinger et al. 2004). In addition, the expression of *npr-1*(+) in the *npr-1* expressing neurons (AQR, PQR, and URX), that is sufficient to rescue the social behavior phenotype of *npr-1(lf),* was unable to rescue the acute locomotor sensitivity phenotypes of *npr-1*. This suggests that there is a distinct difference between the locomotor speed phenotype and the clumping and bordering phenotype. NPR-1 is a regulator of the neuroadaptive process in response to ethanol and other social behaviors. Natural variation between wild-type strains N2 and CB4856 portrayed differences in the development of AFT and has been mapped to a single gene, *npr-1*.

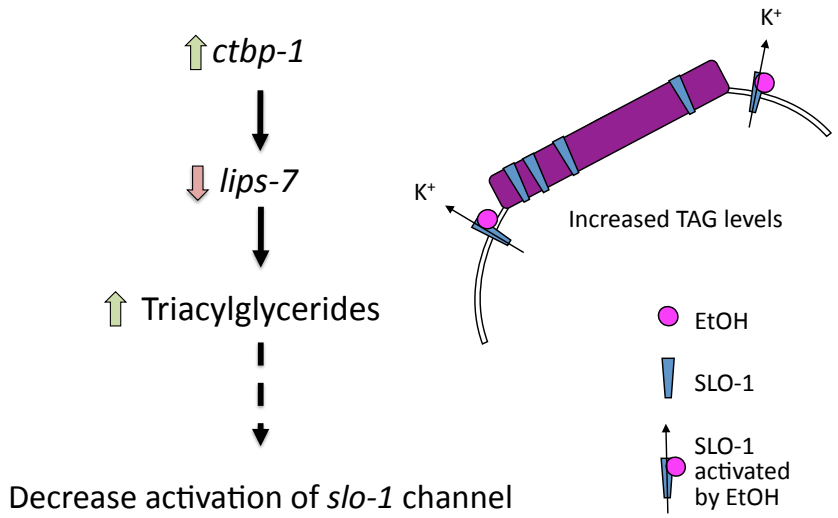
Caenorhabditis elegans as a model organism

Caenorhabditis elegans (*C. elegans*) are an excellent model to study the molecular basis of alcohol tolerance, abuse, and alcoholism. In their natural habitat, they are found in the soil of temperate regions or within rotten fruit and feed on bacteria. Within a laboratory, they are easily and inexpensively cultured and maintained on agar plates and feed off on *Escherichia coli*. Their development and biological aspects are very well characterized and understood. They have a short reproductive cycle, where

they are able to grow from egg to egg-laying adult in 3 days. With a short generation time, a self-fertile hermaphrodite can also produce a brood size of approximately 300 allowing for a large-scale production of animals in a short period of time. Furthermore, *C. elegans* are transparent, which provides a good canvas for detection of *in vivo* fluorescence markers; this aids in the visualization of neurons, processes such as axonal growth, embryogenesis, and fat metabolism. While *C. elegans* are multicellular organisms, hermaphrodites contain only 959 somatic cells and 302 neurons. Also, their entire genome has been fully sequenced, with approximately 20,470 protein-coding genes. Importantly, it has been shown that 40% of the *C. elegans* genome is homologous to humans making them a very attractive model organism to use (Hodgkin 1998). All of these features make *C. elegans* a good tool for genetic manipulation and can be used for early research in understanding the molecular mechanism of many human diseases including alcoholism.



Increase activation of *slo-1* channel
Ethanol Sensitive



Decrease activation of *slo-1* channel
Ethanol Resistance

Jill Bettinger

Figure 1. Mediating effects of triacylglyceride levels on ethanol sensitivity

lips-7, a lipase that regulates the triacylglyceride levels in the lipid bilayer is inversely modulated by *ctbp-1*. It is proposed when triacylglyceride levels are low, ethanol is able to activate more SLO-1 channels, which mediates the effects of sensitivity to ethanol. In contrast, when triacylglyceride levels are high, more SLO-1 channels are immersed within lipid bilayers and fewer channels are activated, thus producing a resistant response to ethanol.

Chapter 2: *bet11* regulates the development of acute functional tolerance

Specific Aim 1: Identification of the gene that is disrupted by the *bet11* mutation; this gene is a regulator of acute functional tolerance.

Rationale: The nematode neuropeptide receptor (NPR-1) antagonizes the development of acute functional tolerance (AFT) by ethanol, however evidence suggests it is not the sole regulator of tolerance. While there has been an abundance of research investigating the behavioral effects of ethanol, the various molecular mechanisms underlying these effects are still not very well understood. Utilizing a forward genetics approach, we probed the entire *C. elegans* genome to screen for genes involved in the development of AFT, by investigating animals that were unable to develop AFT. Specifically, we identified mutations that caused animals to become slow developers of AFT in a genetic background that was a fast developer of AFT.

Materials and Methods:

Nematode maintenance and strains

Nematodes were maintained on nematode growth medium (NGM) agar plates seeded with OP50, *E. coli* food, and kept at 20°C. The strains that were used in this study were: N2, CB4856, *npr-1(ky13)*, *bet8;npr-1(ky13)*, *bet11;npr-1(ky13)*, *bet14;npr-1(ky13)*, *eg626;npr-1(ky13)*, F23C8.6(*ok3325*), F23C8.7(*tm3394*). Strains were

provided by R. Barstead (Gene Knockout Consortium), J. Bettinger (Virginia Commonwealth University, Richmond, VA), S. Mitani (National Bioresource Program and Tokyo Women's Medical College, Tokyo, Japan), and the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN).

Mutagenesis

Mutagenesis was performed on *npr-1(ky13)* animals. The *npr-1(ky13)* strain contains a null mutation at *npr-1* and causes a fast development of AFT phenotype. In order to locate genes that disrupt AFT, we utilized and mutated *npr-1(lf)* animals, that are fast developers of AFT, and screen for slow developers of AFT or animals that have an interrupted development of tolerance. In order to robustly visualize the development of AFT, *npr-1(ky13)* were used as a sensitized background. The *npr-1(ky13)* mutation was used to sensitize the strain to be mutagenized, so that the slow developers of AFT could be identified in the progeny and differentiated from the parental *npr-1(ky13)* fast developers of AFT. *npr-1(ky13)* animals underwent ethyl methanesulfonate (EMS) mutagenesis, which generally induces single base pair mutations or small deletions/insertions to produce varied mutant phenotypes; slow developers of AFT animals were specifically selected from the progeny of the mutagenized animals as follows. *npr-1(ky13)* worms were grown normally on seeded OP50 (NGM) agar plates at 20°C until most animals grew to the L4 stage and then were washed off the plate with M9 (42.3 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 85.6 mM NaCl, 0.1 mM $MgSO_4$) into a plastic 15 mL conical tube. Worms were spun down at maximum speed in a clinical centrifuge for 30 seconds and the supernatant was discarded. The worm pellet was washed with M9, inverted several times, and re-spun. The supernatant was discarded

and the pellet was then resuspended in 2 mL of M9. The 2 mL of worms were treated with 50 mM liquid EMS. The conical tube was placed on a rocker for 4 hours at 20°C. After mutagenesis, worms were washed twice with M9 and then transferred to NGM plates with OP50 for 15 minutes – 2hrs, at which point healthy looking worms were picked and bred for two generations. The F_2 progeny was used to ensure that any induced mutations would be homozygous in a quarter of the animals tested and the animals were screened for ethanol tolerance (see below).

Ethanol administration

Ethanol was administered to the worms through NGM agar plates. Plates were dried at 37°C with lids off and then cold 100% ethanol (200 proof) was added to the appropriate final concentration (weight to volume). Plates were sealed with parafilm and allowed to equilibrate for 2 hours prior to transferring the worms onto the plate. Worms were exposed to ethanol by crawling on top of the agar and through a thin layer of the liquid extruded from the plate

Ethanol chemotaxis assay

Mutagenized animals were subjected to a chemotaxis assay on ethanol in order to separate and isolate animals that displayed a change in the development of AFT. The F_2 progeny of mutagenized *npr-1(ky13)* animals were incubated in 300 mM ethanol (ethanol:M9 solution) for 90 minutes to develop tolerance before being placed at one spot (origin) of a 300 mM ethanol NGM plate. On the opposite side of the plate was a spot of attractant (butanone:EtOH (1:1000) and NaAzide). Worms were allowed to move freely for 90 minutes; animals are attracted to butanone and in these conditions, *npr-*

1(ky13) animals have developed sufficient tolerance to be able to chemotax to the attractant within 90 minutes. After 90 minutes, animals that were not able to chemotax to the attractant spot effectively were animals that we believed were hypersensitive to the effects of ethanol and unable to develop tolerance. These affected animals were individually picked, allowed to produce self-progeny, and the progeny was tested for ethanol sensitivity and the development of tolerance through at least two more trials of ethanol sensitivity experiments to verify the altered response to the effects of ethanol. Strains with a confirmed altered ethanol response phenotype were maintained and the position of the induced mutation was identified using genetic mapping (see below). False positive ethanol sensitive results could be due to environmental factors or injury to the animal through handling; such animals were discarded following subsequent testing.

Ethanol sensitivity and acute functional tolerance assay

Animals were tested for their ethanol response phenotype visually as follows: Animals were age-matched in the L4 stage, and allowed to mature overnight into adults. Assay plates were dried by incubation at 37°C with lids off for 90 minutes the day before the assay. Copper rings, up to four per plate, were melted into the dried agar plates and used as corrals for different strains within a single plate to allow for direct comparison. Ethanol plates were treated with an exogenous dose of 300 mM ethanol. First day adult worms to be assayed were acclimated off of food for 30 minutes on dried plates immediately prior to ethanol exposure. Animals were analyzed visually at time-points during ethanol exposure: 10, 20, 30, 40, and 50 minutes. Speed, body bends, forward progression, and body movements were observed and described.

Creating recombinants for genetic mapping

We performed genetic mapping using a recombination strategy between the mutant strain (in an N2 background) and a genetically polymorphic mapping strain, CB4856. In order to perform genetic mapping, recombinant mutant animals were created. The mutant *bet11*, a slow developer of AFT was identified through the mutagenesis screen and was used to create recombinants in order to identify the location of the mutation responsible for the worms' suppressed ethanol response. Recombinants were created in a background of N2 and CB4856 since both strains are fully sequenced and contain natural variations between them (Figure 2A). They contain approximately one single nucleotide polymorphism (SNP) every 800 bp on average. The *bet11* mutant was generated in the *npr-1(ky13)* strain, which is in the N2 genetic background. The *npr-1(ky13)* mutant animals are fast developers of AFT, whereas the *bet11* mutation causes them to become slow developers of AFT. The CB4856 strain has a low-functioning isoform at *npr-1* and is a fast developer of AFT. Normally, the progeny of *C. elegans* hermaphrodite self-fertilization are almost all hermaphrodites, but we can identify successfully mated animals because they will generate approximately a 50% male progeny and the progeny will be heterozygous animals. *npr-1(ky13)* animals were mated with CB4856 males and we picked heterozygous F_1 animals and allowed them to self-fertilize to produce the F_2 generation (Figure 2B). In the F_2 generation, the animals will have a random assortment of genotypes. F_2 animals were then screened for the mutant *bet11*-like phenotype, which is a slow development of AFT. Animals that were slow developers of AFT were individually picked and allowed to self-fertilize to generate populations. These populations were retested to make certain that the slow

development of AFT bred true. Eighty-four recombinants were picked in order to determine the location of the mutation.

DNA isolation and genetic mapping using SNP markers

DNA from *bet11* recombinants was isolated for DNA by washing worms off highly populated, healthy plates using 1.7 mL ddH_2O and transferring into 1.5 mL Eppendorf tubes. Isolated worms were centrifuged for 1 minute, the supernatant was discarded and the pellet was washed and resuspended with ddH_2O and centrifuged again for 1 minute. The supernatant was discarded leaving 0.1 mL of worm plus water mixture. Tubes were frozen quickly at -80°C to burst open the isolated worms. Frozen worms were then further lysed using 35 uL lysis buffer (200 mM KCl, 40.1 mM Tris, 21.4 mM $MgCl_2$, 0.164 mM Gelatin, 64.7 mM NP-40, 15.5 mM Tween-20) and 0.42 uL proteinase K (0.37 mM) solution per sample. Lysis was performed in a 60°C water bath for 90 minutes and then at 95°C in a heat block for 25 minutes to inactivate the proteinase K. The resulting DNA-containing solution was used in genetic mapping.

