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Investigating a Model for Fetal Alcohol Damage in *Caenorhabditis elegans*

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

By

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List of Abbreviations

AA	Alcoholics Anonymous
ADH	Alcohol dehydrogenase
ADHD	Attention Deficit Hyperactivity Disorder
AFT	Acute Functional Tolerance
ARBD	Alcohol-Related Birth Defects
ARND	Alcohol-Related Neurodevelopmental Disorder
AWC ^{OFF}	Non- <i>str-2</i> ::GFP expressing AWC cell
AWC ^{ON}	<i>str-2</i> ::GFP expressing AWC cell
BK	Large Conductance Calcium and Voltage-Sensitive Potassium Channel (BK is for <u>B</u> ig <u>K</u> ⁺ Channel)
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic Adenosine Monophosphate
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CGC	<i>Caenorhabditis elegans</i> Genetics Center
CI	Chemotaxis Index
CNS	Central Nervous System
ddH ₂ O	Double Deionized Water
DIC	Differential Interference Contrast

DMF	Dimethylformamide
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
<i>E. coli</i>	<i>Escherichia coli</i>
EGF	Epidermal Growth Factor
EtOH	Ethanol
F ₁	First Filial Generation
F ₂	Second Filial Generation
F ₃	Third Filial Generation
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorder
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
L1	<i>C. elegans</i> Larval Stage 1
L2	<i>C. elegans</i> Larval Stage 2
L3	<i>C. elegans</i> Larval Stage 3
L4	<i>C. elegans</i> Larval Stage 4
LDL	Low-Density Lipoprotein
mmBCFA	Monomethyl Branched-Chain Fatty Acid
MRI	Magnetic Resonance Imaging
NaN ₃	Sodium Azide

NGM	Nematode Growth Medium
NIAAA	National Institute on Alcohol Abuse and Alcoholism
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PUFA	Polyunsaturated Fatty Acid
SNP	Single Nucleotide Polymorphism

Abstract

INVESTIGATING A MODEL FOR FETAL ALCOHOL DAMAGE IN CAENORHABDITIS ELEGANS

Lindsay Miyoko Kondo

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

Virginia Commonwealth University, 2012

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Alcohol use and abuse has many harmful effects, especially to children exposed prenatally, including fetal alcohol spectrum disorders (FASDs). The disabilities due to fetal alcohol exposure continue throughout life and cause major financial burdens to society. The molecular mechanisms underlying FASDs are not well understood. We have taken a genetic approach to characterize ethanol's effect on changing a discrete cell fate decision during embryogenesis in the nematode, *Caenorhabditis elegans* (*C. elegans*). Our preliminary data suggest that ethanol can affect the development of

AWC neurons, a pair of olfactory neurons in *C. elegans*. We suggest that lipids can protect AWC neurons from ethanol's effects. Importantly, we show that altering the metabolism of triacylglycerols (TAGs) can rescue this cell fate change in behavioral assays. By identifying molecular causes of fetal alcohol damage in humans we hope to be able to develop a greater understanding of how to prevent these detrimental effects.

Chapter 1: Introduction

Alcohol

Alcohol is a widely accepted drug in today's modern society. Alcohol abuse causes many detrimental socioeconomic consequences including increases in criminal activity, loss of employment and productivity, increases in comorbidity with other disorders and increases in healthcare costs that affect all social and ethnic groups. Even with the numerous negative consequences associated with alcohol, people continue to drink. This can then lead to the development of alcohol addiction. As described in DSM-IV, alcohol abuse is "a maladaptive pattern of drinking, leading to clinically significant impairment or distress, as manifested by at least one of the following occurring within a 12-month period: recurrent use of alcohol resulting in failure to fulfill major role obligations at work, school, or home, recurrent alcohol use in situations in which it is physically hazardous, recurrent alcohol-related legal problems, or continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol (American Psychiatric Association, 1994)." Alcohol abuse can then lead to alcohol addiction with continued alcohol use, which is characterized by repetitive alcohol drinking patterns that leads to a loss of control over alcohol consumption (Moonat et al., 2010). Alcoholics suffer negative social consequences such as losses of a partner, friends, family and their job (Spanagel, 2009). Alcoholics are dependent on the drug with features including

excessive drinking, tolerance, withdrawal if alcohol is removed, a persistent desire for alcohol and impaired social, occupational, and recreational activities (DSM-IV, 1994). Prolonged alcohol use can also lead to organ damage of the brain, heart, liver, pancreas and intestines. The Central Nervous System (CNS) can be severely affected with sulcal widening, ventricular enlargements and amygdala, hippocampus, and white matter deterioration leading to deficits in memory, learning, abstraction, problem-solving, and sleep (Schuckit, 2009; Spanagel, 2009). Chronic and heavy alcohol drinking can also cause increased blood pressure, high LDL (Low-Density Lipoprotein) cholesterol, pancreatitis, liver cirrhosis, and decreased bone density (Schuckit, 2009).

Additionally, alcohol abuse has a high comorbidity with other psychiatric disorders including anxiety, attention, and major depressive disorders and nicotine dependence. Studies have shown that panic disorder, bipolar disorder, antisocial personality disorder, posttraumatic stress disorder, and depression are all tightly linked to alcohol abuse (Raimo and Schuckit, 1998; Schuckit et al., 1997; Spanagel, 2009; Swendsen et al., 1998). Furthermore, alcoholism, along with anxiety and depressive disorders, are the most common psychiatric syndromes cited in community surveys and pose a significant health concern (Kessler et al., 1994). Similarly alcoholism has been closely associated with nicotine addiction where as many as 90% of alcoholics are cigarette smokers which can lead to a higher incidence of head and neck cancers (Miller et al., 1998). Chronic alcohol use along with nicotine-use or psychiatric disorders

overall causes an additive effect and leads to increased mortality rates (Miller et al., 1998).

Molecular effects

Ethanol is a very simple and highly diffusible molecule making it difficult to isolate its specific molecular mechanisms and interactions. Ethanol has two reactive sites, the hydroxyl group and the short carbon backbone, and poor reactivity resulting in low potency (Lovinger and Crabbe, 2005). Therefore, low potency allows for a wide range of effects of ethanol ranging from intoxication to anesthesia. Low potency also prevents binding studies to be conducted to identify occupancy of a specific molecular site. In addition, ethanol distributes into many cellular compartments and has a large range of physiologically significant concentrations making it difficult to study.

Ethanol affects many neural mechanisms, which contribute to a person's acute sensitivity to alcohol, development of tolerance and dependence, and a strong desire or craving for alcohol. Alcohol has both direct and indirect targets that are currently being studied (Lovinger and Crabbe, 2005). Various studies have shown an array of proteins, encompassing neurotransmitter receptors, ion channels and neurotransmitter transporters, and processes are affected by ethanol. GABA_A receptors are major targets for ethanol and their function has been shown to be potentiated by ethanol (Grobin et al., 1998). Ethanol has also been shown to increase the activity of SLO-1/BK channels.

