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Identification and Characterization of Ethanol Responsive Genes in Acute Ethanol Behaviors in Caenorhabditis elegans

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Identification and Characterization of Ethanol Responsive Genes in Acute Ethanol Behaviors in *Caenorhabditis elegans*

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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Table of Contents

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Chapter 4: Chung Chan and Dr. Ian Martin performed the behavioral analysis of *exc-4* and *exl-1*. Gina Blackwell performed the behavioral analysis of *unc-68*.

List of Tables

List of Figures

List of Abbreviations

AA	Alcoholics Anonymous
ADH	Alcohol Dehydrogenase
AFT	Acute Functional Tolerance
ALDH	Aldehyde Dehydrogenase
aRNA	Amplified Ribonucleic Acid
ANOVA	Analysis of Variance
AUD	Alcohol Use Disorder
BAC	Blood Alcohol Concentration
CNS	Central Nervous System
COGA	Collaborative Study on the Genetics of Alcoholism
cDNA	Complementary Deoxyribonucleic Acid
Ct	Cycle Threshold
cAMP	Cyclic Adenosine Monophosphate
CRE	cAMP Response Element
CREB	cAMP Response Element Binding
DAVID	Database for Annotation Visualization and Integration Discovery
DSS	Dent's Saline Solution
DNA	Deoxyribonucleic Acid
GABA	γ-aminobutric Acid
GC	Gas Chromatography
GWAS	Genome Wide Association Study
ID	Identification Extensions

IMS	Imaging Mass Spectrometry
LR	Level of Response
LORR	Loss of Righting Reflex
mRNA	Messenger Ribonucleic Acid
MAS5	Microarray Suite Software Version 5
NGM	Nematode Growth Media
NMDA	*N*-methly-D-aspartate
NAC	Nucleus Accumbens
PCR	Polymerase Chain Reaction
PFC	Prefrontal Cotex
PKA	Protein Kinase A
PKC	Protein Kinase C
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
RMA	Robust Multichip Analysis
RNA	Ribonucleic Acid
SAM	Statistical Analysis of Microarrays
TAG	Triacylglycerol
TMeV	TIGR MultiExperiment Viewer
VTA	Ventral Tegmental Area
YLD	Years Lost to Disability

Abstract

IDENTIFICATION AND CHARACTERIZATON OF ETHANOL RESPONSIVE GENES IN ACUTE ETHANOL BEHAVIORS IN CAENORHABDITIS ELEGANS

By: Joseph Thomas Alaimo

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Directed by: Jill C. Bettinger, Ph.D.

Associate Professor, Department of Pharmacology and Toxicology

Alcohol abuse and dependence are complex disorders that are influenced by many genetic and environmental factors. Acute behavioral responses to ethanol have predictive value for determining an individual's long-term susceptibility to alcohol abuse and dependence. These behavioral responses are strongly influenced by genetics. Here, we have explored the role of genetic influences on acute behavioral responses to ethanol using the nematode worm, *Caenorhabditis elegans*. First, we explored the role

of ethanol metabolism in acute behavior responses to ethanol. Natural variation in human ethanol metabolism machinery is one of the most reported and reproducible associations found to alter drinking behavior. Ethanol metabolism is conserved across phyla and alteration in this pathway alters acute behavioral responses to ethanol in humans, mice, rats, and flies. We have extended these findings to the worm and have shown that loss of either alcohol dehydrogenase or aldehyde dehydrogenase results in an increase in sensitivity to the acute effects of ethanol.

Second, we explored the influence of differences in basal and ethanol-induced gene expression in ethanol responsive behaviors. We identified a set of candidate genes using the basal gene expression differences in *npr-1(ky13)* mutant animals to enrich for genes involved in AFT. This analysis revealed ethanol changes to the expression of genes involved in a variety of biological processes including lipid metabolism. We focused on a gene involved in the metabolism of fatty acids, *acs-2*. *acs-2* encodes an acyl-CoA synthetase that activates fatty acids for mitochondrial β-oxidation. Animals carrying mutant *acs-2* have significantly reduced AFT and we explored the role of genes in the mitochondria β-oxidation pathway for alterations in ethanol responsive behaviors. We have shown that knockdown of *ech-6*, an enoyl-CoA hydratase, enhances the development of AFT. This work has uncovered a role for fatty acid utilization pathways in acute ethanol responses and we suggest that natural variation in these pathways in humans may impact the acute alcohol responses to alcohol that in turn influence susceptibility to alcohol abuse and dependence.

Chapter 1

Background and Significance

Alcohol Use, Abuse, and Dependence - Epidemiology

Alcohol abuse and alcohol dependence are commonly referred to as alcohol use disorders (AUDs), and are serious, pervasive, and debilitating disorders. Alcohol misuse is responsible for 2.5 million or 3.8% of total deaths each year worldwide and individuals suffering from an AUD live 10 to 15 years shorter than those who do not have an AUD (WHO, 2010; Schuckit, 2009). It is estimated that as many as 80% of men and 60% of women in developed countries drink at some time during their lives (Tesson *et al.,* 2006) and that AUD are more prevalent in men than in women (Tesson *et al.,* 2006; Hasin *et al.,* 2007; Mertens *et al.,* 2005; Schuckit, 2009). In the United States, 8.5% or 17 million people either abuse or are dependent on the drug, where as 76 million are suffering from an AUD globally (Grant *et al.*, 2004).

Alcohol use is a leading contributor to the global burden of disease. The degree of disease burden is measured as years lost to disability (YLD) and AUDs rank 4th amongst low and middle-income countries and 3rd for high-income countries for YLD (WHO, 2008) The high number of YLD due to alcohol use stems from a variety of different disease and injury conditions including infectious disease, cancer, diabetes, cardiovascular disease, and unintentional and intentional injuries (Rehm *et al.*, 2011)

Impact of Alcohol Use Disorders in Costs to Society

The costs associated with AUDs are astronomical, accounting for 1- 3% of the gross domestic product in high-income countries (WHO, 2010; Rehm *et al.*, 2009). In 2006, AUDs cost the United States $223.5 billion and 40% of these costs were paid by the government (Bouchery *et al.*, 2011). This cost far out paced the excise tax revenue generated from the sale of alcoholic beverages. The Federal Government received about $8.9 billion from alcohol excise taxes, while State level governments collected an additional $5.1 billion (Xu and Chaloupka, 2011). The majority of the economic cost was due to lost productivity, increases in health care costs, and increases in criminal justice costs. Lost productivity accounted for a total of $161.3 billion. Impaired work productivity was the top cost and a national survey has found that 19.2 million workers, or 15% of the work force, drink enough alcohol to lead to workplace impairment. The top two behaviors that contribute to this impairment are employees working with a hangover (11.6 million) and drinking during the workday (8.9 million) (Frone, 2006). Health care costs accounted for $24.6 billion and the majority of the cost was for specialty care for abuse and dependence. Criminal justice costs accounted for $20.9 billion and the majority of costs came from increases in police protection. The government covered 99% of the increase in costs for health care and criminal justice (Bouchery *et al.*, 2011).

There is also a range of social distress caused by alcohol use. When one individual in a marriage suffers from an alcohol use disorder, the decision to divorce or separate is about 10% higher than couples where an individual does not suffer from an alcohol use disorder (Ostermann *et al.,* 2005). The major contributor to divorces rates is the lack of participation of the alcoholic spouse in everyday household tasks and the

financial stress they place on the relationship (Zewben 1986; Leonard and Roberts, 1998; Lichter *et al.,* 1992). In addition, alcohol use is correlated with child abuse (Miller *et al.,* 1993). Studies have shown that parental alcohol abuse is associated with physical abuse of a child (Miller *et al.,* 1997; Vogeltanz *et al.,* 1999). Also, children who have been sexually abused were more likely to have a family history of alcoholism and a majority of the victims were abused by another family member or by a stranger who was intoxicated (Miller *et al.,* 1997).

Neuropharmacology of Ethanol

Acute Ethanol exposure results in a wide range of neurobehavioral effects. At high doses ethanol produces sedative effects and at low doses produces stimulating effects (Pohorecky 1977; Holdenstock and de Wit 1998). Over the decades, many studies have been completed to determine the neuropharmacological mechanisms of action of ethanol. These studies have implicated from *in vitro* and *in vivo* work that several neurotransmitter systems, including the GABAergic system, glutamatergic system, opioid system, the mesocorticolimbic dopaminergic system, and many intracellular signaling pathways are modulated by ethanol.

GABA is an inhibitory amino acid that acts to decrease a neurons action potential and therefore inhibits neurotransmission. Acute ethanol primarily affects the GABA system by activating $GABA_A$ receptors. $GABA_A$ receptors are heteropentameric ligand-gated ion channels that are composed of 2 α(1-6), 2 β(1-3) and either a γ (1-3), δ, ε, θ, π, or ρ(1–3) subunit (Olsen and Sieghart 2008; Sieghart and Sperk 2002). $GABA_A$ receptors can exist as either synaptic or extrasynaptic receptors. When GABA binds to

$GABA_A$ receptors, the conformation of the channel changes and allows chloride ions to pass down an electrochemical gradient. The influx of chloride ions hyperpolarizes the membrane leading to neuronal inhibition. (Kumar *et al.,* 2009). The synaptic $GABA_A$ receptor α1 subunit is the most abundant α subunit in the brain and is expressed thought out most brain regions. In addition, 50% of all synaptic $GABA_A$ receptors contain the α1 subunit (Kralic *et al.,* 2002). The α4 subunit predominantly occurs in extrasynaptic $GABA_A$ receptors (Olsen and Sieghart 2009).

Genetically modified mouse models have contributed greatly to uncovering the role of $GABA_A$ receptors in acute behavioral responses to ethanol. In particular, many studies have focused on synaptic $GABA_A$ receptors subunit composition and their effects on ethanol sensitivity at high and low doses of ethanol (Wallner *et al.,* 2003). Knockout of the α1 subunit of $GABA_A$ receptors results in an increase in ethanol's stimulating effects (Blendov et al., 2003; Karlic *et al.,* 2003) However, knockout of the α1 subunit of $GABA_A$ receptors in male mice displayed a reduced loss of right reflex (LORR) duration to a sedative dose of ethanol (Blendov *et al.,* 2003), but this result has not been replicated in other studies (Karlic *et al.,* 2003). Knockout of the α2 subunit of $GABA_A$ receptors in mice also display a reduced LORR to a sedative dose of ethanol (Boehm *et al.,* 2004). Collectively these studies suggest that synapses containing α1 or α1 subunits are important for specific ethanol-induced behavioral effects.

In addition, work has implicated the role of specific extrasynaptic $GABA_A$ receptor subunits in the effects of ethanol. Several studies have shown that α4 containing extrasynaptic $GABA_A$ receptors have an enhanced inhibition to low dose ethanol, but γ2 containing extrasynaptic $GABA_A$ receptors are less sensitive to low dose ethanol

(Wallner *et al.,* 2003; Wei *et al.,* 2004). α4 and δ containing extrasynaptic $GABA_A$ receptors have been reported to be inhibited at a high dose of ethanol (Liang *et al.,* 2008). Finally, knockout mice of the β2 subunit of extrasynaptic $GABA_A$ receptors are less sensitive to the sedative effects of ethanol measured by LORR (Blendov *et al.,* 2003). Together these studies indicate a role for extrasynpatic $GABA_A$ receptors as a molecular target for ethanol and particular subtypes are involved in ethanol-related behaviors.

The expression of $GABA_A$ receptor subunits is regulated by ethanol. Studies have shown that the $GABA_A$ receptor α1 subunit expression is decreased following chronic ethanol exposure (Devaude *et al.,* 1997), while acute ethanol causes increases in expression of $GABA_A$ receptor α1 subunits (Kumar *et al.,* 2012; Liang *et al.,* 2007). This suggests that different doses of ethanol change the expression of different receptor subtypes of $GABA_A$ and further suggests that ethanol induced changes in expression can lead to genes that are involved in ethanol's mechanism of action.

Glutamate receptors are ligand-gated ion channels that are widely expressed throughout the brain. There are two types of glutamate receptors; ionotropic and metabotropic. Ionotropic glutamate receptors excite neurons during fast synaptic transmission and modulate the strength of neurotransmission. Metabotropic glutamate receptors have several effects including reducing synaptic transmission (Ozama *et al.,* 1998). *N*-methyl-d-aspartate (NMDA) receptors are heterotetrameric receptors that contain two obligatory GluN1 subunits and any combination of the following: GluN2A-D and GluN3A-B (Collingridge *et al.,* 2009; Traynelis *et al.*, 2010). NMDA receptors contain a Mg^{2+} block within the ion channel and the Mg^{2+} block is removed by

depolarization of the neuron. Removal of the block allows the channel to pass Ca^{2+} ions and Ca^{2+} influx serves as an indicator of the strength of synaptic transmission (Möykkynen and Korpi 2012).

The effects of ethanol on ionotropic glutamate receptors, especially NMDA receptors have been widely studied. Studies have shown that acute ethanol inhibits NMDA receptors in a dose dependent manner over a range of 5-50 mM (Lovinger *et al.,* 1989). A study using brain slices of the hippocampus of adult rats with intact NMDA receptors observed potent inhibition at 25mM ethanol (Lovinger *et al.,* 1990). In addition, the subunit composition of NMDA receptors respond differently to acute ethanol. GluN2A-B subunits are more sensitive to ethanol inhibition than GluN2C-D subunits (Kuner *et al.,* 1993). This suggests that like $GABA_A$ receptors particular NMDA subunits are important in ethanol effects.

There is evidence that intracellular calcium plays a role in ethanol inhibition of NMDA receptors. The obligatory GluN1 subunit at the C-terminal tail contains a C0 domain that binds calmodulin and α-actinin-2. Both of these proteins compete to bind to the C0 domain of the GluN1 subunit and elevated calcium levels results is α-actinin-2 displacement from the C0 domain and allows calmodulin to bind (Krupp *et al.,* 1999). Studies have shown that ethanol inhibition is stronger if calcium and calmodulin dependent inactivation of NMDA receptors occurs (Anders *et al.,* 2000). Other studies have tried to identify the binding site of ethanol within NMDA receptors by inducing point mutations that alter the ethanol sensitivity of the receptor. Point mutations Met813Ala and Leu819Ala in transmembrane region 4 of the GluN1 subunit enhances ethanol

inhibition, where as point mutation Phe639Ala in transmembrane region 3 reduces ethanol sensitivity (Smothers *et al.,* 2006; Ren *et al.,* 2003).

Genetically modified mice have contributed greatly to understanding ethanol's effects on NMDA receptors. Acute ethanol administration has shown to cause an enhancement of NMDA receptor function by phosphorylation of the GluN2B subunit of the receptor. Mice lacking a functional tyrosine kinase, Fyn, are more sensitive to the depressive effects of ethanol. These animals displayed enhanced sensitivity in a dose dependent manner using the LORR assay (Miyakawa *et al*., 1997). Pharmacological inhibition of NMDA receptor glutamate biding sites in mice and rats results in an enhanced sensitivity to ethanol measured by LORR assay (Yaka *et al.,* 2003; Ramirez *et al.,* 2011). In addition, studies have that shown NMDA antagonists reduced the development of acute functional tolerance (AFT) (Khanna *et al.,* 2002). Taken together these results suggest that ethanol directly affects NMDA receptors by inhibiting their function and the inhibition is occurring in a subunit specific manner, which can alter ethanol-related behaviors.

The midbrain dopamine system has been widely studied for its role in reinforcement (Olds and Milner 1954, Schultz 2007; Wise 2004). The mesocorticolimbic dopaminergic pathway consists of three major brain regions: the ventral tegmental area (VTA), nucleus accumbens (NAC), and prefrontal cortex (PFC). Midbrain dopamine neurons involved in the initiation of reinforcement processes originate in the VTA and project to structures closely associated with the limbic system, most prominently the NAC shell region and the PFC. Acute ethanol administration stimulates dopamine release from the NAC shell region (Di Chiara and Imperato 1988; Imperato and Di

Chiara 1986, Pontieri *et al.,* 1995) and studies have shown that acute low dose ethanol causes increases in the firing rate of dopaminergic neurons in the mesolimbic pathway (Di Chiara and Imperato 1988; Gessa *et al.,* 1985). In addition, studies have shown that alcohol preferring rats will directly self-administer alcohol into the posterior region of the VTA (Gatto *et al.,* 1994; Rodd *et al*., 2004). This suggests that ethanol can modify the function of the dopaminergic neurons in the mesolimbic pathway. Self-administration of ethanol into the VTA can be decreased when dopamine neurons in the VTA are inhibited by quinpirole. Rats treated with the quinpirole had a decrease in the firing rates of the VTA dopamine neurons suggesting that alcohol is reinforcing by activation of these neurons (Rodd *et al.,* 2004). Together, this data indicates that ethanol can induced the release of dopamine, which plays a role in the reinforcing effects of the drug.

The opioid system is widely distributed thought the CNS and is involved in learning and memory, emotional behavior, and reward processing (Bodnar 2012) Opioid receptors and peptides can be found in the PFC, hypothalamic nuclei, NAC, VTA, and the amygdala (Mansour *et al.,* 1994; Mansour *et al.,* 1995). All opioid receptors are G-protein-coupled receptors and act mainly via inhibitory G proteins leading to a reduction in neurotransmitter release and inhibition of neuronal activation (Kieffer and Evans 2009). Acute ethanol has been shown to stimulate the release of the endogenous opioid peptides β-endorphin, enkephalin and dynorphin (Marinelli *et al.,* 2003; Marinelli *et al.,* 2005; Marinelli *et al.,* 2006). β-endorphin and enkephalin bind to μ and δ receptors, respectively and can decrease the release of dopamine from the NAC (Hillmacher *et al.,* 2011). Ethanol consumption causes an increase in extracellular dopamine levels and

when the μ and δ receptors are antagonized, the dopamine enhancing effect of ethanol is significantly decreased (Acquas *et al.,* 1993; Benjamin *et al.,* 1993) (See Current Treatments for Alcohol Disorders). This suggests that the dopamine enhancing effect of ethanol is dependent on opioid induced neurotransmitter reduction. This points to the complexity of the molecular mechanisms induced by ethanol where different but multiple neuronal systems are required to produce cellular effects. μ-receptor knockout mice do not display the reinforcing effects of ethanol and ethanol induce dopamine in the NAC is attenuated in these animals (Roberts *et al.,* 2000). δ-receptors knockout mice show elevated ethanol consumption (Roberts *et al.,* 2001). Dynorphin binds to the κ-receptor and pharmacological inhibition of the κ-receptor shows no effect on alcohol self-administration, but studies have suggested that the κ-receptor may be involved in the negative reinforcing effects of alcohol (Williams and Woods 1998, Doyon *et al.,* 2006; Nguyen *et al.,* 2012). Together, these results suggest that the opioid system is an integral part for the reinforcing effects of ethanol and that alterations in this system can limit ethanol's rewarding effect.

There are a numerous signaling pathways that are affected by acute ethanol. The signal transduction within the NAC following the release of dopamine enhances the activity of adenylyl cyclase, which is coupled to the stimulatory G protein $G\alpha_s$ (Neer 1995, Diamond and Gordon 1997; Ron and Jurd 2005). Acute ethanol exposure (20 – 500 mM) potentiates the receptor-mediated stimulation of adenylyl cyclase activity (Saito *et al.,* 1985.). Adenosine triphosphate becomes converted to cyclic adenosine monophosphate (cAMP) by adenylyl cyclase, which subsequently activates protein kinase A (PKA) signaling (Diamond and Gordon 1997). PKA is a tetramer composed of

a homodimer of regulatory subunits (RIα-β and RIIα-β) and two catalytic subunits (Cα and Cβ) (Mckinght 1991). Mice haploinsufficient for $G\alpha_s$ have low adenylyl cyclase activating and voluntarily consume less alcohol (Wand *et al.,* 2001). PKA RIIβ knockout mice have decreased ethanol induced LORR, but increase ethanol consumption (Thiele *et al.,* 2000). *Drosophila* mutants carrying a hypomorphic PKA RII also have a reduced sensitivity to the depressive effects of ethanol (Parke *et al.,* 2000), Recent work has shown that acute ethanol administration produces dose-dependent increases in the expression of PKA and $GABA_A$ receptors α1 subunits and that pharmacologically blocking PKA inhibited the ethanol induced increases in $GABA_A$ receptors α1 subunits suggesting that both PKA and $GABA_A$ receptors may function in network communication (Kumar *et al.,* 2012).

PKA signaling leads to the phosphorylation of the transcription factor cAMP response element-binding protein (CREB). Phosphorylation of CREB results in increased transcription of genes containing cAMP response elements (CRE) within their promoter region (Lonze and Ginty 2002; Moonat *et al.,* 2010). CREB regulates many genes, which include genes that control neurotransmission, cell structure, signal transduction, transcription and metabolism (Lonze and Ginty 2002). Acute ethanol treatment has been shown to lead to increased expression and phosphorylation of CREB (Yang *et al.,* 1996), but chronic ethanol treatment has been shown to decrease CREB phosphorylation (Yang *et al.,* 1998). Mice halploinsufficient for CREB display increased ethanol preference and have decreased expression of Neuropeptide Y (NPY) and brain-derived neurotrophic factor (BDNF) (Pandey *et al.,* 2004). Together this work suggests that both acute and chronic ethanol can induce changes in gene expression

that lead to widespread cellular changes. This population of genes that have differential expression due to ethanol may reveal mechanisms by which cells are changing their function due to ethanol exposure that may provide insight into ethanol responsive behaviors.

In addition to PKA signaling, the protein kinase C (PKC) signaling pathway is stimulated by acute ethanol exposure (25-200 mM)(Diamond and Gordon 1997; Depetrillo and Swift, 1992; Kumar *et al.,* 2006). There are several isoforms of PKC that are expressed thought the brain (Naik *et al.,* 2000; Choi *et al.,* 2002). PKC α, β, γ are activated by calcium and diacylglycerol where as PKC δ, ε, η, θ is only activated by diacylglycerol (Newton and Ron 2007). PKC signaling has been widely studies for its regulatory role of $GABA_A$ receptor function and various PKC isoforms associate with $GABA_A$ receptor. PKCβ has been shown to associate with $GABA_A$ receptor β subunits (Connolly *et al.,* 1999) and studies have shown by western blot analysis that acute ethanol administration causes a decrease in phosphorylation of $GABA_A$ receptor β subunits in rats (Kumar *et al.,* 2006). Reduced phosphorylation of the $GABA_A$ receptor γ2 subunit enhances the action of ethanol and mice lacking PKCε have reduced phosphorylation at this subunit (Qi *et al.,* 2007). Genetic manipulation of PKC isoforms has been fruitful in delineating the role of PKC activity in acute ethanol effects. PKCγ null mice display reduced sensitivity to the effects of ethanol on LORR (Harris *et al.,* 1995). PKCε knockout mice display increased ethanol sensitivity to the effects of ethanol measured by LORR (Hodge *et al.,* 1999). PKC δ null mice showed a reduced ataxia to ethanol (Choi *et al.,* 2008). Taken together these studies suggest that PKC isoforms have distinct roles in mediating acute ethanol behavioral effects.

Current Treatments for Alcohol Use Disorders

Despite the negative physiological and socioeconomic consequences, individuals continue to drink. Currently, three government approved pharmacological therapies designed for treating AUDs are available; disulfiram, naltrexone, and acamprosate (Krishnan-Sarin *et al.,* 2008)

Disulfiram targets ethanol metabolism by inhibiting aldehyde dehydrogenase (ALDH) enzyme function. When an individual drinks alcohol while on this drug, ethanol is successfully converted into acetaldehyde, but inhibition of ALDH results in an increase in acetaldehyde concentrations. The increases in acetaldehyde concentrations cause extremely unpleasant effects, such as difficulty breathing, tachycardia, nausea, vomiting, and headaches that could last for days. These adverse effects result in very low rates of medication adherence, and therefore this method of drug treatment is ineffective without supervision (Fuller *et al.*, 1986, Buonopane and Petrakis, 2005).

Naltrexone antagonizes the μ-opioid receptor. This causes a decrease in the positive reinforcing effects of alcohol (Douaihy *at al.*, 2013). Clinical trials using naltrexone for treatment of AUDs have manifested mixed results. Naltrexone has been shown to reduce drinking and increase abstinence in non-treatment seeking heavy drinkers (Garbutt, 2009), but treatment amongst more severe alcohol dependent patients was ineffective (Krystal *et al*., 2001). As in the case for disulfiram, adherence is an issue with naltrexone treatment and low adherence significantly promotes higher risk for returning to heavy drinking (Minozzi *et al.*, 2006; Swift *et al.,* 2011). To circumvent this, a monthly injection that causes slow release of the drug has been shown to be

effective in reducing heavy drinking outcomes (Garbutt *et al.*, 2005). However, the effectiveness of naltrexone as a treatment for an AUD is dependent on an individual's genetic background. Genetic variation at the μ-opioid receptor can either enhance or suppress the effects of naltrexone treatment for some individuals (Arias *et al.,* 2006). In addition, a major drawback for patients using naltrexone is the ineffectiveness of opioid-based analgesics.

Acamprosate is thought to act primarily by modulating glutamatergic transmission and acts as a coagonist or modulator of NMDA receptors (Kiefer and Mann, 2010, Olive *et al.*, 2012). Clinical trials have reported mixed results. In a US study, acamprosate was not effective for alcohol dependent patients, but in a European study, acamprosate was extremely effective in maintaining abstinence (Anton *et al*., 2006, Mason *et al*., 2006). Taken together, the three government-approved treatments provide evidence that improved and perhaps even personalized pharmacological interventions are needed. One way to facilitate this approach is to uncover and better understand the mechanisms of action of alcohol.

There are other types of therapies used to treat AUDs. One of the most popular platforms of intervention is Alcoholics Anonymous (AA). As of 2013, there are 59,321 AA groups and 1,295,656 members in the United States (AA, 2013). AA is a self-driven 12-step recovery program for individuals that want to stop drinking. Individuals attend meeting sessions at their own discretion and engage in group discussions. Studies have shown that the frequency of attendance is highly correlated with alcohol abstinence (Kaskutas *et al.*, 2005). There are also behavioral treatments that are commonly used to deter alcohol use. Behavioral therapies for couples and families focus on the

incorporation of positive activities, communication skills training, and identification of potential relapse trigger points (McCrady, 2012). This treatment has shown to significantly reduce the frequency of use (Powers *et al.,* 2008). Coping skills training and cognitive behavioral therapy help individuals to cope with risky situations that promote alcohol-seeking behaviors. These therapies have also been shown to reduce alcohol use (Magill and Ray, 2009).

Behavioral programs and therapy have seen more success with managing an individuals drinking problem, however our work will help contribute to informing patients on their potential abuse liability. Informing patients about their abuse liability may allow individuals to take precautionary behavioral measures possibly before or when drinking becomes an issue.

Genetic Susceptibility of Alcohol Use Disorders

Susceptibility to developing an AUDs has a significant genetic component. Familial inheritance of alcoholism is a well-known and documented factor of risk for alcoholism (Cotton, 1979, Kendler *et al.*, 1997, Lieb *et al.,* 2002). Initial studies compared the concordance rates for alcohol abuse and dependence amongst monozygotic twins, which have identical genomes, and dizygotic twins who only share on average half of their genetic material. These studies have shown higher concordance rates amongst monozygotic twins, indicating that there is a genetic contribution to the risk for alcohol dependence (Pickens *et al.*, 1991, Agrawal and Lynskey, 2008). This research and other studies have suggested that 40-60% of the risk is inherited (Prescott and Kendler, 1999; Heath *et al.,* 1999; Schuckit *et al.,* 2001;

Schuckit and Smith, 2006). However, these studies have suggested that other factors such as environmental influences also contribute to this risk. Some of these factors include peer influence and availability of alcohol (Prescott and Kendler, 1999; Schuckit and Smith, 2006, Youg-Wolff *et al.*, 2011).

Since the familial and twin studies have suggested that genetics plays a role in risk for developing AUD, researchers have employed three different approaches to identify which genes in particular influence this risk. These approaches are linkage studies, genetics association studies.

Initially, linkage studies were performed on multiple families having two or more alcoholic members. DNA was isolated from families and genotyped for genetic markers to identify loci that cosegregated with the disorder. Multiple studies have revealed regions of the genome that contribute to the risk of alcoholism and these regions of the genome are referred to as quantitative trait loci (QTL). The Collaborative Study on the Genetics of Alcoholism (COGA) was one of the first large-scale initiatives to define QTLs contributing to alcohol dependence (Begleiter *et al.,* 1995). COGA and others have shown evidence that multiple regions from across the genome contribute to this risk suggesting that alcohol dependence is a complex disorder. There have been 12 different chromosomes implicated in AUDs that include chromosomes 1, 2, 4, 6, 7,10, 11, 12,14,16,17 (Hill *et al.*, 2004; Dick *et al.*, 2002; Hill *et al.*, 1999; Edenberg and Foroud 2006). Over the years of collecting linkage results for families with multiple alcoholic members or for other phenotypes including alcohol dependence and alcohol consumption, chromosomal region 4q22-4q32 is the most consistently reported region across studies (Prescott *et al.,* 2004, Hill *et al.,* 2004*;* Dick *et al.,* 2002; Hill *et al.,* 1999).

This genomic region contains a subset of the alcohol dehydrogenase (ADH) genes (Prescott *et al.*, 2004). There is considerable genetic variation in the ethanol metabolizing enzymes ADH and ALDH and other work has shown that individuals carrying specific alleles either have an increased or decreased risk for developing an AUD (Chen *et al.,* 2009; See Discussion). Despite the identification of ADH in the risk of AUDs there are still major drawbacks to these studies. Most studies have implicated broad chromosomal regions for AUDs still leaving researchers with thousands of candidate genes.

Another approach for identifying specific genes influencing the risk of developing an AUD are genetic association studies. There are two types of genetic association studies; targeted genetic association studies and genome wide association studies (GWAS). Targeted genetic association studies ascertain individuals and test for the frequency of alleles or genotypes in both cases and control groups and are typically used to investigate regions and genes of prior interests or candidate genes. Replication of findings is important to validate since these studies are done with a smaller population. However, there have been reports of subpopulation differences in alleles that contribute to specific phenotypes as possible causes for why some findings from one population cannot be replicated in other populations (Riestchel and Treutlein 2012). One great example of this is the association of an ALDH variant that is only found in Asian populations (See Chapter 2). To date, genes that have shown reproducibility across multiple association studies for alcohol dependence are as follows: dopamine receptor D2 (DRD2), ankyrin repeat and kinase domain containing 1 (ANKK1), serotonin transporter (SLC6A4), catechol-*O*-methyltransferase (COMT), tryptophan

hydroxylase 1 (TPH1) (See *Genes Mediating Acute Ethanol Responsive Behaviors in C. elegans and Other Species* in this chapter), dopamine transporter (SLC6A3), interleukin-1 receptor, type II (IL1RA), interleukin-10 (IL10), tumor necrosis factor (TNF), alcohol dehydrogenase 1B (ADH1B), alcohol dehydrogenase 1C (ADH1C), and aldehyde dehydrogenase 2 (ALDH2) (see Chapter 2) (Rietschel and Treutelin, 2013).

GWAS analyze several hundred thousand or millions of markers across the entire genome to detect difference in allele or genotype frequencies in a systematic manner for cases and controls. One of the major limitations to this approach is the extreme level of significance required because of the level of correction for multiple testing (Cichon *et al.*, 2009). As with the targeted genetic association studies, genes or regions replicated in independent sample sets raises the confidence of the region or the gene in the disorder. Many genes have been implicated through these studies, but only a few have actually reached significance. Those genes include, alcohol dehydrogenase 1B (ADH1B), alcohol dehydrogenase 1C (ADH1C), aldehyde dehydrogenase 2 (ALDH2), near peroxisomal trans-2-enoyl-CoA reductase (PECR), and autism susceptibility candidate 2 (AUTS2) (Rietschel and Treutelin, 2013).

Alcohol Endophenotypes – Low Level of Response

Despite numerous human studies, very few definitive genetic loci have been implicated in AUDs. This is chiefly due to the complex nature of the disorder (Chesler *et al.,* 2005, Koob and Volkow 2010, Schuckit 2002; Schuckit and Smith 2004, Dick and Foroud 2003). The diagnosis of AUDs is based on a range of reported symptoms according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition

(DSM-IV). However, most are typically treated as a binary outcome of affected or unaffected, which can severely limit the detection of more subtle and variable phenotypes. Certain risk factors such as genetics may affect individuals differently, which may results in differences in the severity of the disease or a particular set of symptoms. This most likely is attributed to clinical heterogeneity, polygenic determinants, reduced penetrance, and epistatic effects (Hines *et al.*, 2005). One way to identify biological attributes or intermediate phenotypes is to study a subset of characteristics that are closely related or contribute to the outcome of the disease. This approach is only effective if the studied characteristcs correlate of influcne the outcome of the disease (Schuckit, 1994). This approach focuses on particular components of the disease spectrum and is referred to as endophenotypes. The hypothesis behind using endophenotypes is to increase the ease in identifying genetic and environmental factors that contribute to particular components of the disease spectrum because the number of factors influencing each is fewer than the number affecting the clinical syndrome (Carlson *et al.*, 2004).

One well-known and established AUD endophenotype is level of response (LR) to alcohol. An individual's naive LR to ethanol has been shown to be correlated with their abuse liability later in life (Shuckit, 1994). LR is a measure of the acute effects of ethanol on a variety of measures which include, in different studies, level of change in subjective feelings of intoxication (assessed using the Subjective High Assessment Scale), motor performance, hormone levels, electrophysiological measures observed at specific blood alcohol concentrations, self reporting of the number of drinks required for specific effects (assessed using the Self-Rating of the Effects of Alcohol – SRE), and

the effects of 3 beverages consumed over 10 minutes (alcohol challenge) (Schuckit and Smith, 2000; Schuckit 1998, Schuckit *et al.,* 1997). These models measure a variety of variables, but all measure acute alcohol sensitivity, which is a composite phenotype influenced by acute sensitivity and the development of acute functional tolerance (Newlin and Thomson, 1994, Schuckit 1994). Acute sensitivity is a measure of the maximal effect of the drug. Acute functional tolerance was first described by Mellanby (1919). These studies showed that dogs treated with a single dose of ethanol displayed more motor impairment at a given blood ethanol concentration on the rising portion of the blood ethanol concentration curve than at the same blood ethanol concentration on the falling portion. Therefore, AFT reflects rapid cellular adaptations to the effects of ethanol on neurons that are required for certain behaviors (Mellanby 1919; Keir and Deitrich 1990; Newlin and Thomson 1990; Hu *et al.*, 2008). It is thought that functional tolerance reflects homeostatic adaptations that attempt to restore normal neuronal activity (Le Moal and Koob 2006).

Initially, studies examined participants that were sons of alcoholics and matched comparison subjects for their naïve LR and found that individuals with an initially low LR to alcohol from this cohort were more likely to have developed alcohol dependence than individuals with a high LR. (Schuckit 1994, Schuckit and Smith 1996). Other studies have replicated these findings across different populations (Heath *et al.,* 1999, Volavka *et al.,* 1996). These results suggest that LR of is a strong endophenotype for predicting the long term drinking behavior of naïve individuals. These results also suggest the importance of individual differences in initial alcohol sensitivity and much work has been done to uncover some of the genetic factors associated with the LR in humans. It is

estimated that 40% of offspring of alcoholics have a low LR to alcohol providing further evidence that genetics can influence LR (Schuckit *et al.,* 1996)

Association studies have been completed and a few regions have been implicated in influencing the LR. These regions include genes encoding neuropeptide Y (NPY), the potassium-BK channel (KCNMA1), the alcohol dehydrogenase gene cluster found on chromosome 4, brain-derived neurotropic factor (BDNF), the dopamine-2 receptor (DDR2), catechol-*O*-methyltransferase (COMT), serotonin 1D receptor (5-HT1D), Glutamate receptor 5 (GRM5) and others (Wilhelmsen *et al.,* 2003; Schuckit *et al.,* 2004; Schuckit *et al.,* 2005).

Furthermore, this phenotype has been heavily investigated in model organisms that can recapitulate aspects of the LR phenotype such as initial sensitivity and acute functional tolerance (Newlin and Thomson 1994, Hu et al., 2008; Ponomarev and Crabbe, 2002). Work in animal models has shown that these traits are genetically influenced and therefore model organisms serve as a powerful resource to understand how genes can influence these responses (See *Ethanol Responsive Behavioral Assays in C. elegans* section in this chapter and Chapters 2-4 of this document).

The Utility of C. elegans as a Model Organism for Behavior

Caenorhabditis elegans are an outstanding model organism to study a variety of biological processes and mechanisms. The characterization of worm physiology, development, neurobiology, and genome is very extensive and fundamentally distinguishes the model from any other.

C. elegans naturally dwell in soil and develop rapidly, going from egg to adult in 3 days, and grow in broods of 300-350 animals. Animals grow to 1.5 mm in length and are reared very easily in a laboratory setting on an agar plate that contains a food source of *E. coil* (we use the strain OP50 to feed worms in our laboratory). They can be reared at 20°C or at 15°C for a reduced growth rate. They are cultured using a dissecting microscope for visualization and a platinum pick for transfer. For long-term storage, animals can be frozen in a similar fashion as mammalian cell lines (Sulston and Hodgkin 1988). Stocks have been shown to retain viability for up to 25 years when frozen in liquid nitrogen and 12 years when frozen at -80°C. This is essential because laboratory strains maintained in growing cultures for long periods of time in a laboratory setting can diverge. This occurs through fixation of a random mutation into a population by a population bottleneck. This typically happens by picking an animal that is carrying a mutation and through self-fertilization, expanding the mutation in the population each generation. The costs associated with culturing and storing animals is also extremely inexpensive, another advantage for scientists in these challenging economic times.

There are two *C. elegans* sexes, a self-fertilizing hermaphrodite and males. The possibility of self-fertilization and the ability to cross hermaphrodites with males makes genetic manipulation easy. Hermaphrodites have two sex chromosomes, XX, and males have one, XO. Males occur in a population at a 0.2% frequency by a spontaneous non-disjunction event of one of the sex chromosomes. After mating between a hermaphrodite and a male, 50% of the progeny will be males because 50% of the male sperm are null for the sex chromosome (Akerib and Meyer 1994). The anatomical structure of worms is exquisitely simple. Hermaphrodites contain 959 somatic cells of

which 302 of those cells are neurons and 95 are body wall muscles. The male is slightly different and has 1031 somatic cells and 381 neurons. The increase in cell number in males is concentrated to the tail structure and is required for mating functions (White 1988).

Work completed by Sulston *et al.,* in 1983 revealed the complete lineage of cells during development. This work found that the cell lineage or cell fate decisions are invariant. This provided a great foundation to study the genetics of development since the number and positions of cells are consistent between animals and manipulations that cause subtle changes in the lineage could be detected. Additional work allowed the mapping of all synaptic connections in the animal and the generation the first "connectome" (White *et al.,* 1986). The worm genome was the first sequenced genome of all multicellular species and was completed in 1998 (*C. elegans* Sequencing Consortium, 1998). The worm genome encodes 20,513 protein-coding genes (WormBase, 2013; Release WS238) and about 40% of these genes are orthologous to human genes (Shayne and Greenwald 2011).

There are a variety of genetic tools readily available to the *C. elegans* community. Forward genetic screens are often employed to uncover mechanistic insight into phenotypes of interests. These mutations can be identified through mapping, comparative genomic hybridization, or by whole genome sequencing. There are many ways to manipulate the expression of genes in *C. elegans.* These methods include classic transformation by injection of exogenous DNA, microparticle bombardment of exogenous DNA, and *Mos1*-mediated single copy insertion of exogenous DNA (Boulin and Hobert, 2012). Reverse genetic approaches include utilizing two RNA interference

(RNAi) libraries that are administered to animals by feeding (See Chapter 2 -3 and Appendix B) and cover about 95% of the genome (Kamath *et al.,* 2003; Rual *et al.,* 2004). The community also has a Deletion Mutant Consortium that has created more than 6,841 deletions in 6,013 genes in the genome. Recently, the completion of the million-mutation project provides the community with 2,007 mutagenized strains that have been extensively characterized at the molecular level and contain more than 800,000 single nucleotide variants, 14,800 insertions and deletions, and 1,400 chromosomal rearrangements (Thompson *et al.*, 2013, *C. elegans* Deletion Mutant Consortium, 2012, Fraser *et al.*, 2000, Kamath *et al.,*2003, Rual *et al.*, 2004).

C. elegans have a simple nervous system that contains about 5000 chemical synapses, 2000 neuromuscular junctions and 600 gap junctions (White 1998). Completed sequencing of the worm and human genome reveal conserved gene systems for neurotransmitter biosynthetic enzymes, synaptic release mechanisms, and neurotransmitter receptors, including both ligand-gated ion channels and G-protein-coupled receptors. Worms contain two major classes of potassium channels: the inward rectifier channels and voltage-regulated potassium channels and they also have voltage-activated calcium channels. At the neurotransmitter levels, all major molecules are well represented. *C. elegans* use acetylcholine, dopamine, serotonin, y-aminobutric acid (GABA), glutamate, and neuropeptides. Neurotransmitter synthesis, packaging into synaptic vesicles, and reuptake or destruction is also similar in worms and vertebrates. Ligand-gated ion channels are also represented in the worm (Bargmann, 1998). The conservation of the nervous system between the two species and the overlap of signaling pathways affected by ethanol makes using *C. elegans* as a model

for understanding the genetic contributions to ethanol responses extremely advantageous.

Therefore, we use *C. elegans* to study the genetics of acute ethanol responsive behaviors because animals dose dependently become intoxicated to ethanol for a variety of behaviors at concentrations that are similar to humans (Davies *et al.,* 2003; Alaimo *et al.,* 2012).

Ethanol Responsive Behavioral Assays in C. elegans

Previously studies have shown that worms dose dependently respond to a variety of different measureable phenotypes induced by either acute or chronic ethanol treatment or during withdrawal from the drug. The following are behavioral assays currently used in the field to understand the influence of genes on ethanol responsive behaviors.

Locomotion-based

Locomotion assays are the standard assay for measuring the acute responses to alcohol In our laboratory. These assays involve recording and analyzing two-minute movies of animals in the absence and presence of alcohol and comparing the treated and untreated speeds (See Appendix B, and Methods Section of Chapters 2-4). This assay primarily tests a well defined neuromuscular network and its associated neurotransmitters. Worms move in a sinusoidal wave that requires 95 body-wall muscles cells that receive excitatory inputs at cholinergic neuromuscular junctions and inhibitory inputs at GABAeric neuromuscular junctions (Chalife and White, 1988). To

generate the sinusoidal pattern of movement, the contraction of the dorsal and ventral body muscles must be out of phase. To turn the body dorsally, the dorsal muscles contract while the opposing ventral muscles relax. This pattern of alternating dorsal and ventral contractions is produced by interactions between excitatory cholinergic inputs and inhibitory GABAeric inputs (Riddle *et al.,* 1997). We derive from this assay two measures of the acute locomotor responses to ethanol; initial sensitivity and acute functional tolerance (AFT) (Figure 1), both of which are important components of the mammalian phenotype of LR. We have shown that worms become maximally intoxicated in 6 minutes (See Chapter 2, Figure 3, Alaimo *et al.,* 2012) and that locomotion between 6 minutes and 10 minutes is not different and therefore we measure initial sensitivity at 10 minutes of continuous exposure. We measure the locomotion of the animals at 30 minutes of continuous exposure for their ability to adapt to drug treatment and calculate the degree of AFT (Figure 1), by subtracting the speed at 30 minutes from the speed at 10 minutes.