We began by looking at polymorphisms at three broadly spaced points (markers) on each of the six chromosomes (18-point mapping) and identifying the genotype in each DNA sample at each specific marker. Identification of N2 or CB4856 DNA was determined using snip-SNP or sequencing SNP (Wicks, Yeh et al. 2001). Snip-SNP markers are SNPs that interrupt a restriction site, causing one genotype to have an endonuclease restriction site that the other genotype lacks. A fragment of DNA that contained the SNP marker was amplified using PCR and digested using the appropriate restriction enzymes to distinguish between the genotypes of DNA from the recombinant

line. In the case that the SNP does not lie within a restriction site, the genotype of the SNP was determined using Sanger sequencing of a fragment spanning the SNP that had been amplified using PCR. When all 18-points of genetic mapping were genotyped, an area of high incidence of N2, which should segregate with the *bet11* mutation, identified the mutation location. Finer genetic mapping was utilized to pinpoint the mutation using additional SNPs.

RNAi induction and ethanol assay

RNAi by feeding is accomplished by feeding worms bacteria that produces a dsRNA, that induces an RNAi response by degrading a specific mRNA. Specific clones of the Ahringer library were used to knockdown specific genes that lay within our mapped interval for the *bet11* mutation. *npr-1(ky13)* animals were used with the expectation that if the gene affected by RNAi is the same gene affected by *bet11* then we would expect to see a reproduction of the *bet11* mutant phenotype, that is, a slow development of AFT. Samples from frozen RNAi cultures were inoculated in LB and 50 μg/μL ampicillin, shaken at 37°C overnight, and stored at 4°C for up to a week. RNAi agar plates were prepared using a NGM agar media with IPTG (to induce production of the double stranded RNA) and ampicillin (for selection to maintain the plasmid) added to it. RNAi liquid cultures (100 μL) were spread onto agar plates and allowed to grow overnight before use. Four to five late L3 or young L4 animals were moved onto a seeded RNAi plate, and allowed to continue to develop into adults. Animals were moved 36-40 hours later to freshly seeded RNAi plates and allowed to lay eggs for 2 hours. Adults were removed and the F_1 progeny that had grown in the presence of the RNAi-containing bacteria were tested for ethanol sensitivity and AFT to 400 mM ethanol.

Transformation by microinjection to rescue phenotype

Yeast artificial chromosomes (YACs) and cosmids were microinjected directly into the distal gonad of the *C. elegans* to transform progeny of the injected animals with random extrachromosomal DNA arrays containing the injected DNA (Evans 2006). Cosmid DNA was purified using an alkaline lysis kit. The *bet11;npr-1(ky13)* animals were injected using a pulled borosilicate glass capillaries with a fine internal glass filament. Animals were submerged in halocarbon oil and then adhered and immobilized on 2% agarose pads. Injections were done using a Zeiss Axiovert inverted DIC microscope and a micromanipulator was used for fine positioning of the needle. The flow of the DNA was regulated by a pressurized injection system. Once the needle was inserted into the distal gonad of the worm, the DNA solution (YAC: 130 ng/μL and H20::gfp: 10 ng/μL; Cosmid: 30 – 45 ng/μL and H20::gfp: 10 ng/μL) was injected so that the solution flows freely in both direction and the gonad begins to swell up. Animals were recovered from immobilization using M9 solution and injected P_0 animals were transferred to seeded NGM plates. F_1 animals were checked for transformation (H20 – pan-neuronal GFP marker); if the marker was present, animals were individually picked as an individual transformed line and F_2 animals carrying the extrachromosomal arrays were tested on 400 mM ethanol for rescue.

Whole genome sequencing

First day starved adult worms were washed from multiple plates with M9 into a 15 mL plastic conical tube in order to obtain at least 500 μL of worms. Worms were pelleted and washed with M9. This was repeated two more times and then flash-frozen

in the -80°C freezer. DNA was isolated using Puregene Core Kit A for isolation from tissue (Qiagen). Isolated DNA samples were sequenced using next generation whole genome sequencing at the Center for the Study of Biological Complexity (VCU) using a single channel.

Results:

***bet11;npr-1(ky13)*, a slow developer of AFT**

npr-1(ky13) animals are fast developers of AFT and were mutagenized in order to create and identify animals with disrupted development of tolerance to ethanol (slow developers of AFT). The F_2 progeny of the mutagenized animals were screened and individually isolated by a chemotaxis assay in the presence of ethanol. The progeny of these slow developers of AFT were analyzed several times by visual ethanol assays and only animals that continued to exhibit reduced AFT to ethanol were kept and individually maintained. Animals that failed to reproduce the slow development of tolerance phenotype could have been previously injured or have been inadvertently picked due to environmental factors. Out of the 2600 mutant genomes screened, 16 animals that had increased ethanol-sensitivity, relative to *npr-1(ky13)*, were isolated. From this screen, *bet11;npr-1(ky13)* was isolated and further investigated as a mutant defective in the development of AFT (Figure 3).

***bet11* genetically mapped to chromosome I**

To locate the gene that mediates ethanol sensitivity in *bet11;npr-1(ky13),* recombinants were created by crossing the mutant to a mapping strain, CB4856. Single nucleotide polymorphisms between *npr-1(ky13)* (N2 genetic background) and CB4856

(low-function *npr-1* allele) were utilized as markers to map the *bet11* ethanol-sensitivity mutation. Initially mapping at three broad points within each of the six chromosomes (18-point mapping), the position of the *bet11* mutation was narrowed to the left arm of chromosome I. After finer SNP mapping, the mutation was narrowed further to an interval between -12.0 and -7.31 *C. elegans* map units (Figure 4). This interval was approximately 900 kb and contained roughly 150 genes.

F23C8 rescued *bet11* through transformation rescue

Different yeast artificial chromosomes (YAC) or cosmids containing wild-type genomic DNA from within the narrowed *bet11* mapping region were used to rescue the mutant's slow development of AFT phenotype to a fast development of AFT. I was able to inject wild-type copies of these genomic DNA constructs into *bet11;npr-1(ky13)* and produce extrachromosomal DNA arrays (Figure 5). Transformed *bet11* animals were assessed to determine if the transformed DNA rescued the phenotypic fast development of AFT. Within the interval, the following YACs and cosmids were successfully transformed into *bet11;npr-1(ky13)*: Y39G10, C55C2, F12B6, F23C8, T16H5. All transformants were subjected to a 400 mM visual ethanol assay. Y39G10AR, C55C2, F12B6, and T16H5 failed to rescue *bet11*'s slow development of AFT. F23C8 was able to partially rescue *bet11* and was further investigated. Genes within the F23C8 cosmid were filtered and narrowed by their position within coding regions. Two mutants, F23C8.6(*ok3325*), which contains a 702 base pair deletion and F23C8.7(*tm3994*), which has a 224 base pair deletion were tested on 400 mM ethanol at 10, 20, 30, 40, and 50 minutes to see if either mutation could phenocopy the *bet11* mutation phenotype. The deletion mutants were visually compared to wild-type N2, *npr-*

1(ky13), and *bet11;npr-1(ky13)* to determine their ethanol response. F23C8.6(*ok3325*) and F23C8.6(*ok3325*);*npr-1(ky13)* showed phenotypes on ethanol similar to N2 and *npr-1(ky13)* respectively, whereas F23C8.7(*tm3994*) showed a slower development of AFT phenotype to ethanol relative to N2, but did not phenocopy *bet11*'s ethanol-sensitivity phenotype. The *tm3994* animals had a unique response to ethanol, where there was little forward movement, but an increase in head and tail movement. The strain F23C8,7(*tm3994*) has yet to be tested in an *npr-1(ky13)* background. Due to F23C8.7(*tm3994*) increased ethanol sensitivity, F23C8.7 is a possible gene candidate for mutant *bet11.* Additional studies will need to be conducted to verify if F23C8.7 is the gene mutated by *bet11*.

Two gene targets identified through whole genome sequencing

We performed whole genome sequencing on *bet11;npr-1(ky13)* and *bet14;npr-1(ky13)* simultaneously. The *bet14;npr-1(ky13)* mutant was isolated as a slow developer of AFT from the same mutagenesis screen as *bet11;npr-1(ky13*), but it has been mapped to a separate location and chromosome. Both mutants were generated in the same *npr-1(ky13)* strain, which allowed for common variations found in both mutants to be attributed to the parent strain *npr-1(ky13)*. By eliminating these common variants, we are left with variants that are unique to *bet11* and *bet14*. The analysis of *bet11* found that there were two coding region mutations within two separate genes in the mapped area of interest (Figure 6). A putative histone methyltransferase found homologous to other *C. elegans* and human genes (Y39G10AR.18) was located at CI:-8.83 and contained a proline to serine missense mutation. The second mutation was located at CI:-12.32 and contained a proline to leucine missense mutation within the gene

Y20F4.4. The Y20F4.4 gene is uncharacterized in the worm, but was found to be homologous to the human gene SRRM2, a serine/arginine repetitive matrix protein 2. The SRRM2 protein is known to show high alternative RNA splicing in neuronal sources and blood of Parkinson's disease patients (Shehadeh, Yu et al. 2010). The gene mutated by *bet14,* which has been mapped to chromosome II has not been further identified.

Discussion

An ethanol sensitive mutant *bet11;npr-1(ky13)* was isolated following EMS mutagenesis of a fast developer of AFT, *npr-1(ky13)*. The gene that mediates the slow development of AFT and ethanol sensitivity in *bet11* was localized by genetic 18-point mapping using SNPs. Mapped to the left arm of chromosome I, *bet11* was further investigated by finer genetic mapping within this area of interest. The interval of interest was narrowed down between map units -12.0 and -7.31 as the potential location of *bet11.* This allowed for the utilization of transformation rescue. Transformation of cosmid F23C8 by microinjection partially rescued the *bet11* phenotype from a slow developer of AFT to a faster developer of AFT. Deletion alleles within the F23C8 cosmid, F23C8.6(*ok3325*) and F23C8.7(*tm3994*) were analyzed by testing them on a visual ethanol assay. If the *bet11* mutation lies within F23C8.6 or F23C8.7, the deletion allele strains in a *npr-1(ky13)* background would phenocopy the *bet11* slow development of AFT. Both F23C8.6(*ok3325*) and F23C8.6(*ok3325*);*npr-1(ky13)* failed to phenocopy *bet11's* slow development of AFT phenotype, nor suppress the *npr-1(ky13)* fast development of AFT, suggesting that *bet11* does not lie within the F23C8.6 gene. F23C8.7(*tm3994)* was sensitive to ethanol, but F23C8.7(*tm3994*);*npr-1(ky13)* has

not been tested for its ethanol response. If *tm3994* is able to suppress *npr-1(ky13)*, the fast developer of AFT phenotype, then F23C8.7 is a potential gene candidate for *bet11.* This coincides with the *bet11* whole genome sequencing results, where no mutations were found within the open reading frame of F23C8 and there was a decrease in the ethanol sensitivity response of F23C8.6(*ok3325*) deletion allele. It is however possible that a F23C8.7 mutation was not detected in the whole genome sequencing results if the mutation is located within the promoter region or contained a deletion that was not detected through the sequencing analysis program. In contrast, another possibility since F23C8 was able to partially rescue *bet11*, that there is an over-expression of a gene with a role in AFT other than the gene affected by the *bet11* mutation. This can be further investigated and clarified by identifying the F23C8.7(*tm3994*);*npr-1(ky13)* ethanol sensitivity. Sequencing identified two functional mutations in Y20F4.4 and Y39G10AR.18 were found within the mapped chromosome I region. These two genes provide us with a promising lead to a potential regulator of AFT to ethanol, where Y39G10AR.18 is a more likely candidate. This is due to its location closer to the center of our genetically mapped area of interest.