SLO-1, or large conductance, voltage and calcium sensitive potassium (BK) channels, are essential in controlling neuronal excitability, synaptic strength and

plasticity and smooth muscle tone. These channels are important in regulating neurotransmitter release and repolarize active neurons by conducting potassium ions through cell membranes. SLO-1 channels are found at presynaptic nerve endings and are activated by membrane depolarization and calcium. BK channels have been shown to be activated by ethanol at clinically relevant concentrations (Dopico et al., 1996, 1998). Additionally, BK channel subunit composition as well as the lipid environment surrounding BK channels has been shown to affect alcohol tolerance (Feinberg-Zadek et al., 2008).

NMDA receptors, glycine receptors, neuronal nicotinic receptors, CREB proteins, VTA dopaminergic neurons and the mesocorticolimbic and extended amygdala circuitry are affected by ethanol (Harris et al., 2008; Lovinger and Crabbe, 2005; Spanagel, 2009). Furthermore, alcohol has been implicated in effects on other enzymes and systems including alcohol dehydrogenase (ADH), adenylyl cyclase, the serotonin system, the dopamine system and the neuropeptide Y receptor (Davies et al., 2004; Harris et al., 2008; Lovinger and Crabbe, 2005). By having a better understanding of ethanol's molecular targets, there is a greater chance of creating new and improved drug treatments to prevent relapse and maintain abstinence for alcoholics.

Current treatments for alcoholism

Currently there is no cure for alcoholism. Abstinence maintenance and prevention of relapse are the major goals for treatment of alcohol-dependent individuals; reducing the incidences of drinking can decrease alcohol-related consequences and

improve quality of life. FDA-approved drug treatments to enhance abstinence include Disulfiram (Antabuse), Naltrexone (Revia, Vivitrol), and Acamprosate (Campral) are used along with peer support groups to encourage and help a person's recovery from alcoholism.

Peer support groups such as Alcoholics Anonymous (AA) and Smart Recovery are 12-step programs to help alcoholics in their desire to stop drinking. It has been shown that regular weekly participation in 12-step programs are effective in maintaining a high rate of alcohol abstinence (Fiorentine, 1999). Also, Blonigen et al. found that alcoholics attending AA had decreased impulsivity associated with fewer alcohol use problems, increased self-efficacy, and better coping and social support after 1 and 8 years. Another important feature of 12-step programs is that recovering alcoholics can help other alcoholics maintain sobriety which benefits both people involved (Pagano et al., 2004).

Along with peer support groups, pharmacotherapy has been shown to be beneficial in alcohol abstinence. Disulfiram was first developed as a rubber vulcanizer, and considered as a treatment when it was discovered that rubber industry workers had an adverse reaction to alcohol by 1910 (Krampe and Ehrenreich, 2010). Disulfiram was not an FDA-approved drug used for alcoholism until the 1940s when it was found to inhibit the metabolism of alcohol. Two enzymes in the liver metabolize alcohol. Alcohol dehydrogenase first reversibly metabolizes alcohol to acetaldehyde. Acetaldehyde is then further broken down into acetate by aldehyde dehydrogenase. Disulfiram inhibits

the liver enzyme aldehyde dehydrogenase leading to an accumulation of acetaldehyde. Acetaldehyde build-up starts as soon as 10 minutes after alcohol consumption and can cause unpleasant aversive effects ranging from facial flushing, sweating and headache to dizziness, nausea, tachycardia, increased pulse and respiration to vomiting and respiratory depression (Krampe and Ehrenreich, 2010). Studies have shown that Disulfiram increases both abstinence and the number of days until relapse (Jørgensen et al., 2011; De Sousa and De Sousa, 2004, 2005, 2008; De Sousa et al., 2008; Laaksonen et al., 2008).

Naltrexone is a competitive antagonist at μ -, κ -, and δ -opioid receptors with the highest affinity for μ -opioid receptors (Garbutt, 2010). Alcohol affects the endogenous opioid system by causing the release of endorphins, which enhance dopamine signaling thereby triggering alcohol reinforcement and provoking a person to drink more (Anton, 2008). Although Naltrexone's mechanism of action in alcoholism treatment is not fully understood, it is believed to block alcohol-induced release of dopamine leading to the reduction of stimulus and reinforcing effects of alcohol and reducing craving and loss of control (Sinclair, 2001). Naltrexone comes in two routes of administration, oral Naltrexone (Revia) or injectable Naltrexone (Vivitrol). Oral Naltrexone has been shown to decrease alcohol craving and relapse but the studies have been confounded by small sample sizes, a large variety in dosage and low levels of medication compliance (Bouza et al., 2004; Volpicelli et al., 1997). Intramuscular injectable Naltrexone was developed to maintain constant plasma levels, increase medication compliance, and increase

exposure to the therapeutic dose (Johnson et al., 2008). Studies have shown that high-dose Vivitrol is efficacious in preventing heavy drinking in subjects by reducing both frequency and quantity of alcohol consumption (Garbutt, 2010; Johnson, 2008; Lee et al., 2010).

Acamprosate is the newest drug to be approved by the FDA for maintenance of abstinence in alcohol dependent patients. Acamprosate has structural similarity to the neurotransmitter GABA (Kennedy et al., 2010). Acamprosate has unique mechanisms of action that are still being elucidated. Chronic alcohol use facilitates inhibitory GABAergic neurotransmission and attenuates excitatory glutamatergic neurotransmission (De Witte et al., 2005). To compensate for this change, there is an increase in the number of NMDA receptors as well as an increase in their sensitivity (Tsai et al., 1995, 1998). Therefore, when a person goes through alcohol withdrawal there is an excessive increase in excitatory neurotransmitters which activate receptors at a higher than normal level. Acamprosate acts to normalize a hyper-glutamatergic state by inhibiting mGluRs and modulating NMDA receptor function thus decreasing neuronal hyperexcitability seen during early abstinence. (De Witte et al., 2005; Johnson, 2008; Kiefer and Mann, 2010). Acamprosate decreases alcohol craving and relapse behavior, has a good safety record, has a superior compliance rate among patients, and is the most widely prescribed drug for the treatment of alcoholism (Bouza et al., 2004; Mason and Heyser, 2010).

Another new drug, Topiramate, is currently being tested and has shown promise in being used for the treatment of alcoholism. Topiramate is an anti-epileptic medication that is being investigated as a treatment for alcohol dependence due to its dual ability to antagonize glutamate receptors and inhibit dopamine receptors, which are important in reward and reinforcement of alcohol use (Johnson, 2010; Olmsted et al., 2008). Clinical trials have shown that Topiramate reduces the number of heavy drinking days and increases days of continuous abstinence from alcohol (Johnson et al., 2007). The NIAAA Medications Development Team has recently identified three new goals for the advancement of alcohol treatment research. These three goals are: develop new approaches to make the development of alcohol-dependence medications more efficient, develop strategies to increase the effect size of compounds in clinical trials and to facilitate the use of alcohol medications in today's clinical practice (Litten et al., 2012). To accomplish these three goals for alcohol treatment they have identified objectives such as discovering and validating new molecular targets and implementing animal and human laboratory research paradigms to screen new drugs to make alcohol drug development more efficient and improve clinical testing (Litten et al., 2012).