A variation of the above locomotion assay, commonly referred to as a dispersal assay or food race assay, measures the ability of the animals to navigate towards the food source, *E. coli* OP50. In this assay, animals are placed at the opposite side of the plate from a point source of food. Over time the worms navigate towards the food and the efficiency of navigation can be quantified by counting the percentage of worms that reach the food at a given time interval. This behavioral measure requires animals to rely on sensorimotor processing, but the food race assay analysis does not take basal speed differences of mutant animals into account in the data analysis (Mitchell *et al.,*2010). In addition, chemosensory neurons required for odorant detection in the

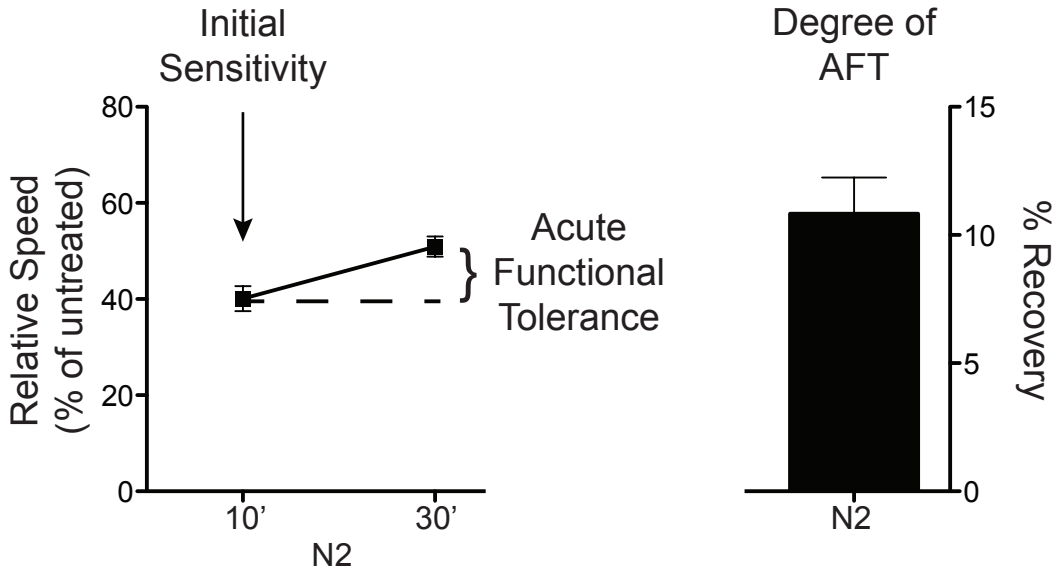


Figure 1. Acute locomotor response to 400mM continuous ethanol exposure of wild type N2 animals. The x-axis represents the relative speed expressed as percent of untreated (treated speed/untreated speed x 100). The y-axis represents time of continuous ethanol exposure. By 10 minutes animals become maximally intoxicated and severs as a measure of initial sensitivity. By 30 minutes of continuous exposure, animals are moving significantly faster. The observed behavioral change is called acute functional tolerance. In the right panel we calculate the degree of AFT animals develop by subtracting their relative speed at 30 minutes from their speed at 10 minutes. N2 typically recover about 8-12% of their untreated speed (n = 4) Error bars are standard error of the mean.

environment may be affected by ethanol and may complicate the analysis (Kapfhamer *et al.,* 2008.

The food race assay has also been used to measure a withdrawal-like syndrome. In this assay, animals are pretreated with ethanol for at least 6 hours and immediately after treatment are tested in the food race assay. During the assay, few animals reach the food source. However, when a low dose of ethanol is added to the plate, a significant number of animals are able to reach the food source (Mitchell *et al.,* 2010). This is interpreted as ethanol induced relief of withdrawal and most likely reflects chronically induced neuroadapations. This suggests that the chronic ethanol exposure has altered the balance of neuronal signaling required for locomotion. The addition of ethanol may add relief because it is restoring the balance of neuronal signaling.

A second measure of withdrawal-like behavior has been studied by our laboratory. Wild type animals were exposed to 350 mM exogenous ethanol for 18-22 hours. When these animals were removed from the drug, there was a significant increase in bordering and clumping behavior (Davies *et al.,* 2004). Wild-type animals are solitary feeders and therefore have little tendency to engage in social feeding behavior such as clumping and typically do not prefer the thicker parts of the bacterial lawn (bordering) (de Bono and Bargmann, 1998). The increase in bordering and clumping after withdrawal of long-term ethanol exposure of wild type animals photocopies neuropeptide Y like receptor 1 (*npr-1)* loss of function animals. This work suggests that the NPR pathway is involved in this withdrawal phenotype (Davies *et al.,* 2004).

A distinct locomotion-type assay of ethanol responses is to measure the rate of swimming or ("thrashing") of a worm in liquid. Swimming is the behavior of lateral movements or head bends that the animals make while immersed in liquid and this behavior requires dopamine and serotonin. These assays can be done visually under a microscope or by recoding movies and analyzing the behavior in a high-throughput manner using ImagePro software. (See Chapter 2 Methods) (Buckingham and Sattelle, 2009, Vidal-Gadea *et al.,*2011).

Egg-laying

C. elegans can produce about 300 progeny over the course of their adult life. A wild type young adult hermaphrodite will store about 10-15 eggs in its uterus at any given time and it continuously generates new eggs and lays the oldest eggs. The egg-laying motor circuit requires synaptic input from two classes of motor neurons and 16 muscle cells. This circuit is modulated by serotonin and requires acetylcholine (Schfer, 2005). Egg laying frequency is typically decreased by ethanol intoxication (Davies *et al.,* 2003), but in some cases, egg laying is stimulated by low doses of ethanol. Assays are performed on and off ethanol in the presence of food. The number of eggs laid are determined at 45 minutes.

Pharyngeal Pumping

The *C. elegans* pharynx consists of radial muscle, which rhythmically pumps (coordinated waves of contraction and relaxation) to maintain the feeding activity of the animals. The pharynx uses 3 different groups of neurons that are modulated by

serotonin, glutamate and acetylcholine (Franks *et al.,* 2006). Electropharyngeogram (EPG) recording of the activity of the pharyngeal muscle in the presence of serotonin and presence and absence of ethanol has been performed over a time course of 3-5 minutes (Mitchell *et al.,*2007) and a low dose of ethanol has been shown to inhibit pharyngeal pumping.

Chemotaxis/Preference Assays

The preferences assay is a variation of a simple chemotaxis assay. Animals are pre-treated with ethanol for 4 hours and then removed to a plate in which they are given the option to discriminate between an ethanol containing quadrant and a non ethanol-containing quadrant. The attraction to ethanol is quantified by measuring the preference index which is the number of animals in the ethanol containing quadrant minus the number of animals in the non-ethanol containing quadrant divided by the total number of animals tested. A positive preference index is indicative of more animals in the ethanol containing quadrants and a negative preference index indicates more animals in the non-ethanol containing quadrants. A preference index close to zero indicates no preference (Lee *et al.,*2009).

Genes Mediating Acute Ethanol Responsive Behaviors in C. elegans and Other Species

SLO-1

The voltage -and calcium- activated potassium (BK) channel, SLO-1, is a major ethanol target protein in worms. Previous work has shown that ethanol activates SLO-1

causing a large efflux of potassium ions, hyperpolarizing the cell and depressing neuronal excitability, which is a major cause of intoxication (Davies *et al.,* 2003). *slo-1* loss-of-function animals are profoundly resistant to the sedative effects of ethanol and do not develop significant acute functional tolerance, where as *slo-1* gain-of-function animals off of ethanol display some phenotypes of intoxication (Davies *et al.*, 2003, Bettinger *et al.*, 2012). *slo-1* null animals do not display resistance to the acute effects of ethanol in the food race assay nor does it exhibit an altered ethanol withdrawal phenotype (Mitchell *et al.,* 2010). This suggests that the food face assay may not be sensitive enough to capture particular phenotypes of mutant animals.

The *Drosophila* ortholog of *slo-1*, *slowpoke,* which gave the gene its name, has also been characterized for its role in ethanol responses. A loss of function of *slowpoke* in *Drosophila* results in the failure of animals to develop rapid tolerance to ethanol (Cowmeadow *et al.*, 2005, 2006). The protein structure of SLO-1 is different between invertebrates and vertebrates. In vertebrates, SLO-1 contains α and β subunits. The α domains contain 7 transmembrane regions, 2 regulator domains that are involved in pore formation, and a calcium binding region. There are 4 β subunits and each channel exists as an assembly of 4 α subunits and 4 β subunits (Treistman and Martin 2009). In mice, knockout of the β4 subunit of the channel results in the enhancement of tolerance to ethanol, suggesting that this subunit inhibits acute tolerance (Martin *et al.*, 2008). The human ortholog to *slo-1,* KCNMA-1, has also been shown to be associated with alcohol dependence and LR in human studies (Kendler *et al.*, 2011, Schuckit *et al.*, 2005).

NPR-1

The G protein-coupled neuropeptide Y receptor-like protein, *npr-1*, negatively regulates AFT in worms. Loss-of-function *npr-1* mutants display an enhanced development of AFT to ethanol and are initially resistant (Davies *et al.,* 2004; Bettinger *et al.,* 2012). Overexpression of *npr-1* results in opposite ethanol responsive behaviors where these animals display a reduced AFT phenotype relative to wild type (Davies *et al.*, 2004). In the food race assay, *npr-1* mutants did not exhibit any behavioral differences from wild type nor did *npr-1* mutants respond differently to ethanol withdrawal (Mitchelle *et al.*, 2010). However, earlier studies have implicated *npr-1* signaling in ethanol withdrawal showing that N2 animals exposed to ethanol for 18 hours and subsequently withdrawn from the drug phenocopy *npr-1* mutant bordering and clumping behavior (Davies *et al*., 2004). This strongly suggests that ethanol is acting to down regulate the *npr-1* pathway.

A role for of neuropeptide Y-like signaling in acute behavioral responses to ethanol is very well conserved across flies and mice. The *Drosophila* homolog of neuropeptide Y is the neuropeptide F, NPF, and its receptor is neuropeptide F receptor, NPFR1. NPF acutely mediates sensitivity to ethanol sedation. Knockdown of NPFR1 or NPF by RNA interference showed decrease alcohol sensitivity, and overexpression results in opposite behaviors (Wen *et al.,* 2005). In vertebrates, neuropeptide Y (NPY) null mice show decreased sensitivity to ethanol on LORR and increased ethanol consumption. Overexpression of NPY results in opposite phenotypes (Thiele *et al.*, 1998). Genetic manipulation of NPY Y_1 and Y_2 receptors in mice modulate ethanol intake. Knockout Y_1 mice display an increase in consumption of ethanol and are less

sensitive to the sedative effects of ethanol (Thiele *et al.,* 2002). Y_2 knockout mice drink significantly less ethanol and have normal sensitivity to sedative effects of ethanol (Thiele *et al.,* 2004). Finally, in human association studies, NPY receptor polymorphisms are significantly associated with alcohol dependence and alcohol abuse (Lappalainen *et al.*, 2002, Schuckit *et al.*, 2004).

Synaptic Transmission Machinery - RAB-3, AEX-3, UNC-18, JUD-4

In *C. elegans, rab-3* encodes a protein that is associated with synaptic vesicles and is required for proper synaptic transmission (Nonet *et al.*, 1997, Mahoney *et al.*, 2006). Animals carrying a loss-of-function mutation of *rab-3* display a decrease in sensitivity to the sedative effects of ethanol. Animals carrying a loss-of-function mutation of *aex-3*, a guanine triphosphate exchange factor that interacts with *rab-3* to promote synaptic transmission (Iwasaki *et al,* 1997, 2000) results in a decrease in sensitivity to ethanol, implicating synaptic transmission in acute ethanol responses (Kapfhamer *et al.*, 2008, Davies *et al.*, 2012). In mice, animals that are either null or haploinsufficient for *Rab3A* demonstrate a decrease to the ataxic effects of ethanol in the stationary dowel test and also display resistance in the loss of righting reflex assay. Interestingly, *Rab3A* haploinsufficient mice voluntary increase their ethanol intake while *Rab3A* null mice do not (Kapfhamer *et al.*, 2008).

In worms, *unc-18* encodes a protein that functions as a chaperone for syntaxin and enables the docking of vesicles to synaptic regions before priming and fusion (Weimer *et al.,* 2003). Animals carrying a point mutation in *unc-18* are resistant to the sedative effects of ethanol for swimming behavior (Graham *et al.*, 2009). However at

lower concentrations of ethanol, *unc-18* mutants display a loss of ethanol-induced hyperactivity. Interestingly, the point mutation induced at the *unc-18* locus in this study is from a previously mapped QTL for ethanol preference drinking in mice (Fehr *et al.,*2005). It should be noted that another study that measured swimming/thrashing behavior did not observe the ethanol induced activation effect of low dose ethanol treatment (Mitchell *et al.,*2007). The sole difference between these two studies was the wild-type animal of the first study carried a control transgene (Graham *et al.*, 2009) and the other wild-type strain was unmodified. This suggests that the observed ethanol induced behavioral activation effect may be an artifact of the type of transgene insertion. We have shown that the osmolarity of the solution used in the measure of the intoxicated effects on swimming, Dent's saline solution, can alter swimming behavior (Alaimo *et al.,* 2012). In *Drosophila,* mutations in *Syntaxin 1A,* a gene that encodes a protein that is part of the soluble N-ethlymaleimide-sensitive factor attachment protein receptor (SNARE) and is required for synaptic vesicle fusion, is required for the development of rapid tolerance. A mutation in *Dynamin,* a gene involved in synaptic vesicle recycling, is also required for the development of rapid tolerance to ethanol (Krishnan *et al.*, 2012).

Human studies have looked for changes in the protein levels of synaptophysin I, syntaxin 1A, synaptsome associated protein 25 (SNAP-25), and vesicle-associated membrane protein (VAMP) in the prefrontal and motor cortices between chronic alcoholics and control subjects. This work reports only an elevation of synaptophysin I protein levels (Henriksson *et al.,* 2008).

In worms, *jud-4* encodes a protein of unknown function, but putative loss-of-function mutants display profound resistance to a lethal dose of ethanol measured by swimming assays. *jud-4* is weakly homologous to the mammalian Home protein which is integral to the assembly of proteins regulating glutamate signaling and synaptic plasticity. In *Drosophila*, *homer* mutants display an enhanced sensitivity to the sedative effects of ethanol and are impaired in their ability to develop rapid tolerance (Urizar *et al.*, 2007) In mice, *Homer2* knock-out mice do not develop place preference or locomotor sensitization to repeated ethanol exposures (Szumlinksi *et al.*, 2005).

SEB-3

In worms, *seb-3* encodes a corticotropin-releasing factor (CRF) G-protein coupled receptor that is homologues to mammalian CRF. CRF receptors are typically involved in the negative emotional state induced by stress in vertabrates, which has been implicated in excessive drinking and withdrawal. Worms harboring a gain-of-function mutation of *seb-3* have an enhanced development of AFT to the sedative effects of ethanol using standard locomotion assays and during swimming(Jee *et al.,* 2012). A loss-of-function mutation in *seb-3* results in the failure to develop AFT during swimming (Jee *et al.,* 2012). Interestingly, *seb-3* gain-of-function mutants display an increase in body tremors while in liquid. This effect can be induced in wild type animals exposed to ethanol for 4 hours and subsequently withdrawn from the drug. The withdrawal effect was diminished when ethanol was added back to the liquid. In mice, *seb-3* is orthologus to CRF1. Mice treated with a selective pharmacological CRF1

receptor antagonists displayed similar initial sensitivity, but developed less AFT than wild-type mice (Jee *et al.*, 2012).

UNC-79

In worms, *unc-79* encodes a protein that localizes and regulates the activity of the NCA cation channel (Speca *et al., 2010)*. Animals carrying the *e1089* allele of *unc-79* display an enhanced sensitivity to the depressive of effect of ethanol during swimming. Work has also shown mutants in either of the NCA cation channels, *nca-1* and *nca-2,* are resistant to the effects of ethanol in swimming (Speca *et al*., 2010). A different allele of *unc-79*, *ec1*, displays a hypersensitivity to the low dose effects of ethanol when measured by locomotion, but does not alter behavioral responses at higher doses of the drug (Davies *et al.,* 2011). In vertebrates, mice that are null for *unc-79* are lethal. Haploinsufficiency at this locus results in altered acute responses to ethanol as well. These mice display a similar hypersensitivity to the acute sedative effects of ethanol relative to wild type littermates measured by the loss of righting reflex test (Speca *et al.*, 2010).

LIPS-7

In worms, *lips-7* encodes a lipase that is involved in the cleavage process of fatty acids from triacylglycerols (TAGs) complexes. TAGs can consist of a combination of any 3 fatty acids linked to glycerol backbone and are utilized in cellular membrane structure and function (Coleman and Mashek, 2011). Animals carrying a loss-of-function mutation for *lips-7* display a reduced initial sensitivity and an enhanced development of

AFT (Bettinger *et al.,* 2012). Previous studies have shown that *slo-1* gain-of-function animals display a phenotype that mimics some aspects of intoxication. These animals have reduced locomotion off of ethanol (Davies *et al*., 2003; Bettinger *et al.,* 2012). However, animals harboring a loss-of-function of lips-7 and a gain-of-function of *slo-1* display an increase in basal speed (Bettinger *et al.,*2012). Previous studies have shown *in vitro* that the components of the lipid bilayer can alter ethanol access to *SLO-1* in a variety of ways including through membrane thickness and structure shape changes due to phospholipid features (Treistman and Martin, 2009; Crowley *et al.,* 2009; Yuan *et al.,* 2008; Yuan *et al.,* 2007; Lin *et al.,* 2006). Together this suggests that *lips-7* can alter the structure of the lipid bilayer, which can affect the function of proteins in the cellular bilayer structure.

TPH-1, SER-4

In *C. elegans*, *tph-1* encodes tryptophan hydroxylase. This enzyme is required for the rate-limiting first step in serotonin biosynthesis. Animals harboring a loss-of-function mutation of *tph-1* display a reduced sensitivity to the depressive effects of ethanol during locomotion analysis, suggesting that serotonin plays a role in this response (Wang *et al.,* 2011). *ser-4* encodes a G-protein metabotropic serotonin receptor and animals harboring a deletion of this receptor also display a reduced sensitivity to the depressive effects of ethanol (Wang *et al.*, 2011). Studies in mice have shown that knockout of the D_2 serotonin receptor results in a reduced sensitivity to the depressive effects ethanol (Phillips *et al.,* 1998; Palmer *et al.,* 2003).

After a single drinking session, levels of serotonin metabolites can be found in human urine and blood suggesting that ethanol can induce the release of the neurotransmitter. (LeMarquand *et al.,*1994). Serotonin has been widely implicated in human association studies for AUDs (See *Genetic Susceptibility of Alcohol Use Disorders* in this section).

The Utility of Gene Expression Microarrays in Ethanol Research

The traditional approaches for dissecting genes involved in complex disorders typically rely on gene mapping. These studies have been used to identify susceptibility loci by linking genetic variants to the disease (See *Genetic Susceptibility of Alcohol Use Disorders*), but fail to get at the underlying biological mechanisms induced by alcohol use.

Microarrays allow for the unbiased and parallel monitoring of mRNA expression across the whole genome. The application of microarrays to study complex disease has dramatically increased over the years and has been essential in identifying gene expression that is correlated with the diseases or subtypes of the disease (Ginsberg *et al*., 2012, Colombo, *et al.,* 2011, Seo *et al.,* 2006; Middleton *et al.,* 2002; Latil *et al.,* 2003). These disease-related genes are likely to reflect a combination of genetic factors such as individual variation and the results of dynamic interactions of environmental factors that ultimately contribute to the disease state (Chesler *et al.,* 2005). Thus, the gene expression response holds valuable information regarding underlying cellular mechanisms that contribute to disease pathology and etiology.

Over the past decade, this approach has been used in AUD studies. These studies include postmortem analysis of specific brain regions of alcoholic and non-alcoholic individuals (Lewohl *et al.,* 2000; Mayfield *et al*, 2002) and examining the genetic differences between inbred strains of mice that have opposite ethanol response phenotypes such as initial sensitivity, acute tolerance, and alcohol consumption (Xu *et al.,* 2001, Tabakoff *et al.,* 2003; Mulligan *et al.,* 2008, 2011; Treadwell and Singh 2004; Wolstenholme *et al.*, 2011, Kerns *et al.,* 2005).

Each of these studies is unique and has taken different approaches in identifying genes involved in ethanol behavioral responses. Naïve inbred strains of mice that display differences in their initial sensitivity to ethanol showed differences in basal gene expression of 41 genes (Xu *et al.,* 2001) These early results linked the idea that basal gene expression differences can possibility explain the differences in behavior observed between these two mice. Basal gene expression differences in specific brain regions that contribute to ethanol behavioral phenotypes are also being investigated (Kerns *et al*., 2005; Letwin *et al.,* 2006), however whether these can altered the acute behavioral responses to ethanol has not been reported.

Acute behavioral responses to alcohol have been shown to correlate with abuse liability and dependence in humans (Schuckit, 1994). Studies have focused on the acute ethanol induced gene expression changes because it is hypothesized that they represent a measure of signaling events leading to changes in ethanol response behaviors (Kerns *et al.,* 2005). Therefore, other studies have focused on ethanol induced gene expression differences in animals that have basal genetic differences. Studies have been completed using two inbred strains of mice, DBA/2J and C57BL/6

mice that differ greatly in acute locomotor responses and ethanol consumption (Phillips *et al.,* 1994). Whole brain studies of these mice acutely treated with a high dose of ethanol identified 61 genes that were differentially expressed (Treadwell and Singh, 2004). This small number of genes that responded to ethanol was found to be involved in cell signaling, gene regulation, and homeostasis and stress responses. Other studies have focused on basal and acute ethanol-response differences in the ventral tegmental area, nucleus accumbens, and prefrontal cortex between DBA/2J and C57BL/6 mice. This study highlighted that acute ethanol induces coordinated changes in gene expression that have an overall functional role in neuroplasticity (Kerns *et al.,* 2005).

To date, there is a void in studies characterizing the role of genes whose expression changes due to acute ethanol treatment and their involvement in mediating acute behavioral responses to ethanol. Other studies have established that ethanol treatment causes changes in gene expression in mostly humans and mice. Investigating the role of these genes in ethanol responsive behaviors has been limited because of the time and cost to generate mutant mice of interest. Using worms to study ethanol mediated gene expression and the effects of these genes on behavior is advantageous compared to other model organisms because of the large and readily available supply of animals carrying a range of mutations in a majority of genome. Furthermore, worms display two key components of LR, which is a predictive measure of long-term abuse liability, initial sensitivity and AFT. Establishing the role of acute ethanol responsive gene expression in acute ethanol responsive behaviors will provide candidate genes involved in influencing risk for alcohol abuse and dependence in humans. Furthermore establishing a link between ethanol gene expression changes and acute ethanol

behaviors may also reveal novel cellular mechanism involved in ethanol response. The work in this dissertation is designed to identify novel ethanol responsive target genes by performing a whole genome gene expression microarray analysis in *Caenorhabditis elegans*. We hypothesized that ethanol responsive genes would provide a basis of the underlying biology by which cells are compensating due to ethanol exposure. We predict that these compensatory changes are important mechanisms the cell induces to limit the depressive effects of ethanol. Identify genes that contribute to the compensatory changes are ideal candidates to test for their role in the acute behavioral responses. We also predict that genetic alterations of ethanol responsive genes would affect behavioral responses to ethanol by altering pathways that are involved in a specific ethanol response. The genetic alterations induced may change the initial function of that pathway and therefore alter how the cell compensates to limit the depressive effects of the drug. Other work in this dissertation was set out to understand the contributions of ethanol metabolism to acute behavioral responses to ethanol and to dissect tissue ethanol concentrations in our model.

Chapter 2

Ethanol Metabolism and Osmolarity Modify Behavioral Responses to Ethanol in *C. elegans*

A majority of this material has appeared in the article “Ethanol metabolism and osmolarity modify behavioral responses to ethanol in *C. elegans*” by J.T. Alaimo, S.J. Davis, S.S. Song, C.R. Burnette, M. Grotewiel, K.L. Shelton, J.T. Pierce-Shimomura, A.G. Davies, J.C. Bettinger (2012) in Alcoholism Clinical and Experimental Research 36(11): 1840-1850 and is used here by permission.

Introduction

Alcohol abuse is a common disorder influenced by both genetics and environment. Despite strong evidence of a role for genetics in abuse liability, few specific susceptibility candidate genes for alcoholism have emerged. Natural variations in components of the ethanol metabolism machinery are among the very few identified genetic causes of the variation in alcohol abuse liability in humans (recently reviewed by Pautassi et al., 2010). Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH); subsequently, acetaldehyde is metabolized to acetate by an aldehyde dehydrogenase (ALDH). Certain isoforms of ADH that are more enzymatically active are protective for susceptibility for alcoholism. Similarly, ALDH isoforms with decreased enzymatic activity also decrease disease liability. Both types of alleles are

predicted to increase acetaldehyde levels, which appear to be aversive, suggesting a mechanism for the decrease in susceptibility to alcohol abuse disorders in individuals carrying them. While there are significant correlations between inheritance of these ADH or ALDH alleles and rates of alcohol abuse (Chen et al., 2009; Crabb et al., 2004; Edenberg et al., 2006; Kuo et al., 2008), there is little experimental detail on the behavioral consequences during acute ethanol treatment of variation in alcohol metabolism.

The nematode worm, *C. elegans*, has been increasingly exploited as a behavioral model for understanding the genetic contributions to ethanol responses (Bettinger and McIntire, 2004; Davies et al., 2003, 2004; Davis et al., 2008; Graham et al., 2009; Kapfhamer et al., 2008; Lee et al., 2009; Mitchell et al., 2007, 2010; Morgan and Sedensky, 1995; Speca et al., 2010). Worms are an excellent model for this work because of the extremely well conserved neurobiology between worms and humans (Bargmann, 1998). Worms show a dose-dependent depression of several behaviors when treated with ethanol (Davies et al., 2003, 2004; Morgan and Sedensky, 1995). Several laboratories are now exploring the mechanisms by which ethanol exerts its behavioral or developmental effects, but little effort has been made to determine if metabolism has a significant role in modulating behavioral responses to ethanol in this model. Here, we directly examine the effect of altering ethanol metabolism on the behavioral response to ethanol in the worm.

Materials and Methods

Nematode culture and strains

C. elegans were maintained using standard methods (Brenner, 1974). Strains used were: N2 var. Bristol, *sodh-1(ok2799), sodh-1(bet20).*

Phylogenetic analysis

Human liver ADH proteins were accessed using PubMed. ADH1A (Accession: NP_000658.1), ADH1B (Accession: NP_000659.2), and ADH1C (Accession: NP_000660.1) were BLAST against the *C. elegans* genome and all protein sequences were loaded into CLC Sequences Viewer (version 6.4). All protein sequences were aligned using default program conditions for the most accurate alignment. We used this alignment to infer evolutionary relationships between Human and worm ADHs by using the programs tree view feature. When creating the phylogenic tree we used the neighbor joining algorithm, which infers that evolution rates are free to differ in different lineages.

RNAi

RNAi induction was performed as described (Kamath et al., 2001). Cultures of bacteria containing RNAi vectors from the RNAi feeding library generated by J. Ahringer at the University of Cambridge (Geneservice, Cambridge, UK) were grown in LB supplemented with 50 µg/mL ampicillin for 12 - 18 hours at 37˚C with shaking. Nematode Growth Medium (NGM) plates containing 1mM IPTG and 25 µg/mL ampicillin were seeded with bacteria containing RNAi vectors, and were incubated at room temperature for 24 hours. 3-5 L4 stage wild-type N2 or *sodh-1(ok2799)* worms were placed on the seeded plates and incubated at 20˚C for 36-40 hours. Adult worms were

moved to new RNAi plates and allowed to lay eggs for 1-2 hours. Plates were incubated at 20˚C to allow worms to develop to adulthood. First day adults were collected and subjected to allyl-alcohol survival assays. Knockdown of target gene expression levels was confirmed using quantitative RT-PCR (Table 1). Primers used in quantitative RT-PCR experiments are in Table 2.

Allyl-alcohol survival assays

Allyl-alcohol survival was used to assay the function of ADH as described (Williamson et al., 1991).

Analysis of Locomotion (crawling)

Age-matched 55 hour-old adult animals reared at 20˚C were used. Locomotion on plates was assayed as described (Davies et al., 2003). Ten worms for each strain were tested. Two minute movies were recorded, and movies were analyzed using ImagePro Plus Version 6 (Media Cybernetics, Bethesda, MD, U.S.A.). To account for differences in basal speeds, we calculated a relative speed (ethanol-treated average speed / untreated average speed x 100).

Test strains were compared to controls assayed simultaneously on the same plates. A one-way ANOVA was performed with a significance value of $P < 0.05$ with Dunnett's post-hoc tests comparing each mutant and knockdown strain to N2. *t*-tests were performed to determine significant differences between timepoints.

Time course analysis was performed as above except that single animals were placed on plates and video images were recorded every 0.5 seconds for 15 minutes.

Table 1. Gene expression ratios of wild type worms treated with RNAi targeted for ADH or ALH

Strain	Target gene	Gene expression ratio	SEM
sodh-1(RNAi)	*sodh-1*	0.30	0.09
D2063.1(RNAi)	D2063.1	0.02	0.01
H24K24.3(RNAi)	H24K24.3	0.39	0.04
alh-1(RNAi)	*alh-1*	0.17	0.01
alh-3(RNAi)	*alh-3*	0.08	0.03
alh-5(RNAi)	*alh-5*	0.25	0.03
alh-6(RNAi)	*alh-6*	0.40	0.04
alh-7(RNAi)	*alh-7*	0.47	0.08
alh-8(RNAi)	*alh-8*	0.09	0.01
alh-9(RNAi)	*alh-9*	0.02	0.01
alh-10(RNAi)	*alh-10*	0.50	0.07
alh-11(RNAi)	*alh-11*	0.45	0.06
alh-13(RNAi)	*alh-13*	0.33	0.05

Table 2. Primer sequences used for detection of mRNA levels by qRT-PCR

Gene symbol	Sequence name	Forward primer	Reverse primer	Amplicon size
act-1	T04C12.6	GCTGGACGTGATCTTACTGATTACC	GTAGCAGAGCTTCTCCTTGATGTC	114
sodh-2	K12G11.4	GATCACGCATGGATGTTGATGA	ATTGGAACATGCACAATTCCACG	66
D2063.1	D2063.1	AGCCGTCAATGTCCCATTAG	CTTTCCATCGTGCATTTTTGTG	77
H24K24.3	H24K24.3	GCAGGGAAATGGATTTATGC	TTCACTGAACGTGGAGCATC	94
alh-1	F54D8.3	GGCATCTTTGGAATCTTTGGA	TCCGGCGTAATAACGAAGAG	97
alh-3	F36H1.6	TTCCCACCAACGATTCTGTC	AAACTCCAGCAGCAAGTCC	160
alh-5	T08B1.3	ATGCCAAGGAGAGCAAAGAC	CATTTCCAGCAAGCACGAC	105
alh-6	F56D12.1	TCGCCGAGAATTTGGATAAC	GGATGGATGCACAAAGTGG	86
alh-7	F45H10.1	CTTGATGTCGCTGTGAATGGAA	CATGGACGTAGATTCGATTGGC	98
alh-8	F13D12.4	CGGAATCCAGTTCTACACTC	CTGTGGGAACGACATTTGTG	91
alh-9	F01F1.6	TGGAGGCAGTACATGAGACG	TTAATTCCCTGAGCCAAAGG	74
alh-10	C54D1.4	CAGTTTGGTGCAACACTTGG	TCGTGGAGACCTTCTCTTCC	91
alh-11	F42G9.5	CAGGATTCGGACGTGAAAAC	TTGACAAAGACCGACTTCAGG	70
alh-13	T22H6.2	TTCCAACAAAGAACGTGGTG	ATCGAGTCCAGCAGATTTGG	121

The speed for each interval was averaged for 6 worms. The speeds were binned for each 1-minute period and an average was calculated. A one-way ANOVA was performed with a significance value of $P < 0.01$ using Prism v5.0 (GraphPad Software), with Bonferroni post-hoc tests comparing all bins. The effects of osmolarity on crawling were assessed as above, except that assay plates were either made of standard NGM or of Dent's Saline Solution (10 mM D- glucose, 10 mM HEPES, 140 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) with 2% agar.

Analysis of Locomotion (swimming)

The effect of ethanol on swimming was assessed for both individuals and groups of animals. Individuals were video recorded (30 frames per second) in liquid medium (3 mL) over a 2% agarose surface in 6 cm diameter plates to promote continuous swimming. The midlines of the worms were determined with a custom-written image analysis macro using ImagePro as described (Pierce-Shimomura et al., 2008). Animals were gently transferred to identical conditions that contained 400 mM ethanol. Groups of animals were video recorded in identical conditions. The number of head bends made by each individual in a 30-second time window was quantified at 5, 10, 15 and 20 minutes of exposure to ethanol.

Internal Ethanol Concentration calculations

Exposure to ethanol:

55-hour old worms reared at 20˚C were used. Several hundred worms were placed on unseeded plates containing the appropriate concentration of ethanol (prepared as for

locomotion assays, see above) for 10 or 50 minutes. Worms were photographed for subsequent size analysis. Exactly 200 worms were picked from plates to a tube containing 20 µl ddH_2O. Tubes were placed at -80˚C until analysis. Worms were thawed on ice and ground in the tube with a pestle (Kontes pellet pestle, Fisher Scientific, USA). Worm homogenate was stored at -20˚C.

For 200 worms: Picking took an average of 1.5 minutes per condition. Picking itself caused a loss of between 0 µL and 0.25 µL of the water, evaporation in that time caused an undetectable loss of volume.

Size calculation:

Photographs were analyzed using ImagePro Plus v6. Ten worms were used per strain per condition. A midline was drawn to determine the height, h. Three diameters were drawn, one at the vulva (the approximate center of the animal), one each at the approximate midpoint between the vulva and head or tail (Figure 2a), these lengths were averaged and used as the diameter, ½ of this length was the radius, r. Volume was determined as for a cylinder: volume = $\pi r^2 h$.

Calculation of internal ethanol concentration:

We calculated the internal ethanol concentration using the equation $C_1V_1 = C_2V_2$, where C_1 = concentration of ethanol in homogenate, V_1 = (20 µL + volume of 200 worms), V_2 = volume of 200 worms. We solved for C_2.

Gas Chromatography ethanol concentration analysis

Homogenates were tested for ethanol concentration using a Hewlett Packard model 5890A gas chromatograph (GC) equipped with a flame ionization detector and 2 meter 5% Carbowax 20M 80/120 mesh packed column (Restek, Bellefonte, PA). Samples were kept frozen at -20˚C until analysis. Injections were accomplished manually with a 10 µl gas-tight glass syringe. The GC parameters were: 5 µl injection volume, 7 minute sample run time, injector temperature 200˚C, oven temperature isothermal 90˚C, detector temperature 220˚C, helium carrier gas flow rate 30 ml/min, hydrogen flame flow rate 25 ml/min and air flow rate 400 ml/min. Data were collected and analyzed by Clarity GC software (Apex Data Systems, Prague, CZ) using a linear regression analysis with no weighting. A 7 point calibration curve preceded the analysis of ethanol concentrations. Ethanol concentrations were calculated by the external standard method. Quality control ethanol standards preceded and followed each pair of samples. Accuracy of results was inferred if the assayed ethanol concentrations from quality control ethanol standards varied by no more than 10% from actual concentrations. All quality control standards met this criterion. If sufficient homogenate was available, each sample was tested in duplicate and a mean of the two values was used in subsequent calculations. Each ethanol concentration data point represents a mean (±SEM) generated from three independent samples.

Internal Ethanol concentration analysis using spectrophotometric analysis

C. elegans homogenates were tested for ethanol concentration according to the manufacturer's directions using an Alcohol Reagent Kit (Pointe Scientific, Canton, MI, USA). 1.5 µL of worm homogenate was added to 300 µL ice-cold alcohol reagent,

incubated for five minutes at 30˚C, and the reaction was stopped by putting the tube into an ice-cold aluminum block. Alcohol concentration was determined by measuring the absorbance at 340 nm.

Results

Identification and inactivation of alcohol dehydrogenase genes

There are many genes with homology to human ADH in the *C. elegans* genome, and ADH activity has been directly demonstrated in worms using a spectrophotometric assay (Williamson et al., 1991). Our goal was to examine the behavioral consequences of impairing alcohol metabolism, and therefore we chose a subset of the genes with strong homology to human liver ADHs as good candidates for subsequent analysis. We used two methods to identify potential ADHs that metabolize ethanol in the worm. First, we identified *sodh-1*, a gene that showed transcriptional regulation in response to a prolonged treatment with very high concentrations (7%) of ethanol (Kwon et al., 2004) and has been annotated as an ADH. We also tested the two genes with highest homology to *sodh-1*: *sodh-2* and *D2063.1*. Second, we used the primary amino acid sequence of human liver ADH (Human liver ADH proteins, ADH1A (Accession: NP_000658.1), ADH1B (Accession: NP_000659.2), and ADH1C (Accession: NP_000660.1)) in BLAST searches to identify homologous *C. elegans* proteins. We then created a dendogram to identify which protein were the most evolutionally conserved (Figure 2). *H24K24.3* was selected as a candidate because it had the strongest relationship with human liver ADH proteins. *sodh-1, sodh-2,* and *D2063.1*

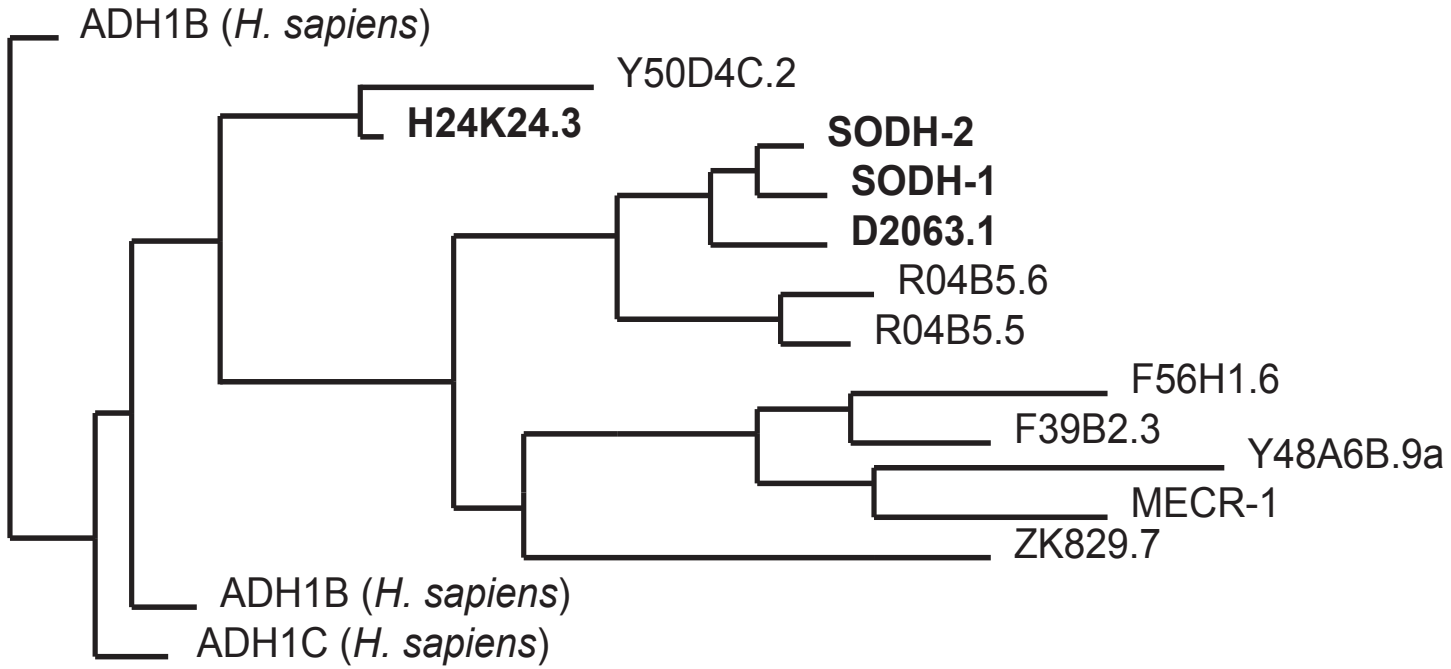


Figure 2. Phylogenic tree of *Homo sapiens* Liver ADH and *C. elegans* ADH. The evolutionary relationship between Human Liver ADH proteins and *C. elegans* ADH proteins are depicted. *C. elegans* proteins found in bold were candidate genes tested in allyl-alcohol assays.

were also identified by using this approach. Previous work had identified transcripts from both *sodh-1* and *H24K24.3* as being expressed from ADH-encoding genes, these genes have not previously been functionally characterized (Glasner et al., 1995; Waterston et al., 1992). The phylogenetic analysis identified other genes, but they were not selected because of reagent availability or lethality phenotypes.

Disruption of either of two ADH genes,* sodh-1 *or* H24K24.3*, confers resistance to allyl-alcohol toxicity

We tested a subset of the genes identified by homology above for their ability to metabolize ethanol using an allyl-alcohol toxicity assay that has been previously used extensively to identify mutants with defects in ethanol metabolism in worms and in other organisms (Williamson *et al.,* 1991). Allyl-alcohol is metabolized by ADH into the toxin acrolein, and wild-type worms that are grown on allyl-alcohol plates die within 24 hours, whereas worms with defects in ADH function are resistant to allyl-alcohol toxicity ((Williamson et al., 1991) and Table 3). Inactivation of *sodh-1* conferred profound resistance to allyl-alcohol-induced lethality (Table 3). Previously, Williamson *et al.,* (1991) performed a genetic selection using allyl-alcohol survival to isolate mutations in putative ADHs in the worm, and identified AL2B, and we replicated the allyl-alcohol resistance of this mutant (Table 3). We identified a G to A point mutation in *sodh-1* in AL2B, which would result in a Glycine 158 to Glutamate missense mutation (for consistency of nomenclature, AL2B is hereafter referred to as *sodh-1(bet20)*). Additionally, RNA inactivation of *H24K24.3* conferred resistance to allyl-alcohol, however, knock-down of function with RNAi of neither *sodh-2* nor *D2063.1* was able to

confer strong resistance to allyl-alcohol (Table 3). It is important to note that these RNAi experiments do not unambiguously rule out a role for *sodh-2* or *D2063.1* in the metabolism of ethanol. This RNAi treatment does not completely eliminate the mRNA for either candidate ADH (Table 1), and the residual mRNA may be sufficient to confer function of the candidate. In addition, *C. elegans* tissues are differentially susceptible to RNAi. Worm neurons are quite refractive to RNAi using this method, and it is possible that expression of the candidate gene is preserved in neurons. In this case, expression of the gene in neurons may be sufficient to provide function. Because inactivation of both *sodh-1* and *H24K24.3* altered allyl-alcohol sensitivity, we therefore used strains inactivated for *sodh-1* and *H24K24.3* in our subsequent studies.

Determination of the timecourse of intoxication in wild-type animals

To determine the appropriate time points during ethanol treatment at which to assess the biochemical and behavioral effects of compromising ADH function, we observed the kinetics of the onset of intoxication in wild-type animals. We placed individual worms (n=6) in a copper ring on a plate with 500 mM exogenous ethanol, and recorded their locomotion continuously for 15 minutes, starting immediately after the animals had been placed on the plate. Animals rapidly decrease speed in the first few minutes of ethanol exposure, and reach a plateau at 6 minutes, suggesting that the animals accumulate enough ethanol in this time to become maximally impaired (Figure 3A). When we examined the data in 1-minute bins, we found that the neighboring bins were not different after 6 minutes ($P > 0.05$, Figure 3B). We chose 10 minutes exposure as an

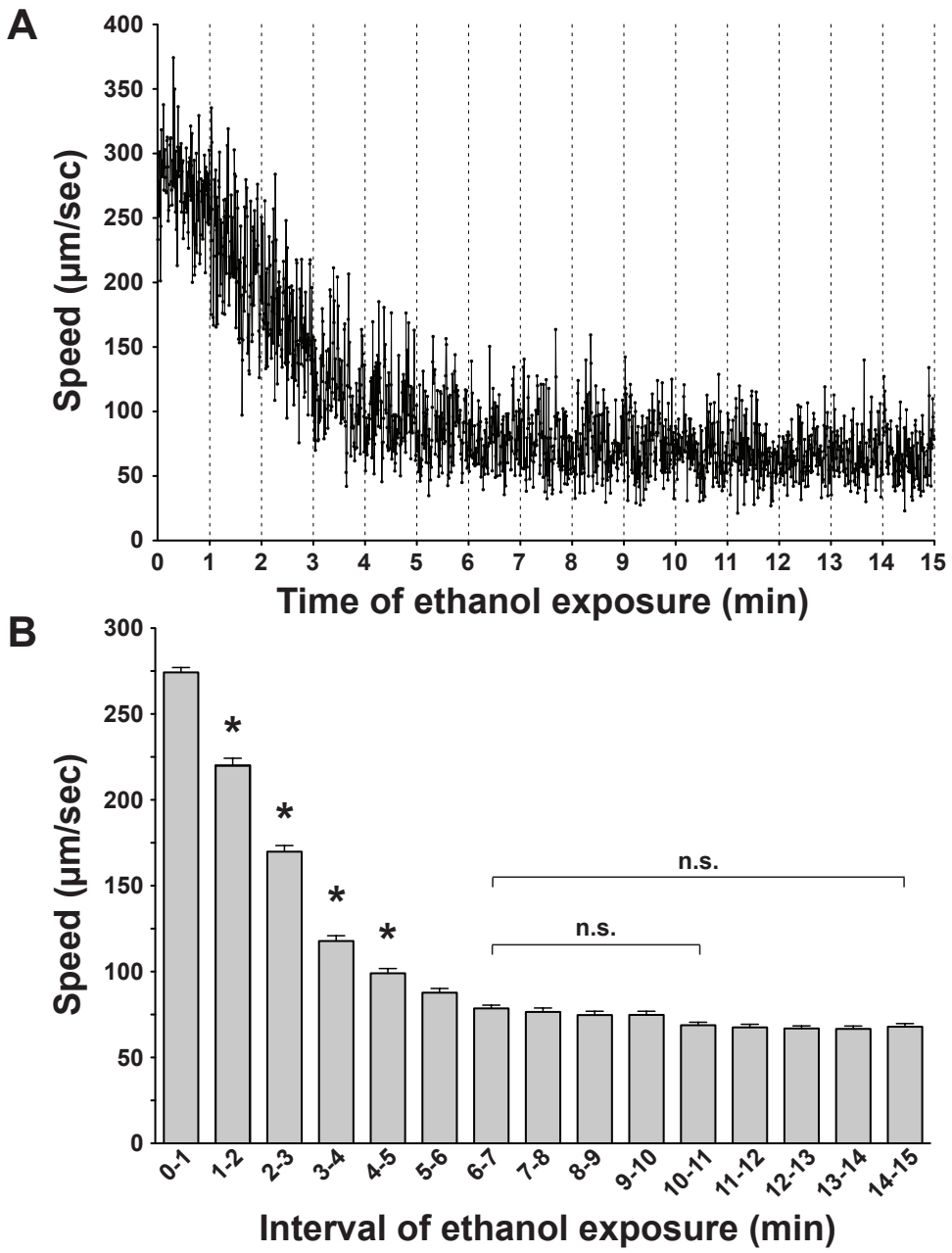


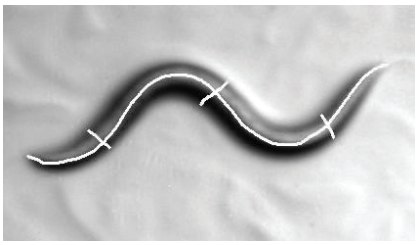
Figure 3. Time course for ethanol effects on speed of locomotion. (A) The mean speed (µm/sec ± SEM) of young adult wild-type animals (n = 6) exposed to 500 mM exogenous ethanol in the absence of food is shown for every 0.5 seconds over a 15-minute period beginning immediately after ethanol exposure. (B) Speeds within each minute of ethanol exposure were binned and a mean (n = 120) calculated for each bin. Significant decreases in speed are only seen in the first 5 minutes when each bin is compared with the previous bin (*, $P < 0.01$). Bin 6-7 minutes is not significantly different from any bin that follows. n.s., not significantly different.

initial time point for biochemical and behavioral testing for convenience and consistency with previous experimental designs (Davies et al., 2003, 2004; Kapfhamer et al., 2008).