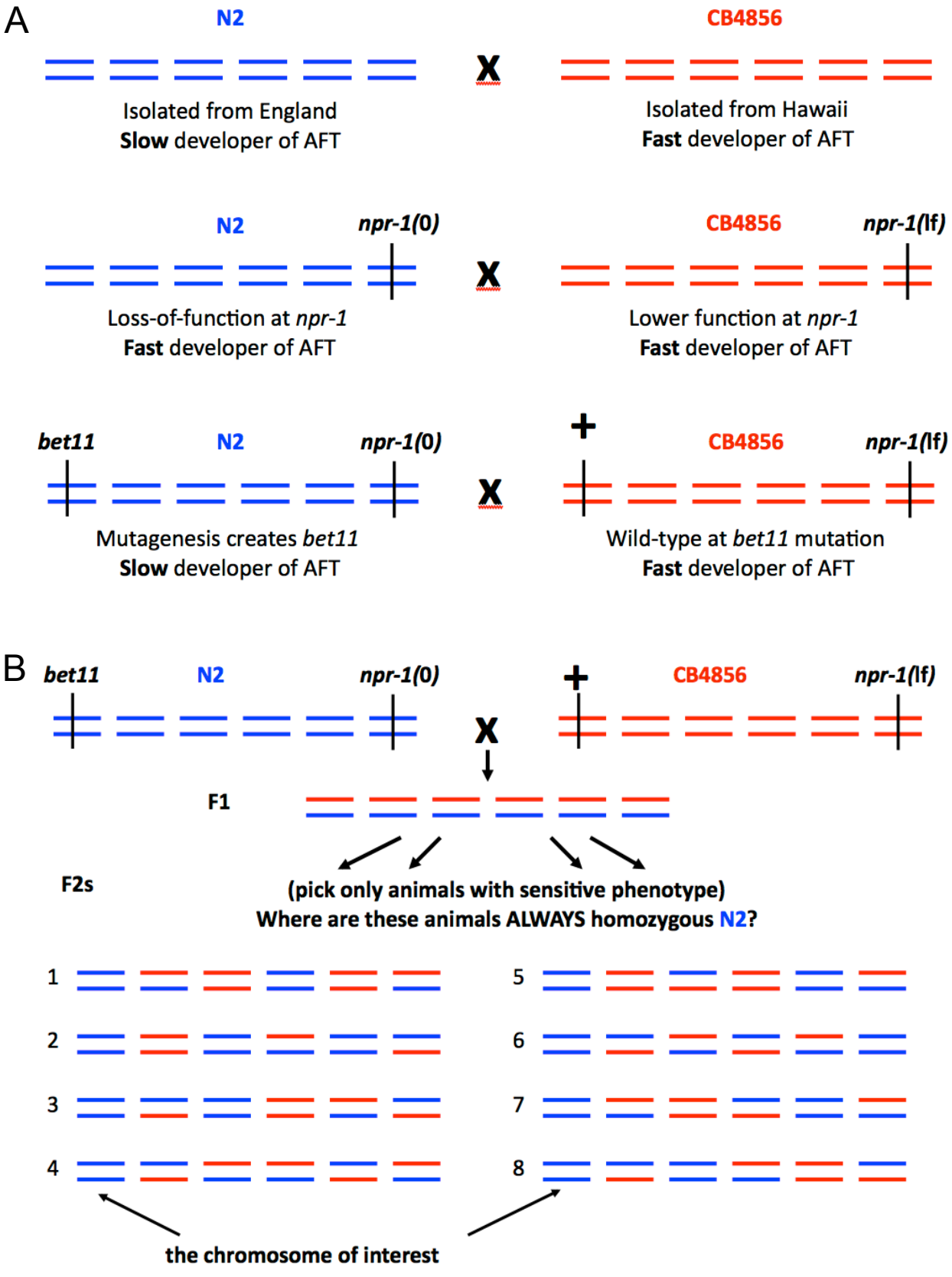


Figure 2. Identifying and genetically mapping *bet11* recombinants

(A) **Basis of genetic mapping.** N2 and CB4856 were crossed in order to create *bet11* recombinant animals in order to locate the *bet11* mutation. N2, a slow developer of AFT, has a null mutation at *npr-1* and becomes a fast developer of AFT. When *npr-1(0)* is mutatgenized creating a mutation at *bet11*, it changes this N2 from a fast developer of AFT to a slow developer of AFT. CB4856 has a low-functioning allele at *npr-1* and is wild-type wherever *bet11* is located. (B) **Creating *bet11* recombinants for genetic mapping.** The mutant *bet11;npr-1(0)*(N2) was crossed to CB4856;*npr-1(lf)* and in the F_2 generation there were a random assortment of genotypes. By isolating only animals that had a similar phenotype as *bet11*, after creating a map, there will be one identifiable location where *bet11* mutation lies.

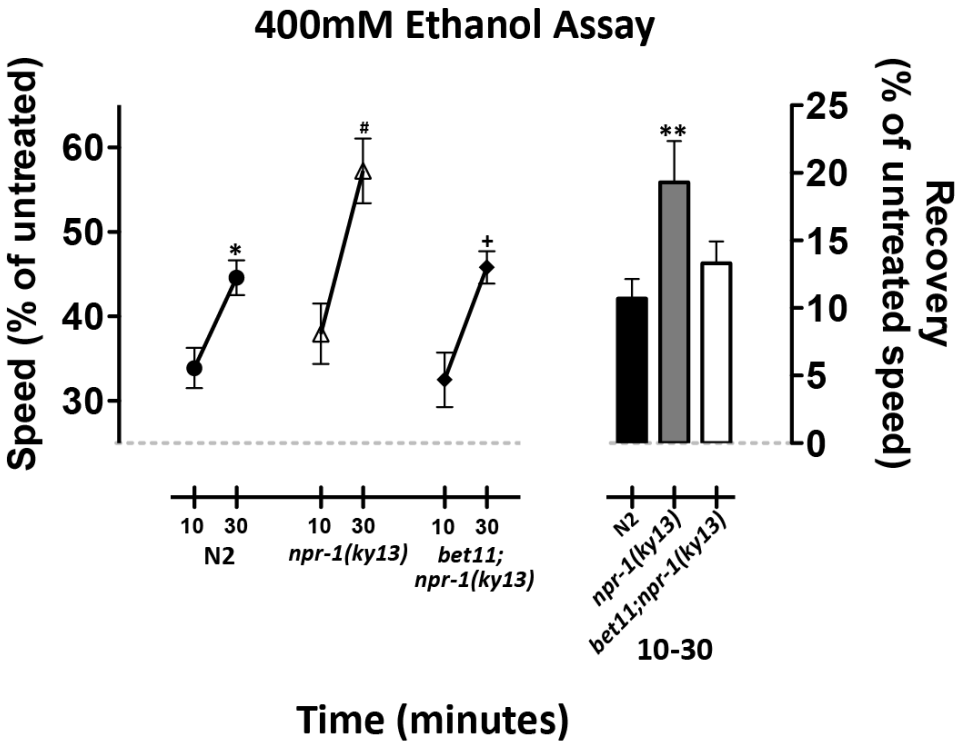


Figure 3. *bet11;npr-1(ky13)* ethanol sensitivity

Mutant *bet11;npr-1(ky13)* is a slower developer of AFT relative to *npr-1(ky13).* N2 has similar ethanol sensitivity to *bet11;npr-1(ky13)* and *npr-1(ky13)* recovers and develops tolerance much faster than N2 by 30 minutes.

One-way ANOVA
p-value<0.05
post-hoc – Bonferroni test
*, #, + relative to 10 minute time point within strain
** relative to N2

	Y48G1C	F58C11	Y71G12A	Y39G10AL	F32B5	Y71F9B.7	W03D8	D1007	Y105E8B
DNA Sample	-20	-19	-12	-9.86	-8	-7.31	-6	-1	26
J 4-3-47		Het	N2	N2	CB	CB	Het	CB	CB
4-3-7	Het	N2	Het	Het	N2		Het	Het	Het
4-3-16	Het	Het	Het	Het	N2	N2	Het	Het	N2
4-3-18	N2	Het	N2	Het	N2	N2	N2	Het	Het
4-3-19	Het	Het	Het	Het	N2	N2	Het	CB	CB
4-3-21	Het	Het	Het	Het	N2		Het	Het	Het
4-3-22	Het	Het	Het	Het	N2	Het	Het	Het	CB
4-3-23	N2	Het	N2	Het	N2	Het	N2	Het	Het
4-3-24	N2	Het		Het	N2	Het	N2	Het	N2
4-3-25	Het	Het	N2	Het	N2	N2	Het	Het	Het
4-3-26	Het	Het	N2	Het	N2	N2	Het	Het	Het
4-3-27	Het	Het	Het	Het	N2	N2	Het	N2	N2
4-3-28	Het	Het	Het	Het	N2	N2	N2	Het	Het
4-3-31	Het	Het	N2	Het	N2	N2	Het	Het	N2
4-3-32	N2	Het	N2	Het	N2	N2		Het	N2
4-3-33	N2	N2	N2	Het	N2	N2	N2	Het	Het
4-3-37	Het	Het	Het	Het	N2	Het	N2	CB	Het
4-3-38	N2	CB	N2	Het	N2	N2	N2	Het	N2
4-3-43		Het	Het	Het	N2	Het	Het	CB	CB
4-3-45	N2	N2	Het	Het	N2	N2	Het	Het	Het
J4-3-1	N2	CB	CB	Het	N2	N2	Het	Het	N2
J4-3-15	N2	Het	N2	Het	N2	N2	Het	Het	N2
J4-3-18		N2	Het	Het	N2	HET	Het	Het	Het
J4-3-25	Het	Het	Het	HET	N2	N2		Het	Het
J4-3-42	CB	CB	N2	Het	N2	N2	Het	Het	Het
J4-3-44	N2	CB	N2	Het	N2	N2	N2	CB	CB
J4-3-28	N2?	Het	Het	N2	N2	Het	N2	Het	N2
J4-3-31		Het	N2	N2		Het	Het	Het	CB
J4-3-40	N2	N2	N2	N2	N2	Het	Het	Het	Het
4-3-8	N2	N2	N2	N2	N2	N2	N2	N2	Het
4-3-13	N2	N2	N2	N2	N2	N2	N2	CB	N2
4-3-14	N2	N2	N2	N2	N2	N2	N2	N2	N2
4-3-15	Het	N2	N2	N2	N2	N2	Het	Het	CB
4-3-17	Het	Het	Het	N2	N2	N2	N2	N2	N2
4-3-20	N2	Het		N2	N2	N2	Het	Het	CB
4-3-30	N2	N2	N2	N2	N2	N2	N2	CB	Het
4-3-34	Het	Het	Het	N2	N2	N2	N2	N2	N2
4-3-35	N2	Het	Het	N2	N2	N2	N2	N2	CB
4-3-36	N2	N2		N2	N2	N2	N2	CB	Het
4-3-39	N2	N2	N2	N2	N2	N2	N2	N2	N2
4-3-40	N2	N2	N2	N2	N2	N2	N2	N2	Het
4-3-41	N2	Het	N2	N2	N2	N2	N2	N2	Het
4-3-42	Het	N2	N2	N2	N2	N2	Het	CB	CB
4-3-46	N2	N2	N2	N2	N2	N2	Het	Het	Het
J4-3-4	Het	CB	Het	N2	N2	N2	Het	N2	N2
J4-3-6	Het	Het	N2	N2	N2	N2	N2	N2	CB
J4-3-8	N2	N2	N2	N2	N2	N2	N2	N2	Het
J4-3-11		Het	N2	N2	N2	N2	N2	Het	Het
J4-3-12	N2	N2		N2	N2	N2	N2	Het	N2
J4-3-13	Het	N2	N2	N2	N2	N2	N2	N2	Het
J4-3-17		N2	N2	N2	N2	N2	N2	N2	N2
J4-3-19	Het	Het	Het	N2	N2	N2	Het	Het	Het
J4-3-20		N2	N2	N2	N2	N2	N2	N2	CB
J4-3-21		Het	Het	N2	N2	N2	N2	N2	N2
J4-3-27		Het	Het	N2	N2	N2	N2	Het	N2
J4-3-29	N2	N2	N2	N2	N2	N2	N2	N2	CB
J4-3-32		N2	N2	N2	N2	N2	N2	Het	Het
J4-3-37	N2	N2	N2	N2	N2	N2	N2	N2	N2
J4-3-41		Het	N2	N2	N2	N2	Het	N2	CB
J4-3-45	N2	N2	N2	N2	N2	N2	N2	N2	N2
J4-3-46	Het	N2	N2	N2	N2	N2	N2	Het	Het
J 4-3-50	N2	N2	N2	N2	N2	N2	N2	N2	CB
J 4-3-51		Het	N2	N2	N2	N2	Het	Het	CB
J 4-3-52	N2	N2	N2	N2	N2	N2	Het	N2	CB
J 4-3-53	Het	N2	N2	N2	N2	N2	Het	N2	N2
J 4-3-56	Het	CB	Het	N2	N2	N2	Het	N2	N2
J 4-3-57	N2	N2	N2	N2	N2	N2	Het	N2	Het
J 4-3-59	Het	Het	Het	N2	N2	N2	Het	N2	N2
J 4-3-62	N2	N2	N2	N2	N2	N2	Het	N2	N2
J 4-3-63	Het	Het	N2	N2	N2	N2	Het	N2	CB
J 4-3-64	N2	Het	N2	N2	N2	N2	N2	Het	HET
J 4-3-65	N2	N2	N2	N2	N2	N2	N2	N2	N2
4-3-9	N2	N2	N2	N2	N2		N2	N2	Het
4-3-49	N2	N2	N2		N2		N2	Het	CB
J4-3-2	Het	Het	N2		N2	N2	N2	N2	N2
4-3-12	Het	N2	N2		N2		N2	N2	N2
4-3-1	N2	N2	N2		N2		N2	CB	CB
4-3-2		N2	N2		N2		N2	N2	CB
J4-3-24		N2	Het		N2	N2	Het	Het	CB
4-3-3	N2	N2	N2	N2	N2		N2	CB	N2
4-3-4		CB	N2	N2	N2		N2	N2	N2
4-3-5	N2	N2	Het		N2		N2	N2	Het
4-3-6	Het	N2	Het		N2		N2	N2	Het
J4-3-35	N2	N2	N2	N2		N2		CB	CB

Figure 4. *bet11* genetic mapping

Using SNP markers for genetic mapping, the *bet11* mutation was mapped to the left arm of chromosome I between map units -12.00 and -7.31. *bet11* mapped to the CI:-8.00 SNP 100%.