Prenatal alcohol exposure

Alcohol causes numerous negative consequences to the person drinking but can cause more severe brain and behavioral effects to the child of an alcoholic mother. Currently, prenatal alcohol exposure is the leading preventable cause of developmental disorders and birth defects in the United States (Bailey and Sokol, 2008). Deficiencies

due to prenatal alcohol exposure are estimated to occur in 1 to 5 percent of live births and create devastating emotional and financial burdens (May et al., 2009; Sampson et al., 1997). In 1973, Jones and Smith first described the teratogenic effects of alcohol on children born to alcoholic mothers, which have now become the characteristic signs of Fetal Alcohol Syndrome (FAS). The three defining signs of FAS include facial abnormalities such as a smooth philtrum and short palpebral fissures, both prenatal and postnatal growth deficits, and CNS abnormalities that can be structural, neurological, behavioral or any combination thereof (Warren et al., 2011). FAS is the most severe effect of prenatal alcohol exposure. FAS has a prevalence of 0.5 to 7.0 per 1,000 live births in the United States (May and Gossage, 2001; May et al., 2009). However, prenatal alcohol exposure does not affect each child in the same way.

Due to the wide variety of negative effects fetal alcohol exposure causes, specific terms were created to describe the consequences seen in children including alcohol-related birth defects (ARBD), alcohol-related neurodevelopmental disorder (ARND), and fetal alcohol spectrum disorders (FASDs). ARBD refers to children with alcohol-related physical anomalies only (Stratton et al., 1996). ARND is diagnosed in a child with confirmed prenatal alcohol exposure who has CNS neurodevelopmental, cognitive, or behavioral abnormalities (Stratton et al., 1996). In 2004, FASD was developed as an umbrella term to describe children who were prenatally exposed to alcohol and have a spectrum of deficits that may include physical, mental, behavioral, and/or learning disabilities with lifelong repercussions (Bertrand, 2004). FASD has a higher estimated

prevalence than FAS of 1 to 5 percent of live births in the United States (May et al., 2009; Sampson et al., 1997,). Currently there are no generally accepted standard recommendations for diagnosing FASD. Clinicians tend to use the 4-Digit Code, a revised version of the Institute of Medicine guidelines or a Canadian set of diagnostic criteria to diagnose FAS and other adverse effects due to prenatal alcohol exposure (Astley and Clarren, 2000; Chudley et al., 2005; Hoyme et al., 2005).

Disabilities due to prenatal alcohol exposure persist throughout life and produce major societal and financial burdens due to health care costs, residential care, productivity losses and special education services. Overall, the estimated cost of FASD in the United States is \$6 billion per year (Lupton et al., 2004). Even though widespread efforts have been made to inform and educate women on the harmful effects of drinking during pregnancy, 120 million women in the United States consume alcohol and about 10 percent continue to drink even after learning they are pregnant (Centers for Disease Control and Prevention 2002).

Diagnosis of FASD in the clinic as well as in research has greatly improved due to enhancements in detection criteria. Nonetheless, there still is failure to diagnose and underreporting of prenatal alcohol exposure due to subtle signs and/or similarities with other disorders such as ADHD. The earlier affected individuals are diagnosed and receive the necessary medical and social services needed the greater the improvement for the quality of life for these people and their families. Today a vast amount of research is being conducted on all aspects of FASD including maternal risk factors,

detection of biomarkers of alcohol in fetuses, improving physical detection in children, elucidating alcohol's neuropathological effects, and improving learning and behavioral deficits.

Current research on FASD

Prenatal alcohol damage in humans ranges from mild to severe and does not consistently produce the same effects in different individuals. Current studies have shown that the most significant contributors to the variability in dysmorphology and developmental deficits are due to the quantity, frequency and timing of alcohol exposure (May et al., 2007, 2008; May and Gossage, 2011). Other maternal risk factors include age, number of pregnancies, number of pregnancies that progressed past 24 weeks, and nutritional and socioeconomic states (Bingol et al., 1987; Keen, 2010; May et al., 2004, 2007, 2008; May and Gossage, 2011).

A majority of children prenatally exposed to alcohol may not present with obvious characteristics of prenatal alcohol exposure. Therefore, diagnosis of FASD requires maternal confirmation. Yet maternal confirmation of alcohol consumption during pregnancy and drinking histories are usually unreliable and more sensitive and reliable biomarkers of alcohol use are needed. One biomarker is fatty acid ethyl esters (FAEEs) that are metabolites formed when alcohol combines with free fatty acids (Laposata, 1998). It has been shown that FAEEs can be extracted and detected in meconium, the first stool of a newborn, to determine prenatal alcohol exposure (Bearer et al., 1999, 2003). Also FAEE detection is sensitive enough to identify moderate and binge-drinking

patterns but is confounded by the small window for specimen collection, unavailability of the meconium sample, and the process of freezing the specimen within the first 12 hours (Bearer et al., 1999, 2003). Recent research includes hair FAEE analysis and dried blood spot specimen analysis used to detect phosphatidylethanol (Bakhireva and Savage, 2011). Present studies are also investigating detection in children prenatally exposed to alcohol using specific facial measurements.

A major distinguishable hallmark of FAS and FASD are facial dysmorphologies, however, subtle facial characteristics are not very easy to detect in the clinic. Currently, research is being done on using three-dimensional computer recognition imaging as a means to detect FAS (Wetherill and Foroud, 2011). Once various images are taken of a child's face, images can be analyzed to measure precise length, width, and height of certain areas of the face and precise shapes can be generated from specific landmarks. Studies have found differences in eye width and shapes of certain facial regions between children not prenatally exposed to alcohol and children diagnosed with FAS (Klingenberg et al., 2010; Moore et al., 2007). It has been proposed that computer recognition imaging may be a promising tool in the clinic to diagnose FASDs. Moreover, magnetic resonance imaging (MRI) in fetal mice exposed to alcohol are showing results consistent with human data on facial and brain malformations (Parnell et al., 2009; Sulik, 2005).

MRI is being used to detect structural brain and facial dysmorphologies due to prenatal alcohol exposure. Studies have found holoprosencephaly, the abnormal

median joining of the right and left cerebral hemispheres, and reductions in corpus callosum, olfactory bulbs, hippocampus, and cerebellum tissues in fetal mice prenatally exposed to ethanol (Parnell et al., 2009; Sulik, 2005). Facial MRI in fetal mice of ethanol-exposed mothers has found close positioning of the nostrils and a long upper lip, consistent with findings in humans with FAS (Sulik, 2005). Other MRI techniques such as diffusion tensor imaging and magnetic resonance spectroscopy are also being utilized and refined to study alcohol's adverse effects on the CNS. Due to these devastating CNS defects, children with FASD show many behavioral and learning difficulties.