Determination of the internal ethanol concentration in wild-type and ADH-compromised animals

Worms generate a cuticle that is impermeable to many pharmacological agents (Burns et al., 2010; Cox et al., 1981), and we have previously measured internal concentrations approximately 20x lower than the exogenous dose (Davies et al., 2003, 2004; Kapfhamer et al., 2008). However, the determination of internal ethanol concentration has been the subject of some conflicting reports; using different methodology, others have reported that more than half of the exogenous ethanol accumulates in internal tissues within 20 minutes (Mitchell et al., 2007). To unambiguously determine the internal concentration of ethanol that animals achieve in our behavioral assays, we exposed animals to ethanol on plates as we would for behavioral assays, picked exactly 200 worms from the plate into a known volume of liquid, homogenized them, and performed gas chromatography analysis on the homogenate. All ethanol in the solution would come from the worms, and all ethanol that was brought with the worms should be captured in the solution, so by determining the volume of the worms we could calculate the ethanol concentration of the worms. This calculation of internal ethanol concentration is dependent on the size of the worms tested and animals of different genotypes differed considerably in size. We estimated the volume of the worms by calculating the volume of a cylinder of the length and average diameter of the worms we tested (Figure 4A). Consistent with our previously

A



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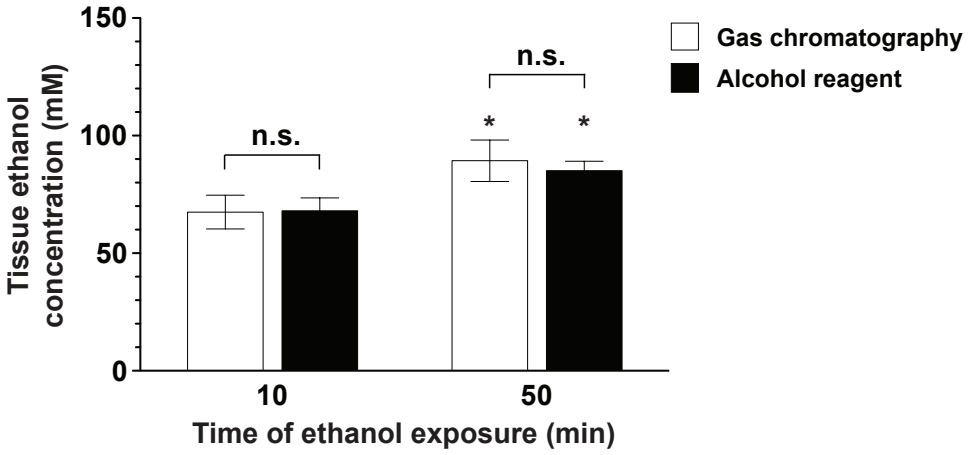

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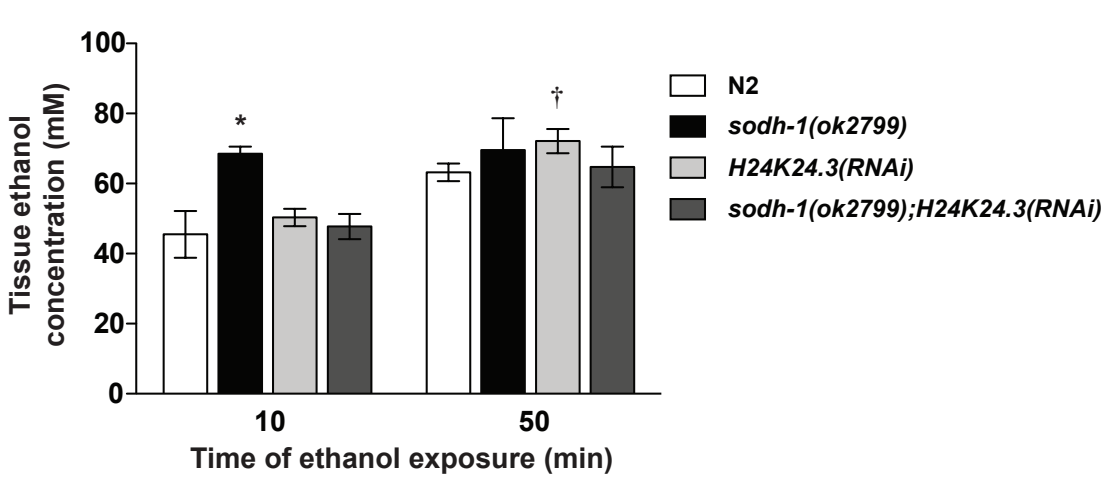


Figure 4. Determination of internal ethanol concentration in wild-type and ADH compromised animals. (A) An example of worm volume measurements. The volume of a worm was determined by taking photographs of animals immediately before they were used for the biochemical analysis. Each animal was traced (n=10 for each strain) to determine the length, *h*. The diameter of the animal, *d*, was the average of three widths, one at the vulva, and one each at the midpoint between the vulva and head or tail. The volume was calculated using formula for the volume of a cylinder (volume = $\pi r^2 h$). (B) There is no difference in internal ethanol concentration measurements using gas chromatograph (GC) and spectrophotometric analysis at 500 mM when actual worm volume was used in the calculation (n = 3). A significant difference is observed between time points (GC: 10 minutes vs. 50 minutes $P < 0.05$; Spectrophotometric analysis: 10 minutes vs. 50 minutes $P < 0.05$). (C) Internal ethanol measurements of 200 worms, treated with 400 mM exogenous ethanol, calculated from spectrophotometric analysis (n = 4). *sodh-1(ok2799)* was different from N2 at 10 minutes but not at 50 minutes. *H24K24.3(RNAi)* was not different from N2 at either timepoint. s*odh-1(ok2799);H24K24.3(RNAi)* was not different from N2 at either timepoint. * Significantly different from N2 at the same timepoint ($P < 0.05$) † Significantly different from the same strain across timepoints ($P < 0.05$). Error bars are SEM.

reported results, we found that internal ethanol concentration remains much lower than the exogenous dose. However, using this very precise method, we determined that the internal concentration of ethanol in these experiments is approximately 2x what we have observed in the past using different tissue preparation methods. For wild-type N2 animals exposed to 500 mM exogenous ethanol, at 10 minutes of exposure the tissue concentration was 67.5 ± 7.1 mM. The tissue concentration of ethanol increased over time; at 50 minutes of exposure, the tissue concentration was 89.3 ± 8.8 mM (P = 0.02 vs. 10 minutes; Figure 4B). This indicates that the development of acute tolerance that we observe over 50 minutes of exposure (Davies et al., 2004) does not reflect a decrease in internal tissue ethanol.

We sought an explanation for the differences in our previously published concentrations and what we have found here using this very sensitive protocol (Davies et al., 2003, 2004; Kapfhamer et al., 2008). Examining our previous protocol, we identified a major confound in the estimation of worm volume in the assay; we made an assumption that, in a pellet of worms that remains following centrifugation and removal of the supernatant, worms made up the vast majority of the volume of the pellet. However, when we calculated the volume of worms in a pellet by counting the worms and determining their volume, we found that this volume could vary by more than 2 fold depending on the size of the pellet and adult age of the worms, and at the most accounted for less than half of the volume (data not shown). When we used a known number of worms of known volume, our original spectrophotometric assay protocol closely recapitulated the internal ethanol concentration determined by gas

chromatography (Figure 4B), demonstrating that use of this simple protocol is acceptable for future internal ethanol concentration measurements in *C elegans*.

We predicted that the internal concentration of ethanol would be higher in animals in which ethanol metabolism is compromised. We determined the effect of inactivation of the two ADHs both singly and in combination on the accumulation of ethanol at the dose of exogenous ethanol at which we tested behavior, 400 mM. We determined the internal ethanol concentration after 10 and 50 minutes of ethanol exposure, to observe an acute accumulation of ethanol (10 minutes) as well as accumulation that could potentially be altered by the development of acute tolerance (50 minutes). Loss of *sodh-1* significantly increased the internal ethanol concentration in animals relative to wild type (Figure 4C). However, inactivation of *H24K24.3* did not significantly increase the internal ethanol concentration (Figure 4C). Interestingly, when we examined animals in which both ADHs were inactivated, *sodh-1(ok2799);H24K24.3(RNAi)*, the concentration of ethanol that the animals accumulated was similar to that of wild type at 10 minutes (Figure 4C), suggesting the possibility that loss of *H24K24.3* may trigger a compensatory response that minimizes the effect seen for loss of *sodh-1* alone.

Assessment of the ethanol-responsive behavioral consequences of compromising ADH function

We assessed the behavioral effects of altering ethanol metabolism on the locomotion of worms. We exposed wild-type and ADH-defective animals to exogenous ethanol and recorded their locomotion on plates after 10 minutes of exposure. 400 mM

was chosen as the upper dose for locomotion analyses using mutant or knockdown strains rather than 500 mM because we hypothesized that the metabolism-defective animals might show hypersensitivity to ethanol and we wanted to eliminate possible floor effects at 500 mM. Loss of *sodh-1* conferred mild but significant hypersensitivity to ethanol when the animals were tested on low (200 mM) and high concentrations (400 mM) of the drug (Figure 5A and 5B). Reduction of function of *H24K24.3* did not change the effects of ethanol on locomotion significantly, although there may be a trend towards increased sensitivity. Inactivating both *sodh-1* and *H24K24.3* did not increase the effect of ethanol on locomotion compared with the *sodh-1(ok2799)* mutation alone (Figure 4A). These results indicate that the difference in ethanol accumulation seen with *sodh-1(ok2799)* mutant animals is sufficient to alter the behavior of the animals when they are exposed to ethanol. Surprisingly, the measured internal ethanol concentration for animals with a loss of both *sodh-1* and *H24K24.3* was not different from wild-type animals (Figure 3C) and yet these animals show a significant increase in ethanol sensitivity compared with wild-type animals (Figure 5A).

Assessment of the ethanol-responsive behavioral consequences of compromising ALDH function

The second step of ethanol metabolism is the oxidation of acetaldehyde to acetate, catalyzed by aldehyde dehydrogenase (ALDH). We tested the effects of

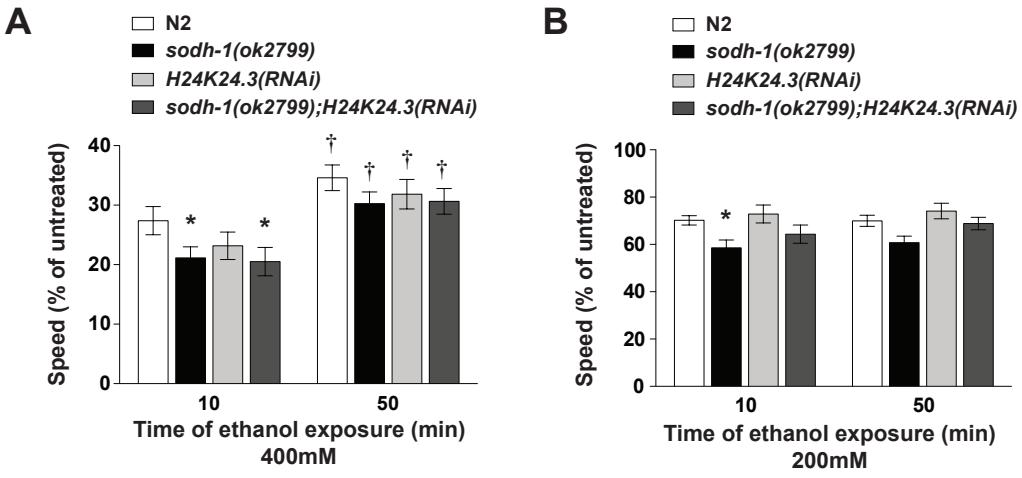


Figure 5. ADH compromised animals demonstrate behavioral sensitivity to ethanol, and develop acute functional tolerance to ethanol. Animals were treated continuously with exogenous ethanol, beginning at 10 minutes and at 50 minutes of exposure, two-minute digital movies were recorded, and speed was determined by ImagePro image analysis software. A % relative speed was calculated by dividing treated speed by untreated speed, to account for any baseline speed differences. (A) Animals treated with 400 mM exogenous ethanol (n = 12). Locomotion of wild-type N2 worms is strongly suppressed by this dose of ethanol. *sodh-1(ok2799)* and *sodh-1(ok2799);H24K24.3(RNAi)* strains are more strongly affected than N2 by ethanol at this dose, but *H24K24.3(RNAi)* animals are not significantly different from N2. Additionally, at this dose of ethanol, ADH mutant animals develop acute functional tolerance; each strain moved significantly faster at 50 minutes than at 10 minutes. (B) Animals treated with 200 mM exogenous ethanol (n = 14). At this dose, *sodh-1(ok2799)* and *sodh-1(ok2799);H24K24.3(RNAi)* strains showed enhanced behavioral sensitivity to ethanol relative to N2, but *H24K24.3(RNAi)* was not different from N2. At this dose, we do not observe development of acute functional tolerance. Error bars are SEM.

knocking down function of each of 10 *C. elegans* ALDH-like enzymes using RNAi. Inactivation of either *alh-6* or *alh-13* caused ethanol hypersensitivity (Figure 6A).

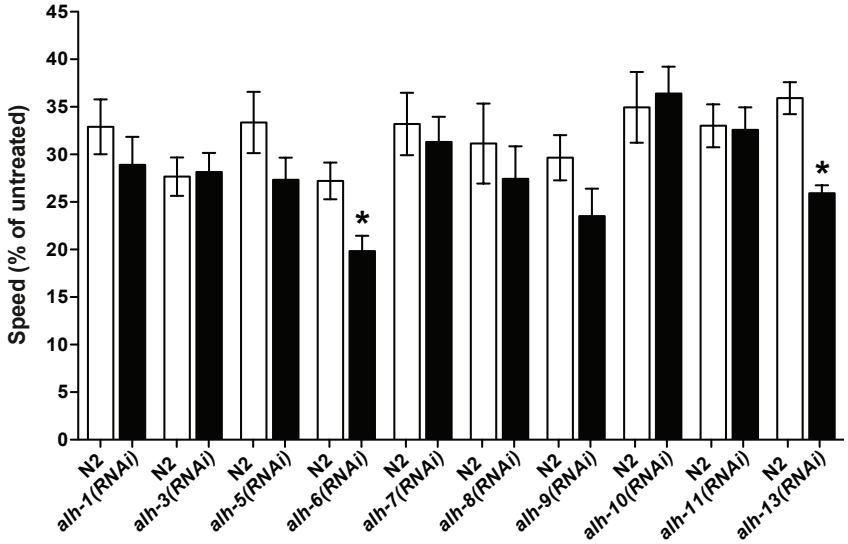
If ADH and ALDH act in a linear pathway, the phenotype of loss of the upstream gene should not be enhances by loss of the downstream gene. We tested this by knocking down function of either *alh-6* or *alh-13* in a *sodh-1(ok2799)* background, and found that the phenotype of the combinations was not different from that of *sodh-1* alone (Figure 6B, 6C). This result provides further support for the linear nature of the metabolism pathway, and further strongly suggests that the ethanol hypersensitivity phenotypes of *sodh-1(ok2799)*, *alh-6(RNAi)* and *alh-13(RNAi)* are due to changing ethanol metabolism.

The action of ADH is reversible; one possibility for the increase in ethanol sensitivity in *alh-6* and *alh-13* knock down animals is that the excess acetaldehyde in these animals is converted back to ethanol, thereby increasing the internal ethanol concentration. We observed an increase in internal ethanol concentration *alh-6(RNAi)* and *alh-13(RNAi)* but not *alh-1(RNAi)* (which did not show a change in behavior on ethanol) (Figure 7), suggesting that one effect of loss of ALDH function may be through increasing the effective ethanol dose.

Ethanol responses in* C. elegans *are sensitive to osmolarity

We next determined how ethanol affects *C. elegans* swimming in liquid. We found that ethanol inhibits swimming, although at a substantially higher dose

A



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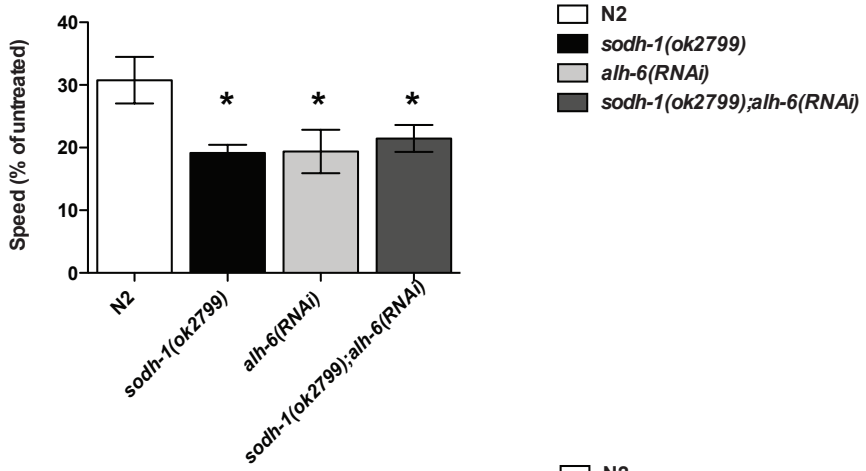

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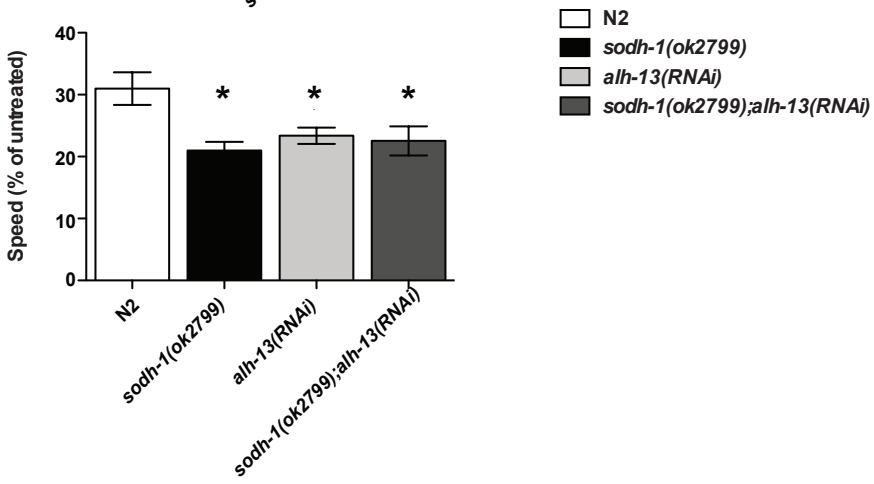


Figure 6: Behavioral effects of intoxication in wild-type and ALDH compromised animals. Animals were treated with exogenous ethanol for 10 minutes, two-minute digital movies were recorded, and speed was determined by ImagePro image analysis software. A % relative speed was calculated by dividing treated speed by untreated speed, to account for any baseline speed differences. In each case, the *alh* knockdown strain was compared to N2 animals tested on the same plates. (A) Knockdown of either *alh-6* or *alh-13* conferred hypersensitivity to the effects of ethanol on locomotion (*, $P < 0.05$). (B, C) Knockdown of neither *alh-6* (n = 6) nor *alh-13* (n =10) in the background of *sodh-1(ok2799)* was able to enhance the ethanol sensitivity beyond that of *sodh-1(ok2799)* alone. Error bars are SEM

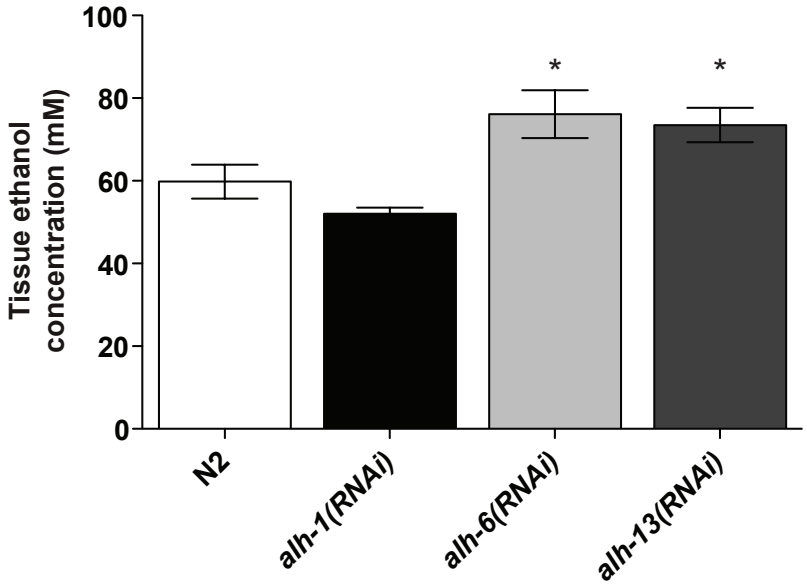


Figure 7. Determination of internal ethanol concentration in wild-type and ALDH compromised animals. Internal ethanol measurements of 200 worms, treated with 500 mM exogenous ethanol, calculated from spectrophotometric analysis (n = 3). *alh-1(RNAi)* served as a control for RNAi delivery and was not different from N2 at 10 minutes. *Alh-6 (RNAi)* and alh-13(RNAi) are significantly different from N2. * Significantly different from N2 ($P < 0.05$). Error bars are SEM.

than is required to inhibit crawling (Figure 8). While our results are in agreement with the first study of ethanols effects on swimming in *C. elegans* (Morgan and Sedensky, 1995), they differ significantly from those reported by Mitchell and colleagues (2007), who used slightly different conditions. We found that differences in our results from those of Mitchell and colleagues (2007) could be explained by the buffers used in our swimming analyses. Our study used NGM, which contains the same salts as the medium that animals are cultured on (Brenner, 1974), whereas the Mitchell study used Dent's buffer, a physiological saline normally used to record the electrical activity of dissected muscle (Avery *et al.,* 1995). While the basal swimming behavior of worms in the two buffers was indistinguishable (data not shown), we observed a striking difference in behavior in the two buffers when ethanol was added. Figure 8A shows the time course of intoxication in 500 mM ethanol in the two buffers. Swimming movement was quantified as the number of head swings during a 10 second time window. The motion of the animals exposed to ethanol decreased in both conditions by 5 minutes of exposure. However, animals assayed in Dent's buffer became essentially immotile by 10 minutes, whereas animals assayed in NGM buffer decreased motion but remained motile during the entire 20 minutes of treatment.

To examine the time course of intoxication during swimming more closely, we plotted matrices that represent body curvature along the anterior-posterior

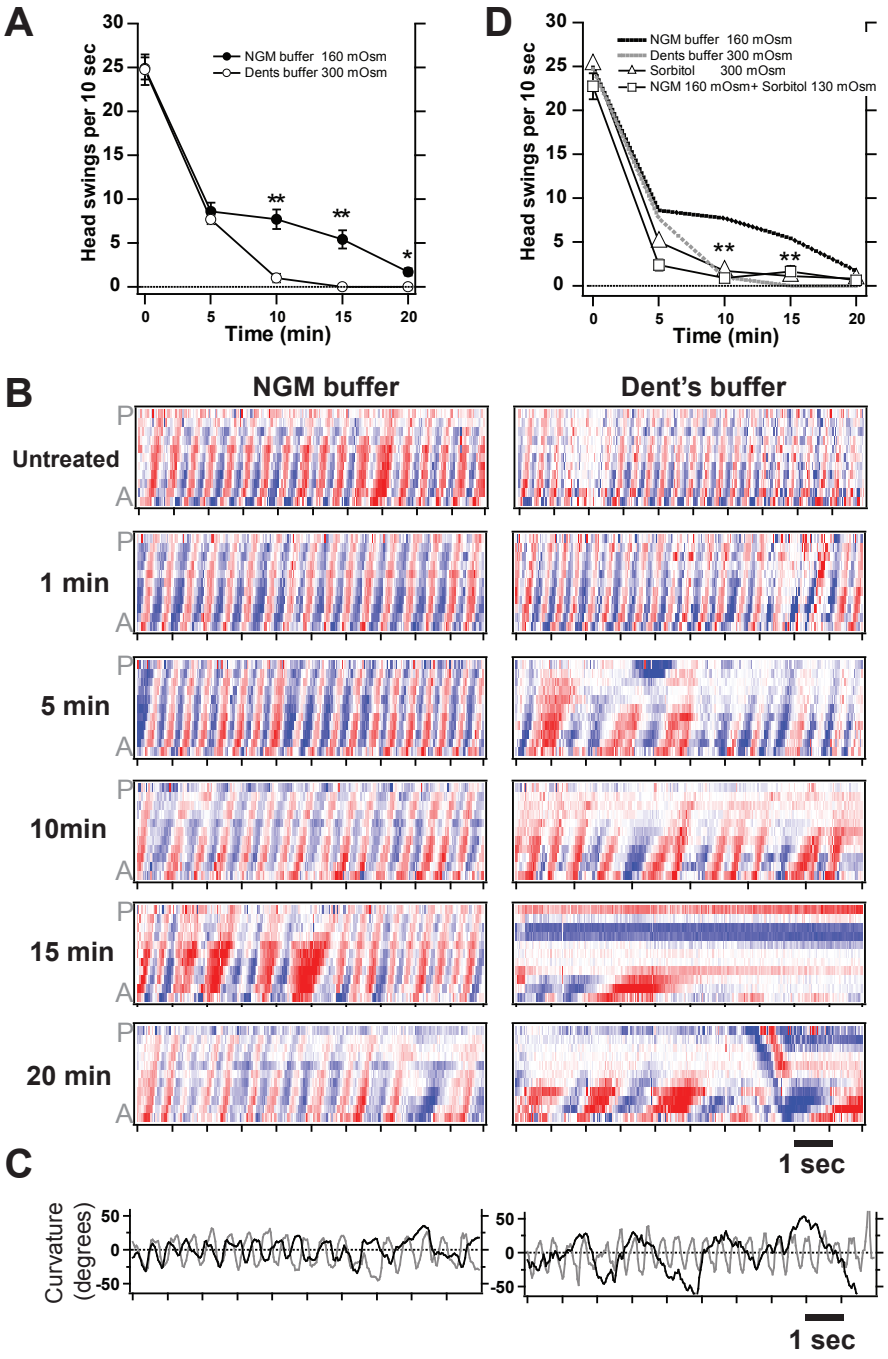


Figure 8. Sensitivity to intoxication while swimming depends on exogenous osmolarity. (A) Time course of intoxication while animals were swimming in 500 mM ethanol. Animals become significantly more immobilized by the same concentration of ethanol in Dent's buffer than in NGM buffer (**, P <0.001). (B) Body curvature matrices during intoxication for one representative animal in NGM buffer and one representative animal in Dent's buffer. Color intensity along the anterior-posterior (A-P) axis versus time represents the amount of bending at given points along the body (red = ventral, white = no bend, blue = dorsal). (C) Plots of neck curvature versus time. Untreated (grey) and 20 minute treatment in ethanol (black). (D) Time course of intoxication while animals were swimming in 500 mM ethanol. NGM and Dent's buffer data are replotted from panel (A). Animals treated with NGM + sorbitol or sorbitol alone were as sensitive to ethanol as those animals treated in Dent's buffer.

axis versus time (Figure 8B). In this scheme, upward slanting red and blue "waves" in the matrix represent ventral and dorsal bends that pass from head to tail to generate forward motion. The representative plot for an animal swimming in NGM buffer (Figure 8B, left column) shows little difference from its untreated condition even after 10 minutes in ethanol aside from a slight decrease in frequency and dampening of bends, which is evident in the muted red and blue color code. By contrast, the representative plot for an animal swimming in Dent's buffer (Figure 8B, right column) shows severe impairment of coordination, beginning at 5 minutes of exposure, reflected in the fact that many of the bends fail to propagate fully from head to tail. Portions of the animal also become immobile by 15 minutes, which is reflected in the fixed color pattern versus time. We also compared the swimming of untreated (grey line) versus 20-minute exposure to ethanol (black line) by plotting the "neck" curvature versus time (Figure 8C). The animal treated with ethanol in NGM buffer showed a slowing in frequency, while the animal in Dent's buffer showed a much slower bend frequency and a rise in maximal bend amplitudes.

While searching for factors that could explain the vastly different results obtained in the different buffers, we noticed that the two buffers differed greatly in osmolarity: NGM is 160 mOsm, while Dent's buffer is 300 mOsm. We tested if *C. elegans'* sensitivity to intoxication while swimming depends on osmolarity, and found that worms became rapidly intoxicated when assayed in NGM buffer in which we had adjusted the osmolarity to match Dent's buffer by adding 130 mOsm sorbitol (Figure 8D). Assaying worms in only 300 mOsm sorbitol without any salts reproduced a dose response for intoxication that was characteristic of Dent's buffer (Figure 8D). Moreover, we found that

pre-incubation for 20 minutes in Dent's buffer or 300 mOsm sorbitol conferred enhanced sensitivity to intoxication when the animals were assayed in 150 mOsm NGM buffer (data not shown). Together, these results suggest that acute sensitivity to exogenous ethanol in *C. elegans* depends on osmolarity, that this sensitivity can be dynamically adjusted, and that this is an explanation that can resolve the conflicting reports of dose response sensitivity to ethanol while swimming.

We tested the effect of osmolarity on the dose effect for ethanol on crawling, and found that the effect was significant, but more subtle than for swimming. At 10 minutes of exposure to 100 mM ethanol, the behavior of worms tested on NGM was less affected compared with worms tested on plates made with Dent's Saline (Figure 9A). We asked if the effect of osmolarity on the dose response for ethanol was due to altering the acute accumulation of ethanol. At 10 minutes of 100 mM ethanol exposure, worms on NGM plates accumulated significantly less ethanol than did animals on Dent's saline plates (Figure 9B).

Discussion

The ADH enzymatic pathway is shared throughout evolutionary history. Modulation of ethanol metabolism has been shown to have a variety of biological effects across phyla when animals ingest large quantities of exogenous ethanol. In *Drosophila melanogaster*, functional variations in both ADH (Geer et al., 1989; Ogueta et al., 2010) and ALDH (Wolf et al, 2002; Fry and Saweikis, 2006; Fry et al., 2008) contribute to variation in ethanol sensitivity. In humans, functional variants of both ADH and ALDH

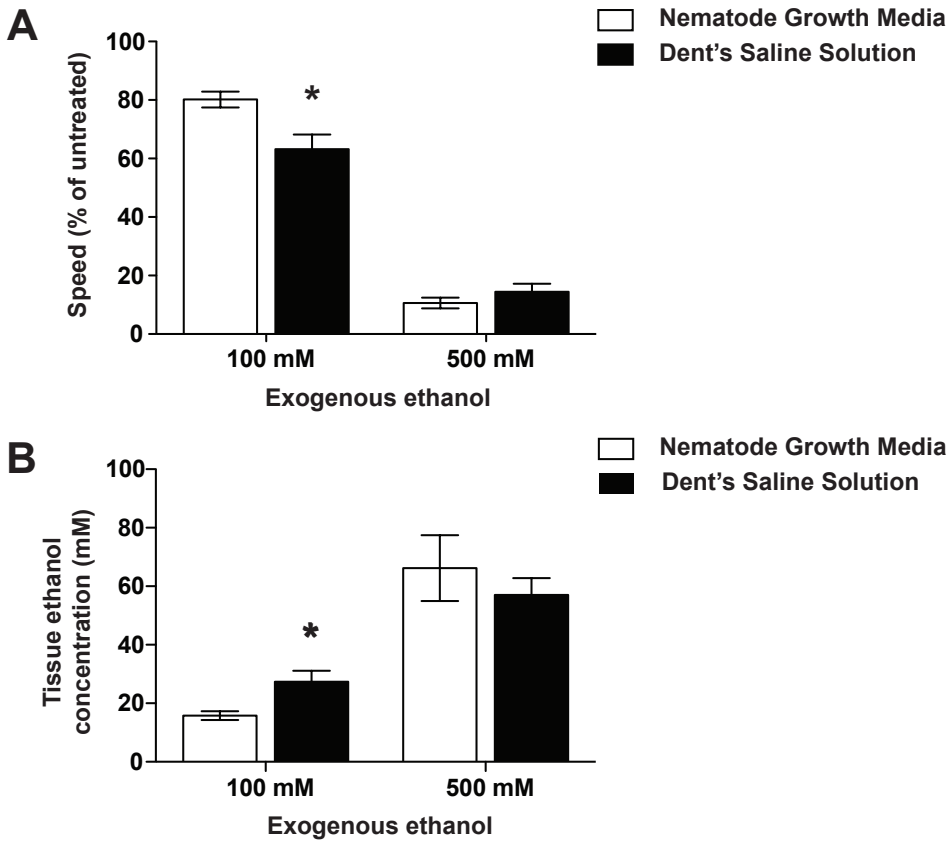


Figure 9. Sensitivity to intoxication and tissue accumulation of ethanol while crawling depends on exogenous osmolarity. (A) Animals were treated with exogenous ethanol for 10 minutes, two-minute digital movies were recorded, and speed was determined by ImagePro image analysis software. A % relative speed was calculated by dividing treated speed by untreated speed, to account for any baseline speed differences. Animals exposed to 100 mM exogenous ethanol for 10 minutes on NGM plates were less affected than animals exposed on Dent's Saline plates (100 mM n = 13; 500 mM n = 7). (B) Animals exposed to ethanol on NGM plates accumulated significantly more tissue ethanol than animals exposed on Dent's Saline plates (n = 3). * Significantly different from nematode growth meida ($P < 0.05$). Error bars are SEM.

have been correlated with altered susceptibility to becoming alcoholic. We have assessed the behavioral effects of altering alcohol metabolism in *C. elegans*.

We identified two ADHs in worms using a method described by Williamson *et al.,* (1991), in which we exploited the fact that ADH metabolizes allyl-alcohol to the toxin acrolein. Inactivation of either *sodh-1* or *H24K24.3* them conferred resistance to allyl-alcohol – induced lethality. We inactivated these two genes, both singly and in combination, in our analysis of ADH pathway function in the behavioral responses to ethanol.

To determine if altering ADH function results in differences in tissue levels of ethanol, we developed a method to accurately measure the internal ethanol concentration in ethanol-exposed *C. elegans*, in which we used a known volume of worm tissue and measured ethanol concentration using gas chromatography. The concentrations that we measured reflect the relative lack of permeability of these nematodes to chemicals in their environment; for 500 mM exogenous ethanol, the wild-type internal concentration is in the range of 70–90 mM (Figure 4B). This translates to blood alcohol concentration (BAC) values of 0.32–0.41%, which would cause profound intoxication in a naïve human drinker. The degree of intoxication associated with these concentrations in *C. elegans* is also profound, on an agar medium worms exposed to 500 mM ethanol move at approximately 20% of their untreated speeds (Davies et al., 2003, 2004 and see Figure 5A) the amplitude of their body bends is significantly reduced (Davies et al., 2003), and their movement becomes severely uncoordinated.

Previously, we reported internal ethanol concentrations that were approximately half of the concentrations that we observe here (Davies et al., 2003, 2004; Kapfhamer et al., 2008). We found that we had overestimated the volume of worms in a pellet derived from spinning worms out of a solution because we assumed that the vast majority of the pellet was made of worm tissue, which we have found here to be incorrect. This observation can also explain why Mitchell *et al.,* (2007) vastly overestimated the internal concentration in their animals; in their paradigm, they incubated worms in a high concentration of ethanol and then tested a pellet consisting of the resulting worm + ethanol solution. Our results suggest that the mixture probably contained relatively less worm tissue and more ethanol solution than they estimated, which would have contributed a significant amount of ethanol to the final concentration.

There is substantial evidence from human studies that an individual's naïve level of ethanol response is a predisposing factor in the development of alcoholism (Heath et al., 1999; Rodriguez et al., 1993; Schuckit et al., 2001; Schuckit, 1994; Volavka et al., 1996). Factors influencing an individual's LR to ethanol include both acute sensitivity as well as the magnitude and rate of development of acute tolerance during the ethanol exposure (Hu et al., 2008; Ponomarev and Crabbe, 2002). Here we show that altered ethanol metabolism in *C. elegans* affects the acute sensitivity of the worms but not the development of acute tolerance. First, ethanol tissue concentrations do not decrease during a single constant exposure (Figure 4B), so metabolism is not sufficient to reduce the concentration of ethanol below that seen at an early time point. Therefore, any reduction in the behavioral effects over this time course is likely to be due to compensatory mechanisms that act in the opposite direction to ethanol or directly limit

the action of ethanol. Second, ADH mutant animals develop acute tolerance similar to that displayed by wild-type animals showing that a reduction in metabolism does not impact the mechanisms of acute tolerance development (Figure 5A). This provides further support for the idea that acute tolerance is occurring at the level of the affected tissues, which in *C. elegans* includes the nervous system (Davies et al., 2003) rather than through a systemic metabolic process.

Our investigation into the reported differences in ethanol sensitivity for swimming behavior led to the unexpected finding that the worms' sensitivity to ethanol depends on the osmolarity of the external medium, and that the worms are able to change their sensitivity to the effects of osmolarity based on experience. Therefore, the constituents of the exogenous medium are critical to note in future alcohol studies using *C. elegans*. We speculate that the permeability of the worm to exogenous ethanol might change depending on the history of exposure. Dynamic permeability may be adaptive for an animal that must occasionally encounter dangerous chemicals, such as ethanol, while roaming through its natural soil environment. Future study of this phenomenon in *C. elegans* may give rise to novel strategies to alter excess permeation of ethanol into specific tissues to prevent toxicity.

In both vertebrates and *C. elegans*, ethanol metabolism makes important contributions to ethanol responsive behaviors. Our results suggest that both metabolism and environmental conditions should be considered in the analysis of mechanisms that contribute to ethanol responsive behaviors.

Chapter 3

Microarray Analysis of Ethanol Responsive Genes Reveals Role for ACS-2 in Acute Functional Tolerance in *C. elegans*

Introduction

Gene expression microarrays have been used extensively to capture mRNA changes associated with autism, breast cancer, cardiovascular and neurological diseases and many others (Ginsberg *et al*., 2012, Colombo, *et al.,* 2011, Seo *et al.,* 2006; Middleton, *et al.,* 2002). The goal of these studies is to identify genes whose expression correlates with the disease. Among these will be disease-related genes that are likely to reflect a combination of genetic factors such as individual variation and the results of dynamic interactions of environmental factors that ultimately contribute to the disease state (Chesler *et al.,* 2005). Thus, the gene expression response holds valuable information regarding underlying cellular mechanisms that contribute to disease pathology and etiology.

Over the past decade the approach of surveying gene expression has been taken with AUD. These studies include postmortem analysis of specific brain regions of alcoholic and non-alcoholic individuals (Lewohl *et al.,* 2000; Mayfield *et al*, 2002), and examining the genetic differences between inbred strains of mice that have opposite ethanol behavioral phenotypes such as initial sensitivity, acute tolerance and alcohol consumption (Xu *et al.,* 2001, Tabakoff *et al.,* 2003; Mulligan *et al.,* 2008, 2011;

Treadwell and Singh 2004; Wolstenholme *et al.*, 2011, Kerns *et al.,* 2005). The latter of the studies has taken an approach to study a specific phenotype or endophenotype that is related to alcohol dependence to uncover specific mechanisms involved in ethanol responses. In addition, these studies have implicated the importance of basal gene expression differences that contribute to ethanol induced gene expression.

Low level of response (LR) to the initial acute effects of ethanol has been shown to correlate with abuse liability and dependence in humans (Schuckit, 1994). This phenotype is significantly influenced by genetics (Heath *et al.,* 1999) and has been under intense investigation to understand how genetic differences can contribute to alterations in levels of response. LR is a composite phenotype consisting of at least two components, initial sensitivity and the development of AFT (Hu *et al.,* 2008; Ponomerev *et al.,* 2002).

The nematode worm, *C. elegans*, has been increasingly exploited as a behavioral model for understanding the genetic contributions to ethanol responses (Bettinger and McIntire, 2004; Davies *et al.,* 2003, 2004; Davis *et al.,* 2008; Graham *et al.,* 2009; Kapfhamer *et al.,* 2008; Lee *et al.,* 2009; Mitchell *et al.,* 2007, 2010; Morgan and Sedensky, 1995; Speca *et al.,* 2010). In *C. elegans*, acute behavioral responses to ethanol are influenced by genetics. In particular, genetic differences in neuropeptide Y receptor-like protein, *npr-1*, results in differences in the magnitude of AFT. AFT reflects a neuronal compensatory response that reduces the depressive effects of the drug (Davies *et al.,* 2004).

In this study, we compared the basal gene expression response of *npr-1(ky13)* animals with gene expression changes in *npr-1(ky13) and* wild type animals treated with

acute ethanol and then looked at the contribution of these differences in acute ethanol response behaviors. We found a significant overlap of ethanol gene expression between wild type and *npr-1(ky13)* mutants that related to a variety of biological processes including lipid metabolism. We focused on characterizing the role of *acs*-2, a gene involved in fatty acid activation for mitochondrial β-oxidation, in acute behavioral responses to ethanol and show that loss of function of *acs-2* results in reduced AFT. This work implicates the role of fatty acid metabolism and mitochondrial β-oxidationin cellular response to ethanol.

Materials and Methods

Nematode Culture and Strains

C. elegans were maintained using standard methods (Brenner, 1974). Strains used in this study were: N2 (var. Bristol), *npr-1(ky13)* (6x outcrossed), *acs-2(ok2457)* (2x outcrossed), *npr-1(ky13)*; *acs-2(ok2457)* and gpls1[*hsp-16-2*::GFP]. We generated double mutants by standard genetic crosses. Genotyping was as follows: the *ky13* allele generates a restriction fragment length polymorphism (RFLP) and the locus was amplified using *ky13*_SNP_F: 5' AGTGCGCTACAGCTTACTGACACCC 3' and *ky13*_SNP_R: 5' CGGTGCGATTGCTTTGGATCGGTA 3'. The amplicon was enzymatically digested using DraI. The wild type copy of *npr-1* is negative for digestion and produces a 592 base pair band after gel electrophoresis. The *ky13* allele is positive for digestion and produces a 592 base pair band. The *ok2457* allele of *acs-2* is a 1868 base pair deletion and was amplified using *acs-2*_F1: 5' CCAAATGGCGTTCTCTGCTCAGT 3' and *acs-2*_R1: 5'

CAATCATGTCACGAGTTCTACCG 3'. Animals carrying the wild-type copy of *acs-2* produce a 2367 base pair band and animals carrying *acs-2(ok2457)* produce a 499 base pair band.