Chromosome I – Left Arm
Transformation Rescue DNA Arrays

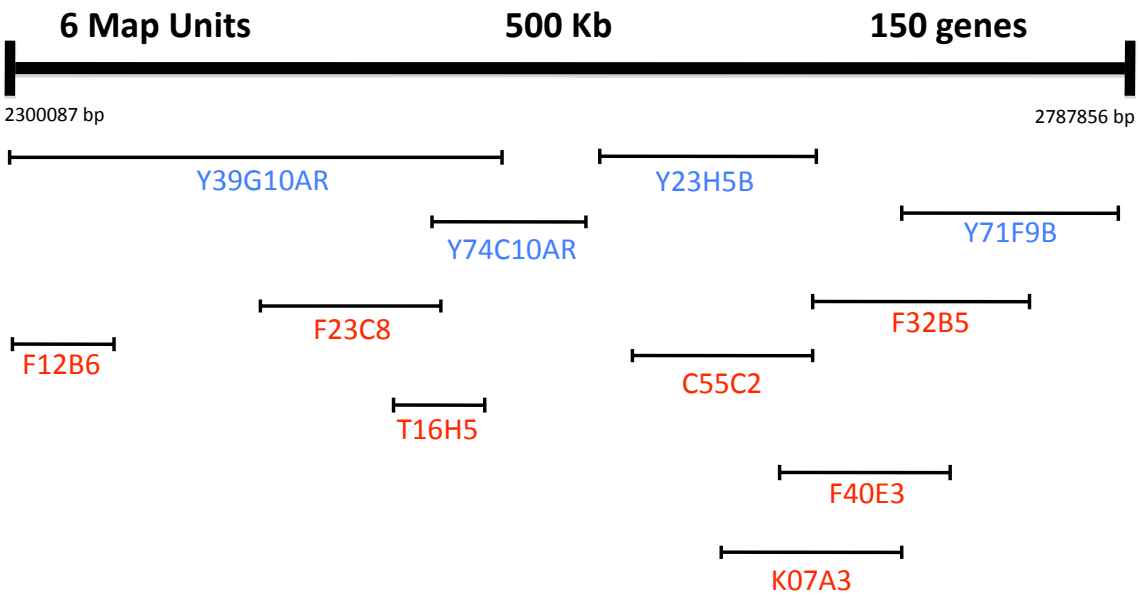
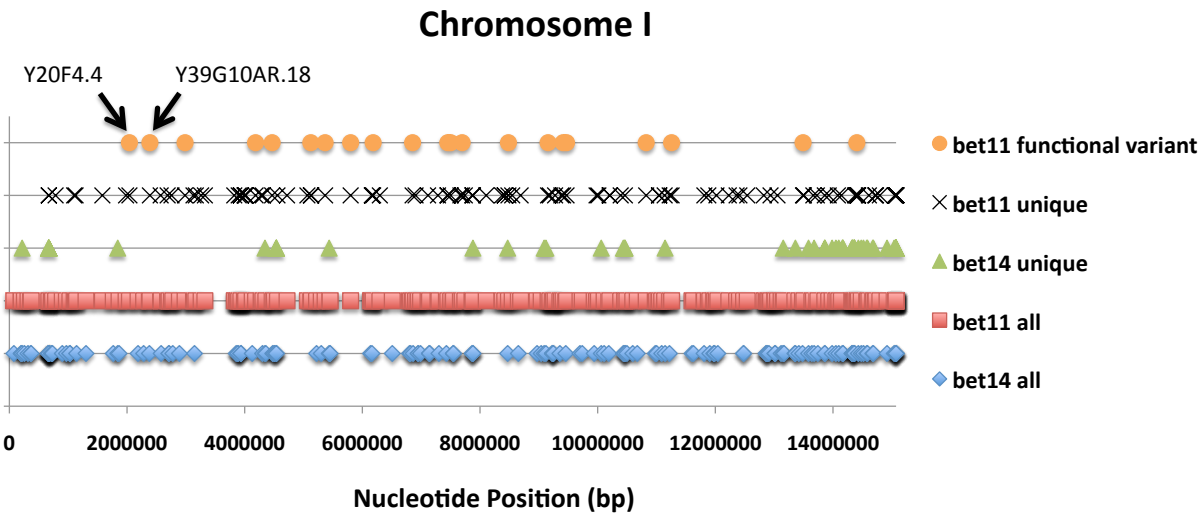


Figure 5. Transformation rescue interval

bet11;npr-1(ky13) animals were microinjected with wild type extrachromosomal DNA of various overlapping YACs and cosmids within the area of interest. Upon successful transformation, animals were analyzed on 400 mM ethanol assay to determine if the transformant was able to rescue *bet11*. Y39G10AR, F12B6, F23C8, T16H5, and C55C2 were transformed into *bet11;npr-1(ky13)* and F23C8 was the only transformed line to be successfully rescued.



(Mia Bolling, Andrew Davies)

Figure 6. *bet11;npr-1(ky13)* whole genome sequencing

Whole genome sequencing was performed on an Illumina platform. *bet11;npr-1(ky13)* and *bet14;npr-1(ky13)* were sequenced simultaneously and mutations that were shared between the two were assumed to be the result of the *npr-1(ky13)* background and mutations were disregarded. Mutations within the genetically mapped area on chromosome I showed two functional polymorphisms, one in Y39G10AR.18 and the other in Y20F4.4.

Chapter 3: Additional contributors to ethanol sensitivity and AFT

Alcoholism is mediated by multiple targets

Alcoholism is a complex disorder and it has become apparent in human and animal genetic studies that there is not a single gene that mediates alcohol-related diseases and alcoholism. The large scale Collaborative Studies on Genetics of Alcoholism (COGA) human genetics study has worked to identify genes that cause vulnerability to alcohol dependence. Advancements by COGA have been useful in identifying and locating some genes associated with alcohol-related problems (Gunzerath, Hewitt et al. 2011). Whole genome linkage studies have identified multiple chromosomal regions that have been linked to alcohol dependence. While there are also several genes that have been consistently shown to play a role in alcohol dependence such as the alcohol metabolizing enzymes ALDH2 and ADH1B, and the neurotransmitter receptor GABRA2, other genes that have been shown to be involved in neural transmission have not been as consistently implicated across studies (Kimura and Higuchi 2011). This is due to the genetic heterogeneity of the human populations within and between different studies. Alcohol has been linked to a number of interactions with neurotransmitters and changes in serotonin, noradrenaline, and dopamine (Nutt 1999). Continuing identification of variants to refine genetic studies by classifying homogeneous phenotypes will allow better identification of alcohol-related

disease associated genes and a better understanding of the interactions between multiple targets.

Previously mapped slow developers of AFT in *C. elegans*

In our laboratory, many mutations that cause slow development of AFT have been isolated and mapped. These mutants were isolated from the same mutagenesis as *bet11* and mutant recombinants were created similarly to how the *bet11* recombinants were made. Mutant animals in a N2;*npr-1(0)* background were crossed with CB4856;*npr-1(lf)* and in the F_1 generation all animals were heterozygous for N2;*npr-1(0)* and CB4856;*npr-1(lf).* In the F_2 generation, animals that had a slow development of AFT were picked. To be a slow developer of AFT, the F_2 animals must be homozygous for the mutation that causes a slow development of AFT. When enough animals have been isolated and genotyped at different marker locations, there should be one location that that contains a high incidence of the mutation's parent genome genotype (N2). This location of the mutation from genetic mapping is hypothesized to only map to one area of interest. However, after mapping multiple, non-allelic mutants, the laboratory noted that in every case, ¾ of the recombinants would map the mutation to one location and the remaining ¼ of the recombinants would map it to an unknown location (Figure 7A). Animals within this ¼ were tested for complementation with the parent mutant and complemented, suggesting that they are not homozygous for the induced mutation and therefore are not defective in AFT due to the induced mutation. These results for genetic mapping were surprising and not very well understood, but the ¾ of recombinants that did generate mapping data to a specific location and allowed the cloning of two non-allelic mutants (Bettinger et al. unpublished). This suggested that the

slow development of these ¼ of recombinants that mapped to another location was slow due to a reason other than the mutation and may have originated from differences in the genetic background.

Synthetic hypersensitive animals were discovered

To better understand what contributes to this unknown slow development of tolerance, the background of the cross was investigated (Figure 7B). N2;*npr-1(0)* and CB4856;*npr-1(lf)* are both fast developers of tolerance and when maintained on their own will always be fast developers of tolerance. A cross was performed, and in the F_1 generation, all animals were heterozygous and in the F_2 generation, a majority ($^{15}/_{16}$) of the animals were fast developers of AFT, but a small subset ($^{1}/_{16}$) were slow developers of AFT. This was intriguing because two parents that are fast developers of AFT were able to generate progeny that were slow developers of AFT. These results correlated with the mutant mapping results, where ¼ of the F_2 progeny had similar tolerance as the mutant and ¼ of those animals genetically mapped to another location, thus generating $^{1}/_{16}$ of the entire F_2 progeny that develops tolerance slower, but its not allelic to the parent mutant (Figure 7A). This allowed us to discover that there is a unique combination of N2 and CB4856 alleles that on their own are fast developers of AFT, but together produce a slow development of AFT phenotype (Figure 8). In order to generate this new phenotype, which we have named the synthetic hypersensitive phenotype, two unlinked homozygous N2 and CB4856 alleles have to come together.

***bet11* produces its own unique genetic mapping pattern**

As described in Chapter 2, *bet11;npr-1(ky13),* a slow developer of AFT was isolated from the genetic background of a fast developer of AFT, *npr-1(ky13)*. *npr-1(ky13)* contains a null mutation on chromosome X in a N2 background and was mutagenized in an EMS screen in order to identify other regulators of AFT besides *npr-1*. To identify the *bet11* mutation, I mapped it by crossing with CB4856;*npr-1(lf).* We made an interesting observation when mapping *bet11*, where instead of the previously observed ¾ mapping to one location and the remaining ¼ map to another unknown location, *bet11* mapped 100% to one particular map location (map unit: -8.00) on chromosome I (Figure 4, Figure 7C). The absence of the remaining ¼ was notable and prompted the following experiments to explain that outcome.

Specific Aim 2: Identify contributing regulators of acute functional tolerance associated with *bet11* though synthetic hypersensitive animals.

Rationale: After isolation of *bet11;npr-1(ky13),* an ethanol sensitive and slow developer of AFT, we propose that the gene affected by the *bet11* mutation may be one of the contributors of the synthetic hypersensitive phenotype. This was suggested by the observation that *bet11* mapped completely to one area of the genome, unlike previously mapped ethanol responsive animals (Figure 4). After crossing two fast developers of AFT, CB4856;*npr-1(ky13)* and N2;*npr-1(ky13)* in a mutagenesis-free analysis, the F_2 generation contained a small portion of animals in the F_2 generation that are slow developers of AFT. We believe that there is a CB4856 allele for one gene and an N2 allele for a different gene, that when each are homozygous together in the same strain,

contribute to an ethanol sensitive phenotype. We believe that the gene mutated by *bet11* described in Chapter 2 identifies the N2 allele described here, and here we utilize a similar approach to identify the CB4856 synthetic hypersensitive allele through forward genetics and genetic mapping.

Materials and Methods

Strains

Nematodes were maintained on nematode growth medium (NGM) agar plates seeded with OP50, *E. coli* food, and kept at 20°C. The strains that were used in this study were: N2, CB4856, CB4856 Recombinant inbred line (RIL) 29, CB4856 RIL30, CB4856 RIL34, CB4856 RIL35, CB4856 RIL36, CB4856 RIL37, *npr-1(ky13)*, *bet11;npr-1(ky13)*. Strains were provided by J. Bettinger (Virginia Commonwealth University, Richmond, VA), J. Kammenga (Univ. of Groningen, The Netherlands) and the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN).