Children with FASD show major behavioral deficits in verbal and spatial learning, memory, planning, problem solving, poor social skills, balance and motor control, and impairments in reading, math, and spelling (Coles, 2011; Paley and O'Connor, 2011; Willoughby et al., 2008). Also FASD children tend to have difficulty maintaining attention and self-regulation. Memory seems to be critically affected by prenatal alcohol exposure and research has found there are complications especially in learning new material. Furthermore, children show deficits in being able to select and employ effective learning strategies (Coles, 2011). Due to these learning deficits, it takes children prenatally exposed to alcohol more trials to master certain material. Most interventions showing positive results include therapists trained in specific tasks working with FASD children individually. O'Connor et al. created a parent-assisted social skills intervention called Children's Friendship Training (CFT) specifically for children with FASD that was

extended to a community mental health center (Frankel and Myatt, 2003). CFT teaches social skills to help children be accepted in society and sessions include instruction on basic rules of social behavior, rehearsing, and coached practice.

Model organisms used for prenatal alcohol studies

Model organisms serve as powerful research tools for studying drugs of abuse, including alcohol. There are a wide variety of model organisms from bacteria to fungi to invertebrates including *C. elegans* and *Drosophila melanogaster* to rodents, fish, and monkeys with each having its own advantages. Different model organisms are studied to understand specific biological mechanistic processes and fundamental consequences of alcohol use, abuse, and dependence with the goal of finding new methods for treating humans who suffer from alcoholism. In alcohol studies, model organisms allow for genetic, dietary, and environmental manipulations.

Many developmental prenatal alcohol exposure studies have been conducted in rodent model organisms. In rodent studies, maternal exposure to alcohol has been shown to increase pup mortality, decrease pup weights, and attenuate crucial growth spurts (Abel and Dintcheff, 1978; Jones and Chernoff, 1978; Middaugh and Boggan, 1995; Singh et al., 1992; Vaglenova and Petkov, 1998). The rotarod and inclined plane tests are used to measure balance, motor coordination, and learning. Offspring of mothers exposed to ethanol during gestation show severe impairments compared to their control counterparts in both the rotarod and inclined plane tests (Abel and Dintcheff, 1978). Similarly, the two-way active avoidance test is used to measure

learning and memory. This test uses a light and sound cue to signify when a foot shock will be applied. Vaglenova et al. found that progeny of ethanol treated Wistar rats had severe learning and memory deficits at both 9 weeks and 5 months of age and had significantly lower numbers of avoidances and were not able to retain avoidance information. Furthermore, similar to human studies, ethanol has been shown to have differential effects due to varying exposure times. The offspring of mice treated with ethanol, who continued to be treated with ethanol after birth, showed hyperactive behavior with increased frequency of exploration and scanning and decreased self-grooming (Cutler et al., 1979). In contrast, juvenile mice treated with ethanol for 5 days showed increased social investigation (Cutler et al., 1979).

Rodent studies have also replicated human findings with prenatal alcohol exposure causing facial dysmorphologies as well as CNS malformations. Studies have shown that alcohol causes inhibition of neuronal growth and decreased brain weight in rat fetuses (Lindlsey et al., 2003; Singh et al., 1992). Moreover, rodent studies have shown prenatal alcohol exposure causes craniofacial abnormalities including short palpebral fissures, a long upper lip, and a smooth philtrum, similar to children with FASD (Sulik et al., 1982; Sulik et al., 2005). Rodent studies have also found similar results to human studies with FASD and found the corpus callosum, olfactory bulbs, hippocampus and cerebellum are severely affected by prenatal alcohol exposure (Sulik et al, 2005).

Similarly, non-rodent animal models have found consistent results with prenatal alcohol exposure. Chick studies have found increased mortality rates, suppression of embryo growth, and inhibition of brain growth in alcohol exposed chick embryos (Boyd et al., 1984; Carver et al., 1999; Pennington et al., 1983; Satiroglu-Tufan and Tufan, 2004). Likewise, Potter et al. found delayed growth and organ development in fetuses of alcohol drinking mothers. Alcohol studies using non-mammalian model organisms including the Japanese medaka, zebrafish, and *Xenopus laevis* showed craniofacial malformations in the head, mouth, lower jaw, eyes and cartilage around the tail region in embryos treated with alcohol (Loucks and Ahlgren, 2012; Nakatsuji, 1983; Wang et al., 2006). Wang et al. further showed that alcohol caused a dose-dependent reduction in survival and hatching rates along with mortality, and cardiovascular defects in medaka. Marrs et al. also showed cardiovascular defects in zebrafish embryonically exposed to ethanol along with uncoordinated movement. Offspring of *Drosophila* exposed to alcohol had decreased hatching, delayed development, and physical malformations of the legs and wings (Ranganathan et al., 1987).

Importantly, many studies across a wide variety of model organisms have replicated human FASD findings of the effects of prenatal ethanol exposure on growth retardation, brain size and development, physical dysmorphologies, behavioral defects and learning and memory deficits. From prenatal alcohol exposure studies using model organisms, various signaling molecules and proteins have been implicated in FASD such as retinoic acid, epidermal growth factor (EGF), and cyclic adenosine

monophosphate (cAMP) (Henderson et al., 1989; Pennington et al., 1983; Satiroglu-Tufan and Tufan, 2004, Shibley and Pennington, 1997).

C. elegans as a model organism for prenatal alcohol studies

Caenorhabditis elegans (*C. elegans*) serves as a powerful genetic tool due to its simple, well-characterized nervous system consisting of 302 neurons and its conserved neurobiology with humans (White et al., 1986). Furthermore, the entire *C. elegans* genome has been fully sequenced (*C. elegans* Genome Consortium, 1998) and each neuron has been described and had its connections mapped (White et al., 1986). Moreover, the developmental fate of each somatic cell has been mapped (Sulston et al., 1983).

Recently, *C. elegans* has emerged as a novel model organism to investigate the effects of ethanol exposure on development. Davis et al. found that larval ethanol exposure in *C. elegans* causes growth and developmental delays. Acute embryonic ethanol exposure significantly increased embryonic lethality and caused mild to severe dysmorphologies (Davis et al., 2008). Chronic ethanol exposure during larval development produced more severe developmental effects including delays in gonadal development and onset of egg laying, smaller brood sizes and decreased lifespan (Davis et al., 2008). In addition, Lin et al. showed that developmental delays in embryonic worms are first observed after 3 hours during chronic ethanol exposure and are most severe during hatching.

SLO-1 BK channels

slo-1 was first identified in *Drosophila* and called *slowpoke* and was later identified in *C. elegans* when Wang et al. conducted a genetic screen to identify regulators of neurotransmitter release. *slo-1* was the sole ion channel isolated suggesting that SLO-1 BK channels are unique in their ability to regulate neurotransmitter release (Wang et al., 2001). Importantly, SLO-1 channels have also been shown to be activated by ethanol and play a significant role in intoxication.