Microarray Analysis

RNA Preparation for Microarray

Prior to performing the microarray analysis, we outcrossed *npr-1(ky13)* animals 6 times to our laboratory wild-type N2 strain to any reduce potential noise that may arise in the downstream analysis due to extraneous mutations in each genetic background. These animals were tested behaviorally for their response to ethanol and were not significantly different than non outcrossed *npr-1(ky13)* animals (data not shown). Age matched first day adult animals were exposed to either 0mM or 500mM ethanol for 2 hours. We chose 2 hours to capture gene expression changes because we predicted that this population of mRNA might represent the genes regulated by the early response genes to ethanol such as transcription factors. Whole animals were homogenized in TRIzol Reagent (Life Technologies, Carlsbad, CA) by freezing and thawing and total RNA was isolated according to the manufacturer's protocol. Total RNA was subsequently purified using RNeasy Mini Kit (Qiagene, Valencia, CA). RNA concentrations were determined by absorbance at 260 nm using NanoDrop 2000 (Thermo Scientific, Waltham, MA) and RNA quality and integrity was assessed by using Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). Total RNA samples were then prepared for hybridization according to manufacture's protocol using GeneChip 3' IVT Express Kit (Affymextrix, Santa Clara, CA). Briefly, total RNA was

converted into single stranded cDNA using T7-Oligo(dT) primers followed by a second strand synthesis step. The double stranded cDNA was then transcribed into complementary RNA (cRNA). cRNA is amplified and biotinylated for 16 hours. Amplified biotinylated cRNA was then purified using magnetic RNA binding beads and cRNA binding buffer to remove enzymes, salts and unincorporated nucleotides. Purifed cRNA was fragmented and both unfragmented and fragmented cRNA was analyzed using the Experion Automated Electrophoresis System (Bio-Rad). We observed size distributions in the range recommended (100-120 nucleotides) by the manufacturer, indicating successful amplification and fragmentation.

Hybridization and Scanning

Each sample (n=16) was hybridized to an individual GeneChip *C. elegans* Genome Array (Affymetrix, Santa Clara, CA) that contains greater than 22,500 transcripts for 16 hours in a hybridization oven at 45 °C using the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). Microarrays were washed and stained using a Fluidics Station 450 (Affymetrix) and the corresponding fluidics protocol for *C. elegans* genome arrays. The microarrays were then scanned using the GeneChip Scanner 3000 (Affymetrix) according to manufactures protocol.

Microarray Data Analysis

Prior to statistical analysis of data, we analyzed a series of quality control metrics to ensure data quality as previously described (Kerns *et al*., 2005; O'Brien *et al.*, 2012). Frist, we processed our data using Microarray Suite software version 5.0 (Affymetrix) to

determine the overall percentage of probes called present. Probes are given a p-value that assesses the reliability of detection. This is done by using a singed rank test to consider the significance of the difference between the perfect-match probe signal value and the mismatch probe signal value. We observed that regardless of treatment and genotype about 54% of probes were called present (data not shown). We then processed our data using Robust Multi-array Analysis (RMA) algorithim to increase sensitivity of detecting small changes between samples (Irizarry *et al.,* 2003). To monitor the entire amplification and labeling process, samples were spiked with ploy-A RNA controls that were provided by the manufacturer. All of the ploy-A controls were called present at the expected order of signal magnitude. We also included hybridization controls supplied by the manufacture to evaluate the sample hybridization efficiency. Hybridization controls were spiked into samples independent of the poly-A controls. All hybridization controls were called present. Both hybridization and labeling controls validated the detection sensitivity of 1:100,000 of the microarrays. We also performed pair-wise linear correlation plots between all microarrays and found a very high Pearson's correlation coefficient value of $r^2 \geq 0.985$ indicating very low technical error (Figure 10). We then filtered our data for all probes sets that were called absent across all microarrays. We used TIGR MultiExperiment Viewer (TMeV) (version 4.8) (Saeed *at al.,* 2003, 2006) and performed a statistical analysis of microarrays (SAM) to identify probe sets with intensity values that were significantly different. We performed a two class SAM for: N2 untreated and N2 treated to produce the N2 ethanol response; *npr-1(ky13)* untreated and *npr-1(ky13)* treated to produce the *npr-1(ky13)* ethanol response; and N2 untreated and *npr-1(ky13)* untreated to produce the *npr-1(ky13)* basal response.

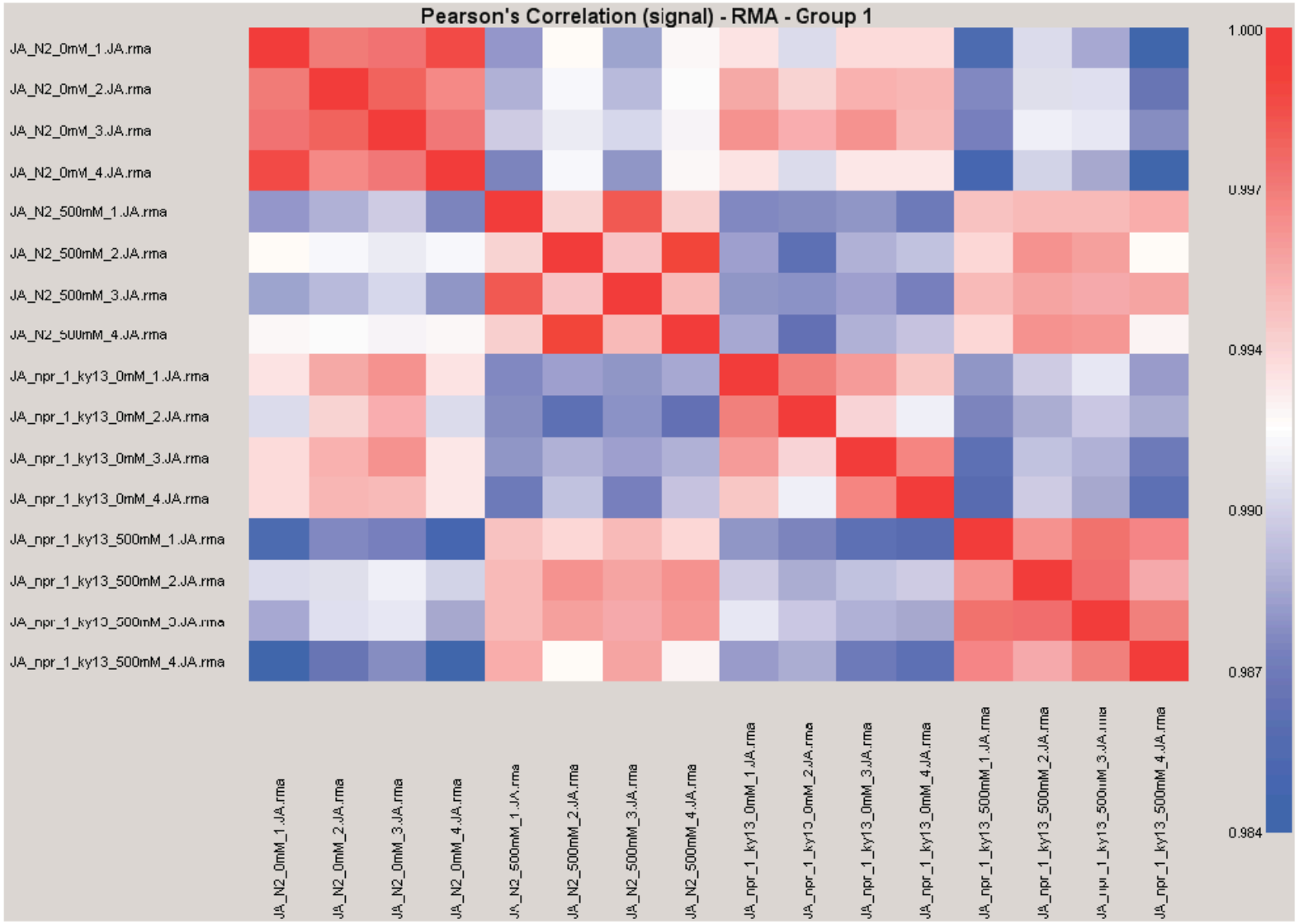


Figure 10. Pearson's correlation coefficient for signal intensity across all microarrays. Correlation coefficients were greater than 0.985 suggesting low technical error (O'Brien *et al.*, 2012). Correlation values are scaled from 0.984 – 1 and are shaded degrees of blue and red to represent the correlation values. A perfect correlation is 1. Squares shaded red indicate correlation values greater than 0.994. Squares shaded blue indicate correlation values less than 0.990 but greater than 0.984.

We used a false discovery rate of (FDR) ≤ 5 %. Genes that showed significant changes were further analyzed for gene ontology and degree of similarity across each treatment.

Bioinformatics Analysis

We analyzed each transcriptional profile to identify any over represented biological themes using the Database for Annotation Visualization and Integrated Discovery (DAVID) (Dennis *et al.,* 2003). We uploaded 442 probe set extensions to DAVID and 441 were accepted for analysis. We used a $P \leq 0.05$ as a cut-off for significant terms. To determine the degree of similarity between transcriptional profiles we used the geneset comparison tool in GeneWeaver (Baker *et al.,* 2012). We also used GeneWeaver to convert Affymetrix probe identifiers (IDs) into Wormbase gene IDs.

Internal Ethanol Concentration

Internal ethanol concentrations were assessed as previously described (Alaimo *et al.,* 2012, Chapter 2, Appendix A). Briefly, age matched worms were photographed and exposed to ethanol for 10 or 30 minutes. Exactly 200 worms were picked into 20 µL ddH_20 and homogenized. Homogenates were tested for ethanol concentration according to the manufacturer's protocol using an Alcohol Reagent Kit (Pointe Scientific, Canton, MI). 1.5 µL of worm homogenate was added to 300 µL alcohol reagent and incubated for 5 minutes at 30°C. To stop the reaction, the tubes were placed on ice and absorbance was measured at 340 nm using a spectrophotometer (Bio-Rad). The

volume of the animals was determined from the photographs by calculating the volume of a cylinder and the $C_1V_1=C_2V_2$ formula was used to calculate the final concentration.

Real-Time Quantitative PCR

Whole animals were homogenized in TRIzol Reagent (Life Technologies) and total RNA was extracted and purified using the RNeasy Mini Kit (Qiagene). 1 ug of total RNA was DNase I treated to remove any contaminating DNA (Life Technologies) and converted into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). Designed primers were tested for efficiency (See Appendix C) and used in the detection of message levels using SensiMix SYBR green (Bioline, Taunton, MA) and the iCycler iQ system. (Bio-Rad). The following primers were use for qRT-PCR for detection of *dlc-2* and *ech-6: dlc-2*_F1 5' ATGGAAGATCCACAACGAGA 3' , *dlc*-2_R1 5' GACTCCCGAAACACTTTCA 3' and *ech-6*_F2 5' CCCACTGTGGCTTTCTCTTC 3' , *ech-6*_R2 5' TCGCTGATCAATCTCCACTG 3' . Final fold changes were calculated using $2^{-\Delta\Delta Ct}$.

Analysis of Speed

Speed of animals was assessed as previously described (Davies *et al*., 2003, 2004, Kapfhamer *et al.,* 2008, Alaimo *et al*., 2012 Bettinger *et al*., 2012 and Appendix A). Briefly, nematode growth media (NGM) plates were dried at 37°C for 2 hours. Copper rings were melted into the surface of the plates and 100% ethanol was added 2 hours prior to the assay to a final concentration of 0mM, 200mM, or 400mM. Age matched animals were acclimated to assay conditions 30 minutes prior to assay. Ten

worms of each test strain were moved into a copper ring on an assay plate and 2-minute movies of their movement were recorded at 10 and 30 minutes of exposure. All assays were recorded using a Retiga4000R camera (Qimaging, Canada) and ImagePro Plus (6.2) (Media Cybernetics, Bethesda, MD) software was used to record and analyze movies.

RNA Interference

RNA interference (RNAi) was performed as previously described (Kamath *et al.,* 2003). Briefly, bacteria containing the L4440 plasmid plus insert from the RNAi feeding library generated by J. Ahringer at the University of Cambridge (Geneservice, Cambridige, UK) were grown with shaking at 37°C in Luria broth supplemented with 50 µg/ml ampicillin salt (LB+AMP) for 18 hours. Cultures were then streaked on NGM plates supplemented with 50 µg/ml ampicillin and 12.5 µg/m tetracycline. Plates were incubated at 37°C for 24-48 hours and single colonies were picked and grown in LB+AMP for 18 hours at 37°C with shaking. RNAi plasmids were isolated using an alkaline lysis miniprep. Briefly, 1.5 mL of pelleted RNAi containing bacteria was incubated in a glucose/tris/EDTA solution for 5 minutes at room temperature. NaOH/SDS was added to the samples, mixed, and incubated on ice for 5 minutes. 5 M potassium acetate was added to the sample, mixed, and incubated for 5 minutes on ice. Samples were incubated for 2 minutes in 95% ethanol to precipitate DNA. Samples were microcentrifuged and resulting pellets were washed with 70% ethanol and resuspended in ddH_20. Isolated plasmids were sequenced using the U19 primer. BLAST was used to confirm the identity of the insert. Once the identity of the insert was

confirmed, NMG plates supplemented with 1 mM IPTG and 25 µg/ml carbinicillin were seeded with bacteria containing L4440 (control) or the RNAi construct to be tested 24 hours prior to use. Three to five L4 stage wild-type N2 animals were placed on seeded plates and incubated for 36 to 40 hours at 20°C. Adult animals were moved to a new RNAi plate and first-day adults from their progeny were assayed.

Transcriptional Inhibition

Age matched adult animals carrying *hsp-16.2*::GFP were heat shocked at 35°C for 1 hour in M9 buffer. Animals were allowed to recover for 60 minutes on NGM plates without food and whole worm images were taken using a GFP microscope. We measured the intensity of GFP expression in the posterior blub, isthmus, and metacorpus of the pharynx using ImagePro Plus (6.2) (Media Cybernetics, Bethesda, MD) software. We choose these three regions because hsp-16.2 is known to be expressed in the pharynx (Strayer *et al.,* 2003) and the structures were easily disguisable.

To determine if actinomycin D inhibited transcription we pretreated *hsp-16.2*::GFP animals with 100 µg/mL of drug for 30 minutes in M9 prior to and during heat shock and allowed animals to recover for 60 minutes on NGM plates without. We chose 100 µg/mL based reports from other studies that have used other transcriptional inhibitors in worms (Rogalski *et al.,* 1990).

Next, we treated both N2 and *npr-1(ky13)* mutants with 100 µg/mL of actinomcyin D for 30 minutes in M9 buffer. Immediately after drug exposure animals were placed on

an acclimation plate for 30 minutes and the speed of locomotion was measured as described above.

Statistics

To determine statistical significance ($P \leq 0.05$) in studies comparing two or more groups, *t* tests, one- and two-way analysis of variance (ANOVA) (Prism, GraphPad, San Diego, CA, USA), followed by Bonferroni multiple comparison tests were performed as appropriate. The SAM was performed as described in Microarray Data Analysis.

Results

Microarray Analysis of Ethanol Responsive Genes in *C. elegans*

Previous studies have shown that NPR-1 antagonizes the development of acute functional tolerance (AFT) to ethanol and that extended exposure to ethanol can alter the function of this pathway (Davies et al., 2004, Bettinger et al., 2012). Prior to performing a microarray analysis of ethanol responsive genes we hypothesized that transcriptional changes that occur during acute ethanol exposure may be required to observe acute behavioral responses to ethanol. Therefore, we globally blocked transcription including the acute ethanol response genes and measured acute behavioral responses to ethanol. We used animals carrying a *hsp-16.2*::GFP to determine the time course of GFP expression of animals heat shocked for 1 hour at 35°C and found a significant increase in GFP expression after 60 minutes post heat shock (data not shown) and therefore used this time point to measure the degree of

transcription. We pretreated *hsp-16.2*::GFP animals with actinomycin D for 30 minutes and during the 60 minute heat shock incubation. We found that GFP expression was significantly reduced in actinomycin D treated heat shocked *hsp-16.2::GFP* animals relative to untreated and heat shocked animals (Figure 11A) ($P < 0.001$) suggesting that 100 µg/mL of actinomycin D can inhibit transcription. Next, we treated wild-type N2 and *npr-1(ky13)* mutants with 100 µg/mL of actinomcyin D and found that both animals did not display any differences in initial sensitivity or the development of AFT (Figure 11B-C). These results suggest that transcription is dispensable for the initial response to ethanol. Furthermore, these results also suggest that basal gene expression may play an important role in behavioral responses to ethanol. Therefore, we hypothesized that basal gene expression differences between N2 and *npr-1(ky13)* may reveal genes that are involved in the acute behavioral differences to ethanol between these animals.

Ethanol regulation of gene expression may also provide insights into the biological mechanisms of cellular responses to ethanol. Identifying ethanol responsive genes may uncover genes involved in a process in a cell that is specific to ethanol. Furthermore, when the basal or initial functional of the gene involved in a specific cellular process is altered it may lead to differences in acute behavioral response to ethanol.

To identify differences in mRNA levels, we performed a whole-genome expression analysis using Affymetix GeneChip *C. elegans* Genome Arrays on wild-type N2 and *npr-1(ky13)* animals exposed to 500 mM exogenous ethanol for two hours. We identified changes in gene expression by performing a two class SAM for: N2 untreated

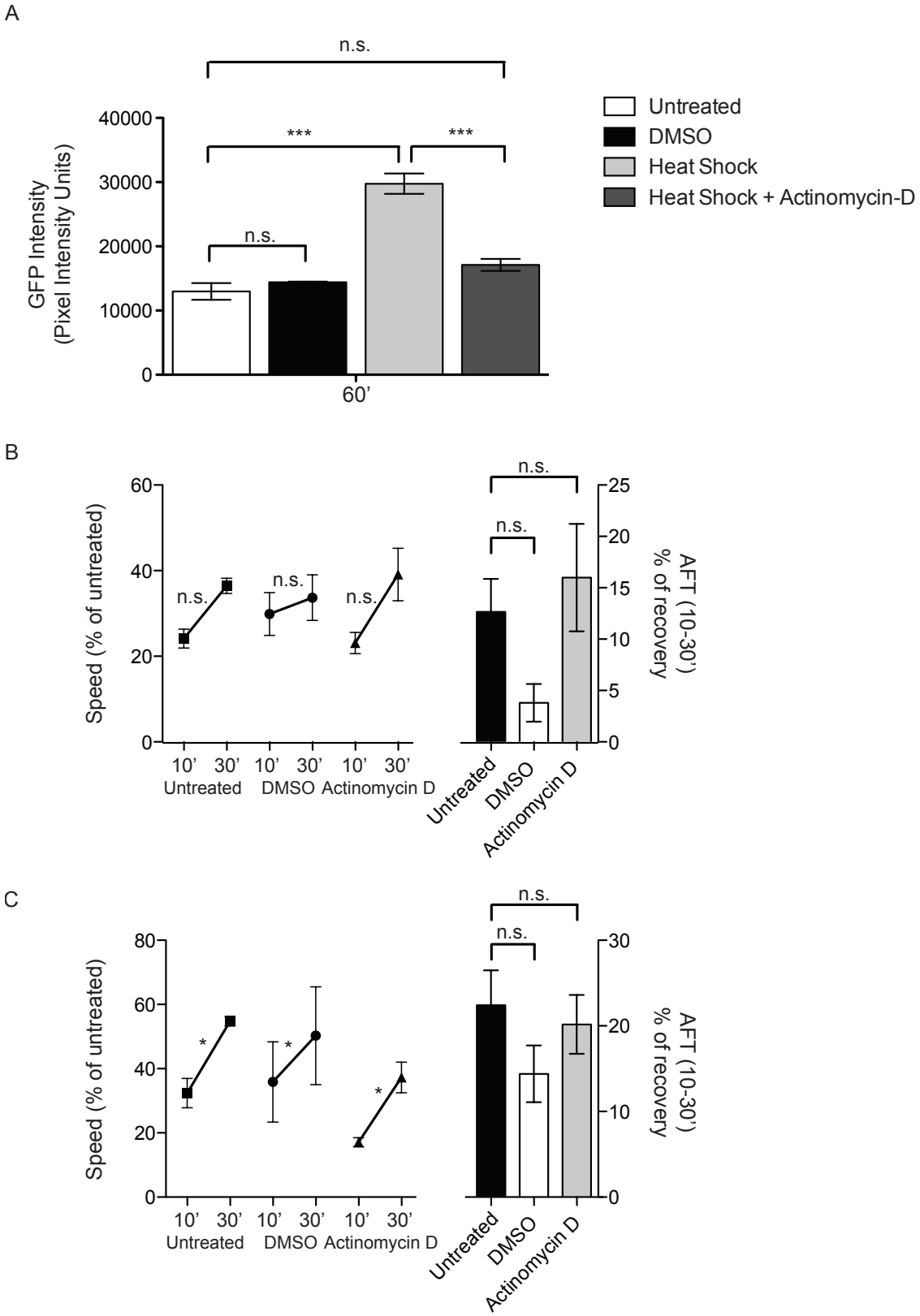


Figure 11. Inhibition of transcription does not alter the development of acute functional tolerance to ethanol. (A) Animals carrying *hsp-16.2*::GFP were heat shocked at 35°C for 60 minutes in the presence or absence of 100 µg/mL actinomycin D and allowed to recover for 30 minutes. 60 minutes post recovery, heat shocked animals had a significant increase in GFP intensity and animals treated with 100 µg/mL of actinomcyin D had similar a GFP intensity as untreated animals and significantly reduced GFP intensity compared to heat shock. The vehicle for actinomycin D, DMSO had a similar GFP intensity compared to untreated (n = 3). (B) Wild type N2 animals were treated for 30 minutes with 100 µg/mL actinomycin D. Inhibition of transcription did not alter the initial sensitivity or the development of AFT relative to untreated (n =3). (C) *npr-1(ky13)* mutant animals treated with 100 µg/mL actinomycin D also did not display a significantly different initial sensitivity or AFT (n = 3). * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. n.s., not significant. Error bars are SEM.

and N2 treated to produce the N2 ethanol response; *npr-1(ky13)* untreated and *npr-1(ky13)* treated to produce the *npr-1(ky13)* ethanol response; and N2 untreated and *npr-1(ky13)* untreated to produce the *npr-1(ky13)* basal response., We found that *npr-1(ky13)* had 221 basal gene expression differences (214 up regulated and 7 down regulated) and ethanol treatment of these animals resulted in expression changes in 611 genes (493 up-regulated and 118 down-regulated). Ethanol treatment of N2 caused changes in 499 genes (442 up regulated and 57 down regulated). Next, we performed an analysis of similarity between all three gene expression profiles (N2 ethanol, *npr-1(ky13)* ethanol, and *npr-1(ky13)* basal) using the geneset comparison tool in Geneweaver (Baker et al., 2012). Ethanol response profiles between N2 and *npr-1(ky13)* had significant overlap of 408 genes (P = 1.332E-15) and 22 of these genes were also basally regulated by *npr-1(ky13)* (Figure 12). In addition, 15 genes were basally different in *npr-1(ky13)*, but regulated ethanol only in N2 (Figure 12).

Assessing neuronal expression

Prior to performing our candidate gene selection analysis, we investigated the sensitivity of the microarray detailing the expression of genes that have expression that is restricted to the nervous system. Since previous studies have implicated a role for neurons in the acute behavioral response to ethanol, we confirmed that we are able to detect neuronally expressed genes in our analysis (Davies *et al.,* 2003, 2004).
We utilized a publically available database that contains the annotations for a broad range of neuronally expressed genes and a literature search for genes with expression that is either pan-neuronal, specific to subsets of neurons, or specific to single neurons

(Lockery Lab Neuron-Specific Promoters Database, 2001, http://chinook.uoregon.edu/promoters.html). The Lockery database contains 218 neuronal markers and studies by Ruvinsky *et al* (2007) have confirmed the expression of 234 broadly expressed neuronal genes. Some of the genes identified in the Ruvinsky *et al.,* 2007 were present in the Lockery Lab database, however the Lockery Lab database contained genes that were expressed in tissues other than neurons.

We tested 28 probe set IDs for detection on wild-type N2 gene expression microarrays and found that 19 of the probes were called present by the MAS5 detection algorithm (Table 4). A majority of neuronally expressed genes that we examined were pan-neuronal or broadly expressed. We were also able to detect genes expressed in neuron pairs AFS, ASI, and BAG. In addition, the probe set IDs for *nhr-38* and *daf-7* are entirely unique for these genes, but the probe set for *gcy-33* is not unique and therefore cross hybridization of genes homologous to *gcy*-33 may also be represented to achieve a signal intensity that allows detection. However, we did not observe detection for some probe set IDs known to be expressed in neurons. This group of genes included both pan-neuronal and individual neurons. There are a variety of explanations for why we may have been unable to detect expression of these genes. One possible explanation is that these particular genes are expressed below detectable levels. These data indicate that we have neuron only gene expression represented in our results and we were able to detect these genes at the level of only two cells for AFD, ASI and BAG neurons.

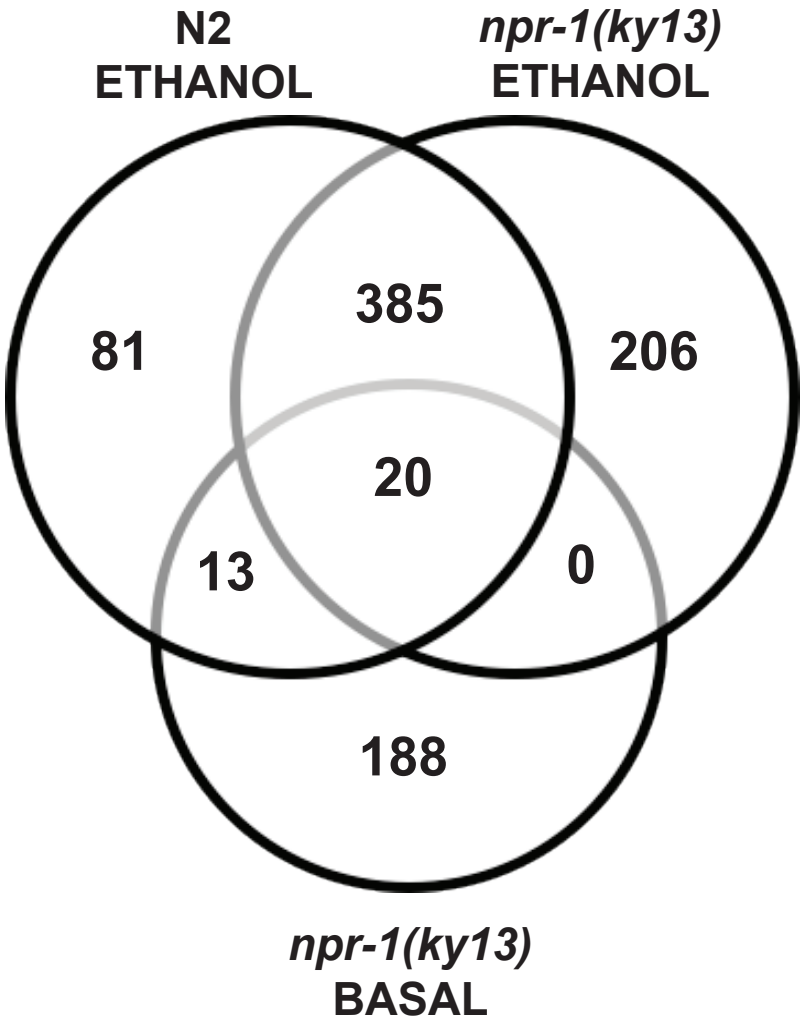


Figure 12. Gene expression overlap between N2 ethanol responsive gene expression, *npr-1(ky13)* ethanol responsive expression, and *npr-1(ky13)* basal gene expression differences profiles. Ethanol treatment resulted in expression changes in 499 genes and 611 genes in N2 and *npr-1(ky13)* respectively. 408 genes were present in both ethanol profiles and the degree of overlap was very significant (*P*=1.332E-15). *npr-1(ky13)* had 221 genes differentially expressed basally and 20 of these genes were ethanol responsive in both N2 and *npr-1(ky13)*. Additionally, 13 genes that were basally different in *npr-1(ky13)* were also ethanol responsive in only N2.

Table 4. Detection of neuronally expressed genes by microarray analysis for wild-type N2

Gene Symbol	Neuronal Location	Probe ID	Detection
jnk-1	All Neurons	175640_at	Yes
		190415_s_at	Yes
rab-3	All Neurons	192427_s_at	Yes
unc-13	Ventral and dorsal nerve cord, nerve ring, head, tail, and touch neurons	171998_x_at	Yes
		191489_s_at	No
unc-76	Ventral and dorsal nerve cord, nerve ring, HSNL, HSNR, CANL, CANR	191703_s_at	Yes
		175598_at	Yes
unc-104	Ventral and dorsal nerve cord, AWC, nerve ring, tail	175742_at	Yes
		188472_s_at	Yes
ehs-1	Ventral and dorsal nerve cord, nerve ring	188120_at	Yes
mec-3	Touch cells, FLP, PVD	194097_s_at	Yes
unc-14	VD and DD – Motor Neurons	174402_at	No
		188044_s_at	Yes
		175683_s_at	Yes
flp-10	AIM, ASI, AUA, BAG, BDU, DVB, PQR, PVR and URX	188393_at	Yes
odr-10	AWA, CEP	187746_at	Yes
ocr-1	AWA, ADL	189270_at	Yes
nhr-38	AFD	193582_at	Yes
daf-7	ASI	193876_at	Yes
gcy-33	BAG	191874_s_at	Yes
		174520_s_at	Yes
str-2	AWC	175756_s_at	No
jkk-1	All Neurons	188020_s_at	No
sng-1	All Neurons	191339_at	No
unc-75	All Neurons	193998_at	No
		183050_at	No
gcy-5	ASER	191609_at	No
gcy-6	ASEL	193179_at	No

Gene ontology enrichment analysis of ethanol responsive genes reveals similar biological processes are induced in wild type and *npr-1(ky13)*

To gain biological insight into the similarity between ethanol response profiles, we performed a gene ontology enrichment analysis for biological process and molecular function using DAVID (Dennis *et al.,* 2003). We found that the overlap between N2 and *npr-1(ky13)* ethanol responsive genes were significantly enriched ($P < 0.05$) for biological processes involved in oxidation reduction, lipid glycosylation and metabolism, transcription, and others (Table 5). The oxidation reduction group included genes from the cytochrome P450 family, flavin-containing monooxygenase family, and multiple types of dehydrogenases. Interestingly, we have previously examined the two dehydrogenases, D2063.1 and K12G11.4 in *C. elegans* for their role in ethanol metabolism and behavior. We found that knockdown by RNAi of either gene did not confer resistance to an allyl-alcohol toxicity assay (Alaimo *et al.,* 2012; Chapter 2). This assay has been used to identify mutants with defects in alcohol dehydrogenase activity (Williamson *et al.,* 1991) suggesting that animals are unable to metabolism alcohol (Chapter 2). The lipid glycosylation and metabolism group consisted of only UDP-glucuronosyltransferases, suggesting that ethanol induces changes in the expression of genes regulating fatty acid modification of lipids. The transcription and regulation of transcription group included genes from the nuclear hormone receptor family.

We also used DAVID to determine if there was over representation of a particular molecular function between both ethanol response profiles. We found that iron ion binding was the most enriched function and 25 of 37 of the genes in this category belonged to the cytochrome P450 family (Table 5). The cytochrome P450 family was

Table 5. Gene ontology enrichment analysis for biological process and molecular function.

Biological Process	Genes	Category Total	*P*-value
Oxidation reduction	41	382	4.3E-16
Lipid glycosylation, modification	13	44	2.1E-10
Response to xenobiotic stimulus	4	6	2.4E-04
Regulation of transcription	24	495	1.2E-03
Cellular homeostasis	7	91	2.0E-02
Glycerol and alditol metabolic process	3	15	4.7E-02

Molecular Function	Genes	Category Total	*P*-value
Iron ion binding	37	262	3.0E-15
Electron carrier activity	32	206	2.5E-14
Heme and tetarapyrole binding	27	145	5.3E-04
Metal ion binding	86	1910	4.0E-08
Steroid hormone receptor activity	24	282	6.1E-05
Cofactor binding	19	226	1.1E-04
Flavin-containing monooxygenase activity	4	7	8.1E-04
Carbohydrate binding	19	177	2.1E-03

also represented in the electron carrier activity, heme binding, and cation binding categories. Carbohydrate binding and steroid hormone receptor activity were also significantly represented. These categories were enriched for genes encoding nuclear hormone receptors (Table 5).

Selection of gene candidates for behavioral testing

We used *npr-1(ky13)* basal gene expression differences as a tool to enrich for candidate genes predicted to be involved in ethanol responsive behaviors. Altering the basal function of *npr-1* results in an enhanced AFT phenotype. This suggests that the functions of the cells in these animals are fundamentally different in their response to ethanol than wild-type animals. Therefore, these genes represent a population of putative candidate genes involved in ethanol responses. 221 genes were differentially expressed in *npr-1(ky13)*. We performed a similarity analysis between the *npr-1(ky13)* basal gene expression profile and both ethanol response profiles and found that 20 genes were differentially expressed basally in *npr-1(ky13)*, but were also regulated by ethanol in both N2 and *npr-1(ky13)* (Figure 11 and Table 6). These genes are the basis of our list of candidate genes that influence ethanol response behaviors because they are basally different, but also ethanol responsive. Additionally we found that 12 genes were differentially expressed basally in *npr-1(ky13),* but only regulated by ethanol in N2. These genes may represent another class of genes involved in AFT. For example, *npr-1(ky13)* animals may respond differently to ethanol because their basal gene expression differences has altered the intrinsic properties of their cells. Therefore, they adapt better

Table 6. Candidate genes from similarity analysis of all expression profiles.

Gene Symbol	N2 Basal RMA Value	N2 Ethanol RMA Value	*npr-1* Basal RMA Value	*npr-1* Ethanol RMA Value	N2 Ethanol Fold Change	*npr-1* Basal Fold Change	*npr-1* Ethanol Fold Change
acs-2	8.876	7.793	9.878	8.267	0.466	1.9831	0.3291
B0564.3	6.190	5.116	7.129	5.571	0.472	1.9519	0.3303
C15C8.3	9.630	10.728	10.328	11.191	2.123	1.6184	1.8193
C25H3.10	7.532	6.382	9.370	6.525	0.444	3.5789	0.1369
cdr-2	6.293	10.498	7.057	10.835	18.328	1.6938	13.7928
dct-7	8.048	6.053	9.393	6.352	0.250	2.6897	0.1167
F15E6.3	9.081	7.616	10.266	7.879	0.361	2.2685	0.1946
F37H8.3	5.765	8.420	6.759	9.051	6.146	2.0110	4.7460
F49C12.7	6.113	9.099	6.783	9.315	7.890	1.5947	5.7561
fmo-2	6.081	11.105	7.207	11.393	31.983	2.1921	17.3623
hpd-1	7.922	10.264	8.567	10.107	5.490	1.5637	2.9332
hrg-1	7.619	9.331	8.562	9.888	3.246	1.9135	2.5100
irg-2	6.673	8.589	7.390	9.324	4.248	1.6521	4.1932
pgp-14	6.813	8.353	7.539	9.128	2.924	1.6489	3.0081
pqn-31	8.319	9.020	8.904	9.547	1.629	1.5036	1.5571
scl-22	6.787	5.766	7.834	6.490	0.492	2.1490	0.3801
T12D8.5	8.637	10.197	9.459	10.372	2.887	1.8229	1.7937
T19D12.4	5.934	6.745	6.516	7.199	1.782	1.5047	1.6036
ugt-5	6.627	8.452	7.243	8.705	3.572	1.5392	2.7408
Y73F4A.2	8.727	9.581	9.444	10.168	1.794	1.6249	1.6524

Table 7. Candidate genes from similarity analysis between N2 ethanol response gene expression profile and *npr-1(ky13)* basal gene expression profile

Gene Symbol	N2 Basal RMA Value	N2 Ethanol RMA Value	*npr-1* Basal RMA Value	N2 Ethanol Fold Change	*npr-1* Basal Fold Change
catp-3	6.027	7.437	7.999	3.007	3.962
cnc-2	5.527	7.331	7.495	3.473	4.822
cth-2	9.490	10.119	10.080	1.550	1.508
nlp-29	8.488	9.859	9.373	2.554	2.129
nlp-30	7.837	8.617	8.705	1.721	1.879
pgp-5	5.755	6.370	6.682	1.526	1.898
ttr-44	8.692	9.277	9.471	1.507	1.725
ugt-29	6.326	7.264	7.092	1.991	1.709
C05D12.3	7.174	7.815	7.867	1.614	1.700
F41E6.5	7.382	8.047	8.066	1.566	1.592
F54B8.4	6.607	7.348	7.205	1.680	1.567
T19B10.2	6.808	7.465	8.471	1.567	1.991
ZK228.4	6.198	7.193	7.132	2.137	1.927

to ethanol exposure. Wild type animals may change their cellular function only after being exposed to the ethanol by modifying gene expression that may result in a more efficient or different adaptive response, like *npr-1(ky13)*. Therefore, genes in common in this class may be good candidates for AFT. We predict that the remaining basal gene expression differences in *npr-1(ky13)* are likely involved in other *npr-1* functions such as pheromone attraction, nociceptive avoidance, oxygen avoidance and feeding behavior.

Another interesting class of genes are the 204 genes that are solely regulated by ethanol in *npr-1(ky13)*. This class represents ethanol changes in gene expression only when *npr*-1 is non-functional, suggesting that this group may be involved in the *npr-1(ky13)* pathway and may also provide insight into how these animals behaviorally respond to ethanol.

Next, we prioritized our list of 22 candidate genes by allele availability and known phenotypes reported to the *C. elegans* online database, Wormbase. In addition, we looked for any gene candidates that had a strong mammalian homolog and neuronal expression. Using this ranking system, *acs-2*, an acyl-CoA synthetase, emerged as our top candidate gene. Previous studies have reported that *acs-2* mutant animals have an increase in lipid content *(*Zhang *et al.*, 2011; Ashrafi *et al.*, 2003). Our laboratory has demonstrated that lipid biology plays an important role in the development of AFT (Bettinger *et al.,* 2012). Animals that have an increase in triacylglycerides (TAGs) are less sensitive to ethanol and display an enhanced development of AFT (Bettinger *et al.,* 2012). In addition, *acs-2* is directly regulated by *nhr-49 (*Van Gilst *et al.,* 2005; Taubert *et al.,* 2006). *nhr-49* loss of function mutants have an increase in fat content (Ashrafi *et al.,* 2003; Horikawa *et al.,* 2008; Xu *et al.*, 2011) and display blunted AFT (Bettinger *et*

al., 2012). Taken together, these data suggest that lipid biology and metabolism are important in ethanol responsive behaviors.

Next, we confirmed the changes in gene expression for *acs-2* found by the microarray analysis using qRT-PCR. We extracted total RNA from independent samples and used primers that were designed to amplify the region captured by the *acs-2* signal on the microarray. Our qRT-PCR results agreed with our microarray findings. *acs-2* gene expression is up regulated in *npr-1(ky13)* mutants. We measured a 1.91 fold up regulation by microarray analysis (Table 6) and 1.95 fold by qRT-PCR (Figure 13). When N2 and *npr-1(ky13)* animals are treated with ethanol, *acs-2* gene expression is down regulated, 0.47 fold in N2 and 0.37 fold in *npr-1(ky13)*. We also observed down regulation for *acs-2* by qRT-PCR. We measured a down regulation of 0.35 fold in N2 ethanol-treated animals, and 0.1886 fold in *npr-1(ky13)* ethanol treated animals (Figure 13). This analysis validates the changes of gene expression for *acs-2* in our microarray studies.

acs-2 modifies the development of AFT in *C. elegans*

acs-2 encodes an acyl-CoA synthetase that activates fatty acids in a two-step irreversible reaction that requires ATP (Li *et al.,* 2010). Loss-of-function of this gene disrupts fatty acid activation and results in fat accumulation(Zhang *et al.*, 2011; Ashrafi *et al.*, 2003). Fatty acids are incorporated into fat stores such as TAGs and genes involved in metabolizing TAGs show altered responses to the effects of acute ethanol (Bettinger *et al.,* 2012). We asked if disruption of fatty acid activation caused by loss-of-function of *acs-2* could alter acute responses to ethanol. Using a locomotion assay

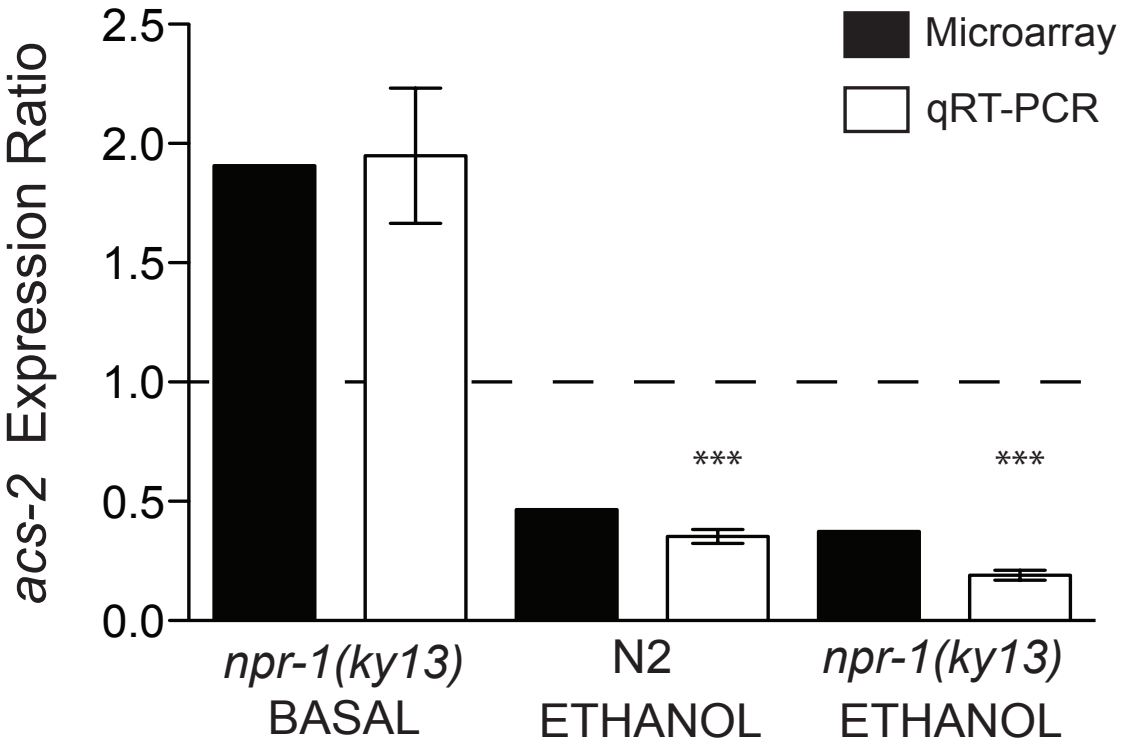
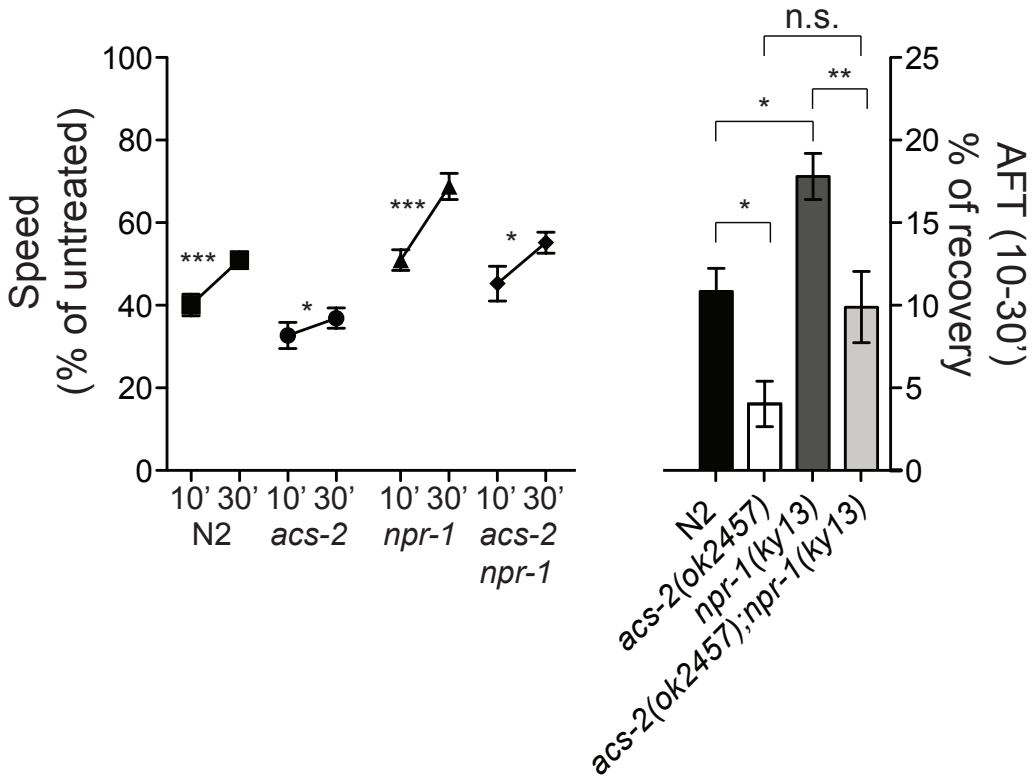


Figure 13. Gene expression confirmation of *acs-2*. We confirmed gene expression of *acs-2* by qRT-PCR in an independent set of samples. A one-way ANOVA showed significant differences between *npr-1(ky13)* basal and N2 ethanol ($P < 0.05$) and *npr-1(ky13)* basal and *npr-1(ky13)* ethanol ($P < 0.05$) by qRT-PCR. N2 ethanol and *npr-1(ky13)* ethanol qRT-PCR results were not significantly different from each other ($P > 0.05$). (n = 4). Error bars are SEM

A



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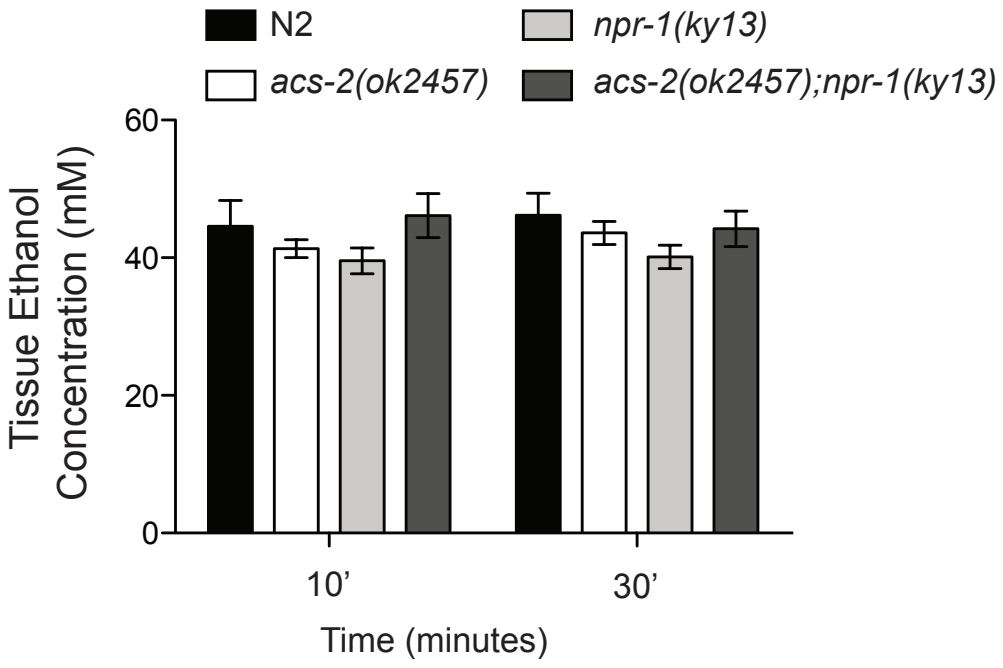

Figure 14. *acs-2* modifies AFT and does not alter ethanol metabolism (A) At 400mM exogenous ethanol, *acs-2(ok2457)* display a similar initial sensitivity to N2, but develop significantly less AFT. *acs-2(ok2457);npr-1(ky13)* animals also have a similar initial sensitivity relative *npr-1(ky13)* and *acs-2(ok2457),* but have a reduced AFT that is significantly different than *npr-1(ky13),* but not *acs-2(ok23457) or* N2. (n = 9)(B). Internal ethanol concentrations are similar across all mutants suggesting the observed behavioral effects are not due to ethanol metabolism. (n = 4). Error bars are SEM. * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$

(Davies *et al.,* 2003, 2004, Appendix A), we found that animals carrying a deletion of *acs-2(ok2457)* display similar initial sensitivity to ethanol as wild-type N2. However, during a continuous exposure to ethanol, *acs-2(ok2457)* mutants have a significantly decreased development of AFT (Figure 14A, right panel) ($P < 0.05$). N2 recovers 10.84% of its untreated speed, but *acs-2(ok2457)* only recovers 4.03% of its untreated speed. This suggests that loss of *acs-2* function modifies the adaptive response of AFT to acute ethanol.