RIL;*npr-1(ky13)* double mutant

CB4856 RILs were crossed with *npr-1(ky13)* males and plated individually. Successfully mated animals were identified, and their progeny were isolated and maintained, and specific RIL and *npr-1(ky13)* SNP markers were used in PCR to verify the generation of the double mutant.

Ethanol sensitivity and acute functional tolerance assay

Animals were tested for ethanol response using the same procedures as described in Chapter 2.

Computer locomotor assay

Assay plates were dried for 90 minutes and L4 animals to assay were picked the day before the assay. On the day of the assay, copper rings were melted into the plate in order to corral different strains of animals within one plate. Cold ethanol (100%) was placed on plates based on their weight to the appropriate final concentration two hours prior to the beginning of the assay and adult animals were acclimated off food for 30 minutes prior to the start of the assay. Each ethanol plate had a parallel control (0 mM ethanol) plate. Movies were made on an Olympus SZX-7 stereo microscope (0.8x magnification and 0.5x objective) using a Retiga 4000R camera (QImaging) and ImagePro Plus (6.2) (MediaCybernetics) software. Ten animals were picked per ring and two-minute recordings (1 frame per second) were captured at 10, 30, and 50 minutes during continuous ethanol exposure. The speed of each worm was tracked using ImagePro plus software and an average speed for each group of ten animals was calculated.

Genetic mapping using SNP markers

DNA from CB4856 RILs was isolated as described in Chapter 2. Identification of specific alleles was determined using snip-SNP markers within chromosome III; SNPs were amplified using PCR and digested using appropriate restriction enzymes in order to differentiate N2 or CB4856 genotype at particular marker.

Results

Homozygous *bet11* is not distinguishable from synthetic hypersensitive animals

The observation of the synthetic hypersensitivity produced by N2;*npr-1(ky13)* and CB4856;*npr-1(ky13)*, in combination with the absence of synthetic hypersensitive animals in the mapping of *bet11*, led us to predict that the fact that we appeared to not be isolating any synthetic hypersensitive animals in the *bet11* mapping could be explained by *bet11* having a distinguishable phenotype. We hypothesized that if *bet11* had a unique or special ethanol sensitive phenotype, that when isolating recombinants, I was able to pick out only true *bet11* recombinants. This would result in a 100% accurate map of the mutation to one locus and select against the synthetic hypersensitive animals. I reanalyzed *bet11*, its recombinants, and the known synthetic hypersensitives identified by mapping other mutants and did not find any noticeable phenotypic difference between all of the animals. This hypothesis therefore was abandoned, since it was not plausible that I could have randomly picked only *bet11* mutant recombinants.

The *bet11* mutation is allelic to the N2 synthetic hypersensitive allele

Since *bet11* was not distinguishable from the synthetic hypersensitives, the most logical explanation would be that the *bet11* mutation affects the same gene as the N2 allele of the synthetic hypersensitive double mutant. I tested this by performing a complementation test between *bet11* and some of the known synthetic hypersensitives generated from the cross of CB4856 and N2 (*npr-1(ky13)*). All synthetic hypersensitive animals failed to complement *bet11,* suggesting that *bet11* and the N2 allele that contributes to synthetic hypersensitivity are located within the same gene (Figure 9). I

further analyzed the known synthetic hypersensitive animals generated during mapping of other AFT mutants by running PCR at the SNP (CI: -8.00) that is tightly linked to the *bet11* mutation (Figure 10). If *bet11* mapped to a different chromosome than the synthetic hypersensitive allele, there would a 50:50 ratio of N2 background or CB4856 background. I found that these animals mapped highly as N2 (95%) to the CI: -8.00 SNP. This suggested that the N2 synthetic hypersensitive allele maps to the same genetic location as the *bet11* mutation. These results strongly support the hypothesis that the *bet11* mutation affects the same gene as one of the genes involved in synthetic hypersensitivity. Therefore, by identifying the mutation that underlies *bet11,* we will simultaneously identify the N2 allele that contributes to synthetic hypersensitivity.

CB4856 synthetic hypersensitive allele is located on chromosome III

While I was able to localize the N2 synthetic hypersensitive allele through *bet11,* to identify the CB4856 contribution our laboratory was fortunate to be able to obtain a subset of CB4856 recombinant inbred lines (RILs) that contain various segments of CB4856 DNA in a N2 background (Figure 11) (Doroszuk, Snoek et al. 2009) The set of 90 lines span all chromosomes and cover 95.91% of the *C. elegans* genome based on chromosomal base pair coverage. The surviving strains (88 out of 90) received were assayed for ethanol sensitivity at 200 mM and 400 mM ethanol (Blackwell, Bettinger et al., unpublished). This enabled us to study the majority of the genome and test certain areas within the genome for genetic variations that affect ethanol sensitivity and tolerance. There was a single distinct group of RILs that portrayed sensitivity to ethanol, where all the other lines had similar or resistant phenotypes relative to N2. This sensitive subset had different fragments and segment lengths of CB4856 DNA, but all

were derived from chromosome III. RIL29, RIL35, and RIL36 were within this subset and each have different levels of ethanol sensitivity and AFT compared to N2 and CB4856 (Figure 12, Figure 13, Figure 14). To verify if these RILs within chromosome III played a role in synthetic hypersensitivity, I put *npr-1(ky13),* a fast developer of AFT into the background of the ethanol sensitive RILs. RIL29;*npr-1(ky13)* and RIL35;*npr-1(ky13)* were visually assayed and video tracked for locomotion to test for ethanol sensitivity at 400mM (Figure 12, Figure 13). *npr-1(ky13)*'s fast development of AFT was suppressed by both RIL29 and RIL35. Conversely, RIL36, which has a CB4856 segment that largely overlaps RIL35, was also assayed on ethanol, but when placed in an *npr-1(ky13)* background, RIL36 did not suppress the *npr-1(ky13)* phenotype (Figure 14). The phenotypes of these strains provided good evidence that the CB4856 allele that contributes to synthetic hypersensitivity lies within the CB4856 segment of chromosome III of the RIL29 and RIL35 sensitive lines.

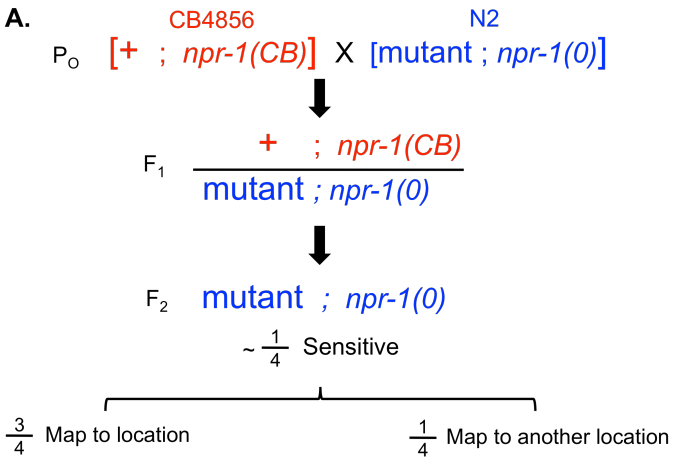
To identify the mutation that causes synthetic hypersensitivity in CB4856, I performed genetic mapping using SNP markers. Ethanol sensitive and resistant RILs were mapped and areas that contained similarities between strains that were able to suppress *npr-1(ky13)*'s fast development of tolerance were identified. Strains that were not able to suppress the fast tolerance were eliminated as the potential location of the CB4856 synthetic hypersensitive allele. Since RIL29 and RIL35 suppressed *npr-1(ky13)*'s tolerance phenotype, the two strains can be used in determining where the allele lies compared with other strains that were not able to suppress the fast development of tolerance phenotype. This elimination method allowed for further

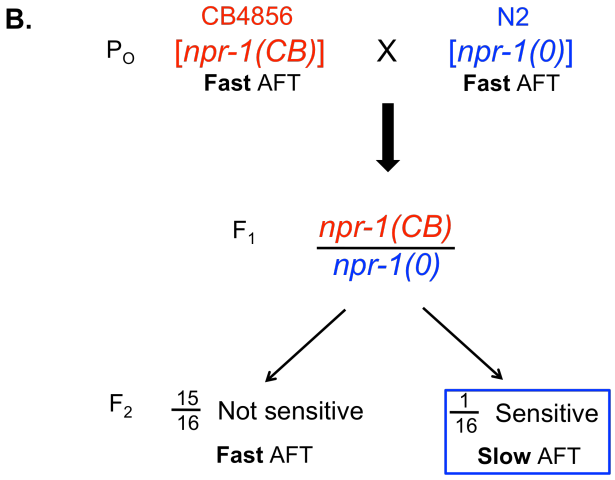
isolation of the CB4856 allele, which has been narrowed down on chromosome III between -16.02 and -12 (Figure 15).

Discussion

Initially, we thought that *bet11* had a unique and distinguishable phenotype that allowed for easy isolation of recombinants and hence produced a 100% mapping data to one locus. After further comparison with synthetic hypersensitive animals and other sensitive mutants, I found that there was no obvious visual sensitive phenotypic difference. This lead us to what we believe is the easiest explanation, that *bet11* contains a mutation in the same gene that has variation that underlies synthetic hypersensitivity and it is the homozygous N2 allele that contributes in combination with the homozygous CB4856 allele to produce ethanol sensitivity. *bet11* failed to complement the synthetic hypersensitive animals suggesting that the variation lies within the same gene. When the synthetic hypersensitive animals were mapped at the *bet11* locus, they mapped highly to the CI: -8.00 locus. The results favor the idea that both the N2 allele and the CB4856 allele are low-functioning alleles that on their own do not produce a detectable sensitive phenotype, but in combination produce an additive and detectable ethanol sensitive phenotype. By identifying *bet11*, it will simultaneously identify the N2 synthetic hypersensitive gene. RILs constructed by Kammenga's laboratory were utilized to identify the CB4856 allele. The RILs contained various size segments of CB4856 DNA within a N2 background. Out of the 88 strains analyzed for ethanol sensitivity, there was only one area that showed increased ethanol sensitivity. The area was located in a large interval on chromosome III. Further investigation within

chromosome III, through genetic mapping of RILs showed that the CB4856 allele lies within chromosome III between map units -16.02 and -12.





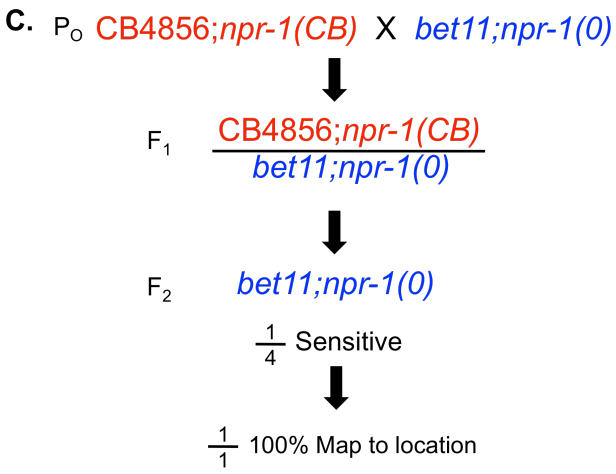


Figure 7. Identifying synthetic hypersensitives

npr-1(0) and CB4856 animals are always fast developers of AFT relative to N2. (A) **Previously mapped mutants.** Through forward genetic mapping, it was identified that when crossing mutagenized animals (in an N2 background) with CB4856, ¼ were ethanol sensitive in the F_2 generation and approximately ¾ would map to one location and approximately ¼ would map to somewhere else in the genome. (B) **Identifying synthetic hypersensitives.** When *npr-1(0)* and CB4856 are mated (without mutagenesis), in the F_2 generation there is mostly animals that are fast developers of AFT, but there is a small portion ($^1/_{16}$) of animals that are slow developers of AFT. (C) ***bet11* mapped 100% to one location.** When *bet11* was genetically mapped, ¼ of the animals in the F_2 progeny were ethanol sensitive and 100% of these sensitive animals identified its mutation to one locus.

$$\frac{\textit{kfg-1}(\text{N2})}{\textit{kfg-1}(\text{N2})} \; ; \; \frac{\textit{kfg-2}(\text{CB4856})}{\textit{kfg-2}(\text{CB4856})} = \text{Synthetic Hypersensitive}$$

(low function)
Fast AFT

(low function)
Fast AFT

Slow AFT

Figure 8. Two unlinked alleles in combination produce a synthetic hypersensitive phenotype