SLO-1 has a high level of sequence homology between mammals such as rodents and humans and *C. elegans* and *Drosophila*. In *C. elegans*, *slo-1* expression is found in both muscles and neurons (Wang et al., 2001). Previously, in ethanol sensitivity screens, Davies et al. isolated multiple loss-of-function mutations in *slo-1* that showed strong resistance to ethanol in locomotion and egg laying assays. Conversely, gain-of-function mutants of *slo-1* looked similar to intoxicated animals. It was also shown through electrophysiology that physiologically relevant doses that causes intoxication in humans activates the BK channel *in vivo* and neuronal SLO-1 produces ethanol sensitivity (Davies et al., 2003). Furthermore, mammalian studies have also shown that BK channels are stimulated by ethanol and develop tolerance (Dopico et al., 1996; Treistman and Martin, 2009).

Membrane lipids affect SLO-1 BK channels

Recently, the lipid environment on the cell membrane has emerged as an important modulator for the interaction between ethanol and proteins. Studies have shown the thickness of the lipid bilayer can modulate basal activity and ethanol

activation of the BK channel (Treistman and Martin, 2009; Yuan et al., 2004, 2007, 2008). BK channels were extracted from human embryonic kidney-293 (HEK-293) cells and reconstituted into different artificial lipid bilayers. In a thin lipid bilayer of phosphatidylcholine (PC), initial ethanol exposure caused strong activation of BK channels whereas in thick sphingomyelin lipid bilayers, BK channels were inhibited and ethanol was not able to activate them (Crowley et al., 2005; Yuan et al., 2004, 2007, 2008). Cholesterol content was also shown to alter BK channel activation by ethanol and acute ethanol tolerance (Bukiya et al., 2011; Yuan et al., 2011). Thus, altering membrane lipid composition modulates initial sensitivity and acute ethanol tolerance in BK channels.

Similarly, Bettinger et al. identified triacylglycerols (TAGs) as an important factor for the development of acute functional tolerance (AFT) to ethanol. Acute functional tolerance is a mechanism of the nervous system to quickly adapt to alcohol's intoxicating effects. *C. elegans* develop AFT during a continuous exposure of ethanol. Previous work has shown that worms become intoxicated in 7-10 minutes to a moderate dose of exogenous ethanol. At this time point, animals have a severely depressed speed of locomotion and have an internal ethanol concentration of 45 mM. After 50 minutes of continuous exposure, animals are moving significantly faster relative to the 10 minute time point. These animals have an internal ethanol concentration about 65 mM. These results suggest that behavioral adaptations induced by ethanol are not

mediated by metabolism but are a compensatory response induced by ethanol that we term AFT (Alaimo et al., 2012; Davies et al., 2004).

CTBP-1, an NAD(H)-dependent transcriptional co-repressor, was shown to negatively regulate LIPS-7, a TAG lipase that metabolizes lipids. Chen et al. found that *ctbp-1* mutants have a 16.8% decrease in TAGs and RNAi knockdown of *lips-7* increased levels of TAGs. *lips-7* mutants were resistant to ethanol and fast developers of AFT and were more resistant to ethanol compared to wild-type N2 (Bettinger et al., 2012). In contrast, *ctbp-1* mutants were slower developers of AFT and more sensitive to ethanol (Bettinger et al., 2012). Taking into account *in vitro* mammalian BK channel activity can be modified by cell membrane lipid composition, these studies suggest that lipids could play a role in the development of tolerance to ethanol by sequestering BK channels (Bukiya, 2011; Crowley et al., 2005; Pietrzykowski et al., 2004; Yuan et al., 2004, 2007, 2008, 2011).

AWC chemosensory neurons in *C. elegans*

C. elegans can sense an extensive variety of odors important to its development and survival due to the expression of several receptor genes (Troemel et al., 1995). AWC cells are a pair of olfactory neurons that together allow *C. elegans* to detect and discriminate between volatile attractive odorants including benzaldehyde, butanone, 2,4,5-trimethylthiazole, and isoamyl alcohol (Bargmann et al., 1990, 1993). Expression of odorant receptors is asymmetrical in the AWCs. One receptor that is asymmetrically expressed is the seven transmembrane G-protein coupled receptor, STR-2. Wes and

Bargmann proposed a model for the use of asymmetry in the AWCs in which it is required for odor discrimination. In this model, one AWC neuron detects benzaldehyde and 2,3-pentanedione whereas the other AWC neuron detects butanone and benzaldehyde and butanone signaling attenuates benzaldehyde signaling (Figure 1, Wes and Bargmann, 2001).

Proper functioning of AWC neurons can be measured using chemotaxis and odor discrimination assays (Bargmann et al., 1993; Wes and Bargmann, 2001). In a chemotaxis assay, worms are tested to see if they have the ability to recognize and travel toward or away from a point source of attractant or repellent. After various time points, worms are counted at the odorant, diluent, and throughout the rest of the plate. The chemotaxis index (CI) is then calculated as the number of worms at the odorant minus the number of worms at the diluent divided by the total number of animals (Bargmann et al., 1993). A CI close to 1 signifies a strong attractant, -1 signifies a strong repellent, and 0 signifies no response. Odor discrimination tests a worm's ability to distinguish between different odors and chemotax towards an attractive odor in the presence of a uniform field of another odorant (Figure 1, Wes and Bargmann, 2001).

AWC chemosensory pathway

AWC neurons make a stochastic decision where a suite of odorant receptors are asymmetrically expressed in 1 of the 2 AWCs (Lanjuin and Sengupta, 2004; Troemel et al., 1999). Early in development, AWC neurons are bilaterally symmetric and have not adopted their final cell fates. During embryogenesis, AWC neurons make an activity

dependent cell fate decision. The cell with less basal activity expresses *str-2* while the cell with more basal activity does not express *str-2* and subsequently specific groups of G protein-coupled receptors are asymmetrically expressed within the two AWCs (Troemel et al., 1999; Wes and Bargmann, 2001). Cell fate decisions can be monitored by the fusion of the *str-2* promoter to GFP (*str-2::GFP*) (Troemel et al., 1999). AWC ON is denoted for the AWC neuron that expresses *str-2::GFP* while AWC OFF is the neuron that does not express *str-2::GFP* (Troemel et al., 1999).

The exact mechanism and subsequent pathway of the AWC ON and OFF decision has not been established. However, mutant screens and epistasis analysis has identified key genes associated with this cell fate decision and a predicted pathway has been suggested (Figure 2). The default state for AWC neurons is OFF and neurons will not become ON in the absence of the other AWC neuron (Troemel et al., 1999). The induction of AWC ON requires the collaboration of *nsy-4* (*nsy*-neuronal symmetry mutant), a claudin-like gene that composes tight junctions, and *nsy-5*, an innexin gene that assembles a gap junction network (Chuang and Bargmann, 2007; VanHoven et al., 2006). First, the future AWC OFF neuron signals to the other neuron to become AWC ON. The original signal is hypothesized to be a voltage signal that regulates membrane potential and travels through gap junctions formed by *nsy-5* (Bauer Huang et al., 2007). This signal suppresses calcium entry by inhibiting UNC-2, UNC-36, and EGL-19, voltage-activated calcium channels and induces high OLRN-1 activity (Bauer Huang et al., 2007; Troemel et al., 1999). OLRN-1 is an uncharacterized protein that shares

distant similarity to *Drosophila melanogaster* RAW which restricts JNK signaling (Bauer Huang et al, 2007). OLRN-1 represses the UNC-43 mitogen-activated protein kinase cascade within the AWC ON neuron and sends information back to the AWC OFF cell (Bauer Huang et al., 2007). AWC ON identity is then maintained by NSY-7, a DNA-binding protein and transcriptional regulator that represses AWC OFF genes (Lesch et al., 2007; Taylor et al., 2010).