Next, we asked if *acs-2(ok2457)* can alter the development of AFT in *npr-1(ky13)* mutants. Since *acs-2* is basally up regulated in *npr-1(ky13)* mutants relative to N2 (Table 6, Figure 13), and *npr-1* animals have enhanced development of AFT, we predicted that altering *acs-2* levels in the opposite direction may result in AFT levels similar to *acs-2*. We found that *acs-2(ok2457);npr-1(ky13)* double mutants displayed similar initial sensitivity to *npr-1(ky13)* and *acs-2(ok2457)* alone (Figure 14A) ($P > 0.05$). Double mutants developed significantly less acute functional tolerance than *npr-1(ky13)* ($P < 0.01$), but similar levels of AFT to *acs-2(ok2457)* and N2 ($P > 0.05$) (Figure 14A, right panel). These results demonstrate that *acs-2* can modify the fast AFT phenotype of *npr-1(ky13)* and it may suggest an this effect is additive.

Paralogs of *acs-2, acs-20* and *acs-22*, have been reported to play a role in cuticle formation of animals (Kage-Nakadai *et al.,* 2010). The cuticle is a waxy outer surface that protects animals from the environment and many pharmacological agents (Burns et al., 2010; Cox et al., 1981). The reduced AFT phenotype of *acs-2(ok2457)* may be due to an increase in ethanol entry due to disruption of the cuticle. To test this, we measured

internal ethanol concentrations and found that the *acs-2(ok2457)* animals had similar internal ethanol concentrations as wild type ($P > 0.05$)(Figure 14B). In addition, *npr-1(ky13)* and *acs-2(ok2457);npr-1(ky13)* all displayed similar tissue ethanol concentrations ($P > 0.05$)((Figure 14B). This suggests that the behavioral effects of *acs-2* are likely to be due to a difference in the pharmacodynamics effects to ethanol.

Impaired mitochondrial β-oxidation may alter acute responses to ethanol

Previous studies have found that loss of function of *acs-2* results in a decrease in mitochondrial β-oxidation rates and loss of function mutations in *nhr-49,* an upstream regulator of *acs-2,* displayed abnormal mitochondrial morphology shown by high pressure freezing transmission electron microscopy (Pathare *et al.,* 2012) To determine if the failure to develop AFT in the *acs-2(ok2457)* mutant is due to a decrease in mitochondrial β-oxidation function, we inhibited gene expression in the mitochondrial β-oxidation pathway using RNAi. We identified genes in the mitochondrial β-oxidation pathway by performing literature searches (Van Gilst *et al.,* 2005a; Van Gilst *et al.,* 2005b; Brock *et al.,* 2007, Zhang *et al.,* 2013) and adapted these finding to show genes involved in the mitochondrial β-oxidation pathway (Figure 15).

First, we asked if inhibiting fatty acid entry into the mitochondria would alter ethanol responses. We tested two genes that encode carinitine palmitoyl transferases, *cpt-2* and *cpt-5*. *cpt-2* is predicted to localize to the inner membrane of the mitochondria and *cpt-5* is positively regulated by *nhr-49* (Van Gilst *et al.*, 2005a; Van Gilst *et al.*, 2005b). We found that when animals were treated with RNAi for *cpt-2* and *cpt-5,* initial sensitivity and AFT were similar to controls (Figure 16A). This suggests that altering the

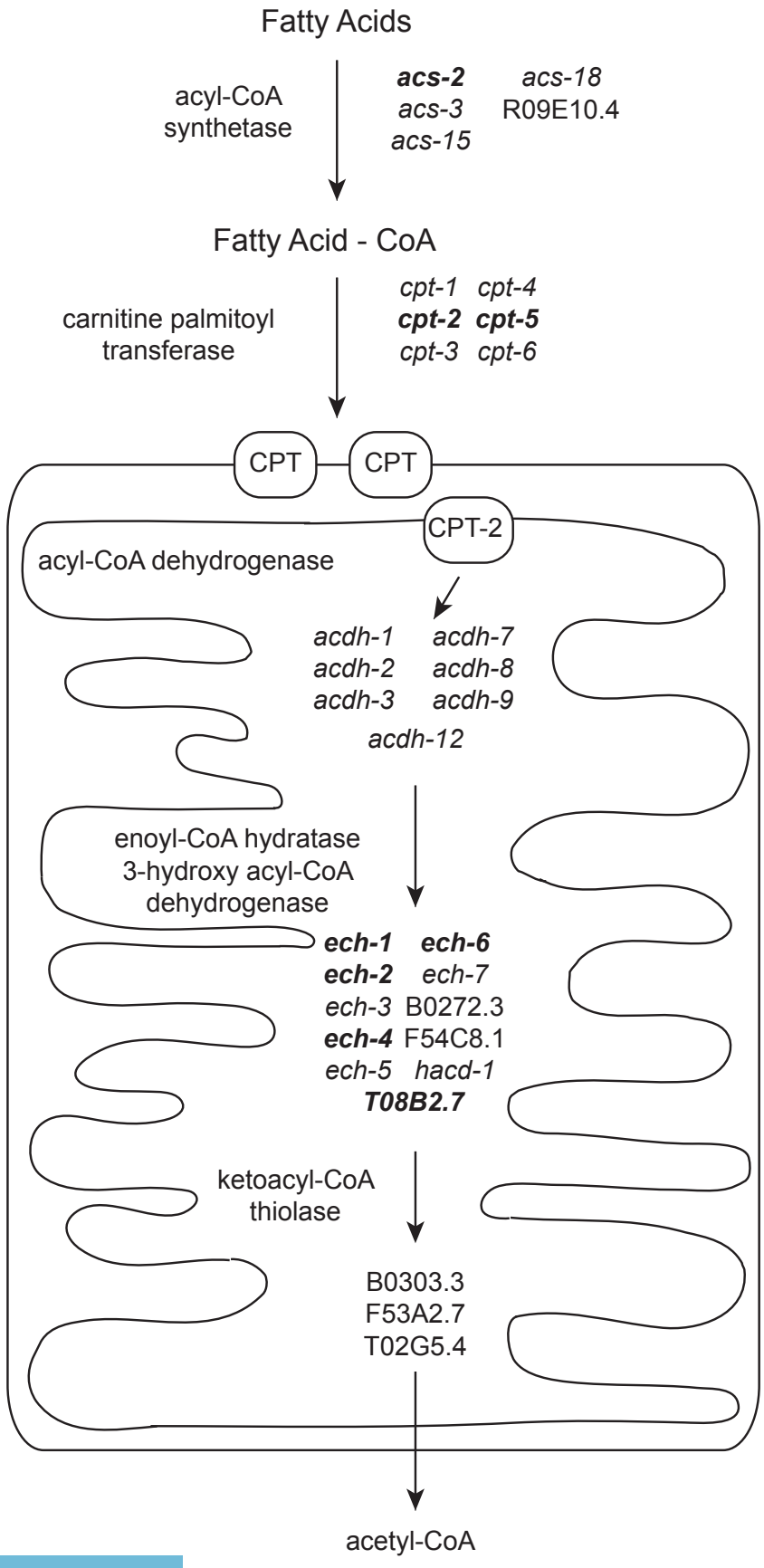

Figure 15. Mitochondrial β-oxidation pathway in *C. elegans*. Fatty acids become activated by the addition of CoA by acyl-CoA synthetase. Activated fatty acids enter the mitochondria from the cytoplasm by carnitine palmitoly transferase and are dehydrogenated by acyl-CoA dehydrogenase. Enoyl-CoA hydratase and 3-hydroxy acyl-CoA dehydrogenase breakdown the fatty acid further where ketoacyl-coA thiolase catalyzes the release of acetyl-CoA from the mitochondria. In bold are the genes tested in acute ethanol responses.

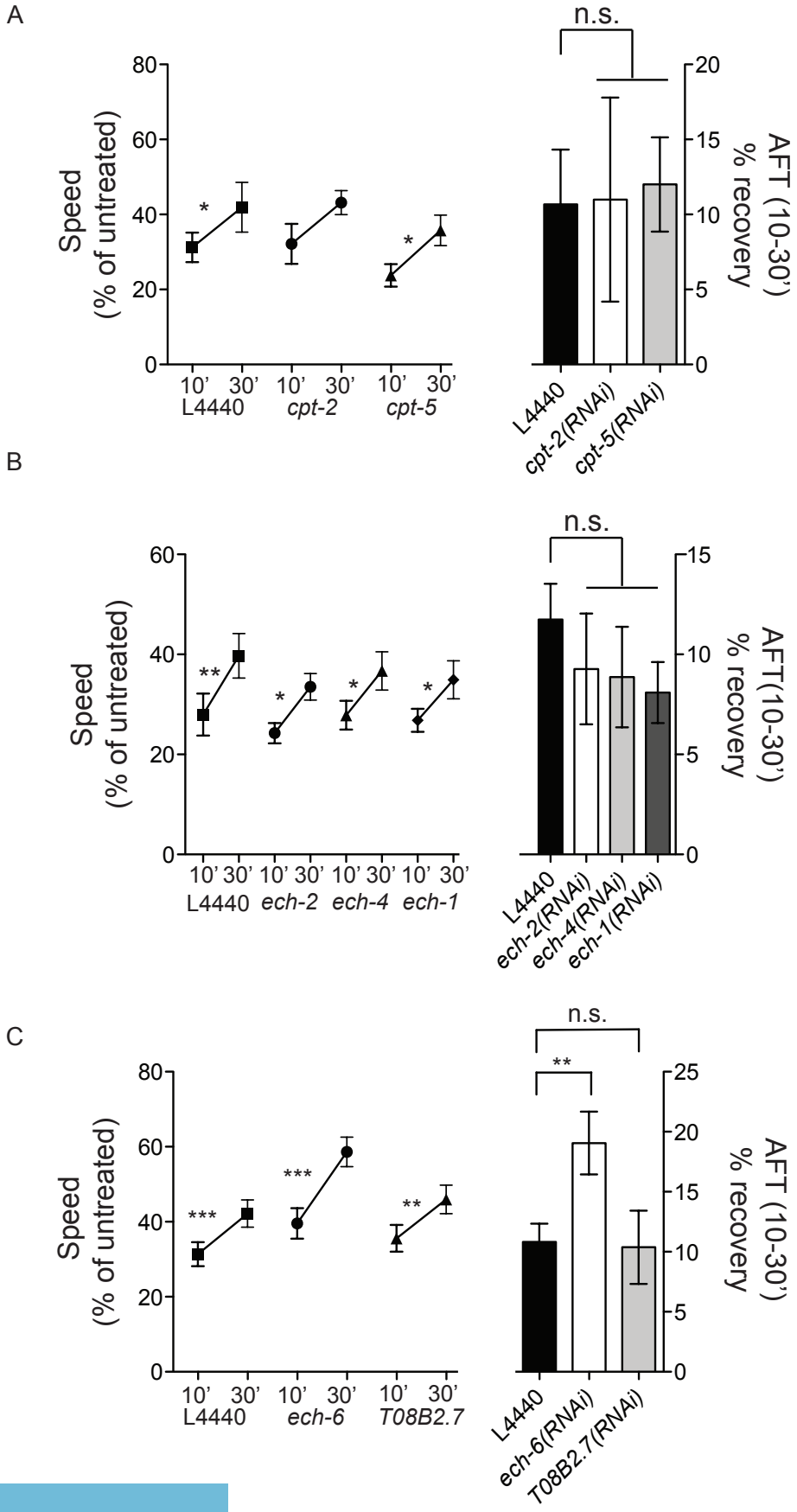

Figure 16. Mitochondrial β-oxidation may influence acute ethanol behaviors. (A) At 400mM exogenous ethanol *cpt-2(RNAi)* and *cpt-5(RNAi)* animals displayed similar initial sensitivity and development of AFT relative to control (n = 4). (B) *ech-2(RNAi), ech-4(RNAi),* and *ech-1(RNAi)* mutant animals also display similar initial sensitivity and AFT relative to control (n = 4). (C) *T08B2.7 (RNAi)* animals were not different than control for initial sensitivity or AFT. *ech-6(RNAi)* mutants displayed an enhanced AFT relative to wild type, but initial sensitivity was not significantly different (n = 8). Error bars represent SEM * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

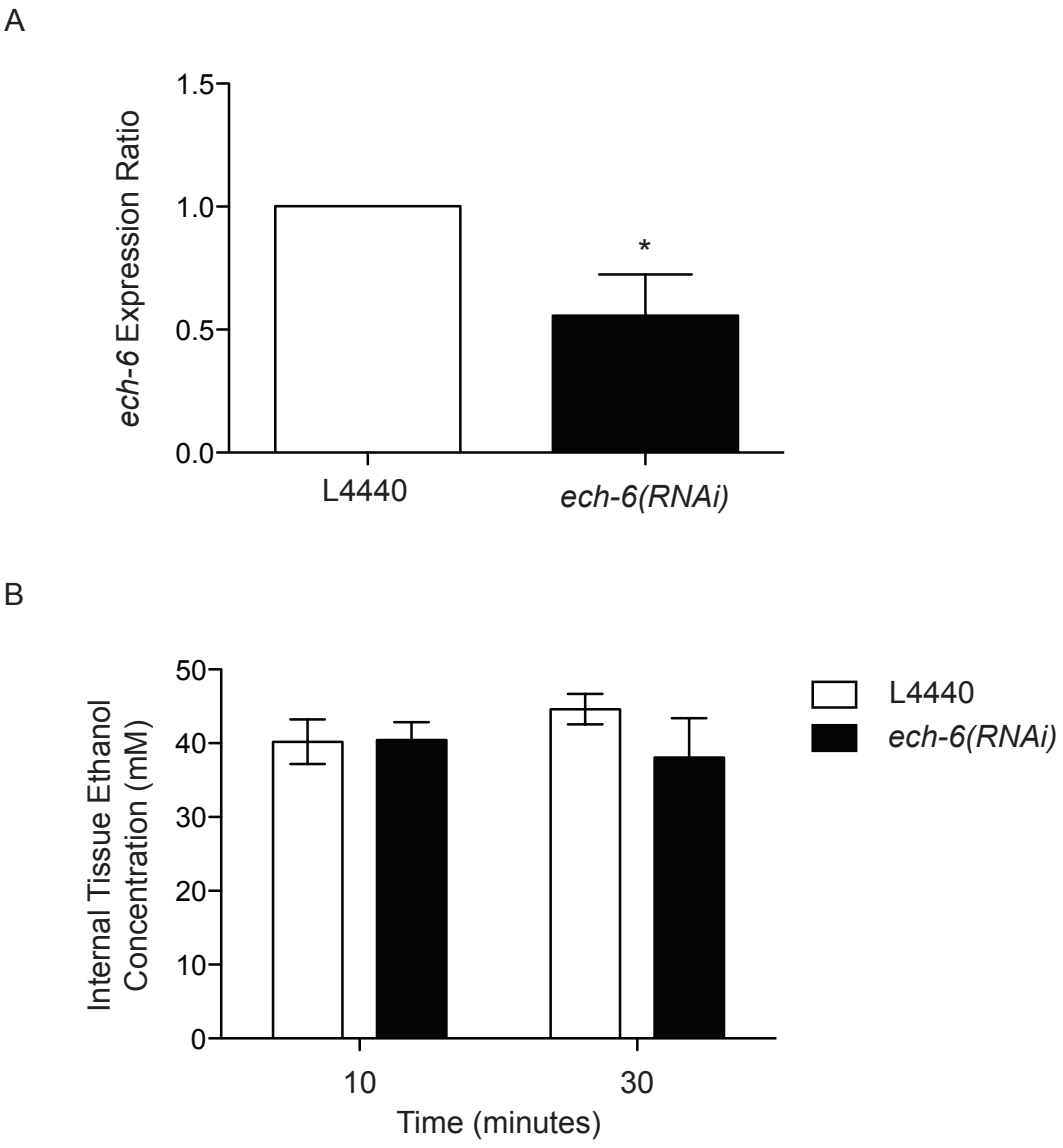

Figure 17. *ech-6(RNAi)* mutants have significantly decreased mRNA levels and do not have altered ethanol metabolism. (A) qRT-PCR of *ech-6* in *ech-6(RNAi)* mutants shows that messages levels are significantly reduced (n = 4). (B) *ech-6(RNAi)* animals have similar internal ethanol concentrations relative to control (n = 3). Error bars represent SEM. * $P < 0.05$

entry of fatty acids into the mitochondria through *cpt-2* and *cpt-5* does not modify behavioral responses to ethanol. We did observe large viability in the development of AFT for *ctp-2(RNAi)* animals, suggesting that the behavior was not consistently observed and more trials are needed. However, there are many reasons that can explain why we were unable to see an effect. One possibility may be that our method of knockdown did not reduce message levels adequately enough to have an effect. Any remaining message that was not degraded may have been enough to provide normal function.

Next, we examined knockdown by RNAi of 4 different enoyl-coA hydratases; *ech-1*, *ech-2*, *ech-4* and *ech-6*. *ech-1(RNAi), ech-2(RNAi),* and *ech-4(RNAi)* animals displayed similar initial sensitivity and AFT to controls. However, *ech-6(RNAi)* animals displayed enhanced AFT to the depressive effects of ethanol. This suggests that the lost of this specific enoyl-CoA hydratase can alter acute behavioral responses to ethanol. In addition this results suggests that mitochondrial β-oxidation may also be involved in acute behavioral responses to ethanol.

Lastly, we tested the effects of knock down of the hydroxyl acyl-CoA dehydrogenase, *T08B2.7*, for acute behavioral responses to ethanol. These animals do not have altered ethanol responses relative to wild type.

We were intrigued by the *ech-6(RNAi)* ethanol response and further examined *ech-6(RNAi)* animals for knockdown of mRNA levels by qRT-PCR. We found that animals treated with RNAi by feeding had significantly less *ech-6* message (0.40 fold) than the L4440 control (paired t-test, $P = 0.0388$). We also tested *ech-6(RNAi)* animals for altered ethanol metabolism and found that they do not display any differences from

controls (Figure 17). Taken together, this suggests that knockdown of *ech-6* can alter acute behavioral responses to ethanol.

Discussion:

In this study, we performed an unbiased survey of gene expression across the whole genome of *Caenorhabditis elegans* for ethanol responsiveness. There is increasing evidence in the literature that ethanol responsive genes are excellent candidates to gain insights into the underlying cellular and physiological mechanisms in ethanol response behaviors (Mulligan *et al.,* 2008, 2011; Treadwell and Singh 2004; Wolstenholme *et al.*, 2011, Kerns *et al.,* 2005). More importantly, studies have shown that some of these genes, when genetically altered, can affect acute behavioral responses to ethanol (Bhandari *et al.,* 2012, Costin *et al.,* 2013, Wolstenholme *et al.*, 2011, Cozzoli *et al.,* 2012). We captured gene expression at a sedative dose of ethanol. We found that we were able to detect tissue specific transcripts down to the level of two neurons suggesting that the microarray analysis was very sensitive (Table 4).

We determined the ethanol responsive gene profile of N2 and animals mutant for *npr-1* and found a significant number of genes commonly regulated by ethanol between both genotypes (Figure 12). A gene ontology enrichment analysis revealed that the common ethanol response was enriched for a variety of biological processes including genes involved in oxidative stress, lipid modification and glycosylation, and transcription (Table 5). Previous work has shown that lipid metabolism is involved in ethanol responsive behaviors (Bettinger *et al.,* 2012). Furthermore, the lipid environment has

shown to affect the function of BK channels (Crowley et al., 2005; Yuan *et al.,* 2008, 2007; Lin *et al.,* 2006, Bettinger *et al.,* 2012)

Recently, Peltonen *et al.,* (2013) determined the ethanol responsive genes of chronic low dose ethanol treatment of worms developing from embryo to L4 stage by RNA seq analysis. Interestingly, they report similar gene ontology categories for biological process as our study, including oxidation-reduction, lipid glycosylation, and lipid modification. This provides further evidence that lipid homeostasis may play an important role in ethanol responses. Additionally, our microarray data set and the Peltonen et al., (2013) RNA seq data set are enriched for similar molecular functions such as monooxygenase activity, heme and tetrapyrrole binding, iron ion binding, and electron carrier activity. This suggests that ethanol may be acting on similar cellular processes during acute and chronic exposure. Another study by Kwon et al., (2004) also looked for ethanol responsive genes, but at a lethal dose of 7% ethanol at 15 minutes and 30 minutes and this data set only showed similarity to 20 genes produced by the RNA seq study by Pheltonen *et al.,* When we compared our wild type ethanol response profile to Kwon et al., 2004, we found 85 genes overlaped between data sets. We predict that these genes may be involved in the stress response. However, it must be noted that the Kwon studies were performed with very limited power.

We determined the basal gene expression profiles of *npr-1(ky13)* animals and used these changes in gene expression to select for candidates for genes involved in ethanol responses. This resulted in 20 candidate genes (Table 6) and we focused on *acs-2* because of its association with lipid metabolism. We found that *acs-2* function is required for normal development of AFT (Figure 14A) and we sought to clarify the

mechanism by which *acs-2* is regulating this process. First, we show that *acs-2* mediated disruption of fatty acid activation in *npr-1(ky13)* animals can modify the rate of AFT. However, *acs-2(ok2457);npr-1(ky13)* still have an intermediate phenotype of each individual mutant suggesting that *acs-2* and npr-1(ky13) together produce an additive response (Figure 14A). Since *acs-2* can modify AFT in a sensitized background, it suggests that the effects of *acs-2* may be an important regulator of adaptive responses. Second, *nhr-49,* a transcriptional regulator of *acs-2*, when mutated results in the failure to develop AFT (Bettinger *et al.,* 2012). *nhr-49* loss of function mutants have a significant increase in fat content which is caused by a repression of *acs-2* expression (Van Gilst *et al.,* 2005a). *nhr-49* is a transcription factor that regulates a variety of energy metabolic processes and mutant animals have an abnormal mitochondrial morphology. It is not known whether *acs-2(ok2457)* mutants have altered mitochondrial morphology, but studies have shown they have reduced rates of **β**-oxidation relative to wild type. This information suggests that mutants in *acs-2* may cause mitochondrial dysfunction and this dysfunction may result in defects in AFT. To test this, we examined genes in the mitochondrial β-oxidation pathway (Figure 15 and Figure 16) for their acute responses to ethanol. None of the genes that we tested altered ethanol responses, except for *ech-6(RNAi)*, which displayed an enhanced AFT (Figure 16C). This result led us to a few possible explanations. First, if loss of *ech-6* function results in a decrease in mitochondrial β-oxidation and mitochondrial dysfunction, we would predict that animals would have displayed responses to ethanol similar to *nhr-49* and *acs-2* loss of function. The opposite phenotype for loss of *ech-6* function may result from an accumulation of its precursor trans-2-enoyl CoA and this precursor may be utilized in other pathways in

the mitochondria, such as mitochondrial fatty acid synthesis (Gurvitz 2009) Taken together, our results indicate that altered mitochondrial β-oxidation alters acute adaptive responses to ethanol and warrants further investigation.

One other possible explanation into the mechanisms of *acs-2* regulating AFT could be through its increase in fat stores. Previous work has investigated the role of fat stores induced by mutations of different genes in the development of AFT or initial sensitivity. These studies concluded that fat stores do not regulate AFT (Bettinger *et al.,* 2012). One could speculate that since *acs-2* mutants have a decrease in mitochondrial β-oxidation (likely due to a decrease in activated fatty acids specific for β-oxidation), that excess fatty acid cellular pools are being utilized by other acyl-CoA synthetases. The *C. elegans* genome is annotated for 23 different actyl co-A syntheases, each of which may activate specific chain length. Activated fatty acids have been shown to be involved in a variety of different cellular process such as vesicle trafficking, signaling, fatty acid desaturation and elongation and TAG synthesis (Ashrafi et al., 2009). Additionally, activated fatty acids also modify ATP-sensitive K+ channels and protein kinase C function (Lester et al., 1990).

Our results implicate a role for lipid homoeostasis in the acute responses to ethanol. In addition to our previous work, we have shown that alterations in different lipid metabolic pathways can influence acute responses to ethanol and may warrant further work elucidating mechanisms in which lipids can alter cellular responses to ethanol.

Chapter 4

Across Species Characterization of Genes Involved in Ethanol-Response Behaviors

A portion of this material has appeared in the article “Chloride Intracellular Channels Modulate Acute Ethanol Behaviors in *Drosophila, Caenorhabditis elegans* and mice” by P. Bhandari, J.S. Hill, S.P. Farris, B. Costin, I. Martin, C.-L. Chan, J.T. Alaimo, J.C. Bettinger, A.G. Davies, M.F. Miles, M. Grotewiel (2012) in Genes, Brain and Behavior 11(4): 387-397 and is used here by permission.

Introduction

Over the last decade, a variety of studies from different species have identified numerous chromosomal regions, single nucleotide polymorphisms (SNPs), and gene expression changes that are correlated with alcoholism and individual traits associated with the etiology of the disease (Reich *et al.,* 1998, Hill *et al.*, 2004, Hitzermann *et al.*, 2004, Kerns *et al.,* 2005, Hoffman and Tabakoff *et al.,* 2005, Johnson *et al.*, 2006, Kuo *et al.*, 2006, Morozova *et al.,* 2007, Rodd *et al.,* 2008, Mayfield *et al.*, 2008). This wealth of information has led to the creation of a comprehensive ethanol-related gene resource (ERGR) and other computation tools such as Geneweaver to help analyze and integrate these different types of data for researchers (Guo *et al.*, 2008; Baker *et al.*, 2012).

These tools provide a great platform for identifying possible candidate genes involved in alcohol responses.

Alcohol dependence is a complex disorder consisting of different components, some of which are physiological and some are psychological. Some of these components, particularly the physiological ones, can be modeled using animal systems. Many studies have shown that a variety of ethanol behavioral responses in humans also are observed in vertebrates and invertebrate animals, including monkeys, mice, rats, honey bees, flies, zebrafish, and worms. (Vivian *et al.*, 2001; Corl *et al.,* 2009, Crabbe *et al.,* 2006; Varlinskaya *et al.,* 2001, Maze *et al.,* 2006; Bhandari *et al.,* 2008, Gerlai *et al.,* Davies *et al.,* 2003, 2004) suggesting that conserved mechanisms exist.

Humans that display a LR to acute ethanol have a significant increase in alcohol abuse liability and this response is strongly influenced by genetics (Schuckit 1994; Schuckit and Smith, 2006). Animal models display conserved acute behavioral responses to ethanol and are very amenable to genetic manipulation. Therefore, utilizing model organisms can facilitate defining genes involved in behavioral responses to ethanol. Furthermore, identifying genes that influence ethanol behavioral phenotypes may give insight into how a particular gene function is involved in cellular responses to the drug.

One approach to uncovering such basic mechanisms of alcohol response is to study the role of a gene in ethanol responsive behaviors across multiple species. This approach is based on the principle that important biological processes are frequently conserved between organisms. To date, only a few studies have taken this approach (Cori *et al.,* 2009; Kapfhamer *et al.,* 2008; Lasek *et al.,* 2011a,b; Schumann *et al.,* 2011,

Bhandari *et al.*, 2012; Jee *et al.*, 2013) and additional studies are needed because it may provide validation of a gene being involved in acute ethanol response behaviors and future risk for dependenace in humans.

We have performed two different sets of experiments that have arisen from a series of cross species collaborative studies at the Virginia Commonwealth University Alcohol Research Center (VCU-ARC). In the first experiment, Dr. Michael Miles analyzed the overlap in several microarray studies designed to identify ethanol-responsive genes in mice and humans. These studies identified gene expression changes in: post mortem samples of frontal and motor cortices of human alcoholics vs. non-alcoholic controls (Mayfield *et al.,* 2002, Liu *at al., 2008*), brains of isogenic strains of ethanol-preferring and non-ethanol-preferring mice that were voluntary consuming alcohol (Mulligan *et al.,* 2006), and ventral tegmental area, prefrontal cortex, and nucleus accumbens brain regions in response to acute ethanol in DBA/2J and C57BL/6 mice (Kerns *et al.,* 2005). Using Geneweaver, an overlap analysis identified the Chloride Intracellular Channel 4 (*Clic4*) as being differentially expressed across all of these studies. *Clic4* also was located within a confirmed quantitative trait locus (QTL) for ethanol drinking behavior in mice (Tarantino *et al.,* 1998). Therefore, *Clic4*'s association with consumption and ethanol response made it the top candidate gene for investigation in this analysis.

Behavioral analysis performed by Drs. Poonam Bhandari and Michael Grotewiel demonstrated that *Drosophila melanogaster* carrying a partial loss of function of the fly *Clic4* homolog, *Clic,* has reduced sensitivity to the acute sedative effects of ethanol (Bhandari *et al.,* 2012). In mouse studies performed by Jennifer Hill, Dr. Sean Farris,

and Dr. Blair Costin, overexpression of *Clic4* in the prefrontal cortex brain region resulted in a decreased sensitivity to high-dose ethanol as tested by the LORR assay (Bhandari *et al.,* 2012). The *C. elegans* genome encodes two *Clic* homologs, *exc-4* and *exl-1* (Berry *et al.,* 2003), and we demonstrated that the involvement of *Clic* in ethanol responses is conserved in worms.

Our second experiment was inspired by results from a human genome wide association study (GWAS) for loci that are associated with alcohol dependence that has recently been completed in the laboratory of Brien Riley. We analyzed the 5 most significantly associated genes from the alcohol dependence GWAS for those genes that had strong homologs in *C. elegans.* Krüpple-Like Transcription Factor 12 (*Klf12*), the second most significantly associated gene from the GWAS, is orthologous to *klf-3* in *C. elegans.* Multiple lines of evidence have implicated *Klf12* in alcohol responses. First, *Klf12* gene expression is significantly correlated with *Clic-4* expression in the prefrontal cortex of saline-treated BXD mice (Bhandari *et al.,* 2012). Second, *Klf12* also interacts with C-terminal binding proteins, *Ctbp1* (Schuierer, *et al.,* 2001) and *Ctbp2* (Lomberk and Urrutia, 2005). The homolog of *Ctbp1* and *Ctbp2* in worms is *ctbp-1,* and loss of function of *ctbp-1* results in altered acute ethanol responsive behaviors (Bettinger *et al.,* 2012). Ryanodine receptor 3 (*Ryr3)* was the fifth most significantly associated gene, and is orthologous to *unc-68* in worms. CLIC proteins interact with *RYR3* to inhibit calcium release in musculature in humans (Board *et al.,* 2004). In addition, *Ryr3* was also represented in a gene set enrichment analysis of a GWAS of variables of LR (Joslyn *et al.,* 2010). Therefore, we examined the roles of *unc-68* and *klf-3* in acute behavioral responses to ethanol in *C. elegans.*

Methods:

***C. elegans* genetics**

C. elegans were maintained using standard methods (Brenner, 1974). Strains used were: N2 var. Bristol, *Clic* mutant strains *exc-4(rh133)* and *exl-1(ok857) exc-4(rh133),exl-1(ok857), unc-68(r1161)*, which is homologous to human *Ryr3*, and *klf-3(ok1975)* which is homologous to *Klf12* mutant strain *klf-3(ok1975)*. The double mutant *exc-4(rh133);exl-1(ok857)* was generated using standard genetic crosses, using the recessive excretory canal phenotype that is associated with *exc-4(rh133)* to detect animals that were homozygous for *exc-4*, and detection of the deletion in *exl-1(ok857)* using PCR. We used these primers to detect the *exl-1(ok857)* deletion mutation: *exl-1(ok857)*_F: 5′-GTGCAATCTCGTCAGGACCAGGC-3′, *exl-1(ok857)*_R: 5′-ATGCGTTACGATGCCCCGACAC-3′.

Ethanol-response behaviors

Ethanol-response assays were carried out as previously described (Davies *et al.,* 2003, 2004), see Appendix A for detailed methods. Briefly, 6 cm petri plates containing standard nematode growth media were dried at 37°C for two hours with no lids. Plates were weighed, and copper rings were embedded into the surface of each plate to act as corrals for each genotype. 100% ethanol was added to each ethanol assay plate to achieve a final concentration of 0mM, and 200 mM or 400 mM (w/v). The ethanol was allowed to equilibrate for two hours at room temperature. 10 age-matched first day adult

animals were moved to plates without ethanol and without food to allow for acclimation to the lack of food for 30 minutes before they were transferred to either a 0 mM or ethanol containing assay plate. When performing assays for *exc-4(rh133)*, *exl-1(ok857)*, and the double mutants, we recorded a ten-minute movie followed by 2-minute movies at 15, 20, 25, 30, 40, and 50 minutes of continuous ethanol exposure. We analyzed the 10-minute movie in 2-minute segments at 2, 4, 6, 8 and 10 minutes. These conditions were imposed because *exc-4(*rh133) has a defective excretory canal and to avoid any potential delay in the intoxicating effects because of drug entry issues, we expanded the analysis to more time points. For example, onemight expect that the defect in the canal may cause ethanol to take longer for it to affect all tissues to produce a response. Therefore, behaviorally, the animals may look resistant earlier time points of the assay and become intoxicated at a later time point. Behavioral assays for *unc-68(r1161)* and *klf-3(ok1975)* consisted of 2-minute movies recorded at 10, 30, and 50 minutes. All assays were recorded using a Retiga400R camera (Qimaging, Canada). Movies were analyzed using ImagePro Plus (v6) (Media Cybernetics, Bethesda, MD) using the recognition function to detect worm-sized objects based on pixel intensity. We used the Track Object function to measure the changes in position of the centers of mass for each consecutive frame of the movie. Speed was calculated as distance traveled per unit time.

Internal ethanol measurements

Age-matched first day adult worms were reared at 20°C and placed on unseeded plates containing 0 or 400 mM ethanol for 5, 10, or 30 minutes. Internal ethanol was

measured as previously described (See Chapter 2 and Appendix A for details). Briefly, 200 animals were collected by picking into a microcentrifuge tube and were frozen at −80°C and homogenized in ddH_2O. Ethanol concentration was determined in the homogenates using a commercially available alcohol reagent (Pointe Scientific, Canton, MI, USA) by generating a standard curve by spectrophotometric analysis. The volume of tissue contributed to the samples was quantified by tracing images of animals for their length and diameter and solving the equation for the volume of a cylinder. We multiplied the ethanol concentration of the homogenates found from the standard curve by the dilution factor to calculate the final internal ethanol concentration for each genotype (volume of worm / volume of worm + 20 µL).

Statistical analyses

To determine statistical significance ($P \leq 0.05$) in studies comparing two or more groups, *t* tests (Prism, GraphPad, San Diego, CA, USA), one- and two-way analysis of variance (ANOVA) (JMP, SAS Institute, Cary, NC, USA) followed by Bonferroni multiple comparison tests were performed as appropriate.

Results

Functional analysis of Clic orthologs in *C. elegans*

In order to determine if the *clic* gene orthologs in worms play a role in behavioral responses to ethanol, we assessed the response of two null alleles in the worm *Clic* homologs, *exc-4(rh133)* (Berry *et al.,* 2003) and *exl-1(ok857)* (Berry & Hobert 2006), to the acute effects of 400mM ethanol using an established locomotor behavioral assay

(Davies *et al.,* 2004). We measured the onset of intoxication over the first 10 minutes of drug exposure; the degree of intoxication at 10 minutes of exposure is our measure of the initial sensitivity of the animals. Worms harboring *exc-4(rh113)* had reduced sensitivity to ethanol in the first 5 minutes (Repeated measures of two-way ANOVA; time $F_{10,120} = 14.66$, $P = 0.0244$, genotype $F_{3,120} = 11.06$, $P = 0.0009$ on locomotor behavior in the presence of ethanol with no interaction between the factors, $F_{10,120} = 1.78$, $P = 0.177$, #, Bonferroni multiple comparison; $t(7) = 2.91–4.92$, $P < 0.05$), however *exl-1(ok587)* mutants displayed wild-type initial sensitivity (Figure 18A). We measured the development of AFT by assessing the increase in locomotor speed after the initial intoxicating effects of ethanol for each mutant over the course of 30 minutes. Animals carrying *exc-4(rh113)* trended towards reduced AFT, but *exl-1(ok587)* mutants displayed an enhanced AFT phenotype (†, Bonferroni multiple comparison; $t(7) = 2.91–4.92$, $P < 0.05$, Figure 18A). This suggests that mutations in *exc-4* and *exl-1* cause effects that are exclusive for initial sensitivity and acute tolerance, meaning that the function of these channels do not completely overlap for their effects on acute behaviors responses in worms. Next, we asked if both *exc-4* and *exl-1* work together and measured the behavioral response of *exc-4(rh113);exl-1(ok587)* double mutants to ethanol. We found that these animals displayed wild-type initial sensitivity, similar to *exl-1(ok587)* single mutants and reduced AFT similar to *exc-4(rh113)* single mutants (Figure 18A). This suggests that the effect of loss of *exc-4* function on initial sensitivity requires normal *exl-1* expression and also that the effect of loss of *exl-1* function on AFT requires normal *exc-4* expression. To determine if there were any drug uptake and

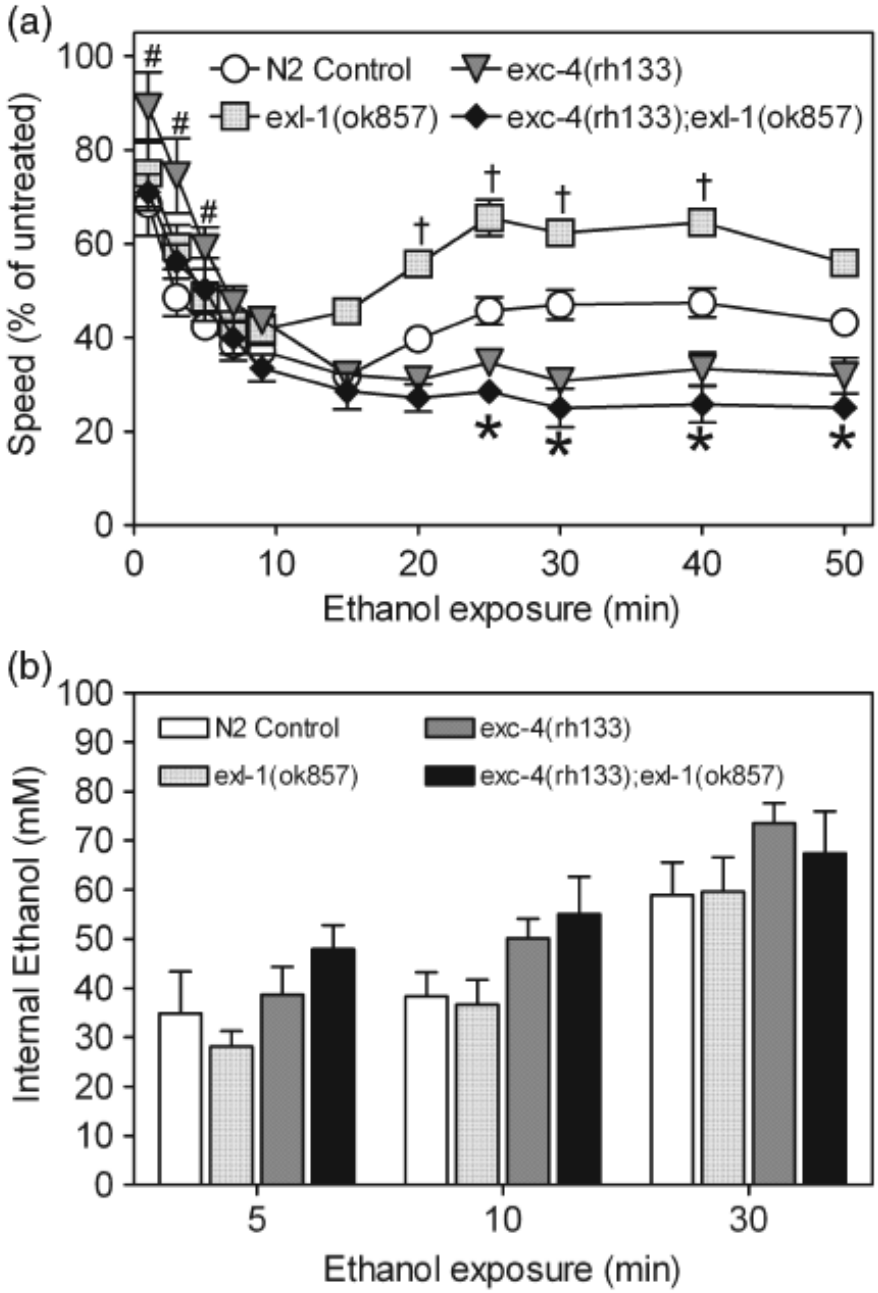

Figure 18. Ethanol sensitivity and acute functional tolerance in *C. elegans* with mutations in *Clic* orthologues. (a) Effect of 400mM exogenous ethanol on relative locomotor speed (percent of untreated animals) in N2 control (open circles), *exl-1*(*ok857*) (light gray squares), *exc-4*(*rh133*) (dark gray triangles), *exc-4*(*rh133*)*;exl-1*(*ok857*) (black diamonds). (b) Internal ethanol concentrations in N2 control and *Clic* mutants exposured to 400mM exogenous ethanol. Neither *clic* ortholog significantly alters tissue concentrations (Bhandari *et al.,* 2012) (n = 6). Error bars are SEM.

metabolic causes of these changes in ethanol responses, we measured internal ethanol concentrations for each mutant. Mutations ineither *exc-4*, *exl-1,* or both genes did not alter internal tissue concentrations relative to wild type, despite increased tissue ethanol concentrations during the time course of exposure, suggesting that the behavioral changes observed are likely to be due to differences in the pharmacodynamic response to ethanol (two-way ANOVA; duration, $F_{2,60}$ = 29.19, P < 0.0001; genotype, $F_{3,60}$ = 3.07, P = 0.0439; interaction between duration and genotype, $F_{3,60}$ = 0.1143, P = 0.951, n.s.; Bonferroni multiple comparisons between N2 and other genotypes, t(11) = 0.0824–1.94, P > 0.05, n.s., n = 6) (Figure 18B).