A unique combination of a low-functioning homozygous N2 allele in one gene and a low-functioning homozygous CB4856 allele in a different gene produces synthetic hypersensitivity. The homozygous N2 allele *(kfg-1(N2))*on its own is a fast developer of AFT and the homozygous CB4856 allele *(kfg-2(N2))* on its own is a fast developer of AFT. Two fast developers of AFT animals create a synthetic slow developer of AFT. *kfg* – Kapo's favorite gene/genes of interest

P_0 *bet11;npr-1(ky13)* / *bet11;npr-1(ky13)* X N2(lf);CB4856(lf);*npr-1*(CB4856*) / N2(lf);CB4856(lf);*npr-1(ky13*)*

F_1 *bet11(0)* / N2(lf) ; N2(+) / CB4856(lf) ; *npr-1(ky13)* / *npr-1*(CB4856*)

Slow development of AFT
Failed to complement

Background

kfg-1	Function Level (%)
bet11(0)	0
N2(lf)	50
CB4856(ff)	100

kfg-2	Function Level (%)
N2(ff)	100
CB4856(lf)	50

kfg-1(bet11) < *kfg-1*(N2) < *kfg-1*(CB4856) *kfg-2(bet11)* < *kfg-1*(CB4856) < *kfg-1*(N2)

Genotype				**Phenotype**
kfg-1	Function Level (%)	*kfg-2*	Function Level (%)	
N2(lf) / N2(lf)	50 %	CB4856(lf) / CB4856(lf)	50 %	Slow AFT Not enough function
N2(lf) / N2(lf)	50 %	N2(+) / CB4856(ff)	75 %	Fast AFT Enough function
CB4856(+) / N2(ff)	75 %	CB4856(lf) / CB4856(lf)	50 %	Fast AFT Enough function
bet11(0) / N2(lf)	25 %	N2(+) / CB4856(lf)	75 %	Slow AFT Not enough function Failed to complement
If complemented				
bet11(0) / N2(+) ; N2(+) / N2(lf)	50% ; 75 %	N2(+) / CB4856(lf)	75 %	Fast AFT Not enough function

Figure 9. *bet11* and synthetic hypersensitive animals failed to complement

The mutant *bet11* and the synthetic hypersensitive animals in the F_1 generation produced a slow development of AFT phenotype. In this scenario for ease we hypothesized that high-function has 100% of function, low-function equaled 50% of function, and null has 0% or no function. We identified that the synthetic hypersensitives have two unlinked low-functioning alleles in two separate genes. The genes of interest are depicted here as *kfg* (Ka-Po's favorite gene). The synthetic hypersensitives are shown to have a high-functioning (full function – ff) CB4856 allele and a low-function (lf) N2 allele in *kfg-1.* In *kfg-2,* CB4856 has a low-functioning allele and N2 has a high-functioning allele. Together the alleles in both genes produce a slow development of AFT phenotype. The *bet11* allele alone is able to produce the same slow development of AFT phenotype, even in the absence of the CB4856 allele, suggesting that it is a strong loss-of-function allele. To analyze this, different combinations of alleles with varied levels of function were assessed by phenotype. The mutant *bet11* crossed with a synthetic hypersensitive produced a slow developer of AFT. The combination that produces a slow developer of AFT must contain *bet11(0)* and N2(lf) in the same gene, thus mutant *bet11* and the synthetic hypersensitives failed to complement. If *bet11* and the synthetic hypersensitves were different genes it would produce a fast developer of AFT F_1 animal.

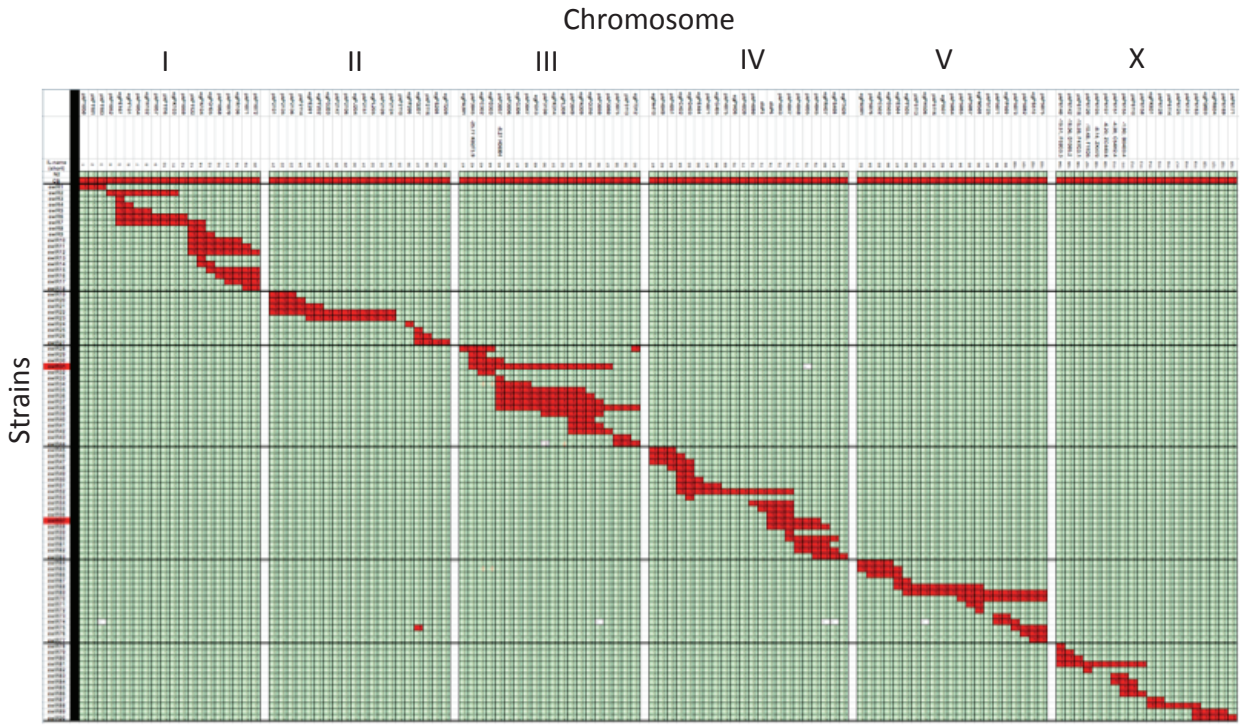
*Could be *ky13* or CB4856 allele

***bet8* Synthetic Hypersensitives**	**CI: -8.00**
4	N2
18	N2
28	N2
49	N2
51	N2
52	N2
55	N2
59	N2

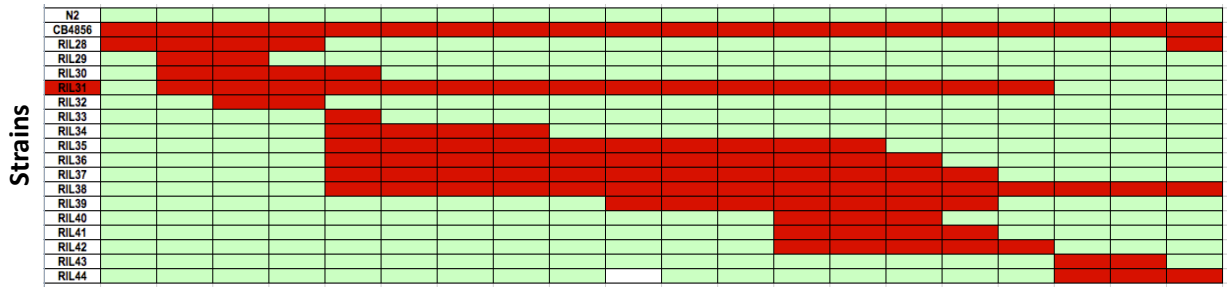
***eg626* Synthetic Hypersensitives**	**CI: -8.00**
6.6.2	N2
8.1.8	N2
10.6.1	N2
9.1.2	N2
8.7.7	N2
10.5.1	N2
10.7.6	N2
10.7.3	CB
115.2	N2
62.1	N2
29.7 (LRC)	N2
53.3 (LRC)	N2
73.3 (LRC)	N2

Figure 10. Synthetic hypersensitive map at CI: -8.00 SNP

Genetically mapping the synthetic hypersensitive animals of other ethanol mutants showed that the N2-ness mapped 95% to the chromosome I -8.00 locus. This suggested that *bet11* mapped directly to the synthetic hypersensitive allele and that *bet11* is a synthetic hypersensitive animal.



Chromosome III



(J. Kammenga, Univ. of Groningen)

Figure 11. Recombinant inbred lines

Ninety recombinant inbred lines were constructed that consist of segments of CB4856 DNA (shown in red) spanned the genome and lies within a N2 background (shown in green). Eighty-eight of the ninety strains were tested for ethanol response and found that throughout the entire genome only strains within a portion of chromosome III showed ethanol sensitivity relative to N2. All other strains showed similar or resistant ethanol responses relative to N2. We specifically analyzed RIL29, RIL35, and RIL36 within chromosome III that portrayed ethanol sensitivity.

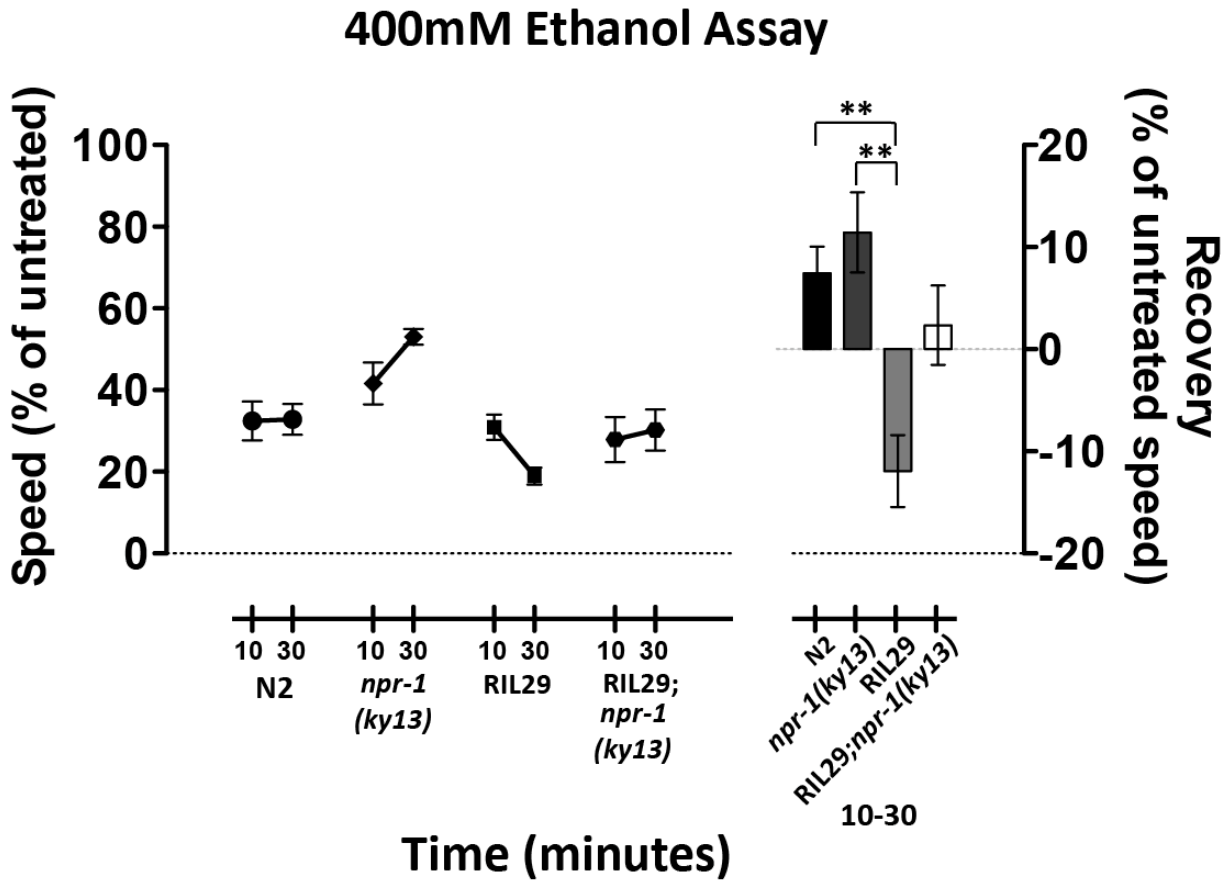


Figure 12. RIL29;*npr-1(ky13)* ethanol response

RIL29 is an ethanol sensitive strain and contains a small portion of CB4856 DNA within a N2 background. It is more sensitive than N2 and does not develop AFT. RIL29;*npr-1(ky13)* suppresses the *npr-1(ky13)* phenotype.