The AWC OFF neuron maintains calcium entry via UNC-2, UNC-36 and EGL-19 (Bauer Huang et al., 2007; Troemel et al., 1999). Calcium then activates UNC-43, a calcium/calmodulin-dependent protein kinase II (CaMKII) which physically interacts with and phosphorylates TIR-1, an adaptor protein (Chuang and Bargmann, 2001; Reiner et al., 1999). TIR-1 localizes and activates NSY-1, a mitogen-activated protein kinase kinase kinase (MAPKKK) (Chuang and Bargmann, 2001; Sagasti et al., 2001). NSY-1 then phosphorylates and activates SEK-1, a mitogen-activated protein kinase kinase (MAPKK) (Sagasti et al., 2001; Tanaka-Hino et al., 2002). Subsequently, it is believed that SEK-1 activates an unidentified *C. elegans* mitogen-activated protein kinase (MAPK) that then suppresses transcription of *str-2* rendering the neuron AWC OFF (Bauer Huang et al., 2007; Tanaka-Hino et al., 2002; Taylor et al., 2010). AWC OFF maintains its identity through HMBX-1, a transcription factor, during the adult stage (Lesch et al., 2010).

Lipids in *C. elegans*

Lipids have a wide range of important functions in mammals, which include being structural components of membranes and sources of energy. Thus, the regulation of lipids is important for vital biological functions. *C. elegans*, similar to mammals, utilize various desaturases and elongases to synthesize fatty acids from their diet of bacteria to produce saturated, monounsaturated, and polyunsaturated fatty acids important for their development, reproduction, and lifespan (Vrablik and Watts, 2012; Wallis et al., 2002; Watts, 2009). Worms have fatty acid synthesis pathways that produce a variety of monomethyl branched chain fatty acids (mmBCFAs) as well as saturated, monounsaturated, and polyunsaturated fatty acids (Figure 3).

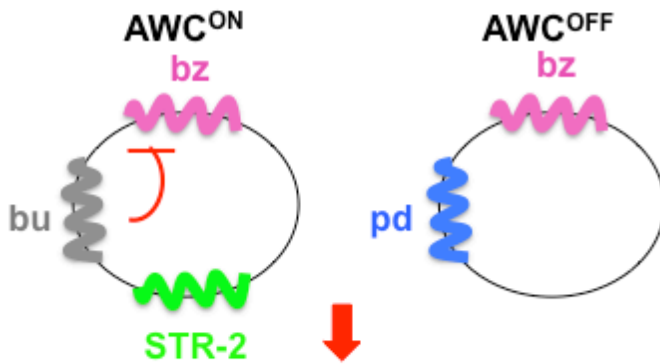
C. elegans also synthesize monomethyl branched chain fatty acids *de novo* from acetyl CoA vital for their development and growth (Kniazeva et al., 2004; Watts, 2009). In addition, worms are cholesterol auxotrophs and consume this sterol through their diet (Ashrafi, 2007). Importantly, *C. elegans* do not require essential fatty acids in their diet like mammals because they have all of the required biosynthetic enzymes. The complexity of their fatty acid pathway allows for flexibility if biosynthetic enzymes are not readily available to produce specific fatty acids.

Hypothesis

Our preliminary data has shown that *slo-1* gain-of-function mutants have 2 AWC neurons with *str-2::gfp* expression (data not shown). Also by adding the *lips-7* mutation, which increases TAG levels, in a *slo-1* gain-of-function background we can suppress the *slo-1* gain-of-function phenotype and animals have 1 AWC neuron with *str-2::gfp*

expression. This suggests that lipid content of the cell membrane can modify SLO-1 activity. Furthermore, by treating *kyIs140* animals to ethanol during embryogenesis we can alter their AWC cell fate decision and animals express 2 apparent AWC neurons expressing *str-2::gfp*. Taken together, we hypothesize that exposing embryonic *kyIs140* animals to ethanol will cause persistent functional consequences due to the altered AWC cell fate decision. Furthermore, by altering the lipid levels of ethanol-exposed animals we may be able to attenuate the effects of ethanol on the cell fate decisions in their embryos.

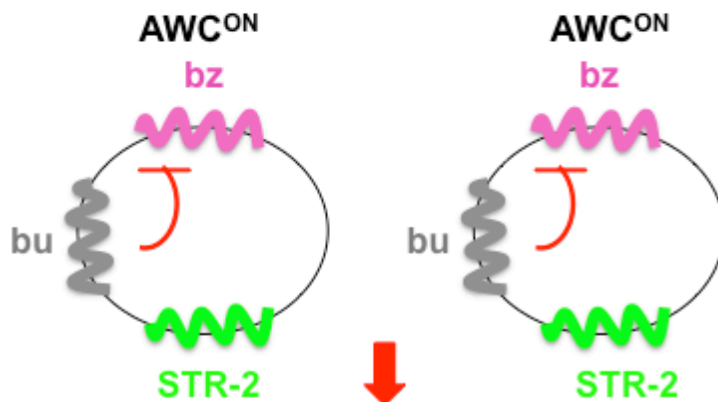
Wild type



bz = benzaldehyde
bu = butanone
pd = 2,3 pentanedione

can detect **benzaldehyde**
in a field of butanone

slo-1(ky389gf)



can NOT detect **benzaldehyde** in
a field of butanone

Figure 1. Model for behavioral defects observed in *slo-1* gain-of-function mutants

In wild-type animals, both AWC neurons recognize benzaldehyde. AWC^{ON} recognizes butanone and AWC^{OFF} recognizes 2,3-pentanedione. Butanone signaling attenuates benzaldehyde signaling in AWC^{ON} (red bar). In *slo-1* gain-of-function mutants, the AWC^{OFF} neuron is transformed into AWC^{ON}, 2,3-pentanedione chemotaxis is lost, and butanone attenuates benzaldehyde signaling in both AWC neurons. (Wes and Bargmann, 2001).