Functional analysis of RYR3 orthologs in *C. elegans*

Human *Ryr3* is expressed in the brain (Hertle and Yeckel, 2007) and is responsible for mediating Ca^{2+} release from the endoplasmic reticulum that is required for neuronal viability and synaptic plasticity (Fitzjohn and Collingridge, 2002). *C. elegans* have a single ryanodine receptor gene , *unc-68*, and we tested mutant animals carrying the loss of function mutation, *r1161*, (Maryon et al., 1996) for altered responses to ethanol. We assayed *unc-68(r1161)* mutants at 200mM exogenous ethanol and found that the animals were significantly resistant to ethanol at 10 minutes of exposure relative to wild type(Figure 19A) (Repeated measures of two-way ANOVA; time $F_{2,56}$ = 3.664, P = 0.032 , genotype $F_{1,56}$ = 18.52, P = 0.0002 interaction between the factors, $F_{2,28}$ = 0.6652 P = 0.5182, *, Bonferroni multiple comparison; t(3) = 3.00 – 4.120, P < 0.05). Internal ethanol concentrations for *unc-68(r1161)* at 10 minutes were not significantly different than N2 (two-way ANOVA; time $F_{1,12}$ = 8.283, P = 0.0139 , genotype $F_{1,12}$ = 13.84, P = 0.00029 interaction between the factors, $F_{1,12}$ = 0.2469 P = 0.1421, **,

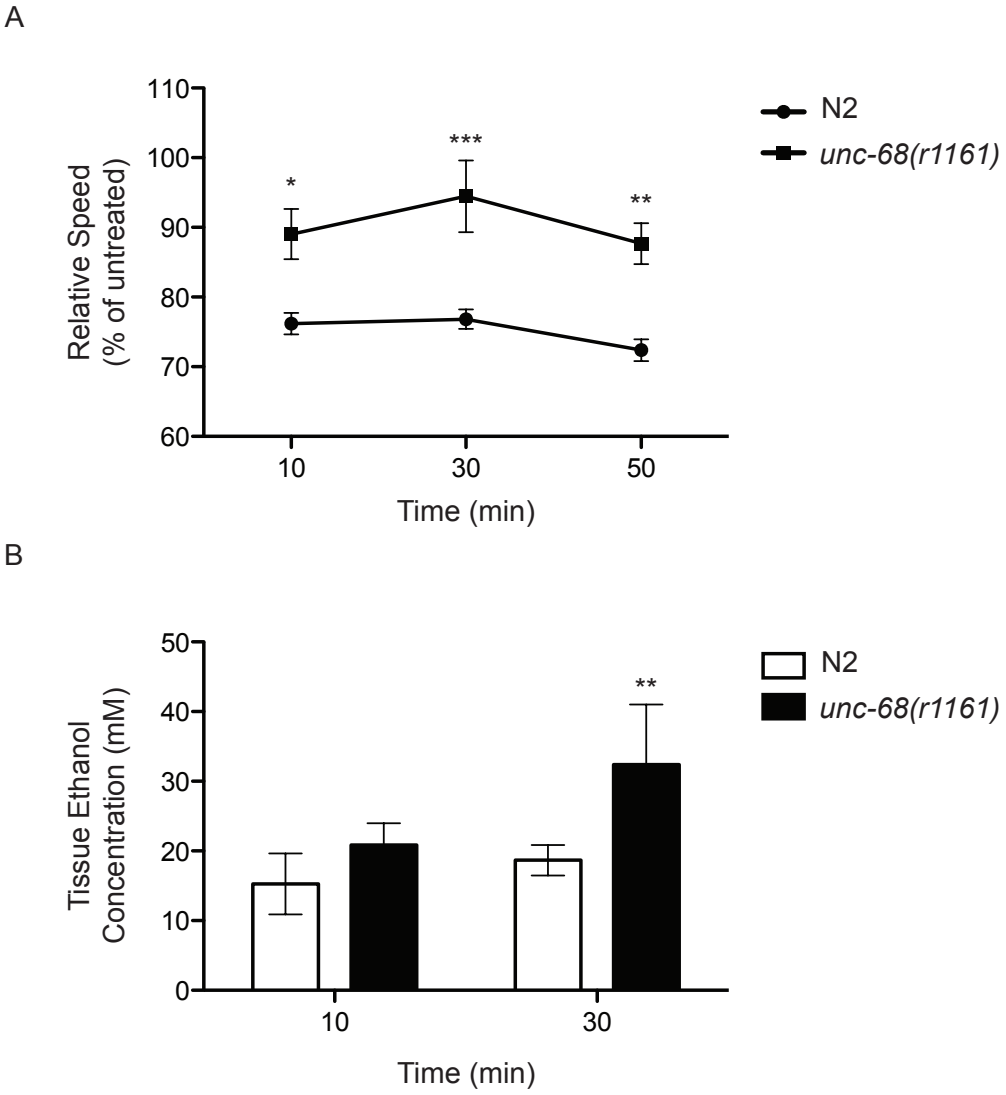

Figure 19. Altered ethanol sensitivity in *C. elegans* with mutations in the mammalian RYR3 orthologue *unc-68*. (A) Effects of 200 mM exogenous ethanol on relative locomotor speeds of wild-type N2 (circles) and *unc-68(r1161)* (squares) (n = 15) (B) Internal ethanol concentrations in N2 control and *unc-68(r1161)* (n = 3). * P< 0.05, ** P<0.01, *** P<0.001. Error Bars are SEM.

Bonferroni multiple comparison; $t(2) = 3.742$, $P < 0.01$). In addition, *unc-68(r1161)* mutants remained significantly resistant throughout the assay despite accumulating higher concentrations of internal ethanol by 30 minutes (Figure 19B). This suggests that the behavioral consequences observed due to loss of *unc-68* function are likely to be due to altered pharmacodynamic responses to ethanol.

Functional analysis of KLF12 orthologs in *C. elegans*

Human *Klf12* encodes a Krüpple-Like Factor zinc-finger transcription factor that is critical for cell differentiation and various physiological functions (Danga *et al.,* 2000, Kaczynski *et al.,* 2003). Worms have multiple Krüpple-Like factors and the worm gene *klf-3* is most closely homologous to human *Klf12.*

We assessed the response of mutant worms carrying the *klf-3(ok1975)* deletion to the acute effects of 400mM ethanol on locomotion (Davies *et al.,* 2003, Appendix A). In this assay, wild-type N2 and *klf-3(ok1975)* animals displayed similar levels of initial sensitivity at 10 minutes (paired T-test $P = 0.8352$), suggesting that *klf-3* does not alter the initial sensitivity to ethanol (Figure 20A). After 30 minutes of continuous exposure, N2 was moving significantly faster than at the previous 10-minute time point (paired T-test $P = 0.0059$), indicating that they had developed AFT. However, worms harboring *klf-3(ok1975)* failed to increase speed over the time of exposure (paired T-test $P = 0.9481$). We calculated the degree of AFT for each strain and found that N2 recovered 17.89 % +/- 2.544 and *klf-3(ok1975)* recovered -0.21 % +/-2.969 of their untreated speed (Figure 20A: right panel). A paired T-test indicated a significant difference between wild-type N2 recovery and *klf-3(ok1975)* ($P = 0.0029$). This suggests that the

loss of *klf-3* function disrupts normal cellular responses to ethanol that results in the failure to develop AFT.

To determine if the ability of *klf-3(ok1975)* to develop AFT was due to an ethanol metabolic defect or entry issue, we performed measurements of internal tissue ethanol concentrations at 10 and 30 minutes. N2 animals accumulated 31.36 mM internal ethanol at 10 minutes and the internal concentration at 30 minutes was 41.1 mM. *klf-3(ok1975)* accumulated similar internal ethanol as N2 at 10 minutes, 35.34 mM and, at 30 minutes, 39.53 (Figure 20B) (two-way ANOVA; genotype $F_{1,18} = 2.107$, $P = 0.1639$, time $F_{2,18} = 3.615$, $P = 0.0479$, interaction between genotype and time $F_{2,18} = 2.74$, $P = 0.6858$, Bonferoni posttest n.s.) This suggests that *klf-3* does not alter the entry of the drug nor does it modify ethanol metabolism and the observed behavioral responses due to the loss of *klf-3* are pharmacodynamic.

Discussion

Using a cross species approach to identify genes that influence ethanol-responsive behaviors using the large number of existing data sets has led to uncovering single gene effects on ethanol responses in *C. elegans*. This approach holds great value for understanding basic mechanisms of ethanol response and provides further evidence for a conserved role of a gene in ethanol responses.

Starting with ethanol-related data sets, mammalian *Clic4* was selected as a candidate gene in ethanol response behavior and genetic manipulations of the gene resulted in altered ethanol responsive behaviors in mice, flies and worms. In *C. elegans*, loss of function of *exc-4* reduced ethanol sensitivity during the first 5 minutes of drug

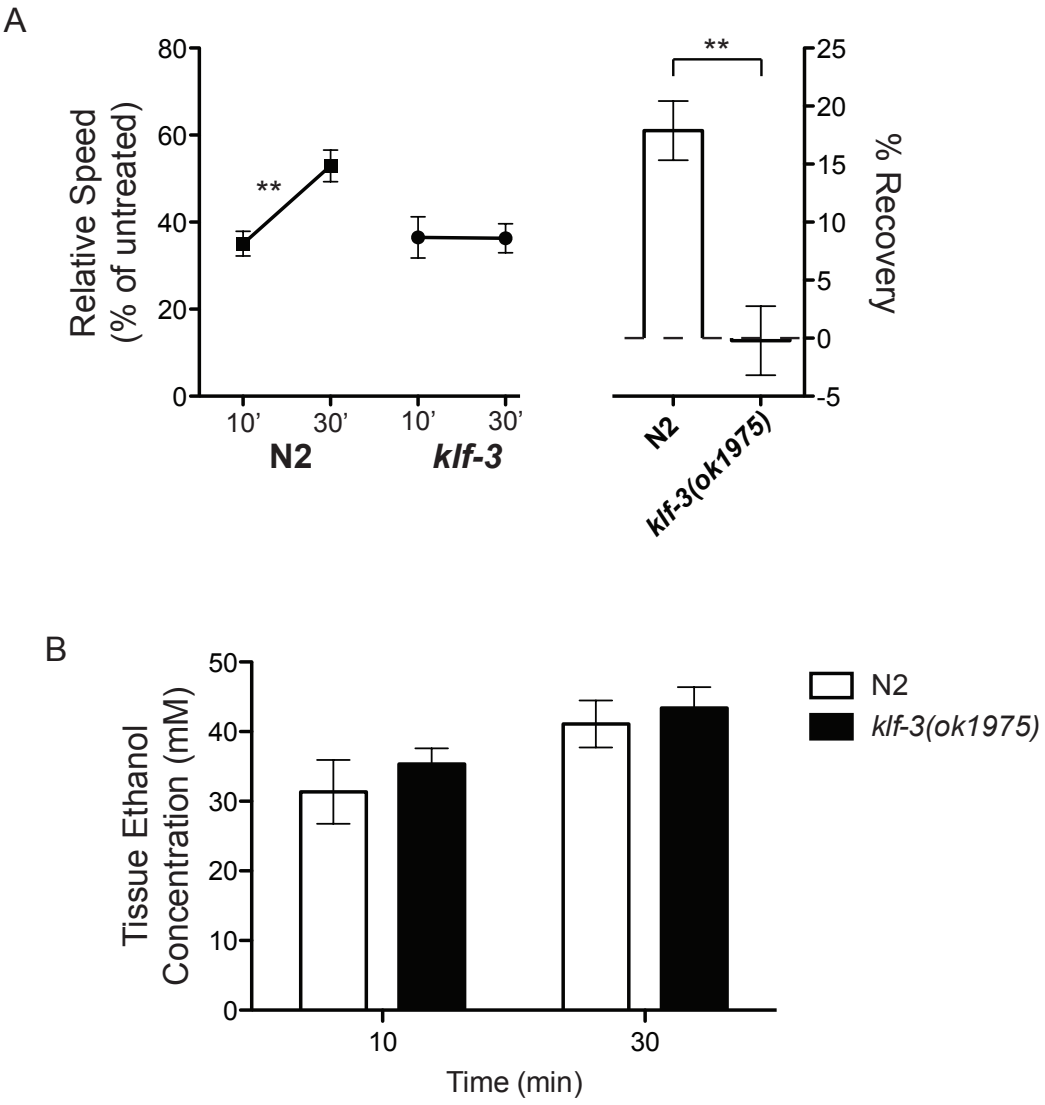

Figure 20. Ethanol sensitivity and acute functional tolerance in *C. elegans* with mutations in the mammalian KLF12 ortholog *klf-3*. (A) Effects of 400 mM exogenous ethanol on relative locomotor speeds of wild-type N2 and *klf-3(ok1975)* (n = 4). (B) Internal ethanol concentrations do not differ between N2 control and *klf-3(ok1975)* at 10 and 30 minutes of exposure (n = 4)

exposure where as the loss of function in another *Clic* ortholog, *exl-1,* enhanced the development of AFT. Genetic manipulation of either *Clic* ortholog caused no alterations to ethanol uptake and metabolism.

Utilizing a different set of ethanol-related data, we focused on the results of a human GWAS that identified loci associated with alcohol dependence. We studied the effects of loss of function of two genes, *Rry3* and *Klf12* because they have shown to interact with other genes mediating acute responses.

Ryr3 encodes an intracellular calcium ion release channel with low Ca^{2+} sensitivity (Nakashima *et al.,* 1997) and is expressed in a variety of tissues including muscle and brain (Hertle and Yeckel, 2007). In vertebrates, ryanodine receptors are known to interact with CLIC proteins and our previous results have shown that altered *clic-4* function affects acute behavioral responses to ethanol (Jalilian *et al.,* 2008, Bhandari *et al.,* 2012 Figure 18). We have shown that the *C. elegans* ortholog of *Ryr3*, *unc-68*, is resistant to the low-dose effects of ethanol despite having a higher tissue ethanol concentration at later time points (Figure 19B). Interestingly, *unc-68* is known to be a major source of Ca^{2+} release required for synaptic vesicle release and muscle contractions (Liu *et al.,* 2005). *unc-68* and a known binding partner, *csq-1*, were found to be significantly up regulated by ethanol in our microarray experiments (see Chapter 3). *csq-1* encodes calesquestrin, a high capacity and low affinity calcium binding protein. This suggests that ethanol may alter calcium release and signaling. This is interesting because a major target of ethanol is the BK channel, which requires calcium for activation and many studies have shown that ryanodine receptors stimulation can

lead to BK channel activation (Xin *et al.,* 2012; Petkov and Nelson 2005; Hristov *et al.,* 2008)

Klf12 encodes a zinc-finger transcription factor and its ortholog in worms, *klf-3,* when non-functional, results in the failure to develop AFT. Internal ethanol data suggests that this behavioral effect is not due to drug uptake or metabolism. In humans, *Klf12* has been shown to bind other Klf-family members and the C-terminal binding proteins, *Ctbp1 and Ctbp2*, to repress transcription (Lomberk and Urrutia, 2005; Schuierer *et al.,* 2001). Interestingly, *Ctbp2* has been identified in an independent GWAS for alcohol dependence (Lind *et al.,* 2010). *Ctbp1* and *Ctbp2* are homologous to *C. elegans ctbp-1*. Previous studies from our lab have shown that animals carrying a loss-of-function of *ctbp-1* have reduced AFT (Bettinger *et al.,* 2012). In addition, a binding partner of CTBP-1, PAG-3, also mediates AFT and animals that carry a loss-of-function mutation of *pag-3* fail to develop AFT (Bettinger *et al.,* 2012). This suggests that binding partners of CTBP-1 that form a transcriptional repressor complex may be involved in mediating AFT. However, it is currently unclear if KLF-3 is acting with CTBP-1 to mediate AFT through similar mechanisms. CTBP-1 regulates approximately 200 genes and represses *lips-7* expression (Chen *et. al* 2009). *lips-7* encodes a lipase that metabolizes triacylglycerides (TAGs). Loss of function of this gene increases TAGs and results in an enhanced AFT phenotype. This supports the idea that TAG levels are important for the development of AFT (Bettinger *et al.,* 2012). To determine if *klf-3* mediates AFT though TAG levels, one simple experiment could be to measure *lips-7* mRNA levels in a *klf-3* mutant. Levels similar to *ctbp-1* would suggest that *klf-3* and *ctbp-1* are acting through similar mechanisms.

Taken together, these data suggest that using a cross species characterization is a powerful approach to identify genes involved in ethanol responsive behaviors. In addition, we show that genes associated with alcohol dependence can modify acute ethanol phenotypes related to LR and these genes, when genetically altered, result in acute behavioral differences in mice, flies and worms.

Chapter V

Discussion and Future Perspectives

Ethanol metabolism is an evolutionarly conserved mechanism across species. This process is governed by at least two enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). In the first step of alcohol metabolism, ethanol is oxidized by ADH to form acetaldehyde. In the second step, acetaldehyde is further oxidized into acetate by ALDH. Both steps, which are reversible, require the reduction of the cofactor NADH.

Multiple studies have shown that genetic variation of these enzymes correlate to AUDs (Chen et al., 2009; Crabb et al., 2004; Edenberg et al., 2006; Kuo et al., 2008). The variants for both ADH and ALDH result in different levels of enzyme activity and therefore differences in the rates of ethanol metabolism. The most notable associations of ADH and ALDH are found amongst individuals of Asian decent. 75% of these individuals carry an allele of ADH (ADH1B*2) that increases the rate of conversion of ethanol to acetaldehyde promoting adverse effects such as facial flushing and vomiting (Eng *et al.,* 2007; Li *et al.,* 2007; Edenberg et al., 2006). Carrying either one or both copies of ALDH1B*2 significantly reduces an individual's risk for alcohol dependence (Chen *et al.,* 2009). In addition, about 30% of the Asian population carries an allele of ALDH (ALDH2*2) that decreases the rate of conversion of acetaldehyde to acetate. Individuals carrying one or both copies of ALDH2*2 are almost completely protected

from developing an AUD (Chen *et al.,* 2009). Conversely, variation at the ADH4 locus is associated with AUDs and has been widely replicated across different populations providing support that variations in ethanol metabolism can differentially influence risk (Endenberg *et al.,* 2006; Luo *et al.,* 2006).

Altered metabolism in other species also affects behavioral responses to ethanol. *Adh-1* knockout mice accumulate more blood ethanol and have an increased sensitivity to the drug as evaluated by loss of righting reflex (Deltour *et al.,* 1999). Mice containing a knock out of *Aldh2* in the high alcohol preferring C57BL/6 background consumed less ethanol and had higher acetaldehyde levels than wild type litter mates (Toyohi *et al.,* 2002; Isse *et al.,* 2005). Studies in high drinking rats have shown that animals carrying both the ADH1B*2 and ALDH2*2 alleles of the ethanol metabolism machinery cause a 60% decrease in consumption, suggesting the aversive effect of excessive accumulation of acetaldehyde can alter consumption (Rivera-Meza *et al.,* 2012). In *Drosophila,* ADH null animals accumulate higher amounts of ethanol and display an increased sensitivity to the drug (Wolf *et al.,* 2002; Ogueta *et al.,* 2010). ALDH null flies also display an increased sensitivity to ethanol, suggesting a role for acetaldehyde in behavioral responses (Fry and Saweikis 2006). Our work has extended the analysis of the role of ADH and ALDH function in acute ethanol response behaviors in *C. elegans*.

By phylogenetic analysis we identified two ADHs, *sodh-1* and *H24K24.3*. Inactivation of either one of these genes resulted in an increase in initial sensitivity, but no alterations to the development of AFT (Figure 5A) Chapter 2. *sodh-1(ok2799)* deletion mutant animals accumulated a significantly higher amount of internal ethanol suggesting that this particular ADH is a key enzyme in ethanol metabolism (Figure 4C).

Internal ethanol concentrations of animals did not change over the time course of exposure, supporting that the AFT observed is likely to be a compensatory response that is limiting the effect of ethanol. Additionally, we resolved a conflict in the literature over the measurement of internal tissue ethanol concentrations in *C. elegans.* Animals exposed to 500mM exogenous ethanol accumulate about 10 – 15% of the exogenously applied dose and our new method accurately accounted for the amount of worm tissue contributed to each sample. Other studies did not account correctly for tissue volumes and reported different internal ethanol concentrations that were both lower and higher than our results (Davies *et al.,* 2003, 2004; Mitchell *et al.,* 2007; Kapfhamer et al., 2008).

We also have explored the role of ALDH function and found that inactivation of either *alh-6 or alh-13* confers an increase in ethanol sensitivity (Figure 6A). There is currently a controversy in the field of the biological role of acetaldehyde in behavior, particularly in the brain. One model which is not widely accepted is that acetaldehyde may act as the molecule responsible for the rewarding and reinforcing effects of ethanol (Rodd-Henriks et al., 2002, Rodd et al., 2005, Hahn et al., 2006, Deng and Ditrich, 2008, Karahanian *et al.*, 2011). Studies in mice and rats have shown that acetaldehyde impairs mobility, coordination, and memory (Dudek and Fuller 1978; Dudek *et al.,* 1984; Tampier and Quintanilla 2003; Quertemont *et al.,* 2004). Our work suggests that knockdown of ALDH results in behavioral sensitivity because of higher internal ethanol concentrations (Figure 6B-C). We interpret this to mean that ADH is oxidizing the excess acetaldehyde that builds up due to the defects in the ALDHs into ethanol, which then exerts behavioral effects. However, our results are not entirely definitive of the role

of acetaldehyde in behavioral responses to ethanol in worms. Ethanol metabolism in the human brain is thought to occur by a combination of the activities of ADH, catalase, and cytochrome P4502E1. Some studies have reported that mammalian brain catalase accounts for 60-70% of ethanol to acetaldehyde conversion (Zimatkin *et al.,* 2006). In *Drosophilia,* 90% of ethanol metabolism occurs by ADH and the remaining 10% by catalase (Geer *et al.,* 1985, 1993). However, ethanol responsive behaviors for catalase mutants in flies have not been characterized. The *C. elegans* genome encodes three catalases, *ctl-1*, *ctl-2*, and *ctl-3* that may contribute to ethanol metabolism (Petriv and Rachubinski, 2004). In addition, exploring ways to increase the level of acetaldehyde in neurons may shed light on the role of the molecule in behavioral responses to ethanol in the worm.

From this work, there are still many unanswered questions in understanding the pharmacokinetics of ethanol metabolism in our model system. Chiefly, the sites of ethanol entry are unknown. Experiments directed at modifying worm anatomy may help answer this question. For example, the worm cuticle is a dynamic structure during development. If environmental cues are unfavorable, animals will go into dauer arrest and produce a thicker cuticle than normal (Golden and Riddle 1982; Cox *et al.,* 1981). If the cuticle acts as a barrier to entry of the drug than less ethanol should be detected in these animals during ethanol exposure. However, the cuticle is not only avenue to explore. There are 4 major exterior openings that may provide a conduit for ethanol entry; the anus, excretory pore, vulva, and the mouth. Work presented in Chapter 4 may provide some insight into the role of the excretory system in ethanol entry. *exc-4* encodes a highly conserved chloride intracellular channel that is required for normal

excretory cell tubulogenesis. When mutated, the excretory canal swells forming a fluid filled vacuole (Berry *et al.,* 2003). The excretory pore itself is structurally normal and presumably still allowing ethanol access into the canal (Buechner 2002). The internal ethanol concentration of this mutant animal is not significantly different than wild-type (Chapter 4), but it is not clear if the altered canal structure causes changes in the distribution of ethanol to other tissues. Providing a basis of ethanol entry will become extremely valuable when performing forward genetic screens or using reverse genetic approaches to detect genes that alter the pharmacodynamics to ethanol.

The exogenous dose used to intoxicate worms is considerably higher than in other model organisms. We predict that the drug must enter through some opening or combination of openings before it can take its effects, but we do not know if ethanol gets distributed equally or is compartmentalized to specific regions in the worm. For example, the intestine is one of the largest organs in the animal and these cells may be exposed to higher concentrations of the ethanol than other cells such as neurons. It is nearly impossible to dissect out specific tissues in *C. elegans* because of their size. . However, recent advances in imaging techniques have shown that live imaging mass spectrometry (IMS) can be used to visually detect the spatial distribution of biomolecules. IMS is a technique bases on two-dimensional mass spectrometry that does not require separation, purification or labeling of target molecules(Kimura *et al.,* 2009). IMS has been used in *C. elegans* to show the differences in biomolecule distribution in wild type animals and *fat-6(tm331)* mutants that lack Δ-9 fatty acid desaturase (Kimura *et al.,* 2009). This technique can also provide answers to other pharmacokinetic questions in a high throughput manner for drug absorption rate,

metabolism, and excretion. Using IMS to characterize mutants of interest will provide a better understanding of that genes role in ethanol pharmacokinetics.

Identifying genes that are causative for complex disorders such as alcoholism has been a difficult challenge. The multiple genetic and environmental factors that can influence the development of this disorder have applied a layer of complexity in defining suitable drug targets (Chesler *et al.,* 2005, Koob and Volkow 2010, Schuckit 2002; Schuckit and Smith 2004, Dick and Foroud 2003). This is mainly because most human studies only reveal regions that are associated with the disease or endophenotypes. Looking beyond the level of associated loci can help uncover the mechanisms in which these genes may act to influence behavioral responses. Gene expression microarrays have been extensively used to study a variety of alcohol use traits (Xu *et al.,* 2001, Tabakoff *et al.,* 2003; Mulligan *et al.,* 2008, 2011; Treadwell and Singh 2004; Wolstenholme *et al.*, 2011, Kerns *et al.,* 2005).). The goal of these studies is to identify genes whose expression correlated with the disease. These expression changes represent a measure of the genetic differences in ethanol induced gene expression, which may influence predisposition to developing alcoholism (Nestler and Aghajanian 1997; Kerns *et al.,* 2005). Individual differences in the initial acute effect of ethanol are correlated with abuse liability and dependence. Therefore, variations in ethanol-induced expression may also underlie predisposition.

We used gene expression microarrays to uncover ethanol responsive genes in N2 and *npr-1(ky13)*. We mined these gene expression profiles for candidate genes that may be involved in modifying acute ethanol response behaviors and used basal gene expression differences between N2 and *npr-1(ky13)* to identify testable candidate

genes. *npr-1(ky13)* animals display an enhanced development of AFT(Figure 14A) and transcription inhibition of these animals did not alter this behavior (Figure 13C) suggesting that *npr-1(ky13)* animals have altered basal gene expression differences that are contributing to its ethanol response behavior. Therefore, we used basal gene expression differences of *npr-1(ky13)* to select candidate genes for behavioral testing.

We found that ethanol responsive gene expression changes in both N2 and *npr-1(ky13)* were similar for a variety of biological processes such as lipid metabolism, transcription, and oxidation reduction. We focused on ethanol responsive genes that were also basally regulated by *npr-1(ky13)* because we predicted that these genes may be involved in mechanism in which cells are compensating to limit the effects of ethanol. We chose *acs-2,* an acyl-CoA synthase, which is required for fatty acid activation (Van Gilst *et al.,* 2005). Loss of function of *acs-2* resulted in significantly reduced AFT (Figure 14A) suggesting that basal levels of this gene are important in the acute responses to ethanol. *acs-2* has been shown to activate fatty acids that are transported into the mitochondria for degradation by a process called β-oxidation (Van Gilst *et al.,* 2005). Animals that are mutant at the *acs-2* locus have reduced mitochondrial β-oxidation rates and we examined the genes downstream of *acs-2* in the mitochondrial β-oxidation pathway (Figure 15) to determine if this process can influence acute behavioral responses to ethanol. We chose to test 7 genes in the mitochondrial β-oxidation (Figure 15) because of their strong homology to mammals and reagent availability. We used RNAi to alter mRNA levels and found that only one gene, *ech-6*, had a significantly enhanced development of AFT (Figure 16C). This Is an interesting result because *acs-2(ok2457)* mutants have the opposite phenotype and one might predict that alteration to

the mitochondrial β-oxidation pathway may result in similar acute behavioral responses to ethanol. One possible explanation is that the accumulation of trans-2-enoyl CoA due to the decrease in *ech-6* function may be utilized in other pathways that are involved in the development AFT. For example, other studies in worms have shown that the mitochondria can participate in fatty acid synthesis and trans-2-enoyl CoA is a molecule that can be utilized and converted into fatty acid in this pathway (Gruvitz, 2009). Other work has implicated that fatty acids are important to the development of AFT in worms. Work in our laboratory has shown that animals deficient in monounsaturated fatty acid synthesis have an enhanced development of AFT (Bettinger *et al.,* 2012). Taken together these results implicate the role of mitochondria β-oxidation pathway in acute behavioral responses to ethanol.

Our microarray work and behavioral analysis has revealed a role for fatty acid metabolism in ethanol responses. However, there are still many other interesting experiments that need to be completed before we can start to understand the role of this complex process in acute ethanol response behaviors. We show that loss of function of *acs*-2 results in reduced AFT. It would be interesting to see the effects of *acs-2* overexpression on ethanol response behaviors. This would reveal the relationship of the gene to AFT. For example, we would predict that *acs-2* overexpression may result in an enhanced AFT phenotype, suggesting that *acs*-2 can regulate AFT and may not just be required to observe the behavior. Therefore, the opposite phenotypes where loss of function mutants display decreased development AFT, but the gain of function mutants display an enhanced development would suggest that the functional level of *acs*-2 can either represses and promotes AFT.

Another experiment that would provide insight into the role of *acs-2* in behavior is to perform tissue specific gene expression experiments. *acs-2* is widely expressed in all tissues of worms including neurons. Restoring *acs-2* expression to wild type levels in all neurons of *acs-2(ok2457)* mutants would provide evidence that AFT has a neuronal basis and may identify the cells that are required to produce this response. If animals display wild type AFT it would suggest that *acs-2(ok2457)* is required in these cells to produce an adaptive response to ethanol. Follow up experiments could then focus on specific neurons that are required for the behavioral rescue. These results would implicate a specific subset of neurons that are mediating the adaptive response and future work exploring how *acs-2* modifies the function of these neurons may reveal new mechanisms into the development of AFT. For example, *acs-2* may be required in neurons that communicate with each other forming a small neuronal network or circuit. *acs*-2 may alter the communication of this circuit or the intrinsic properties of these neurons that are involved in the development of AFT.

Our work has shown the genes downstream of *acs-2* in the mitochondria β-oxidation pathway alters the development of AFT. Our experiments relied on RNAi to determine if a reduction in the mRNA levels alters ethanol responses. However, there are some drawbacks to using RNAi, especially when treatment does not result in a behavioral effect. Frist, neurons are resistant to the effects of RNAi by feeding in worms (Calixto *et al.,* 2010) and therefore gene expression levels of the target gene are normal in this tissue. In our work, treatment of worms with RNAi targeted to *cpt-2* or *cpt-5* did not alter the behavioral response to ethanol (Figure 16A). One interpretation of this result could be that these genes are not involved in ethanol responsive behaviors. .

Another interpretation could be that we were unable to knock down the mRNA enough to have effect on gene function and any residual message in the cell is enough to maintain normal function. To circumvent this issue, using loss of function or null alleles instead of RNAi may result in a different effect since the function of gene is eliminated.

acs-2 is required to activate fatty acids in the cell for a variety of different cellular processes and loss of function of this gene results in fat accumulation (Van Gilst *et al.,* 2005; Ashrafi *et al.,* 2003). This is interesting because it suggests that the fatty acids destined for mitochondrial β-oxidation are being utilized in other pathways in the cell. There is a possibility that the accumulation of fatty acids being produced because of the lack of activation by *acs-2* are only being utilized in the synthesis of TAGs and this would explain why animals stain positive for an increase in fat stores. However, there are at least two other possibilities that can be explored to determine the role of other fatty acid utilization pathways.

First, we can ask the question if altering the fatty acid pool or population in animals alters behavioral responses to ethanol. It has been previously shown that animals carrying a loss of function for *lips-7* have an increase in TAGs (Chen *et al.,* 2009). *lips-7* encodes a lipase that enzymatically cleaves fatty acids from TAGs allowing the fatty acids to be utilized in other cellular pathways such as cell signaling. *lips-7* mutant animals display an enhanced development of AFT to ethanol (Bettinger *et al.,* 2012). Epistasis analysis of *lips-7* and *acs-2* may reveal if the fatty acid pool or population is important in ethanol responses. One possible prediction would be that the *acs-2* mutantion might restore particular fatty acids to the fatty acid population that are otherwise unable to be freed from a TAG and utilized in the cell in a lips-7 mutant

because of the animals deficient lipase function. We would then predict that *lips-7;acs-2* mutant animals would display ethanol response behaviors like wild-type suggesting that *acs-2* is modifying the fatty acid population. The double mutants may not display ethanol response behaviors like wild type and may display behavioral response like the individual mutant, which would suggest that they produce their behavioral effects by different pathways.

Second, we can also look at other acyl-CoA synthetase pathways. Presumably, the loss of *acs-2* function may allow fatty acids to be utilized by other acyl-CoA synthetases in the cell. In mammals, some acyl-CoA synthetases have a broad range of fatty acids preference, while other synthetases do not and will only activate a select type (Fulgenico *et al.,* 1996). This is important, because it implies that there is a level of specificity for a particular fatty acid in a pathway. Many studies have implicated the role of activated fatty acids in a variety of cellular process and pathways such as signaling, vesicle trafficking, fatty acid desaturation and elongation, and sphingolipid synthesis (Coleman *et al.,* 2002). Furthermore, studies have shown that pharmacological inhibition of acyl-CoA synthetases disrupts the budding and fusion process of protein transport from the endoplasmic reticulum to the Golgi (Pfanner *et al.*, 1989). This suggests that determining the identity of these molecules may reveal specific roles for fatty acids in cellular responses and may provide insights into acute ethanol responses.

Overall, the work presented here has shown that the basal expression level of genes is important in behavioral responses to acute ethanol. Furthermore, our data indicate that genes that are ethanol responsive can also identify genes and cellular pathways that are involved in acute behavioral responses to ethanol. Our behavioral

data has validated this approach and this leaves us with a massive resource of gene expression changes that can be mined to uncover novel gene involved in the acute ethanol response, which may have an implication in future risk for developing dependence.

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Appendix A

Detailed Protocols

Analysis of Locomotion:

Plate setup and assay

1.) 12-18 hours before prior to the analysis of locomotion pick 30 L4 staged worms to an OP50 seeded nematode growth media (NGM) plate. Be sure to make the appropriate accommodations for mutant animals that are developmentally delayed relative to wild type animals.

2.) Prior to performing analysis of locomotion dry 4 – 6 NGM plates in the 37°C incubator for 2 hours.

3.) Record the weight of the plates and determine the volume of 100% ethanol to add using the ethanol weight table (Table 8) to achieve your desired concentration.

4.) Heat copper rings for 3-5 seconds over a Bunsen burner using forceps and place on a dried NGM plate.

5.) Melt the appropriate number of rings per plate for your assay. Ensure that the ring has formed a complete seal with the NGM plate surface by looking under the microscope. Also, ensure that the assay area (within the ring) is free and clear of all or any debris.

6.) Next, evenly distribute ethanol throughout the experimental plate and Parafilm both the ethanol and non-ethanol containing plates.

Table 8. Ethanol plate weight calibration for behavioral analysis

Weight (g)	Agar (mL)	50	100	150	200	250	300	350	400	450	500
18.1	10.0	30.1	60.2	90.3	120.4	145.5	180.7	206.8	232.9	262.0	291.1
18.0	9.9	29.8	59.6	89.4	119.3	144.1	178.9	204.7	230.6	259.4	288.2
17.9	9.8	29.5	59.0	88.6	118.1	142.7	177.1	202.7	228.3	256.8	285.4
17.8	9.7	29.2	58.4	87.7	116.9	141.3	175.3	200.7	226.0	254.3	282.5
17.7	9.6	28.9	57.9	86.8	115.7	139.8	173.6	198.6	223.7	251.7	279.7
17.6	9.5	28.6	57.3	85.9	114.5	138.4	171.8	196.6	221.4	249.1	276.8
17.5	9.4	28.3	56.7	85.0	113.4	137.0	170.0	194.6	219.2	246.5	273.9
17.4	9.3	28.0	56.1	84.1	112.2	135.5	168.3	192.6	216.9	244.0	271.1
17.3	9.2	27.7	55.5	83.2	111.0	134.1	166.5	190.5	214.6	241.4	268.2
17.2	9.1	27.5	54.9	82.4	109.8	132.7	164.7	188.5	212.3	238.8	265.4
17.1	9.0	27.2	54.3	81.5	108.6	131.3	162.9	186.5	210.0	236.3	262.5
17.0	8.9	26.9	53.7	80.6	107.4	129.8	161.2	184.5	207.7	233.7	259.7
16.9	8.8	26.6	53.1	79.7	106.3	128.4	159.4	182.4	205.4	231.1	256.8
16.8	8.7	26.3	52.5	78.8	105.1	127.0	157.6	180.4	203.2	228.6	254.0
16.7	8.6	26.0	52.0	77.9	103.9	125.5	155.9	178.4	200.9	226.0	251.1
16.6	8.5	25.7	51.4	77.0	102.7	124.1	154.1	176.3	198.6	223.4	248.2
16.5	8.4	25.4	50.8	76.2	101.5	122.7	152.3	174.3	196.3	220.8	245.4
16.4	8.3	25.1	50.2	75.3	100.4	121.3	150.5	172.3	194.0	218.3	242.5
16.3	8.2	24.8	49.6	74.4	99.2	119.8	148.8	170.3	191.7	215.7	239.7
16.2	8.1	24.5	49.0	73.5	98.0	118.4	147.0	168.2	189.5	213.1	236.8
16.1	8.0	24.2	48.4	72.6	96.8	117.0	145.2	166.2	187.2	210.6	234.0
16.0	7.9	23.9	47.8	71.7	95.6	115.6	143.4	164.2	184.9	208.0	231.1
15.9	7.8	23.6	47.2	70.8	94.5	114.1	141.7	162.1	182.6	205.4	228.3
15.8	7.7	23.3	46.6	70.0	93.3	112.7	139.9	160.1	180.3	202.9	225.4
15.7	7.6	23.0	46.0	69.1	92.1	111.3	138.1	158.1	178.0	200.3	222.5
15.6	7.5	22.7	45.5	68.2	90.9	109.8	136.4	156.1	175.8	197.7	219.7
15.5	7.4	22.4	44.9	67.3	89.7	108.4	134.6	154.0	173.5	195.2	216.8
15.4	7.3	22.1	44.3	66.4	88.5	107.0	132.8	152.0	171.2	192.6	214.0
15.3	7.2	21.8	43.7	65.5	87.4	105.6	131.0	150.0	168.9	190.0	211.1

Table 8. Ethanol plate weight calibration for behavioral analysis

Weight (g)	Agar (mL)	50	100	150	200	250	300	350	400	450	500
18.1	10.0	30.1	60.2	90.3	120.4	145.5	180.7	206.8	232.9	262.0	291.1
18.0	9.9	29.8	59.6	89.4	119.3	144.1	178.9	204.7	230.6	259.4	288.2
17.9	9.8	29.5	59.0	88.6	118.1	142.7	177.1	202.7	228.3	256.8	285.4
17.8	9.7	29.2	58.4	87.7	116.9	141.3	175.3	200.7	226.0	254.3	282.5
17.7	9.6	28.9	57.9	86.8	115.7	139.8	173.6	198.6	223.7	251.7	279.7
17.6	9.5	28.6	57.3	85.9	114.5	138.4	171.8	196.6	221.4	249.1	276.8
17.5	9.4	28.3	56.7	85.0	113.4	137.0	170.0	194.6	219.2	246.5	273.9
17.4	9.3	28.0	56.1	84.1	112.2	135.5	168.3	192.6	216.9	244.0	271.1
17.3	9.2	27.7	55.5	83.2	111.0	134.1	166.5	190.5	214.6	241.4	268.2
17.2	9.1	27.5	54.9	82.4	109.8	132.7	164.7	188.5	212.3	238.8	265.4
17.1	9.0	27.2	54.3	81.5	108.6	131.3	162.9	186.5	210.0	236.3	262.5
17.0	8.9	26.9	53.7	80.6	107.4	129.8	161.2	184.5	207.7	233.7	259.7
16.9	8.8	26.6	53.1	79.7	106.3	128.4	159.4	182.4	205.4	231.1	256.8
16.8	8.7	26.3	52.5	78.8	105.1	127.0	157.6	180.4	203.2	228.6	254.0
16.7	8.6	26.0	52.0	77.9	103.9	125.5	155.9	178.4	200.9	226.0	251.1
16.6	8.5	25.7	51.4	77.0	102.7	124.1	154.1	176.3	198.6	223.4	248.2
16.5	8.4	25.4	50.8	76.2	101.5	122.7	152.3	174.3	196.3	220.8	245.4
16.4	8.3	25.1	50.2	75.3	100.4	121.3	150.5	172.3	194.0	218.3	242.5
16.3	8.2	24.8	49.6	74.4	99.2	119.8	148.8	170.3	191.7	215.7	239.7
16.2	8.1	24.5	49.0	73.5	98.0	118.4	147.0	168.2	189.5	213.1	236.8
16.1	8.0	24.2	48.4	72.6	96.8	117.0	145.2	166.2	187.2	210.6	234.0
16.0	7.9	23.9	47.8	71.7	95.6	115.6	143.4	164.2	184.9	208.0	231.1
15.9	7.8	23.6	47.2	70.8	94.5	114.1	141.7	162.1	182.6	205.4	228.3
15.8	7.7	23.3	46.6	70.0	93.3	112.7	139.9	160.1	180.3	202.9	225.4
15.7	7.6	23.0	46.0	69.1	92.1	111.3	138.1	158.1	178.0	200.3	222.5
15.6	7.5	22.7	45.5	68.2	90.9	109.8	136.4	156.1	175.8	197.7	219.7
15.5	7.4	22.4	44.9	67.3	89.7	108.4	134.6	154.0	173.5	195.2	216.8
15.4	7.3	22.1	44.3	66.4	88.5	107.0	132.8	152.0	171.2	192.6	214.0
15.3	7.2	21.8	43.7	65.5	87.4	105.6	131.0	150.0	168.9	190.0	211.1

7.) . After 1 hour and 30 minutes begin to acclimate animals to assay conditions by moving 10 worms per ring to two non-ethanol containing plates and Parafilm.

8.) After 30 minutes, move the animals to the appropriate assay plate and Parafilm. Move animals in the same order for each plate within a single assay, but alternate the order every assay to control for any bias effect. If you are recording the first 10 minutes of intoxication you will need to offset your acclimation plates by 12-15 minutes, however if you are recording two-minute movies, then you will need to offset each acclimation plate by no less than 2 minutes.

9.) To begin recording locomotion, turn on the camera and open ImagePro.

10.) Adjust the light source to between 70-90 for optimal resolution.