One-way ANOVA
p-value<0.05
post-hoc – Bonferroni test
+ relative to 10 minute time point within strain
** relative to N2

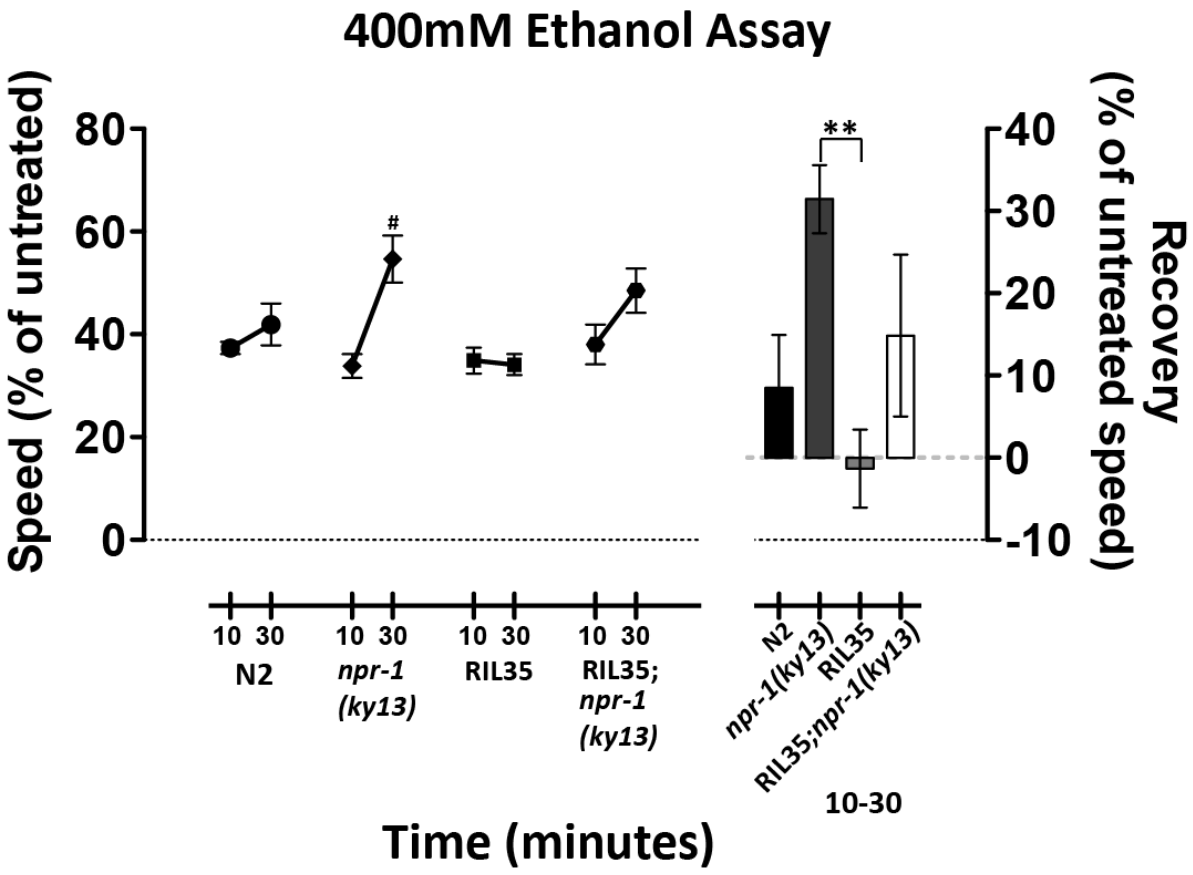


Figure 13. RIL35;*npr-1(ky13)* ethanol response

RIL35 is an ethanol sensitive strain that contains a small portion of CB4856 DNA within a N2 background. It is more sensitive than N2 and does not develop AFT. RIL35;*npr-1(ky13)* suppresses the *npr-1(ky13)* phenotype, as *npr-1(ky13)* is a faster developer of AFT relative to the other strains.

One-way ANOVA
p-value<0.05
post-hoc – Bonferroni test
relative to 10 minute time point within strain
** relative to N2

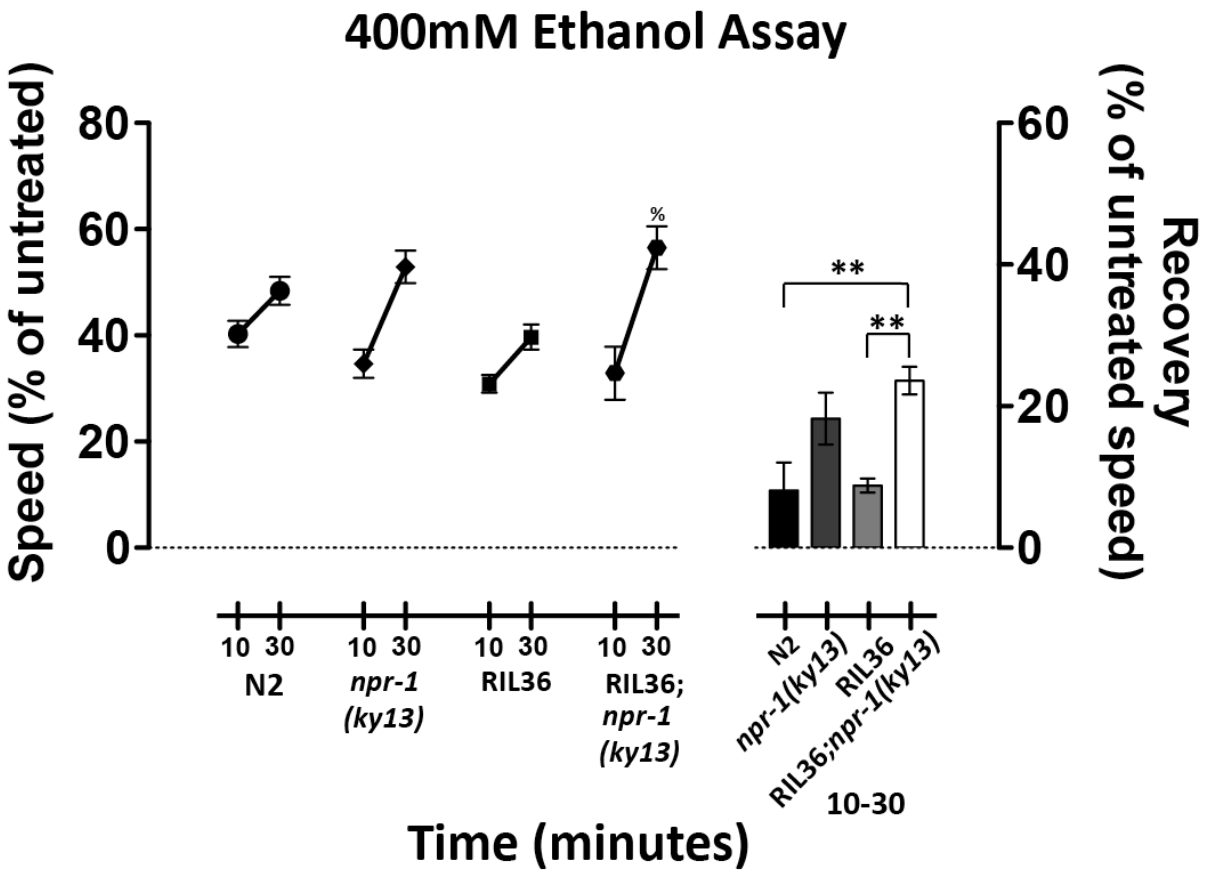


Figure 14. RIL36;*npr-1(ky13)* ethanol response

RIL36 is a line that directly overlaps the CB4856 portion of RIL 35. It is an ethanol sensitive strain and has similar development of AFT as N2. It is slightly more sensitive than N2, but RIL36;*npr-1(ky13)* does not suppress the *npr-1(ky13)* phenotype.

One-way ANOVA
p-value<0.05
post-hoc – Bonferroni test
#, % relative to 10 minute time point within strain
** relative to N2

SNP Location	-25.77	-19	-16.32	-16.02	-15.75	-15.6	-15.52	-15.48	-15.26	-14.93	-14.83	-14.69	-14.06	-12	-8.3	-7	4.13
DNA Sample																	
RIL29	CB	CB	CB	CB	CB	N2	N2	N2	N2	N2	CB	N2	N2	N2	N2		
RIL30	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB		
RIL34		CB	N2	N2	N2	N2		N2	CB	N2	CB	HET	N2	N2		CB	CB
RIL35	N2	N2	N2	N2	CB	N2	N2	N2	N2	N2	N2	HET	N2	CB	CB	CB	CB
RIL36		N2	N2	N2	N2	N2	N2	N2	N2	N2	CB	N2	N2	CB		CB	CB
RIL37		N2	N2	N2	N2	N2	N2	N2	N2	N2	CB	N2	N2	CB		CB	CB

Figure 15. CB4856 synthetic hypersensitive allele genetic map

The RIL's were genetically mapped using SNPs markers. Based on their sensitivity to ethanol, the CB4856 synthetic hypersensitive allele has been narrowed to an interval on chromosome III between -16.02 and -12.00.

Chapter 4: Quantifying lipid levels in respect to ethanol sensitivity

The role of lipids in ethanol sensitivity

Through similar forward genetic screening as described in Chapter 2, we found genes involved in a pathway that putatively regulates triacylglcerides (TAG) through the lipase, LIPS-7 (Figure 1). *ctbp-1*, a transcriptional co-repressor negatively regulates *lips-7*, which in turn modulates the levels of TAG. TAG and lipids have been shown to play a role in mediating the response to ethanol. We know from previous studies that *ctbp-1(eg613)* is a slow developer of AFT and *lips-7(ok3110)* is a fast developer of AFT (Bettinger, Leung et al., unpublished). By better understanding the role of triacylglycerides and how varied levels of lipids can mediate ethanol sensitivity and tolerance, we may be able to identify new mediators and link TAGs to previously identified gene mediators.

Specific Aim 3: Quantifying triacylgylceride levels in varied ethanol responsive animals.

Rationale: Studies have shown that lipids and TAG levels play a role in response to ethanol activation (Treistman and Martin 2009). Lipids levels may modulate the ethanol response through BK and other voltage-gated potassium channels. Understanding the relationship of different levels of TAG of varied ethanol responsive animals will provide a

gateway to understanding the molecular mechanisms of ethanol's action and a possible approach to identifying and characterizing future ethanol sensitive animals.

Materials and Method

Strains

Nematodes were maintained on nematode growth medium (NGM) agar plates seeded with OP50, *E. coli* food, and kept at 20°C. The strains that were used in this study were: N2, *lips-7(ok3110), ctbp-1(eg613).* Strains were provided by J. Bettinger (Virginia Commonwealth University, Richmond, VA) and the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN).

Triacylglyceride level quantification

Triacylglyceride levels were tested on *C. elegans* samples using a colormetric assay, L-Type Triglyceride M by Wako diagnostics (Richmond, VA). Healthy, non-starved populations of synchronized first-day adult worms were washed four times in M9 buffer, then nutated at room temperature with M9 buffer for 30 minutes to allow them to purge any bacteria from their guts. Worms were then washed with ddH_20, pelleted, flash frozen in liquid nitrogen, and stored at -80˚C until further use. Pelleted worms were thawed and ground up using a pestle, and the resulting slurry was dried under vacuum on low speed at room temperature. Worms were dried until the pellet was translucent and powderized. This powder was reconstituted in double ddH_20 and the triaclyglyceride concentrations of the samples were normalized by protein concentration (Bradford Assay). The assay measures the concentration of triacylglycerides in the sample by the absorbance of a blue pigment (Figure 16). A two-

step procedure allows for free glycerol in the sample to not affect the final triacylglyceride level. The free glycerol is decomposed by glycerol kinase (GK), glycerol phosphate oxidase (GPO), and catalase. The triacylglycerides in the sample are hydrolyzed to glycerol and free fatty acids by lipoprotein lipase. Glycerol is then converted to glycerol-3-phosphate by GK and then oxidized by GPO to produce hydrogen peroxide. Hydrogen peroxide causes a quantitative oxidative condensation of N-(3-sulfopropyl)-3-methoxy-5-methylaniline (HHMPS) and 4-aminoantipyrine catalyzed by peroxidase (POD). The assay utilizes the enzymatic method of HHMPS to produce a blue pigment that correlates to the amount of triacylglyceride in the sample. The triacylglyceride concentration can be calculated by dividing the difference in absorbance of triacylglycerides in the sample and free glycerol by a known standards triacylglyceride level and free glycerol and then multiplying it by the known standard concentration (Figure 17).