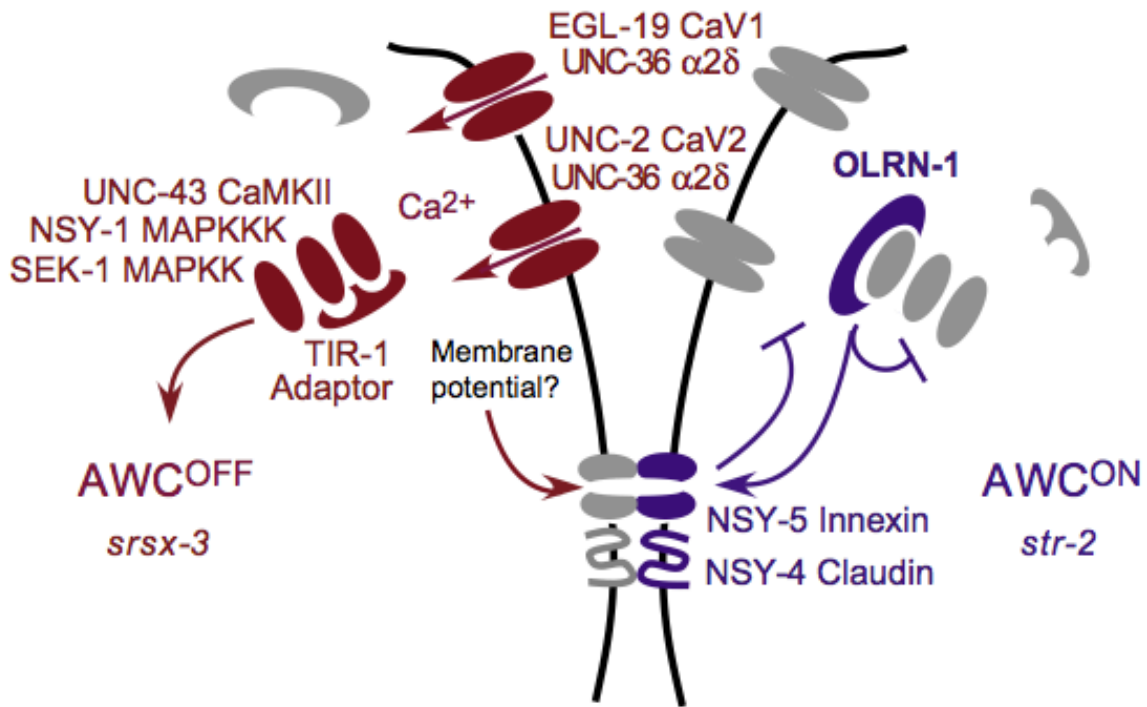


Figure 2. Model for AWC ON and OFF pathways

The future AWC OFF signals to the AWC ON via NSY-5 gap junctions. In AWC ON, the signal suppresses the UNC-2, UNC-36, and EGL-19 voltage-activated calcium channels and allows high OLRN-1 activity. OLRN-1 inhibits the UNC-43 (CaMKII)/NSY-1/SEK-1 kinase cascade within AWC ON. (Bauer Huang et al., 2007)

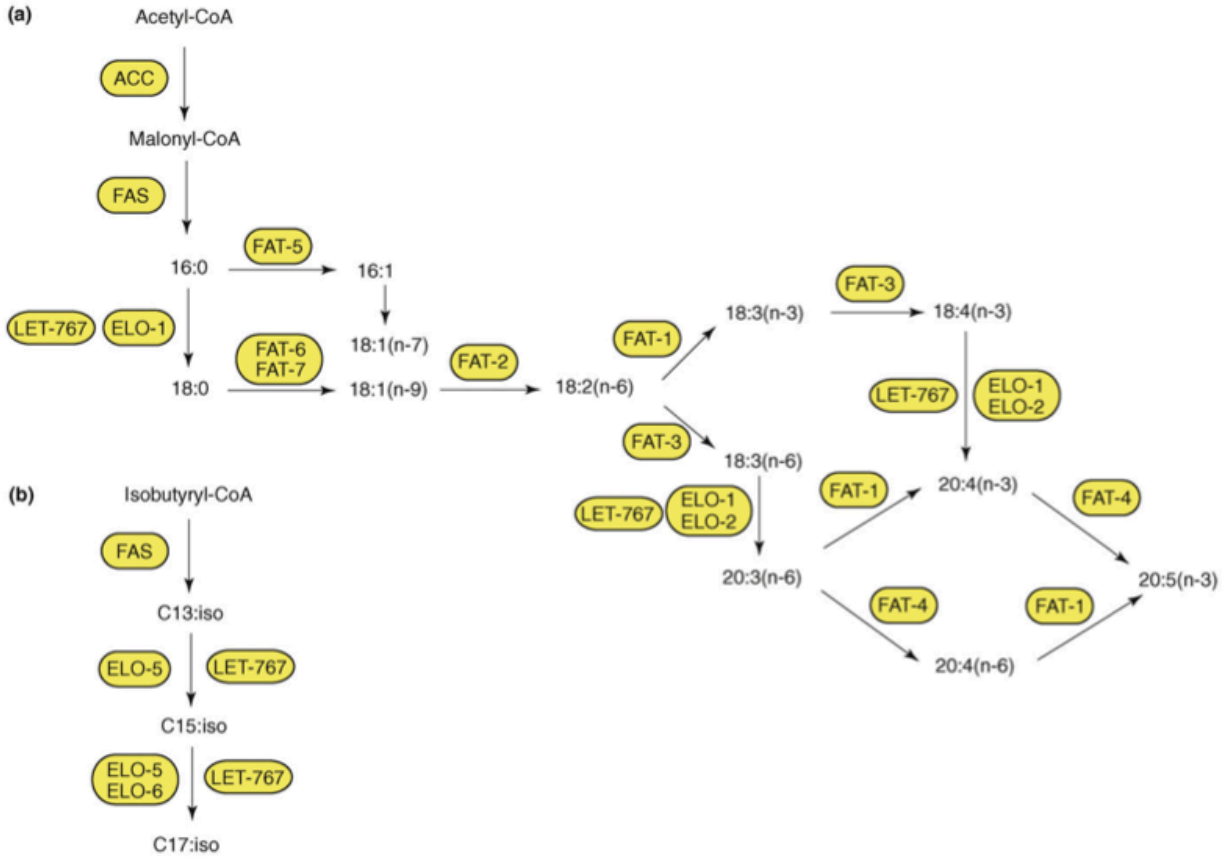


Figure 3. Fatty acid synthesis pathways in *C. elegans*

(a) *De novo* synthesis of polyunsaturated fatty acids (PUFAs). (b) Monomethyl branched-chain fatty acid (mmBCFA) synthesis. (Watts, 2009)

Chapter 2: Materials and Methods

Nematode maintenance and strains

Wild-type was *C. elegans* variety Bristol, strain N2. All other strains contained the integrated *str-2::GFP* transgene *kyls140 (I)* (Troemel et al., 1999). *kyls140* consists of a transgene with the *str-2* promoter inserted and fused into a GFP vector integrated on chromosome I. The strains that were used in these studies were: N2, CX3695 *kyls140*, RB2287 *lips-7(ok3110)*, JCB34 *ctbp-1(eg613)*, KP1097 *dgk-1(nu62)*, BZ142 *slo-1(eg142)*, JCB95 *slo-1(ky389)*, JCB97 *slo-1(ky399)*, JCB96 *lips-7(ok3110);slo-1(ky389)*, JCB98 *lips-7(ok3110); slo-1(ky399)*, JCB78 *kyls140;lips-7(ok3110)*, JCB76 *kyls140;ctbp-1(eg613)*, JCB100 *kyls140;dgk-1(nu62)*, JCB77 *kyls140;slo-1(eg142)*, JCB88 *kyls140;slo-1(ky389)*, JCB97 *kyls140;slo-1(ky399)*, JCB122 *kyls140;lips-7(ok3110);slo-1(ky389)*, and JCB75 *kyls140; lips-7(ok3110);slo-1(ky399)*. Strains were generated in the Bettinger laboratory or were provided by the *Caenorhabditis elegans* Genetics Center (CGC, University of Minnesota, Minneapolis, MN).