11.) Adjust setting of the software to 2x2 binning at 2 frames per second for 120 frames (two minute movie) or 600 frames (ten minute movie) using a 0.5x objective lens and 0.8x magnification.

12.) At the appropriate time points place plates on the light source under the microscope and click record.

13.) Save the file to the external hard drive with a .seq extension.

Computer Analysis

1.) Open the Image Pro software and select file and open.

2.) Select the videos that you will analyze.

3.) Select macro and select flatten magnify add ring.

4.) Using the mouse drag the newly created white ring over a copper ring on the video.

5.) Select measure and track objects. A new window will open. Select track objects automatically.

6.) Select ranges on the count/size window and adjust the intensity of object detection to a level in which all worms within the ring are clearly detectable.

7.) Select count and ensure all worms are included. Next, select continue. When the macro is completed quantitating locomotion, select YES followed by NO.

8.) All detected tracks will appear in the tracking data table. Examine each track individually and ensure that the macro hasn't analyzed extraneous objects and delete any tracks with proper justification

9.) Export data into excel

RNA interference:

Plate preparation

RNA clone selection plates:

The following steps will produce 25 20 cm plates. In a round bottom or Erlenmeyer flask combine the following:

1.) 0.243 L dH_2O

2.) 0.75 g NaCl

3.) 4.0 g Bacto-Agar

4.) 0.625 g Bacto-Peptone

5.) Add stir bar

6.) Cover with aluminum foil and mark with autoclave tape

7.) Use stir plate to dissolve contents

8.) Autoclave using a 30 minute sterilization

9.) Return autoclaved agar to stir plate and allow to cool for 15 minutes

10.) Add 6.25 mL of 1 M K_3PO_4

11.) Add 0.25 mL of 1 M $MgSO_4$

12.) Add 0.25 mL of 1 M $CaCl_2$

13.) Add 0.25 mL of 5 mg/mL cholesterol dissolved in 100% ethanol

14.) Add 125 µL of 100 mg/mL ampicillin dissolved in ddH_2O

15.) Add 500 µL of 5 mg/mL tetracycline HCl dissolved in 70% ethanol

16.) Pour 20 mL into 20 cm plates

17.) Allow 24 hours for plates to dry before use

18.) Store plates at 4°C for up to 1 month

RNA interference plates:

The following steps will produce 75 10 cm plates. In a round bottom or Erlenmeyer flask combine the following:

1.) 0.722 L dH_2O

2.) 2.25 g NaCl

3.) 12.0 g Bacto-Agar

4.) 1.875 g Bacto-Peptone

5.) Add stir bar

6.) Cover with aluminum foil and mark with autoclave tape

7.) Use stir plate to dissolve contents

8.) Autoclave using a 30 minute sterilization time

9.) Return autoclaved agar to stir plate and allow to cool for 30 minutes

10.) Add 0.75 mL of 1 M $MgSO_4$

11.) Add 0.75 mL of 1 M $CaCl_2$

12.) Add 0.75 mL of 5 mg/mL cholesterol dissolved in 100% ethanol

13.) Add 18.75 mL of 1 M KPO_4

14.) Add 0.1875 mL of 100 mg/mL carbenicillin dissolved in 100% ethanol

15.) Add 0.750 mL of 1 M IPTG dissolved in ddH_2O

16.) Pour 10 mL into 10 cm plate

17.) Allow 24 hours for plates to dry before use

18.) Store in 4°C for up to 1 month

Culture Inoculation and Storage

Prior to inoculation prepare the growth media by combining the following:

1.) Autoclave 50 mL of Luria Broth

2.) Add 25 µL of 100 mg/mL ampicillin dissolved in ddH_2O

Inoculation from Library:

1.) Locate coordinates of clone in library

2.) Using a P2 pipette with tip attached gently scrap the well and eject the tip into a culture tube containing growth media

3.) Grow cultures for 12 – 18 hours in a shaker set at 200 RPM and 37°C

4.) Heat an inoculation loop and dip the loop into the newly grown culture

5.) Streak the contents into quadrant 1 (Figure X) of a tetracycline/ampicillin plate

6.) Flame your loop and cool it by touching an area of agar

7.) Streak down from quadrant 1 into quadrant 2 and fill the quadrant

8.) Repeat steps 6 and 7 until all 4 quadrants are filled

9.) Parafilm and label the tetracycline/ampicillin plate

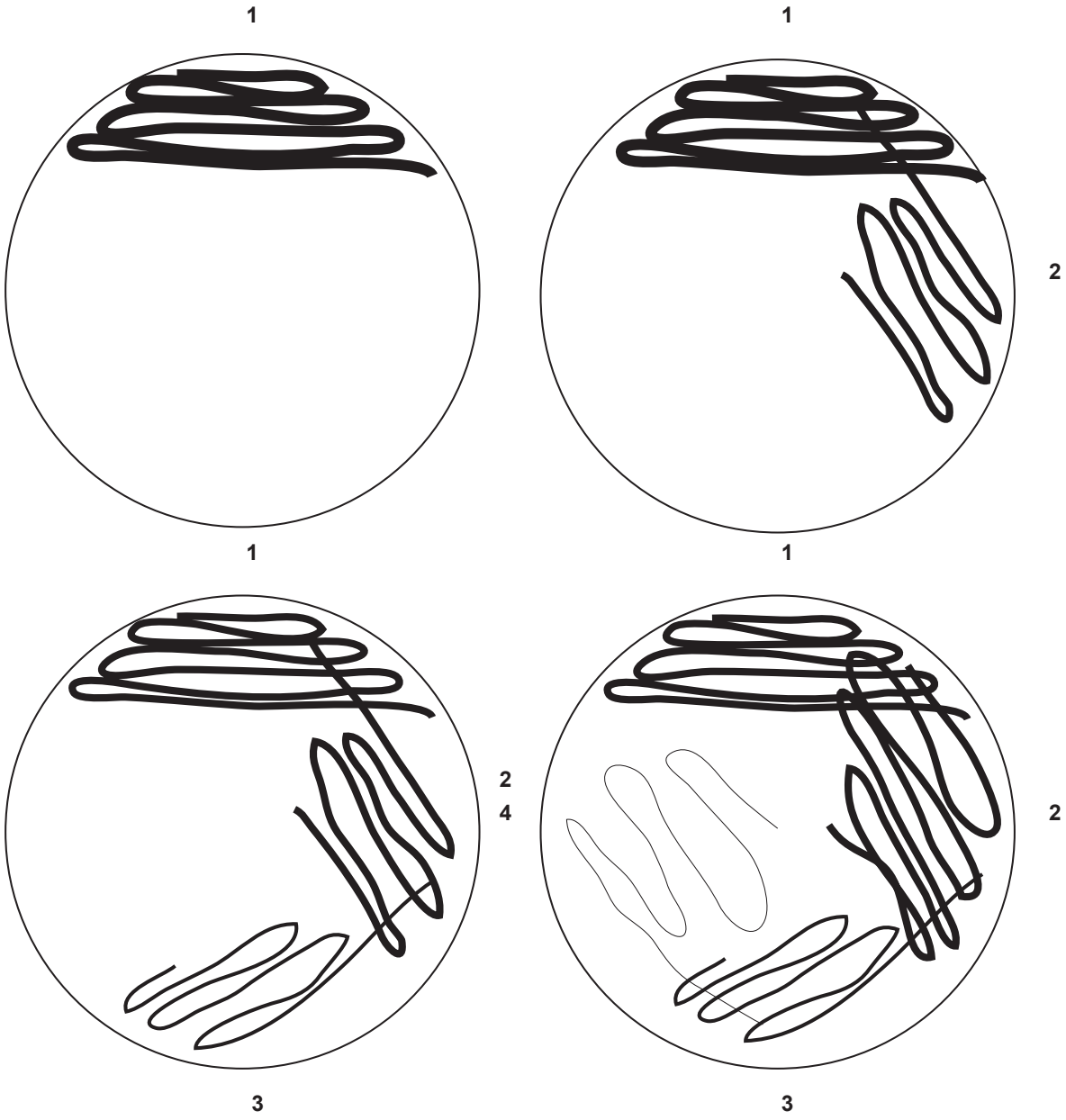


Figure 21: Streaking bacteria. Dip inoculation loop into grown culture and spread in quadrant 1. Heat the inoculation loop and cool by touching agar. Next, streak from quadrant 1 into quadrant 2. Repeat for quadrant 3 and quadrant 4. Single colonies should be visible in quadrant 4 after 24 hours of incubation at 37°C

10.) Incubate the plate at 37°C for 24 – 36 hours or until large single colonies appear

11.) Inoculate a single colony from the plate in 3 mL of growth media

12.) Grow cultures for 12-18 hours in a shaker set at 200 RMP and 37°C

13.) Remove 1.5 mL of culture into a freezing tube

14.) Add 300 µL of 100% glycerol

15.) Mix well and place in -80°C

16.) See *insert Verification* protocol below for the remaining 1.5 mL of culture

Inoculation from Frozen Stock

1.) Add 2 -3 mL of growth media to culture tubes

2.) Remove frozen culture from -80°C

3.) Using a P2 pipette with tip attached scrap the frozen culture and eject the tip into the culture tube

4.) Incubate cultures in a shaker at 200 RPM at 37°C for 12-18 hours

5.) If only one frozen culture is found in the -80°C, freeze another culture. Follow *inoculation from Library* protocol steps 13 -15.

6.) Inoculated culture is stored at 4°C and good for 1 week

Insert Verification

Solution Preparation:

Solution 1

Combine the following into a 100 mL autoclaved bottle:

1.) Add 450.4 mg glucose

2.) Add 50 mL of ddH_20

3.) Mix to dissolve

4.) Add 1.25 mL of 1 M Tris pH 8.0

5.) Add 1 mL of 0.5 M EDTA pH 8.0

6.) Add 0.1 mL of 10 mg/mL RNase A

7.) Store at 4°C

Solution 2

Make this solution fresh and store at room temperature

1.) Add 40 µL of 5 N NaOH per 1 mL of final volume of solution

2.) Add 100 µL of 10% SDS per 1 mL of final volume of solution

Solution 3

Combine the following in a 100 mL autoclaved bottle:

1.) 30 mL of 5 M potassium acetate (49.07 g/100 mL ddH_2O)

2.) 5.75 mL of glacial acetic acid

3.) 14.25 mL ddH_2O

Plasmid Isolation

1.) Using the remaining 1.5 mL of culture from the *inoculation from Library* protocol pellet the culture in a 1.5 mL tube

2.) Remove the supernatant and resuspend pellet in 100 µL of cold Solution I and vortex

3.) Add 200 µL of Solution 2 and invert to mix

4.) Incubate at room temperature for 3-5 minutes

5.) Add 150 µL of cold Solution 3 and invert to mix

6.) Incubate at room temperature for 3 -5 minutes

7.) Spin sample at 13,000 RPM for 5 minutes

8.) Move supernatant to new tube and equal volume of phenol:chloroform and vortex

9.) Spin sample at 13,000 RMP for 2 minutes

10.) Remove the aqueous layer to a new tube and precipitate the DNA by adding 1 mL of 95% ethanol

11.) Vortex for 30 seconds

12.) Incubate at room temperature for 5 minutes

13.) Spin sample at 13,000 RPM for 5 minutes

14.) Look for a clear/white pellet

15.) Carefully remove the supernatant

16.) Add 1 mL of 70% ethanol and invert the tube 4 times

17.) Spin sample at 13,000 RPM for 2 minutes

18.) Carefully remove the supernatant

19.) Dry the pellets by place the tubes with their caps open upside down on a level surface for 20 minutes

20.) Dissolve the pellet in 25 µL of dH_2O and incubate at 37°C for 10 minutes

21.) Quantitate the concentration of DNA of the sample by measure the absorbance at 260 nm

22.) Dilute sample to 100 ng / µL

23.) Send sample to the VCU sequencing center with the U19 primer

24.) When results return, use BLAST to identify if the insert is correct

Generating RNA inactivated strains

When generating knockdown strain also include an L4440 control and a culture that induces a visible phenotype.

1.) 24 hours prior to treatment seed an RNA plate with 50 – 70 µL of culture

2.) Uncover plates halfway and allow them to dry for 15 minutes

3.) The next day plate 3 – 5 L3 or L4 animals

4.) Repeat steps 1-2

5.) 36 – 42 hours later move animals from the first RNA plate to the new RNA plate

6.) Allow animals to develop to desired age

7.) Prior to using animals check your visible control to ensure the plates are working.

Internal Ethanol Concentration Analysis:

Egg Laying

1.) 4 days prior places 8-10 L4 animals on 5 heavily seed NGM plates

2.) Remove adult animals 2 days later

3.) Seed 6 plates per strain to be tested

4.) The next day plate 30-40 gravid adults per plate for 6 plates

5.) Allow animals to lay eggs for 2 hours and remove

6.) Record the time and allow worms to develop to desired hour age

Ethanol Exposure

Prior to ethanol exposure label all tubes needed before starting the assay. Aliquot 20 µL of ddH_2O into each tube and place on ice until collection.

1.) Dry plates for 2 hours at 37°C

2.) Record the weight of the plates and determine the volume of 100% ethanol to add using the ethanol weight table (Table X) to achieve your desired concentration.

3.) Parafilm and allow ethanol to equilibrate for 2 hours

4.) After 2 hours move 10-30 animals to an unseeded plate

5.) Using Image Pro snap pictures of the strain to be tested using the microscope light source set to II, a 1x objective lens at 4x magnification.

6.) Select calculate auto exposure to achieve optimal resolution

7.) Snap picture of 10 different worms and save as a ,tiff file extension

8.) Immediately pellet 3 plates of animals using M9 and a wide bore tip into a tube

9.) Wash the pellet with 1 mL of M9

10.) Remove supernatant and leave about 40 µL of liquid

11.) Using wide bore non-stick pipette tips aliquot 20 µL of worms to one non-ethanol and one ethanol containing plate

12.) Allow 2-3 minutes for the liquid to be absorbed by the plate

13.) Parafilm and start timer

14.) Repeat steps 8-13 for the remaining 3 plates

15.) At collection time, pick 200 worms in 2-3 minutes.

16.) Store worms at -80°C

Spectrometric Analysis

Wear gloves at all times. Keep all reagents on ice unless otherwise noted. Turn heat block to 30°C and fill half way with H_20. Set centrifuge to 4°C. Label all tubes

1.) Create a 1 M ethanol standard (0.584 mL 200 proof ethanol to 9.416 mL ddH_2O)

2.) Perform serial dilutions and make the following standards: 10 mM (100 µL of 1 M ethanol to 9.9 mL ddH_20) 5 mM (5 mL of 10 mM standard to 5 mL ddH_20), 2mM (4 mL of 5 mM standard to 6 mL ddH_20), and 1mM (5 mL of 2 mM standard to 5 mL ddH_20)

3.) Mix each standard and place on ice

4.) Homogenize worms using cleaned and autoclaved blue pestle while samples reside on ice

5.) Spin all samples at full speed fro 1 minute at 4°C

6.) Reconstitute alcohol reagent with 15mL of ice cold ddH_20 and swirls flask to dissolve contents

7.) Aliquot 300 µL of alcohol reagent into 1.5 mL labeled tubes for each sample being tested

8.) Pipette 1.5 µL of homogenized worm sample to 300 µL of alcohol reagent

9.) Mix by flicking twice and immediately place the sample in the heat block for 5 minutes

10.) Keep pace of one sample every 20 seconds

11.) Remove samples and place on ice

12.) Turn on spec and set the wavelength to 340nm

13.) Blank with ddH_20

14.) Spec 100 µL of sample three times and record

15.) Spec the remaining 200uL of sample 1-2 times.

Internal ethanol concentration calculation

1.) Open all images previously recorded in *Ethanol exposure* in ImagePro

2.) Connect the tracing pad via the USB cord

3.) Magnify the image to 100%

4.) Select measure

5.) Using the pen provide with he tracing pad determine the length of the animal by tracing at the head and ending at the whip

6.) Next determine the diameter by tracing the width of the worm at the head, vulva, and tail (Figure 3)

7.) Repeat steps 4-5 3 times for a single worm and average each measure

8.) Repeat steps 4-6 for 10 worms total.

9.) Calculate the volume worm by using the volume equation for a cylinder ($\pi r^2 H$)

10.) For a single worm, use the average length reordered as the height, average all 3 diameters recorded and divide by 2 and use as the radius

11.) Average the volume of each of the 10 worms

12.) Convert the averaged volume from μm^3 to µL by multiplying 10^{e-9}

13.) Multiply the converted number by 200

14.) Calculate the dilution factor by dividing the total volume (20 µL + the volume in step 13) divided by the volume of step 13

15.) Enter the absorbances from *spectrometric analysis* into excel and average all absorbance for each standard and strain

16.) Construct a standard curve and use it to determine the amount of ethanol of each sample.

17.) Multiply the internal ethanol from the curve of each sample by the dilution factor calculated in step 14

18.) This is the internal ethanol of the worm in mM.

Real Time Quantitative PCR:

Primer Design

1.) Access Wormbase and look up gene of interest

2.) Asses how many isoforms exists for the gene and design primers to capture all isoforms

3.) Using the spliced mRNA sequence count back 1 kilobase from the 3'UTR

4.) Copy that portion of the sequence

5.) Access the following website: http://frodo.wi.mit.edu/primer3/

6.) Paste the sequence into the input box

7.) Change the product size range to 75 – 200

8.) Change the number to return to 10

9.) Change the GC clamp to 1

10.) Select "Pick Primers"

11.) Select primer pairs that have the following: equal length, melting temperatures less than 2°C apart, 45-65% GC content, low complementarity (Any and 3' columns), and a GC clamp

12.) Record where the primer pairs fall relative to the exons of the gene

13.) BLAST primers using Wormbase to ensure primers are unique

14.) Access the following website: http://www.premierbiosoft.com/qpcr/index.html

15.) Select "Launch Free Edition"

16.) Log in or create an account

17.) Select "Launch Free Edition" again

18.) Choose SYBR Green and paste the primers into the correct boxes

19.) Change free Mg from 3 to 6

20.) Select “Analyze”

21.) Make sure the primers pairs have low ΔG for Cross Dimer, Self Dimer, and Hair Pin. Do not use any primer with a ΔG greater than 3

Primer Verification and Optimization

Depending on where the primers are located within the gene, you will have to alter the template input from either DNA or cDNA. Use cDNA for optimization when a primer spans an intron-exon junction.

1.) Combine the following reagents into a master mix: For 1 reaction: 2.0 µL 10X reaction buffer, 0.3 µL of X mM MgCl, 0.2 µL of 25 mM dNTP, 0.1 µL of 25 uM Forward primer, 0.1 µL of 25 uM Reverse primer, 0.1 µL Taq, 200 ng/µL final concentration of genomic DNA or 1 µL of cDNA, bring up to 10 µL with nuclease free water

2.) To determine annealing temperature range subtract 5°C from the primer with the lowest melting temperature

3.) On the thermal cycler select the program “Mapping Optimized” and “Edit”

4.) Change the initial temperature to the number you calculated in step 2 and change the end temperature to the number from step 2 plus 11°C

5.) Write down the temperature/column you will use. Ideally use 1°C difference between columns

6.) Go back to the amplification program and change the number of amplification cycles to 35, the 94°C separation step to 15 seconds, the annealing step to 15 second, and the elongation step to the appropriate time.

7.) Run samples on 1.5 – 2.0% agarose gel and score for size

8.) Determine the best band and proceed with that temperature for *qPCR Standard Curve*

RNA Isolation

Worm Growth

1.) One day prior to growth seed 10 NGM plates with 50 µL of OP50 24 per strain

2.) Plate 15 gravid adults per plate

3.) Allow worms to lay eggs for 5-8 hours

4.) When populations reach adult hood begin *Extraction* protocol

Extraction

Wear gloves at all times. Wipe all surfaces and pipettes with 70% ethanol. Wipe down all surfaces, gloves, and pipettes with RNase Zap. Label all tubes needed for isolation process. Turn water bath to 37°C. Make 70% ethanol using RNase free water and store on ice. Only use filtered pipette tips. Keep everything on ice unless instructed otherwise.

1.) Wash each plate with 2 mL of M9

2.) Pellet worms in a 15 mL RNase/DNase free conical

3.) Remove supernatant

4.) Wash the pellet with 5 mL of M9

5.) Remove as much supernatant as possible

6.) Add 1mL of TRIzol reagent to each sample

7.) Place samples in liquid nitrogen for 30 seconds to 1 minute

8.) Thaw samples in the water bath at 37°C for 1 minute

9.) Repeat steps 9-10 until sample contains no intact worms. Check for the level of intact worm tissue under a dissecting microscope. Usually 8-10 freeze thaws will shear all the animals

10.) Let samples sit at room temperature for 5 minutes

11.) While samples are sitting at room temperature transfer the contents to a 2 mL tube

12.) Add 200 µL of chloroform while in a fume hood

13.) Shake samples vigorously for 15 seconds

14.) Let samples incubate at room temperature for 3 minutes

15.) Spin at 10,000 RPM for 15 minutes at 4°C

16.) Remove top/clear phase (about 600 µL) to a new 1.5 mL tube

17.) Add equal volume (600 µL) of 70% ethanol

18.) Vortex samples for 30 seconds

19.) Transfer 700 µL of the sample to a RNeasy Mini spin column with a 2 mL collection tube

20.) Spin samples for 15 seconds at 10,000 RPM at room temperature

21.) Discard flow-though and place the column back into the 2 mL collection tube

22.) Repeat steps 19-21 until the entire volume has been pasted through the column

23.) Add 700 µL of Buffer RW1 to column

24.) Spin for 15 seconds at 10,000 RPM at room temperature

25.) Discard flow-through and place the column back into the 2mL collection tube

26.) Add 500 µL of Buffer RPE to the column

27.) Spin samples for 15 seconds at 10,000 RPM at room temperature

28.) Discard Flow-through and place the column back into the 2 mL collection tube

29.) Add 500 µL of Buffer RPE to the column

30.) Spin samples for 2 minutes at 10,000 RPM at room temperature

31.) Discard 2 mL collection tube and place column into new 2 mL collection tube

32.) Spin at full speed at room temperature for 1 minute

33.) Discard 2 mL collection tube and place column into a labeled 1.5 mL collection tube

34.) Add 30 µL of RNase-free water directly to the center of the column membrane

35.) Spin for 1 minute at 10,000 RPM to elute RNA

36.) Pass the eluted volume thought the column membrane again by spinning for 1 minute at 10,000 RPM at room temperature

37.) Store RNA at -80°C

RNA quantification and cDNA synthesis

Wear gloves at all times. Wipe all pipette and surfaces with 70% ethanol. Wipe down all surfaces, gloves, and pipettes with RNase Zap. Label all tubes need for quantification, treatment, and synthesis process. Remove total RNA, DNase I, and cDNA synthesis kit for the -20°C and place on ice. Keep everything on ice unless instructed otherwise.

1.) Dilute 5 µL of total RNA in 5 µL of RNase free water

2.) Mix and place on ice

3.) Pipette 2 µL of diluted RNA onto the loading chamber of the NanoDrop

4.) Record the concentration (Dilution Factor of 40 ug/µL), 260/280 ratio, and 320/280 ratio.

5.) Dilute the reaming 8 µL of total RNA to 500 ng/µL

6.) Combine the following into a master mix for DNase I treatment: For 1 reaction; 1 µL of reaction buffer, 1.0 µL of DNase I, and 6.0 µL of RNase free water

7.) Pipet 8 µL into a 0.5 mL tube

8.) Pipet 2 µL of 500 ng/µL total RNA into each tube

9.) Mix by pipetting

10.) Incubate samples at room temperature for 15 minutes

11.) While the samples are incubating set the thermal cycler to 65°C

12.) Add 1 µL of 25 mM EDTA to each sample

13.) Incubate samples at 65°C for 10 minutes

14.) Combine the following into a master mix for cDNA synthesis (Bio-Rad iScripts): For 1 reaction; 4.0 µL of reaction mix, 1.0 µL of reverse transcriptase, and 5.0 µL of RNase free water

15.) Pipet 10 µL into each tube

16.) Place samples in thermal cycler and run cDNA synthesis: 5 minutes at 25°C, 30 minutes at 42°C, 5 miutes at 85°C , and hold at 4°C

17.) To test for successful conversion combine the following into a master mix: For 1 reaction: 1.0 µL 10X reaction buffer, 0.3 µL of X mM MgCl, 0.2 µL of 25 mM dNTP, 0.1 µL of 25 uM Froward primer (dlc-2 or act-1), 0.1 µL of 25 uM Reverse primer, dlc-2 or act-1) 0.1 µL Taq, 1 µL of cDNA, bring up to 10 µL with nuclease free water

18.) Run samples

Detection of mRNA levels by SYBR Green

Wear gloves at all times. Label all tubes needed for PCR. Use tape to lock pipettes. Mix samples by inverting only unless otherwise instructed. Remove SYBR Green and primers from -20°C and store on ice. Keep everything on ice unless instructed otherwise. Turn lamp on 30 minutes before loading samples.

Standard Curve

Refer to Figure Y

1.) Perform a serial dilution of wild type cDNA to the following range: 1:8 (10uL cDNA to 70uL DEPC treated water), 1:16 (40 µL of 1:8 cDNA to 40 µL DEPC treated water), 1:32 (40 µL of 1:16 cDNA to 4 µL DEPC treated water), 1:64 (40 µL of 1:32 cDNA to 40 µL of DEPC treated water), 1:128 (40 µL of 1:64 cDNA to 40 µL of DEPC treated water
2.) Combine the following into a 7.5x master mix for PCR: For 1 reaction 5.0 µL of cDNA, 5.5 µL DNase free water, and 12.5 µL of SYBR Green. Master mix: 37.5 µL of cDNA, 41.25 µL L DNase free water, and 93.75 µL SYBR Green.
3.) Mix sample by pipetting a few times and briefly spin
4.) Aliquot 3.5x of the master mix (80.5 µL) into to tubes. One tube is for detection of the internal control and the other for the target gene of interest
5.) Add 3.5 µL of 5 µM primer to the appropriate tubes
6.) Mix by pipetting and briefly spin
7.) Aliquot 25 µL from each master mix tube into 3 wells of the qPCR plate

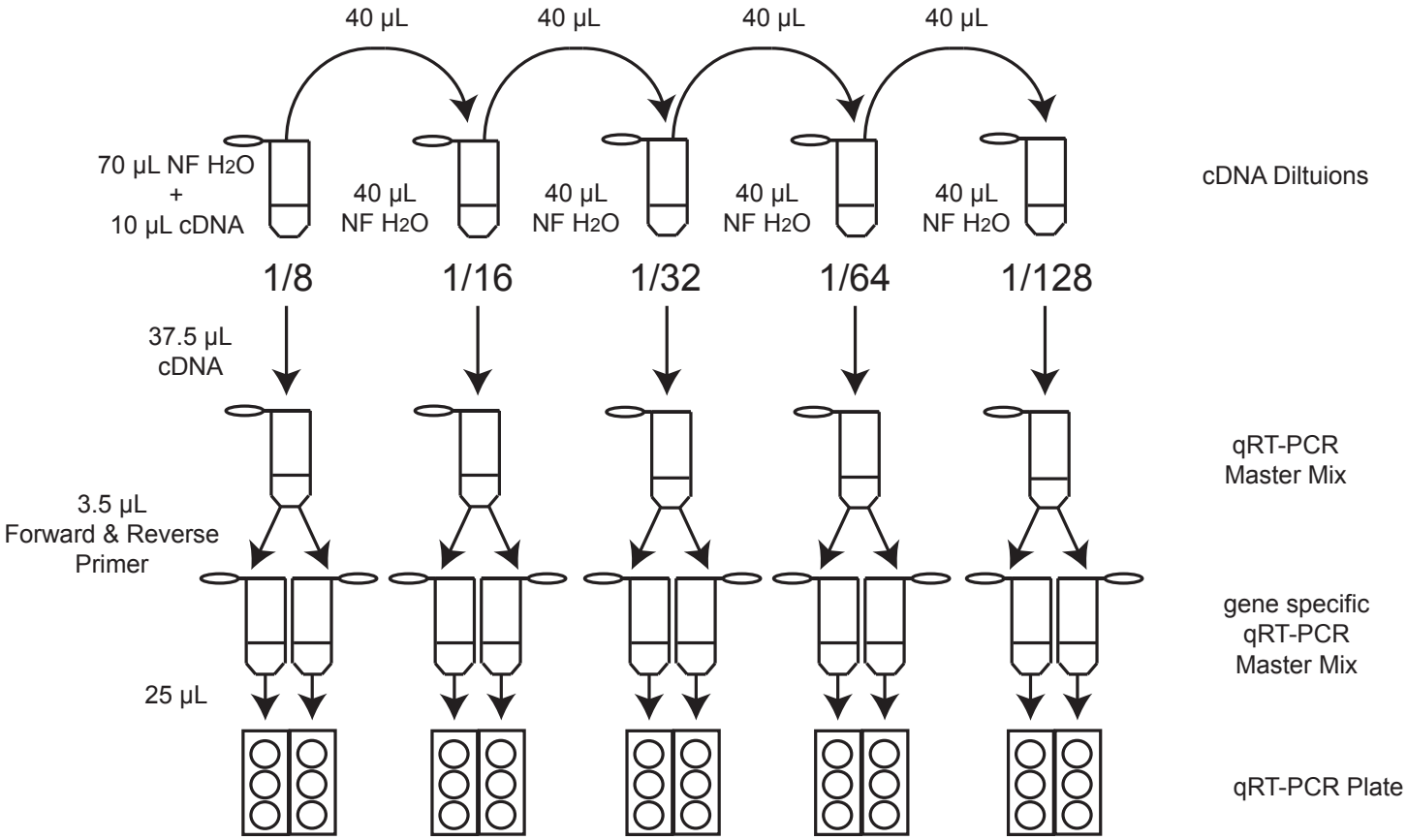


Figure 22. qRT-PCR standard curve work flow. Serially dilute cDNA starting with 1/8 and ending with 1/128. Pipet each dilution into qRT-PCR master mix tube. Split the qPT-PCR master mix into two reactions. Add the appropriate primers to each tube. Next, aliquot 25 µL from each gene specific qRT-PCR master mix into the corresponding well. If you are not performing a standard curve instead of performing serial dilutions you can dilute your cDNA samples 1/8 and proceed with the remainder of the protocol.

8.) Apply PCR film to the plate

9.) Open the Icycler program and use the following conditions for detection: 95°C for 10 minutes, 95°C for 10 seconds and Annealing temperature for 1 minutes for 40 cycles (real-time collection should be enabled during the 1 minute), and 70 cycles of increasing the temperature by 0.5°C every 2 cycles starting at 60°C (melt curve data collection should be enabled during this time)

10.) Select “Plate Setup” and highlight the wells on the plate that contain sample

11.) In the next column select “FAM 490”

12.) In the next column select “Green”

13.) Select “Save” and select “Run Selected Plate Setup”

14.) Change the reaction volume from 50 µL to 25 µL

15.) Review the screen and make sure the amplification profile and plate setup is correct

16.) Select “Start” and save your run

17.) When the run is complete, select “Generate Report” and chose “PCR Baseline”

18.) Print Report

mRNA level

1.) Dilute each cDNA sample 1:8 (5 µL of cDNA to 35 µL of DNase free water)

2.) Mix and briefly spin

3.) Combine the following into a 7.5x master mix for PCR: For 1 reaction 5.0 µL of cDNA, 5.5 µL DNase free water, and 12.5 µL of SYBR Green. Master mix: 37.5 µL of cDNA, 41.25 µL DNase free water, and 93.75 µL SYBR Green.

4.) Mix sample by pipetting a few times and briefly spin

5.) Aliquot 3.5x of the master mix (80.5 µL) into to tubes. One tube is for detection of the internal control and the other for the target gene of interest

6.) Add 3.5 µL of 5 µM primer to the appropriate tubes

7.) Mix by pipetting and briefly spin

8.) Aliquot 25 µL from each master mix tube into 3 wells of the qPCR plate

9.) Apply PCR plate film

10.) Spin plate and ensure no bubble are present

11.) Open the Icycler program and use the following conditions for detection: 95°C for 10 minutes, 95°C for 10 seconds and Annealing temperature for 1 minutes for 40 cycles (real-time collection should be enabled during the 1 minute), and 70 cycles of increasing the temperature by 0.5°C every 2 cycles starting at 60°C (melt curve data collection should be enabled during this time)

12.) Select “Plate Setup” and highlight the wells on the plate that contain sample

13.) In the next column select “FAM 490”

14.) In the next column select “Green”

15.) Select “Save” and select “Run Selected Plate Setup”

16.) Change the reaction volume from 50 µL to 25 µL

17.) Review the screen and make sure the amplification profile and plate setup is correct

18.) Select “Start” and save your run

19.) When the run is complete, select “Generate Report” and chose “PCR Baseline”

20.) Print Report

Calculation of PCR efficiency and correlation coefficient – Standard Curve

1.) Open excel and label six columns the following: Dilution, Input, Log of Input, Ct values Primer Pair 1, Ct Values Primer Pair 2.

2.) In the dilution column enter the value in triplicate of your standard curve dilutions

3.) In the input column, convert your standard curve dilutions to nanograms by multiplying your dilution by 1000

4.) In the log of input column, calculate the log of input

5.) In the Ct value columns, enter in the obtained Ct values from the standard curve report

6.) Create an XY scatter plot and set the x-values as the Log of Input and your y-values and the Ct values obtained for each primer pair

7.) Add a trendline to the graph and display equation and R2 values

8.) To calculate the PCR efficiency select the slope of the line (from the equation) into the following efficiency equation: $((10^{-1/slope}) -1) \times 100$

9.) In order to compare to separate primer pairs using the Livak method the PCR efficiency of both the internal control and the experimental must but in a range of 90 – 110%.

10.) The R^2 values will display your correlation coefficient. This assesses the variability within your triplicate. To be able to compare primer pairs, R^2 values must be > 0.98

Calculation of mRNA levels using Livak Method

1.) Open excel and label six columns the following: Sample, Internal Control Gene (act-1 or dcl-2), Experimental Gene, Δ Ct, Mean Δ Ct, Δ Δ Ct, and Expression Ratio

2.) Fill in the appropriate genotype under the sample column in triplicate. Fill in the Ct values observed from the qRT-PCR report for each gene.

3.) Calculate the Δ Ct by subtracting the internal control gene Ct values from the experimental gene Ct values

4.) Calculate the mean Ct values for each sample

5.) At the bottom of the excel sheet calculate the mean N2 Δ Ct by averaging all N2 mean Δ Ct values.

6.) To calculate the Δ Δ Ct subtract your newly calculated mean N2 Δ Ct from the mean Δ Ct of each experimental sample

7.) To calculate the expression ratio for both the N2 samples and the experimental sample, use the following equation: $2^{-(\Delta\Delta Ct)}$

8.) Average all expression ratios of each sample genotype together and calculate the SEM. Your control expression ration will always be 1.

Microarray

Prior to growing animals for microarray analysis, set up experimental design to avoid any particular bias or batch effects that may complicate downstream analysis. In the case of Chapter 3, an A.M. exposure and P.M. exposure was performed each day (Monday – Friday) for 3 weeks. During the 2 hour A.M. and P.M. exposure N2 0mM and 500mM and *npr-1(ky13)* 0mM and 500mM under went supervised randomization. For example in the A.M. exposure if N2 500mM was selected first, the second exposure

was N2 0mM, the third exposure was *npr-1(ky13)* 0mM and the fourth exposure was *npr-1(ky13) 500mM.* In the P.M. exposure, *npr-1(ky13)* was selected for the first exposure. The randomization will need to be performed prior to egg laying to ensure proper developmental timing.

RNA collection and Quality Control

1.) 30 gravid adult animals were placed on 15 seeded NGM plates and removed after 2 hours.

2.) After 65 hours, adults were washed into an RNase free tube with M9 and pipetted using a wide bore tip onto large plates for 2 hours.

3.) Large plates were dried for 1 hour. Ethanol was added using Table X except the volume of ethanol was doubled to account for the amount of agar.

4.) After 2 hours of exposure animals were washed with M9 into RNase free tubes and flash frozen in liquid nitrogen

5.) To isolate RNA see protocol in Real-Time Quantitative PCR section

6.) After capture of purified total RNA remove the Experion kit from 4°C and allow the contents to equilibrate to room temperature (20 minutes)

7.) Remove the RNA ladder from the 4°C and store on ice

8.) Fill a cleaning chip with 800 µL of Experion electrode cleaner and place in electrophorese station of machine

9.) Close lid and leave chip in for 2 minutes

10.) Fill another clean chip with 800 µL of DPEC-treated water. Place chip in electrophoresis station.

11.) Close lid and leave chip in for 5 minutes

12.) Open the Experion program on the computer and select “New Run”

13.) Enter your sample names for each well

14.) Remove cleaning chip and empty out the DEPC-treat water

15.) Refill the chip with 800 µL of DEPC-treated water and place in electrophoresis station

16.) Close lid and leave chip in for 1 minute

17.) Remove chip, empty out contents, and close lid

18.) Pipet 600 µL of RNA gel (green cap) into a spin filter tube

19.) Spin at 1,500 g for 10 minutes. Repeat spin if all gel has not passed through the column. Filter gel is good for 1 month.

20.) Finger flick RNA ladder and RNA samples to mix and briefly spin

21.) Dilute RNA samples 1:10 in DEPC-treated water and pipet 3 µL of RNA ladder from the stock tube into a RNase free tube. If you think you have low RNA concentration you may not have to dilute. Check the concentration range of the kit you are using.

22.) Heat samples to 70°C for 5 minutes

23.) Place samples on ice for 5 minutes

24.) Vortex RNA strain (blue cap) and loading buffer (yellow cap) for 5 seconds

25.) Spin all samples, RNA ladder, stain, and loading buffer briefly

26.) Remove 70 µL of loading buffer (yellow cap) into a RNase free tube

27.) Mix 65 µL of filtered gel and 1 µL of RNA strain (cover with foil, light sensitive) to make gel stain. This is enough for 3 chips and must be made fresh each time

28.) Pipet 9 µL of gel stain into highlighted GS lane on the chip. Make sure there are no bubbles

29.) Place the chip primly into the priming station set the pressure setting to B and the time setting 1. Press start to prime the chip

30.) Remove the chip from the priming station and check the backside for any air bubbles. If you find any air bubbles prime a new chip.

31.) Pipet 9 µL of gel stain into the GS lane

32.) Pipet 9 µL of filtered gel into the G lane

33.) Pipet 5 µL of loading buffer into lanes 1-13 and L lane

34.) Pipet 1 µL of RNA ladder into L lane

35.) Pipet 1 µL of each diluted RNA sample into the corresponding well. If you are not using all the wells, pipet 1 µL of DEPC-treated water into the wells.

36.) Place chip in the electrophoresis station and wait for the computer to recognize that the chip has been loaded. Select “Start”

37.) Wait 10 minutes and make sure the assay will run to completion. There are many reason an assay would fail however if the program detects all peaks of the RNA ladder, then the assay will run to completion.

38.) When the assay is complete, select “Generate Report” and make sure the first three boxes are checked

39.) Select “Print to PDF” and save file

40.) Each land will have its own report. You will find the concentration of the RNA, the 28s/18s ratio, and the RQI value. Do not rely on the concentration of the RNA from this assay. Use only the values from the NanoDrop for downstream application.

41.) Proceed with samples that have 2:1 28s/18s ratio and an RQI value 9.5 or higher.

aRNA amplification

Poly-A controls, First and Second-strand cDNA synthesis

1.) Thaw all -20°C reagents on ice and allow 4°C reagents to come to room temperature

2.) Add 100% ethanol to the bottle labeled aRNA wash solution concentrate, mix, and store at room temperature

3.) Make serial dilutions of poly-A RNA control stock if using 100 ng of total RNA input amount as follows: 2 µL of Play-A control stock to 38 µL of poly-A control dilution buffer , add 2 µL of the first dilution to 98 µL of poly-A control dilution buffer, add 2 µL of the second dilution to 98 µL of poly-A control dilution duffer , add 2 µL of the third dilution to 18 µL of poly-A control dilution buffer

4.) Thaw first-strand synthesis reagents and place on ice

5.) Assemble the first strand master mix in a nuclease-free tube using the following: 4 µL of first strand buffer mix, 1 µL first strand enzyme mix (do not vortex), and 2 µL of your 4^{th} poly-A dilution.

6.) Flick mix and centrifuge briefly

7.) Aliquot 7 µL to 3 µL of 100 ng RNA

8.) Mix thoroughly

9.) Incubate samples for 2 hours at 42°C in a thermal cycler

10.) Place second strand reagents on ice

11.) After 2 hours briefly centrifuge samples and place on ice

12.) Construct the second strand master mix on ice using the following: 13 µL nuclease free water, 5 µL second strand buffer mix, and 2 µL second strain enzyme mix (do not vortex).

13.) Aliquot 20 µL of second strand mix to 10 µL of first strand mix

14.) Mix thoroughly

15.) Pre-heat the thermal cycler block to 16C and place samples at 16C for 1 hour followed by 65C for 10 minutes

16.) Centrifuge samples briefly

17.) Samples can be stored at -20C or proceed with IVT

In Vitro Transcription to Synthesize labeled aRNA

1.) Remove IVT master mix reagents and allow reagents to equilibrate at room temperature

2.) Contracture the IVT master mix at room temperature by combining the following: 4 µL IVT Biotin label, 20 µL IVT label buffer, and 6 µL IVT enzyme mix.

3.) Mix thoroughly and spin briefly

4.) Transfer 30 µL of IVT master mix to the double stranded cDNA samples

5.) Incubate the IVT reaction for 16 hours at 40°C in a thermal cycler, followed by 4°C hold.

6.) Remove samples and freeze immediately. Samples can only be stored overnight.

aRNA Purification

1.) Preheat the aRNA elution solution to 60°C

2.) Remove the aRNA binding mix reagents and assemble at room temperature by combining the following: 10 µL RNA binding beads and 50 µL of aRNA biding buffer concentrate.

3.) Add 60 µL aRNA binding mix to each sample and pipet up and down to mix.

4.) Transfer each sample to a well of a U-Bottom plate

5.) Mix by pipetting up and down until solution is brown

6.) Add 120 µL 100% ethanol to each sample

7.) Mix by pipetting

8.) Gently shake for 2 minutes using setting 1 of plate shaker. Tape plate to shaker.

9.) Move plate to the magnet stand and capture the beads. Wait 5 minutes or until the solution in each well is clear.

10.) Carefully aspirate and discard the supernatant without disturbing the magnetic beads

11.) Remove plate from stand

12.) Add 100 µL of aRNA wash solution to each sample and shake using setting 2 on the shakers for 1 minute. Tape plate to shaker

13.) Move plate to the magnetic stand and capture RNA binding beads again. Wait 5 minutes.

14.) Remove aspirate and discard supernatant with disturbing the RNA binding beads.

15.) Remove plate from stand.

16.) Repeat steps 12-15

17.) Move the plate to a shaker and shake the dry plate using setting 3 on the shaker for only 1 minute. Tape down the plate. Start slow, and then ramp to setting 3.

18.) Elute the purified aRNA from the beads by adding 50 µL of preheated aRNA elution solution directly to each pellet

19.) Using the shaker on setting 3-5 shake the plate for 3 minutes. Tape the plate to the shaker

20.) If not all the RNA binding beads are not fully dispersed shake for an additional 5 minutes. If that does not work, use a pipette to dissociate.

21.) Move the pate to the magnetic stand and capture the RNA binding beads for 5 minutes

22.) Transfer the supernatant to a 1.5 mL nuclease free tube

23.) Store aRNA at -80°C or place on ice and proceed with quantitation and fragmentation

24.) Purified a RNA can be stared at -20°C for up to 1 year

Evaluation and Fragmentation of aRNA

1.) Use the NanoDrop to determine the concentration of aRNA

2.) Assemble the aRNA fragmentation mixture to the correct format (*C. elegans uses 100 Format)* using the following: 12 µg aRNA, 6.4 µL 5x array fragmentation buffer, bring up to a final volume using 32 µL of nuclease-free water.

3.) Incubate the fragmentation reaction at 94°C for 35 minutes

4.) Place the reaction on ice immediately, spin if any condensation

5.) Use the Experion to asses RNA quality See “Experion protocol” in this section. Load the unfragmented sample next to the fragmented sample. You should see a

distribution of 35-200 nt in the aRNA fragmented profile with large peak at approximately 100-120 nt

6.) Fragmented aRNA can be stored at -20°C or -70°C for long term or proceed with hybridization

Hybridization

1.) Bring arrays to room temperature

2.) Set heat blocks to 99°C and 65°C

3.) Set hybridization oven to 45°C

4.) Remove DMSO and 2x hybridization mix from Hybridization, Wash, and Stain Kit in the 4C and thaw. Store DMSO at room temperate

5.) Remove control oligonucleotide B2 and 20X hybridization controls from -20°C

6.) Head the 20x hybridization controls to 65°C for 5 minutes before using.