Results

Preliminary data showed that *lips-7(ok3110),* which is a fast developer of tolerance and is resistant to ethanol relative to N2 has a similar level of triacylglycerides as N2. *ctbp-1(eg613),* a slow developer of AFT and sensitive to ethanol compared to N2 showed a trend towards a lower level of triacylglyceride compared to N2 (Figure 18).

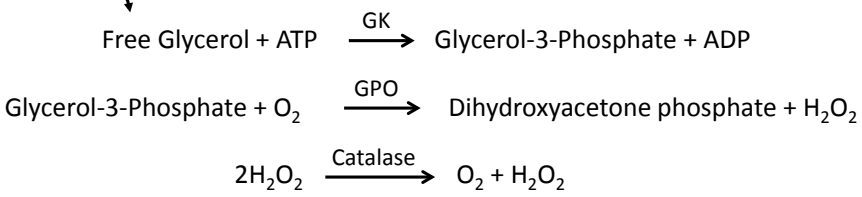
Discussion

Triacylglyceride levels have recently emerged as a likely contributor to ethanol sensitivity and AFT. Our working model proposes that triacylglycerides work in conjunction with other lipids to modulate the lipid membrane composition. By altering

the lipid membrane, our hypothesis is that with an increase in triacylglyceride, there is an increase in the number of BK channels that are sequestered into thicker parts of the membrane. The sequestered BK channels into the lipid microdomains cause a decrease in activation by ethanol and resulted in a resistance response to ethanol (Bettinger, Leung et al., unpublished). *lips-7* has been shown to regulate lipid levels and in response alter the neuronal response to ethanol. After quantifying triacylglyceride levels, it was found that *lips-7(ok3110),* a ethanol resistant strain had similar levels as wild-type N2. On the other hand, *ctbp-1(eg613)*, a sensitive strain had a trend towards lower levels of triacylglycerides relative to N2. These results did not correlate with a previous study identifying a 90% increase in triacylglyceride levels in *lips-7*(C09E8.2) and a 16% decrease in TAG levels in *ctbp-1(ok498)* (Chen, Whetstine et al. 2009). The variation could be attributed to the difference in mutants for *lips-7* and *ctbp-1* that were used to identify TAG levels. RNAi inhibition of the lipase gene, *lips-7*(C09E8.2), resulting in a possibly larger and more pronounce disruption compared to *lips-7(ok3110)* could be responsible for the high TAG levels. We predict that *lips-7(ok3110)* should portray a higher level of TAG and *ctbp-1(eg613)* should have lower levels of TAG compared to N2, which is not seen in our results due to possible limitations in the readout of the mass spectrometer. Having low absorbance outputs could have skewed our results. Another difference is that we quantified TAG levels using a colormetric glycerol based assay, where Chen, Whetstine et al. quantified TAG levels using Nile Red staining. We propose that these limitations and differences can be resolved by measuring the TAG level absorbencies using an apparatus that can measure a larger detectable range and by increasing the sample size. After optimization of quantifying

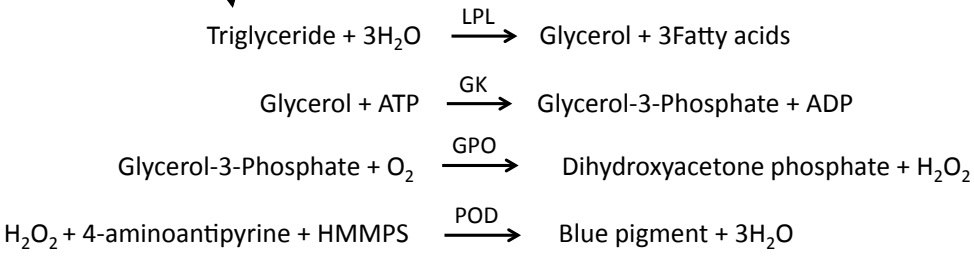
TAG levels, comparing the levels of *npr-1(ky13)* and *bet11;npr-1(ky13*) will add another layer in understanding the effects of ethanol.

Color A



Color A contains glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO), and catalase

Color B



Color B contains lipoprotein lipase (LPL), glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO), 4-aminoanytipyrine, N-(3-sulfopropyl)-3-methoxy-5-methylaniline (HHMPS), and peroxidase (POD)

Figure 16. Triaclyglyceride concentration quantification reaction

Free glycerol in each sample is decomposed in the first step by GK, GPO, and catalase. The triacylglycerides in the sample are hydrolyzed to glycerol and free fatty acids by lipoprotein lipase (LPL). Glycerol is then converted to glycerol-3-phosphate by GK and then oxidized by GPO to produce hydrogen peroxide. Hydrogen peroxide causes a quantitative oxidative condensation of HHMPS and 4-aminoantipyrine catalyzed by POD. HHMPS is utilized to produces a blue pigment that correlates to the amount of triacylglyceride in the sample.

Total Glycerol ↓ Free Glycerol ↓

$$\frac{\text{Sample (Color B)} - \text{Sample (Color A)}}{\text{Standard (Color B)} - \text{Standard (Color A)}} \times \text{Standard Conc.}$$

Figure 17. Triaclyglyceride concentration calculation

Glycerol in the reaction is converted to a blue pigment that correlates to the amount of triacylglyceride in the sample. The concentration of TAG is calculated by the difference in absorbencies of the sample and of the known standard and then multiplied by the known standard concentration.

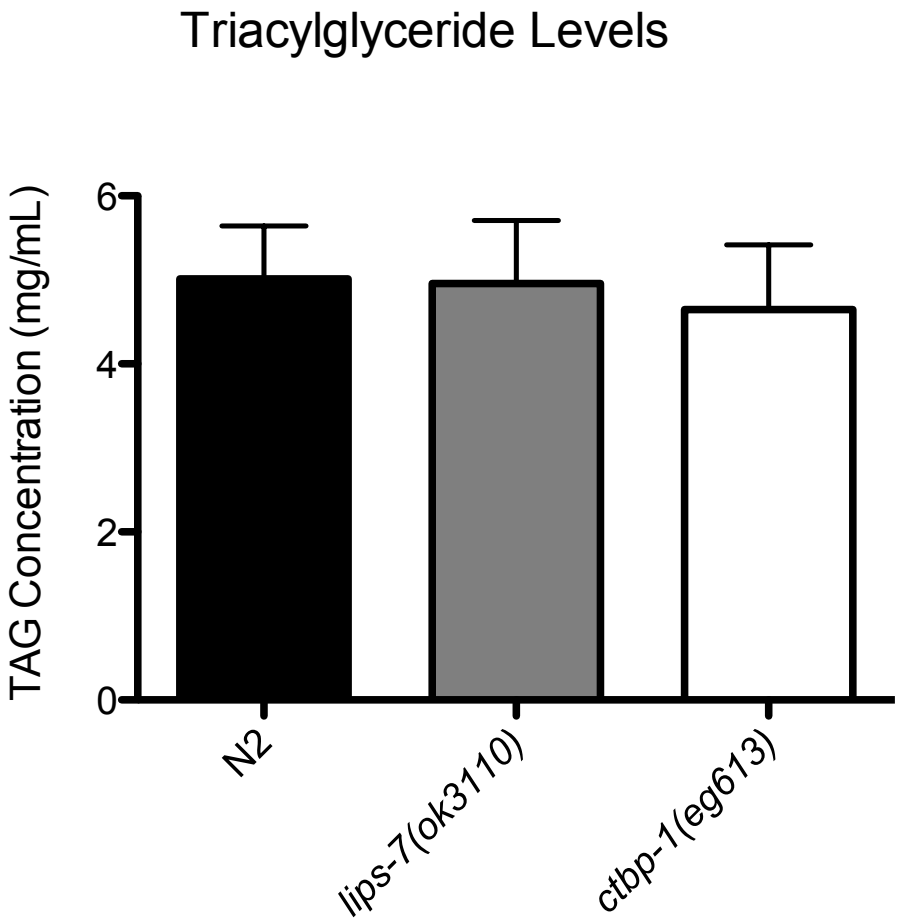


Figure 18. Triacylglyceride levels in ethanol mediated animals

lips-7(ok3110), a fast developer of tolerance and resistant to ethanol relative to N2 has a similar level of triacylglycerides as N2 triacylglyceride levels. *ctbp-1(eg613),* a slow developer of AFT and sensitive to ethanol relative to N2 trends towards a slightly lower level of triacylglyceride compared to N2 levels. There was no significance difference in TAG levels between any of the strains.

One-way ANOVA
p-value<0.05
post-hoc – Bonferroni test

Chapter 5: Conclusions and Future Studies

Alcohol abuse and alcohol dependence are serious disorders and little is known on the molecular mechanism of how ethanol plays a role in the development of these disorders. While there are few and ineffective treatments available, much effort is put forth in hopes to better understand the role of alcohol to effectively treat and prevent these diseases. Using a forward genetic approach, *npr-1(ky13)*, a fast developer of tolerance underwent EMS mutagenesis and mutant *bet11;npr-1(ky13)* was isolated. The mutant *bet11* is a slow developer of AFT and has been narrowed down to a small interval on chromosome I through genetic mapping. Whole genome sequencing identified Y20F4.4 and Y39G10AR.18 as the potential candidates for the gene affected by the *bet11* mutation. Further interrogation of these genes through transformation rescue, RNAi, and GFP promoter localization will uncover an important component to the multi-facetted molecular mechanism of alcohol and the development of alcohol abuse.

bet11 was initially isolated as a mutation that interrupts a gene that is involved in the development of AFT. In the process of identifying *bet11*, an interesting mapping result led us to further investigate the genetic background of the strain used to map *bet11* and found two unlinked alleles that confer synthetic hypersensitivity. Identifying *bet11* simultaneously provides a gateway to identify the *npr-1*(N2) allele of synthetic

hypersensitivity. The contributing synthetic hypersensitive, CB4856 allele has been mapped to an interval on chromosome III. Identifying the paired synthetic hypersensitive alleles and genes that interact in the development of AFT will give new genetic insight on the basis of ethanol response and sensitivity. The wild-type N2 allele will further be identified through *bet11* and the natural variant allele in CB4856 will be further be studied by constructing finer genetic mapping within chromosome III. The RIL's were used as a tool to narrow down an interval and identify the gene that the synthetic hypersensitive CB4856 allele disrupts. Once the CB4856 allele has been narrowed to a smaller interval, transformation rescue and RNAi experiments can be utilized to verify the synthetic hypersensitive allele.

While our laboratory's main focus is identifying protein mediated ethanol response, there has been an increase in interest in understanding how lipid membrane environment may play a role in modulating and interacting with these protein ethanol targets. Previous studies have shown that triacylglyceride levels and lipid membrane thickness may play a role in ethanol sensitivity and tolerance. Quantifying triacylglyceride concentrations of ethanol sensitive and resistant mutants showed that *lips-7(ok3310),* a resistant animal has similar lipid concentrations as N2, where *ctbp-1(eg613)*, an ethanol-sensitive mutant trended towards a lower level of triacylglycerides. Future studies to increase the sample size and to optimize the measurement of triacylglyceride levels due to low absorbance reads will more accurately quantify the differences in lipid levels. Also, identifying triacylglyceride levels of *npr-1(ky13)* and *bet11;npr-1(ky13)* will continue to advance the possible analysis and screens for ethanol sensitivity and tolerance through triacylglyceride level measurements.

Alcohol abuse and dependence are serious and common disorders that reflect the severe addictive properties and easy access of the drug. With the detrimental effects associated with alcoholism along with the rising cost of health care, it is imperative to understand the underlying effects of alcohol. The difficulty arises due to the fact that ethanol is a simple and highly diffusible molecule, thus making it difficult to isolate and identify specific targets and interactions. This has hindered the development of effective treatments. Advancements in the area of alcohol research will help promote the understanding of how ethanol interacts pharmacodynamically and pharmacokinetically. The identification of these ethanol mediators will provide greater insight on ethanol's mechanism of action and help promote the development of effective alcohol-related disease treatments.

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Vita

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Conferences

Poster Presentation. Identification of Genes that Mediate Ethanol-Induced Acute Functional Tolerance in *C. elegans*. **Ka-Po Leung**, Mia Bolling, Gina Blackwell, Jennifer Gardner, Andrew Davies, Jill Bettinger. 18th International *C. elegans* meeting, 2011, UCLA, Los Angeles, CA.

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Jill Bettinger, **Kapo Leung**, Mia Bolling, Andrew Goldsmith, and Andrew Davies (2011). Lipids modulate the development of acute tolerance to ethanol in Caenorhabditis elegans. *In review*.