7.) Mix the following for each target hybridization (For *C. elegans* use 49 Format): 12.5 µg (33 µL) fragmented and labeled aRNA, 4.2 µL control oligonucleotide B2, 12.5 µL 20x hybridization controls, 125 µL 2x hybridization mix, 25 µL DMSO, 50 µL Nuclease-free water.

8.) Heat the hybridization cocktail to 99°C for 5 minutes

9.) Fill array with 200 µL of Pre-hybridization mix by filling it through one of the septa.

10.) Incubate the array at 45°C for 10 minutes with rotation in the hybridization oven

11.) Transfer the hybridization cocktail that has been heated at 99°C to the 45°C heat block for 5 minutes.

12.) Spin the hybridization cocktail at max speed for 5 minutes

13.) Remove arrays from hybridization oven and remove pre-hybridization mix by venting one septa with a clean pipette tip and extract the mix with a pipette. Ensure no bubbles remain. Remove tip

14.) Add the hybridization cocktail to the array. Avoid any insoluble material at the bottom of the tube

15.) Cover septas with lab tape

16.) Place probe array into the hybridization oven and balance appropriately

17.) Rotate at 60 RPM for 16 hours.

Bleaching

1.) 24 hours prior to priming, clean the fluidics station by using the bleaching protocol.

2.) First register your sample information in the Affymetrix GeneChip Command Console (AGCC)

3.) Turn on the fluidics station using the toggle switch on the lower left side of the machine

4.) In the AGCC launcher, click the AGCC fluidics control icon

5.) Make sure both fluidics stations are registered

6.) Select the bleach protocol

7.) Make 500 mL of 0.525% Bleach in a designated container and place all 3 lines into the container

8.) Place an empty container under the waste line

9.) Run the bleach protocol

10.) Follow the prompts on the fluidics station

11.) After the bleach cycler is complete remove the hoses from the bleach container and place them into a container containing 500 mL of deionized water.

12.) Follow the prompts on the fluidics station

Priming, Washing, Staining, and Scanning

1.) To prime the station place hose line A into Wash buffer A and hose line B into Wash buffer B and the waste line into an empty bottle

1.) Place 3 empty 1.5 mL microfuge tubes into the stain holder positions 1,2, and 3

2.) Place the wash block lever into the engaged/closed position. Push the needle lever into the down position

3.) Run the Prime-50 maintenance protocol from the AGCC fluidics control

4.) Prepare the following reagents for using a 49 format array volume (250 µL): stain cocktail 1, stain cocktail 2, and array holding buffer

5.) Gently tap the bottles and mix well

6.) Aliquot 600 µL of stain cocktail1 into a 1.5 mL amber microcentrifuge vial, 600 µL of stain cocktail 2 into a 1.5 clear microcentrifuge vial, and 800 µL of array hold bugger into a 1.5 mL clear microcentrifuge vial

7.) Spin down to remove bubble

8.) Start the AGCC fluidics Control software

9.) Load your saved sample files

10.) Select your protocol, and select compatible, genechip IVT labeling kit, and gene chip HWS kit.

11.) Select Run

12.) Insert the probe arrays into the designed module of the fluidics station while the lever is in the down position

13.) Monitor the levels of your solutions during this process. Make sure there are no bubbles.

14.) When the process is complete, fill the arrays with array holding buffer and wrap in aluminum foil

15.) Turn the scanner on 10 minutes prior to use

16.) Open AGCC scan control software

17.) Select the sample fine name for he probe array you want to scan from the sample file name drop-down list

18.) Click start and load array

19.) Select OK

20.) After scan check in AGCC viewer and check if the array passed internal QC by selecting pass grid alignment. To manually check hit F5,F6,F7,F8 to see all 4 corners. If it failed rescan.

Microarray Analysis

QC reports

1.) In AGCC viewer process samples using the MAS 5 Algorithm.

2.) Select files you want to review in the study dialog box

3.) Select Report followed by View Full Report

4.) Make sure all files are within bounds under the threshold test column

5.) Export and save Full Report

6.) Select Report followed by View Probe Level Summarization Report

7.) Select edit followed by Probe Level Summarization Report Option

8.) Select signal, detection p-value, and detection

9.) Export and Save file

10.) Select Graph and generate the following graphs: signal histogram, box plot – probe cell intensity, box plot – relative probe cell intensity, box plot – signal, box plat-relative signal, MvA plot, Pearson's correlation for signal and detection p-value, and spearman rank correlation for signal and detection of p-value

11.) Export and save all graphs

12.) Reopen your files and process them with RMA

13.) Repeat steps 10 - 11 for the RMA values

14.) Save and export file

Data Analysis - RMA and MAS5

1.) Using excel open the MAS 5 file

2.) Sort your data by probe set ID and Absent Call

3.) Copy the Probe set ID's with an absent call into a new excel spreadsheet

4.) Open the RMA file.

5.) Using the function VLOOKUP in excel remove the probe set IDs from the RMA file that had an absent call in the MAS 5 file

6.) Save the file as a .txt

TMeV

1.) Open TMeV and select file and select load data

2.) Select browse in the select expression data file row

3.) Load your .txt file

4.) Select single color array

5.) Under load annotation data select automatically download *Ceanorhabditis elegans* and underneath select *C. elegans* again

6.) Also check Load Annotation

7.) In the Expression table scroll and select the first RMA value and select load

8.) Under Adjust Data select Gene/Row Adjustments and select Normalize Gene/rows

9.) Under Adjust Data select Sample/Column Adjustments and select Normalize sample/columns

10.) Select Display followed set color scale limits

11.) Under gradient style select Double Gradient

12.) Under color Range Selection set the midpoint value = 0, set the low limit and upper limit values to match. The lower limit will be negative and the upper limit will be positive. MeV will suggest values for you.

13.) Select update limits. Under the color saturation statistics, this will tell you how many probes will be left out because they are out of the range of your scale

14.) Select OK

Statistical Analysis of Microarrays (SAM)

1.) Select analysis followed by statistics followed by SAM

2.) Depending on your comparisons select the apropritte SAM analysis. If you are just comparing to groups of microarrays (control vs. experimental) then perform a two-class unpaired SAM

3.) Assign the control to group A and the experimental to group B

4.) Select yes to calculate q-values

5.) Select construct hierarchical trees for significant genes only

6.) Select OK

7.) If a permutation window pop up select the first option and select OK

8.) Select Pearson's Uncentered

9.) Select OK

10.) When the SAM graph appears select OK

11.) Under Analysis Results select the SAM(1) icon

12.) Under SAM(1) select Delta Table

13.) Determine the FDR(%) Mean you want for your data and record the delta value associated with it in the first column.

14.) Select Analysis, Statistics, followed by SAM

15.) Select the first option and select OK

16.) When the SAM Graph appears enter the delta value for the correct FDR mean

17.) Select OK

18.) Under Table view select ALL significant genes

19.) Copy the Probe ID, the fold change, and the q-value into an excel spreadsheet

Geneweaver

To get a better idea of what genes are actually significant, you can use Geneweaver to convert the probe ID into a Wormbase gene symbol. Currently, this is the best bioinformatics tool to extract precise gene symbols. You can use Geneweaver for a variety of other important tasks such as to identify the similarly between data sets and access a wide variety of genomic data sets across different species.

1.) Access www.geneweaver.org

2.) Create an account

3.) Select manage Gene Sets and upload gene sets

4.) Enter descriptive information in each box

5.) Select species and the appropriate microarray

6.) Paste the probe IDs and the fold change (optional, RMA value is ideal)

7.) Upload gene set

8.) Geneweaver will collapse genes with multiple probe IDs. Select the option show original data to view which genes have multiple probe IDs

9.) Select export data

10.) Save the file

11.) Add the newly uploaded gene list to a Gene set Project for future reference

Hypoxia:

Cobalt Chloride Treatment

Cobalt chloride ($CoCl_2$) is light sensitive. Wrap tubes with aluminum foil when handling the chemical dry or in solution.

1.) Make a 100 mM $CoCl_2$ solution by adding 1.3 grams to 100 mL ddH_20

2.) Filter sterilize the contents into a light protected bottle

3.) Seed a 3-5 day old NGM plate with 50 µL of OP50 24 hours prior to addition of $CoCl_2$

4.) Add 500 µL of 100 mM $CoCl_2$ Allow the solution to equilibrate for 2 hours

5.) Move 50 animals to a plate

6.) Parafilm plates and incubate at 20°C for 16 hours

7.) Move animals to a freshly seeded NGM plate

8.) Score lethality 24 hours post removal of $CoCl_2$

9.) When scoring lethality tap the plate or use your pick to stimulate locomotion. If worms are unable to move then they are scored dead.

Environmental Chamber

1.) 24 hours seed small NGM plates with 20 µL of OP50

2.) Pick 50 worms a plate

3.) Place plates into Bio-Bag

4.) Place an anaerobic indicator, anaerobic generator, and catalysis container into the Bio-bag

5.) Arrange so the arrows shown on the labels of the indicator and generator are pointed up towards the open position of the bag. Position the catalyst so the grid is not in contact with bag

6.) Expel any air form the Bio-bag and use a heat sealer to seal close the contents

7.) Hold bag up right and locate the anaerobic indicate ampule

8.) Crush the indicator ampule using your index finger and thumb

9.) After 30 seconds the indicator should turn pink, if not repeat step 8

10.) Locate the generator ampule

11.) Crush the ampule using your index and thumb.

12.) Gently tap the generate to allow the contents to mix

13.) When you see bubbling within the tube the contents is mixing

14.) Still holding the bag up right, allow the gas in the bag to evolve and 1 minute. If you do not wait the contents will spill into the bag

15.) Place bag at 27°C for 8 hours

16.) 2 hours into the exposure check the bag and make sure the indicator is no longer pink and has turned white. This indicates oxygen levels are less than 1%

17.) Remove animals to a new NGM plate

18.) Score survival 24 hours later

19.) When scoring lethality tap the plate or use your pick to stimulate locomotion. If worms are unable to move then they are scored dead.

Hypoxia Cabinet

Prior to exposure bubble 50 mL of M9 with nitrogen gas for 30 minutes

1.) Allow 30 adult animals to lay eggs for 2 hours

2.) Allow animals to grow to desired age

3.) Pellet animals in non-nitrogen M9

4.) Wash the animals three times with nitrogenated M9

5.) Remove supernatant but leave behind 100 µL

6.) Place worms in cabinets and set oxygen control to 0.1%

7.) Incubate worms for 16 hours

8.) Remove worms from liquid and pipette them onto seeded NGM plates

9.) Score survival 24 hours post removal of hypoxia

10.) When scoring lethality tap the plate or use your pick to stimulate locomotion. If worms are unable to move then they are scored dead.

Allyl-Alcohol Assay:

Plate Preparation:

Refer to Nematode Growth Media plate protocol to generate agar. Add allyl-alochol to agar and pour plates while under the fume hood. Store seeded plates in a box in the fume hood.

1.) Combine the appropriate amount of agar into an autoclaved bottle and add allyl-alcohol to a final concentration 0.35%

2.) Aliquot the allyl-alcohol containing media into plates

3.) Allow plates to solidify for 24 hours

4.) Seed plates with 70 µL of Op50

5.) Leave plates slightly uncovered for 15 minutes to allow food to dry

6.) Allow lawn to grow for 48-72 hours.

Allyl-Alcohol Treatment:

1.) Transfer 25 worms to allyl-alcohol plates

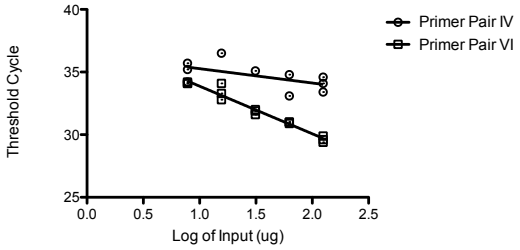
2.) Score survival 24 and 48 hours

3.) To ensure a worm is actually dead tap the plate or tap animals with the pick and assess for movement. All worms unable to move are counted as dead.

Appendix B

Quantitative Real-Time Polymerase Chain Reaction Standard Curves

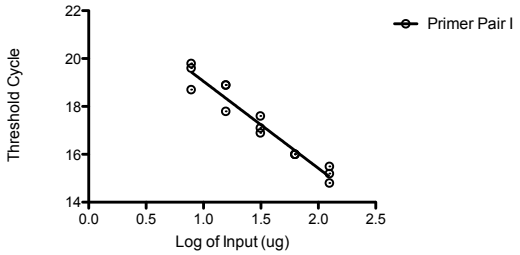
lips-7



Primer Pair IV
Slope = -2.0278
Efficiency < 110%
r^2 = 0.376

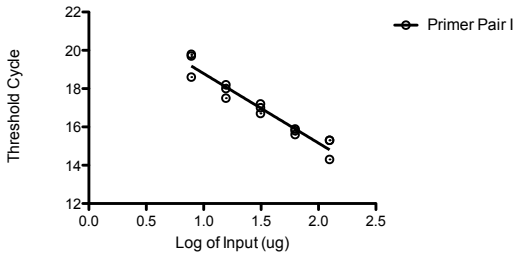
Primer Pair VI
Slope = -3.7981
Efficiency = 83.35%
r^2 = 0.963

act-3



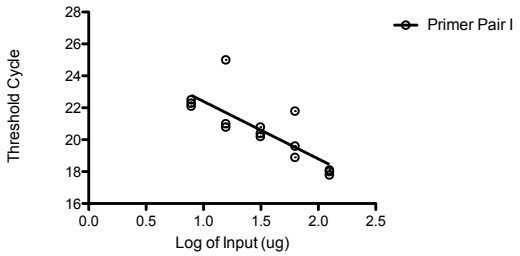
Slope = -3.632
Efficiency = 88.51%
r^2 = 0.941

act-1



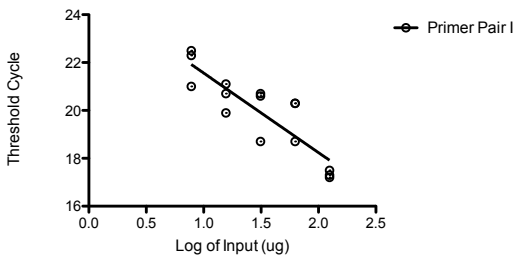
Slope = -3.632
Efficiency = 88.51%
r^2 = 0.939

tba-1



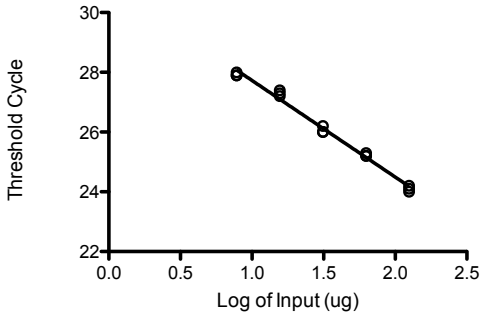
Slope = -3.3219
Efficiency = 100%
r^2 = 0.7511

gpd-2



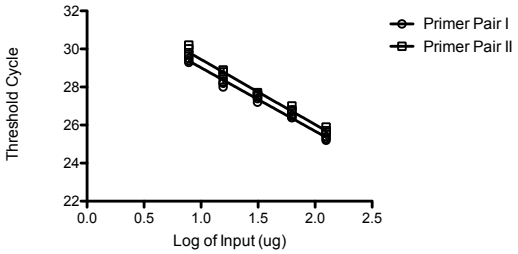
Slope = -3.5988
Efficiency = 89.61%
r^2 = 0.643

dyn-3



Slope = -3.2333
Efficience = 103.8%
r^2 = 0.989

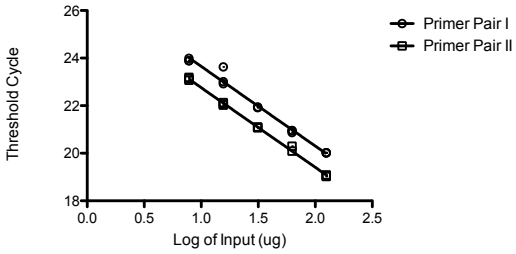
acs-2



Primer Par I
Slope = -3.333
Efficience = 99.5%
r2 = 0.986

Primer Pair II
Slope = -3.4216
Efficience = 96%
r^2 = 0.979

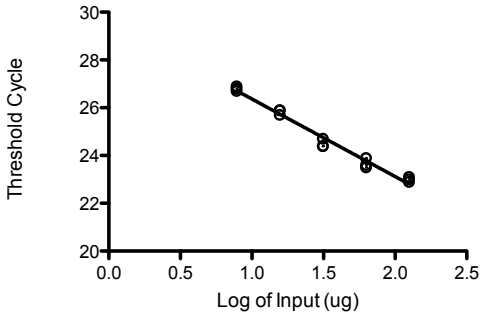
ech-6



Slope = -3.3463
Efficience = 99 %
r^2 = 0.985

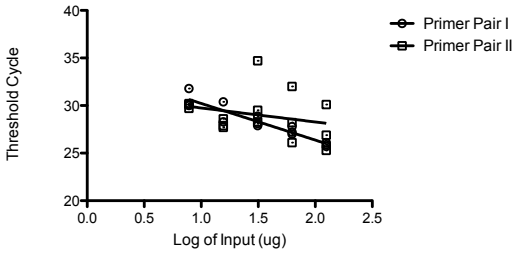
Slope = -3.3253
Efficience = 99.9%
r^2 = 0.997

alh-1



Slope = -3.2444
Efficiency = 103.3%
r^2 = 0.979

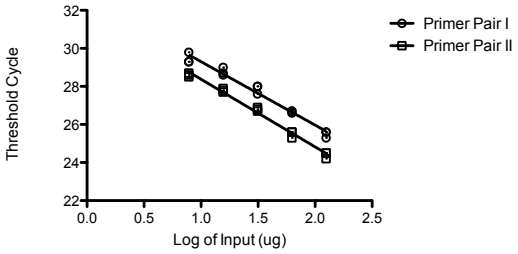
alh-3



Slope = -3.851
Efficiency = 81.8%
r^2 = 0.88

Slope = -2.8596
Efficiency = 123.7%
r^2 = 0.676

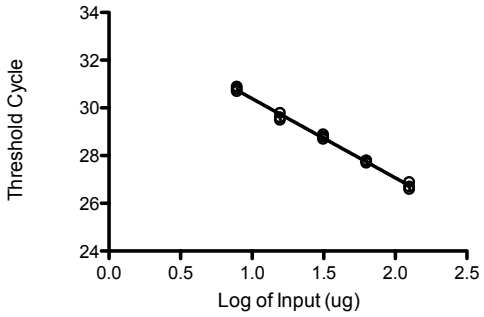
alh-5



Primer Pair I
Slope = -3.333
Efficiency = 99.54%
r^2 = 0.975

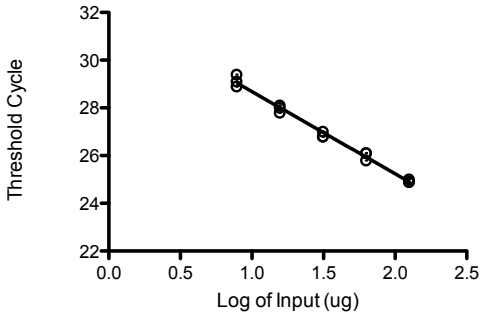
Primer Pair II
Slope = -3.5545
Efficiency = 91.13%
r^2 = 0.988

alh-6



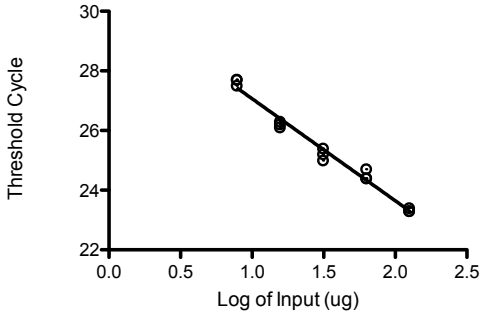
Slope = -3.3219
Efficiency = 100 %
r^2 = 0.994

alh-7



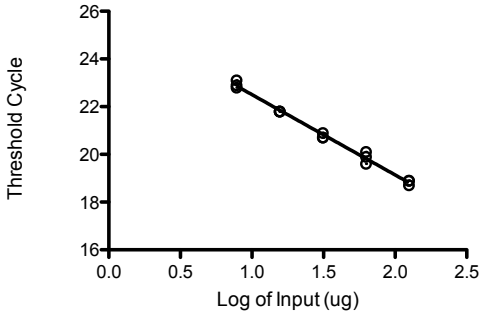
Slope = -3.4437
Efficiency = 95.16%
r^2 = 0.99

ubc-15



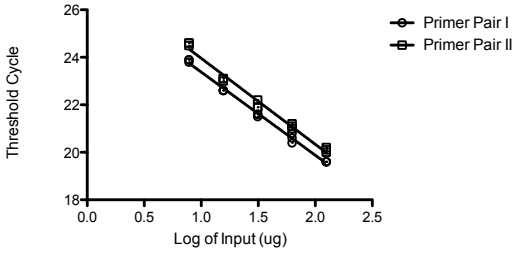
Slope = -3.4216
Efficiency = 96%
r^2 = 0.981

alh-8



Slope = -3.362
Efficiency =98.19%
r^2 = 0.991

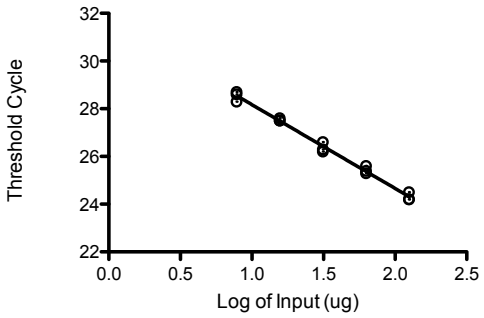
alh-9



Primer Pair I
Slope = -3.4991
Efficiency = 93.1%
r^2 = 0.994

Primer Pair II
Slope = -3.6209
Efficiency = 88.87%
r^2 = 0.986

alh-10



Slope = -3.5102
Efficiency = 92.7%
r^2 = 0.991

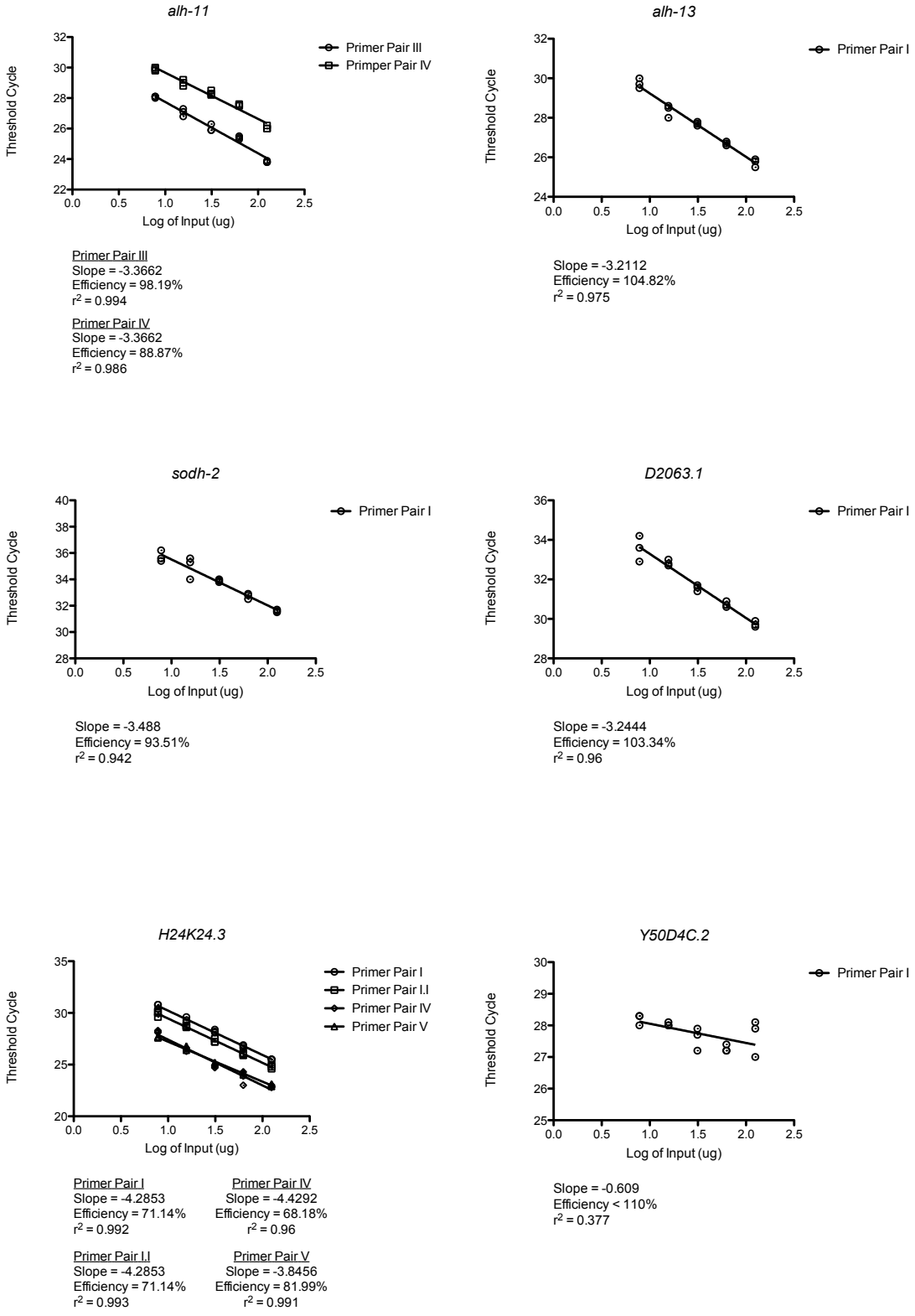


Figure 23. qPCR standard curves with amplificaiton efficiency for genes of interest.

Appendix C

Basal and Ethanol Treated Locomotion Speeds from Mitochondrial β-oxidation Analysis

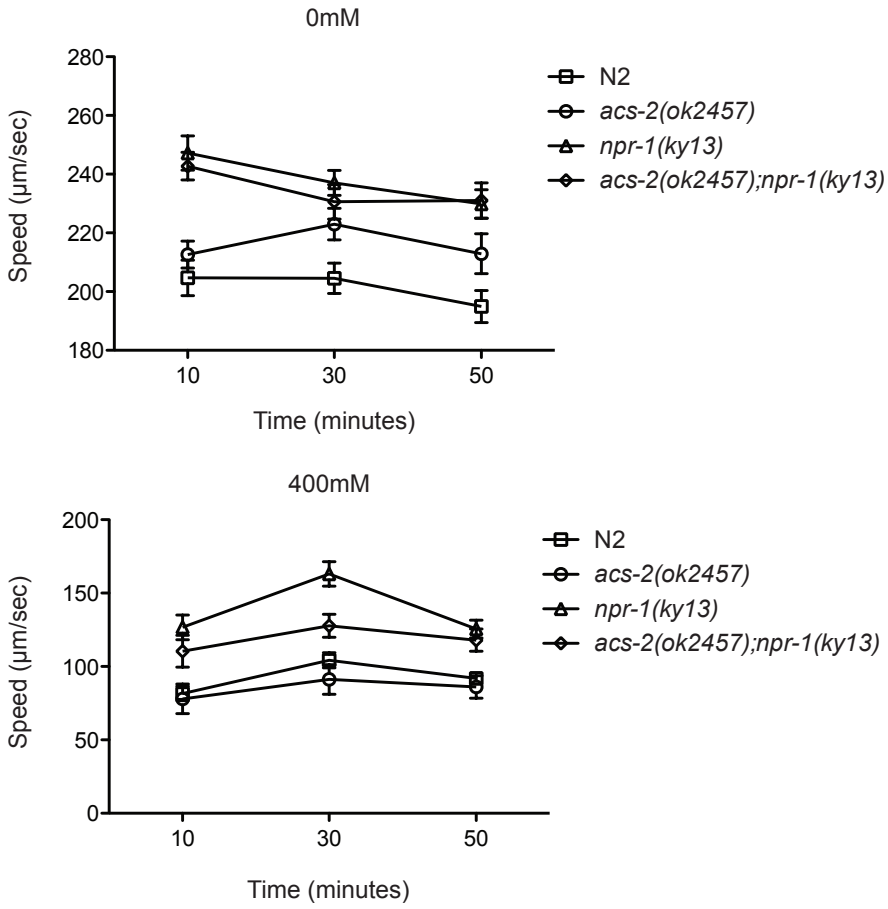


Figure 24. Basal and 400mM ethanol treated speeds for N2, *acs-2(ok2457), nr-1(ky13),* and *acs-2(ok2457);npr-1(ky13).*

Table 9. Basal and ethanol treated locomotion speeds for N2, *acs-2(ok2457)*, *nr-1(ky13)*, and *acs-2(ok2457);npr-1(ky13)*.

N2			*acs-2(ok2457)*			*npr-1(ky13)*			*acs-2(ok2457); npr-1(ky13)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
216.4863	207.2469	217.6313	211.8726	253.6191	239.8671	263.0674	257.2743	248.1266	252.4730	243.2182	234.5098
211.5918	187.9251	197.8545	219.9262	227.5532	218.3555	264.1708	235.0555	229.7523	248.3924	248.7700	255.8309
175.9334	197.0206	190.4412	222.9811	221.6074	201.9263	234.4437	231.5840	241.7696	238.2390	225.0053	238.2327
182.9513	183.3317	175.0890	221.6371	234.3231	207.0946	243.7129	227.4526	209.8827	232.3678	235.8549	226.7354
222.8247	195.6285	176.2952	203.5562	198.4062	170.5336	230.7863	213.8287	221.2226	228.8513	193.4325	204.6477
230.1341	217.3868	177.4747	211.8614	205.9586	206.5642	214.8074	232.2957	206.5642	222.0735	215.3724	200.3795
191.3117	232.9581	214.4544	228.6324	225.8644	235.8575	260.6960	244.5199	235.8575	260.2394	248.1235	238.3481
205.8668	213.0350	207.7867	211.2527	226.1762	219.6535	252.2887	242.3962	242.7555	263.1291	231.6943	244.8679

Untreated Speeds (µm/sec)

N2			*acs-2(ok2457)*			*npr-1(ky13)*			*acs-2(ok2457); npr-1(ky13)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
63.0933	89.0706	101.4540	134.8133	157.5663	135.7179	117.3564	168.0549	127.0079	108.9024	114.6849	119.6731
83.0754	99.0884	103.7877	89.65605	100.6690	81.95131	172.2827	200.7651	146.1612	140.6712	151.2995	135.3994
80.9126	106.179	84.08150	75.12360	90.98722	90.28391	108.3784	152.5554	119.6006	126.6675	128.5483	121.8228
55.3509	87.3857	88.72846	55.88299	80.71304	88.99951	132.1134	162.0141	134.5305	134.8133	157.5663	135.7179
80.6701	91.5989	91.56701	37.00095	54.67430	71.26112	111.4447	141.5022	94.93507	60.23233	88.15572	89.17549
82.9474	97.6662	76.33218	83.25901	89.07432	67.71488	88.54777	120.4315	98.09972	99.02154	117.7731	99.55617
93.8128	122.034	94.45406	81.84748	80.04528	75.17120	155.0675	189.1819	143.1183	137.5475	148.9519	146.9451
91.3309	112.037	86.89993	101.7836	106.8915	104.0266	127.5487	152.1997	136.2232	129.9242	137.5575	131.9408

400mM Treated Speeds (µm/sec)

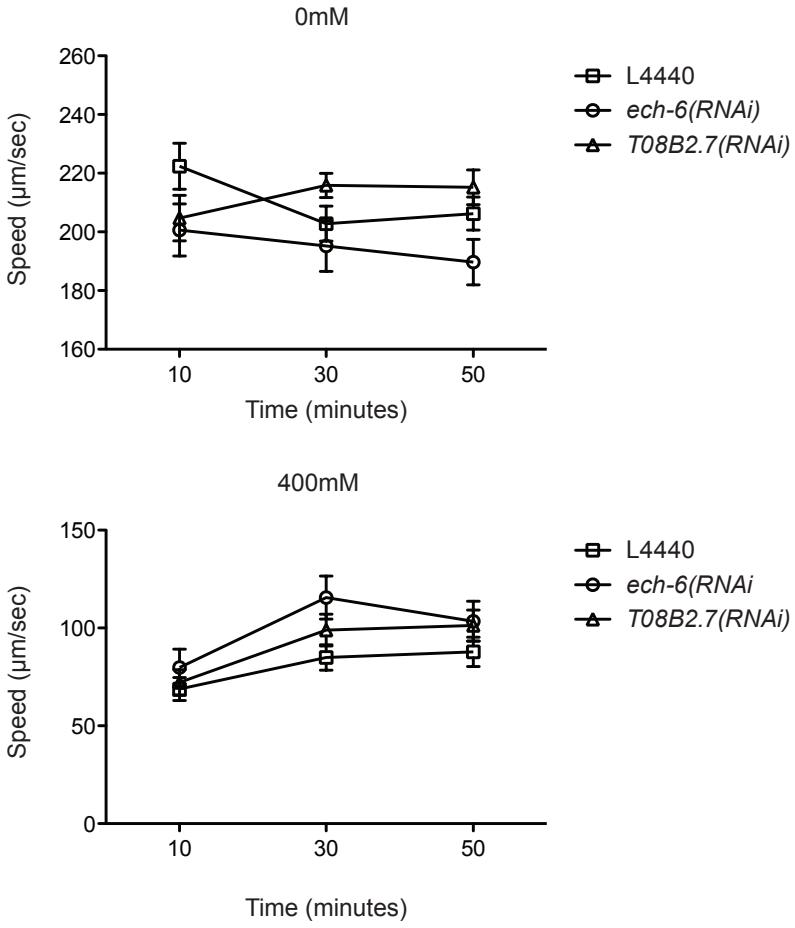


Figure 25. Basal and 400mM ethanol treated speeds for L4440, *ech-6(RNAi)*, *T08B2.7(RNAi)*

Table 12. Basal and ethanol treated locomotion speeds for L4440, *ech-2(RNAi), ech-4(RNAi)*, and *ech-1(RNAi)*

L4440			*ech-2(RNAi)*			*ech-4(RNAi)*			*ech-1(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
220.29	219.43	211.05	239.10	214.86	212.45	195.03	180.27	155.33	177.10	207.38	234.5098
203.02	184.00	186.56	189.94	198.25	184.29	189.49	183.74	174.69	183.61	188.44	255.8309
207.37	193.11	207.47	218.28	188.49	199.53	203.03	189.08	174.75	197.45	172.12	238.2327
245.20	218.00	213.13	217.81	211.70	192.07	214.33	192.42	193.32	230.59	204.66	226.7354

Untreated Speeds (µm/sec)

L4440			*ech-2(RNAi)*			*ech-4(RNAi)*			*ech-1(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
43.27	69.05	79.24	54.62	67.68	82.79	44.41	58.41	61.91	46.13	72.83	72.83
31.25	46.22	46.35	49.76	55.56	43.13	40.22	60.64	68.57	35.47	41.90	60.80
64.75	96.25	73.67	62.44	76.34	62.99	41.89	51.95	51.72	42.91	45.17	50.91
56.94	80.22	69.28	42.14	72.07	65.44	68.88	69.54	65.44	67.76	79.03	63.57

400mM Treated Speeds (µm/sec)

Table 10. Basal and 400mM ethanol treated speeds for L4440, *ech-6(RNAi)*, *T08B2.7(RNAi)*

L4440			*ech-6(RNAi)*			*T08B2.7(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
220.3640	195.2196	196.7132	229.11	216.99	213.88	185.89	217.57	234.29
225.6069	195.4158	208.9918	200.36	189.97	190.61	176.37	223.29	195.89
212.9348	192.6493	201.7841	217.10	222.08	199.00	188.49	214.22	230.91
208.6303	189.9309	177.8582	200.25	204.93	200.60	223.46	209.51	210.14
204.3809	187.7423	206.0131	190.24	173.06	197.08	192.61	196.47	194.34
231.4317	223.0682	205.2702	232.82	221.74	200.25	208.85	207.26	209.20
271.2894	233.9797	229.1633	167.36	165.60	144.06	226.46	224.16	210.05
204.1935	204.6103	224.0458	167.87	167.28	172.23	235.45	234.28	236.60

Untreated Speeds (µm/sec)

L4440			*ech-6(RNAi)*			*T08B2.7(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
67.7600	93.0200	83.5800	66.99	137.61	110.32	63.33	112.31	108.69
58.3600	71.8600	83.1800	85.49	116.24	128.13	86.25	133.67	137.04
58.9200	75.0300	73.1500	119.80	165.25	144.61	74.82	123.57	110.53
56.7800	68.0800	80.4100	72.37	95.24	88.81	72.07	93.39	113.94
88.4100	111.9600	129.8000	46.24	84.49	101.09	39.38	82.25	96.27
50.3100	62.9200	59.6100	111.11	138.61	113.77	104.26	101.12	98.39
72.4900	89.0100	89.6000	48.94	72.17	48.63	69.63	68.35	61.69
97.4100	107.3400	103.2000	87.14	114.58	92.61	67.63	77.09	83.45

Treated Speeds (µm/sec)

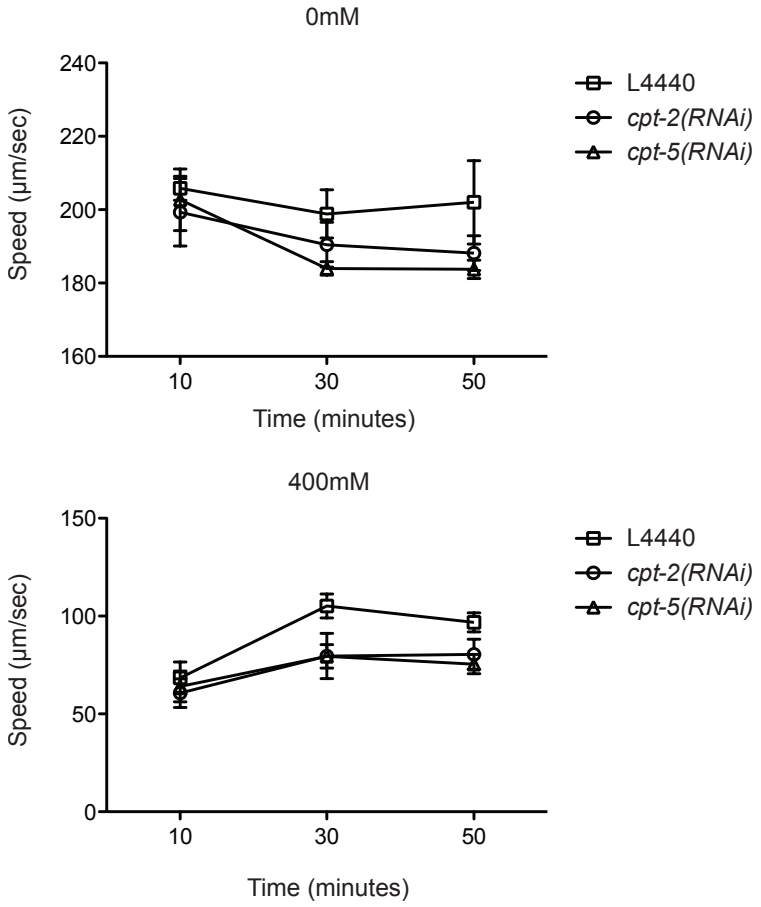


Figure 26. Basal and 400mM ethanol treated speeds for L4440, *cpt-2(RNAi),* and *cpt-5(RNAi)*

Table 11. Basal and ethanol treated speeds for L4440, *cpt-2(RNAi),* and *cpt-5(RNAi)*

L4440			*cpt-2(RNAi)*			*cpt-5(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
209.84	191.84	187.86	196.95	185.75	181.07	211.90	185.75	184.31
197.06	189.46	196.08	199.22	187.75	191.18	200.80	181.64	190.55
211.71	195.98	188.52	222.97	208.06	200.19	179.92	180.25	179.38
204.68	218.12	235.65	178.11	180.27	180.18	218.12	188.35	180.72

Untreated Speeds (µm/sec)

L4440			*cpt-2(RNAi)*			*cpt-5(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
57.91	111.64	187.86	82.54	112.89	102.66	57.03	97.14	87.85
52.24	92.18	196.08	51.10	77.42	67.32	50.14	75.78	73.48
76.74	97.87	188.52	54.54	66.37	78.00	86.39	74.72	76.59
86.87	118.87	235.65	54.38	61.75	74.00	62.74	70.02	63.91

400mM Treated Speeds (µm/sec)

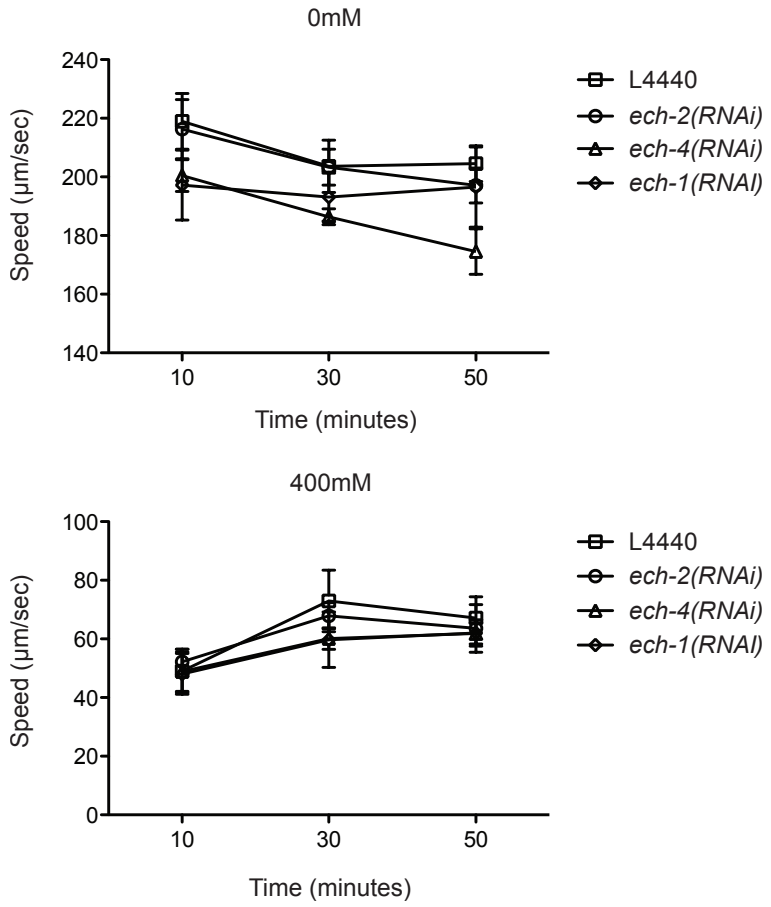


Figure 27. Basal and 400mM ethanol treated speeds for L4440, *ech-2(RNAi)*, *ech-4(RNAi)*, and *ech-1(RNAi)*

Table 12. Basal and ethanol treated locomotion speeds for L4440, *ech-2(RNAi), ech-4(RNAi)*, and *ech-1(RNAi)*

L4440			*ech-2(RNAi)*			*ech-4(RNAi)*			*ech-1(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
220.29	219.43	211.05	239.10	214.86	212.45	195.03	180.27	155.33	177.10	207.38	234.5098
203.02	184.00	186.56	189.94	198.25	184.29	189.49	183.74	174.69	183.61	188.44	255.8309
207.37	193.11	207.47	218.28	188.49	199.53	203.03	189.08	174.75	197.45	172.12	238.2327
245.20	218.00	213.13	217.81	211.70	192.07	214.33	192.42	193.32	230.59	204.66	226.7354

Untreated Speeds (µm/sec)

L4440			*ech-2(RNAi)*			*ech-4(RNAi)*			*ech-1(RNAi)*		
10	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**	**10**	**30**	**50**
43.27	69.05	79.24	54.62	67.68	82.79	44.41	58.41	61.91	46.13	72.83	72.83
31.25	46.22	46.35	49.76	55.56	43.13	40.22	60.64	68.57	35.47	41.90	60.80
64.75	96.25	73.67	62.44	76.34	62.99	41.89	51.95	51.72	42.91	45.17	50.91
56.94	80.22	69.28	42.14	72.07	65.44	68.88	69.54	65.44	67.76	79.03	63.57

400mM Treated Speeds (µm/sec)

Vita

Joseph Thomas Alaimo was born on March 29, 1986, in Scranton, Pennsylvania. He completed his Bachelor of Science degree in Biology and Neuroscience from King's College, Wilkes-Barre, Pennsylvania in 2008. He joined the Molecular Biology and Genetics curriculum in the Pharmacology and Toxicology department in 2008 at the Virginia Commonwealth University School of Medicine.