Nematodes were maintained on 6 cm Petri plates containing nematode growth medium (NGM) agar seeded with *E. coli* food, OP50, at 20°C. Hermaphrodite worms were maintained by self-fertilization.

Generation of *kyls140* double and triple mutants

In order to monitor AWC cell fate changes, all of our strains needed to express *str-2::GFP*. Since *kyls140* animals contain the *str-2::GFP* transgene we used these

animals to generate strains expressing *str-2::GFP*. *lips-7(ok3110)*, *ctbp-1(eg613)*, *dgk-1(nu62)*, *slo-1(eg142)*, *slo-1(ky389)*, *slo-1(ky399)*, *lips-7(ok311);slo-1(ky389)*, and *lips-7(ok311);slo-1(ky399)* strains were each crossed with *kyIs140* males. 2 mutant hermaphrodite L4s were placed with 6 *kyIs140* adult males on a single small plate and left overnight. On day 2, hermaphrodites were removed and plated individually. In the first filial (F_1) generation we can distinguish successfully mated hermaphrodites since they will generate approximately a 50% male progeny and all progeny will be heterozygous. To confirm progeny are heterozygous for *kyIs140*, hermaphrodite worms were selected based on GFP fluorescence in an AWC neuron using a Zeiss Discovery V12 Stereoscope. This would indicate that worms were cross progeny because the GFP construct was donated by the *kyIs140* father. These worms were placed individually on plates and allowed to self fertilize and lay eggs to produce the second filial (F_2) generation. In the F_2 generation, the animals will have a variety of genotypes. F_2 worms were selected based on if they expressed GFP fluorescence in an AWC neuron and if they showed a phenotype similar to the second mutation, for example, *slo-1(ky389)* are slower and flatter compared to *kyIs140*. Selected F_2 worms were individually plated and allowed to self-fertilize to generate the third filial (F_3) generation. If all animals in the F_3 generation had GFP fluorescence in an AWC neuron, it would indicate the F_2 generation was homozygous for *kyIs140*. If only half of the F_3 generation was glowing this would indicate animals are heterozygous for *kyIs140*. If present, homozygous *kyIs140* F_3 progeny were selected and maintained. DNA from subsequent generations

was then isolated and used in Polymerase Chain Reaction (PCR). If needed, single nucleotide polymorphism (SNP) markers were used in PCR. Samples were also sequenced using an ABI 3730xl DNA Analyzer at the DNA Sequencing Core (VCU) to identify if the second mutation was present. If only heterozygous *kyIs140* were present, animals expressing GFP were picked individually and allowed to self-fertilize to generate homozygous *kyIs140* animals. Once *kyIs140* was homozygous, DNA was isolated and PCR or sequencing was conducted to identify the second mutation. From PCR and sequencing results, if animals were both homozygous for *kyIs140* and the second mutation they were selected and maintained. If only animals that were homozygous for *kyIs140* and heterozygous for the second mutation were isolated, animals were picked individually from that generation and allowed to self-fertilize. In the next generation, one-fourth of the progeny are homozygous for the second mutation. Animals are then picked based on phenotype of the mutation and allowed to self-fertilize. DNA from subsequent generations is then isolated and PCR and sequencing are run again. Once animals are both homozygous for *kyIs140* and the second mutation they are maintained.

Chemotaxis and odor discrimination assays

Assay agar (2% agar with 5 mM KPO₄, 1 mM MgSO₄, 1 mM CaCl₂) was melted and 10 ml were aliquoted into 10 cm Petri plates (Fisher). Assay plates were allowed to dry at room temperature overnight on a bench top. For odor discrimination plates, 12 µl or 24 µl of butanone was added to 100 ml of cooled liquid agar before the plates were

poured (see Figure 5). The day of the experiment, assay plates were dried for 1 hour at 37°C with lids off. Two marks were made on the bottom of all plates at opposite sides and about 0.5 cm from the edge of the plate (see Figures 4 and 5). Sodium azide (1 μ l of 1M NaN₃) was placed at both the diluent and odorant spots to immobilize worms when they reached an odorant spot. Sodium azide anesthetized worms within about a 0.5 cm radius of the odorant spots. A spot of the odorant (1 μ l of 1:100 benzaldehyde:EtOH) was placed on the agar over one mark and a spot of diluent (1 μ l of 100% ethanol) was placed over the other mark. About 100-400 well-fed age matched first day adult animals were washed off of culture plates into a 15 mL conical tube with 2 mls of S. Basal (0.1 M NaCl, 0.05 M KPO₄, ddH₂O). Worms were allowed to settle, the supernatant was removed and animals were washed three more times with S. Basal and once with assay buffer (5 mM KPO₄, 1 mM MgSO₄, 1 mM CaCl₂, ddH₂O) to free them of bacteria. Once worms settled in assay buffer, a low retention tip was used to pipet 10-15 μ l of worms. Worms were then placed near the center edge of the plate, equidistant from the diluent and odorant spots and off center at the origin (see Figures 4 and 5). Excess liquid was then wicked off using a twisted Kimwipe. Plates were sealed with Parafilm and arranged as a single layer on the bench top. At 1 hour and 2 hours after the assay began, the numbers of animals at the diluent and odorant spots were counted. Worms were counted at a spot if they were within 1 cm of the center of the odorant spots. The total number of animals in the assay was also determined. A specific chemotaxis index (CI) was calculated as:

CI=

$$\frac{\text{number of worms at odorant} - \text{number of animals at diluent}}{\text{total number animals in assay}}$$

A number close to 1 indicates a strong attractant, -1 indicates a strong repellent, and 0 indicates no response.

Control experiments with wild-type and mutant worms revealed that 2 hours was a sufficient amount of time for all mutants tested to reach odorant sources. Therefore, assays were counted twice, once after 1 hour and once after 2 hours.

Acute embryonic ethanol exposure

NGM agar plates were seeded over half of their surface with *E. coli* OP50. The next day, plates were dried for 1 hour at 37°C before 100% ethanol (at 4°C) was pipetted onto plates based on plate weights to a final total concentration of 500 mM. Care was taken to not get ethanol on bacteria because it will kill bacteria and make it less palatable to the worms. Plates were sealed with Parafilm and left for 2 hours at room temperature to equilibrate.

To generate age-synchronized adult populations of animals that had experienced embryonic exposure to ethanol, we allowed hermaphrodite worms to generate eggs in the presence of ethanol. L4 worms that had been reared on non-ethanol containing plates were placed on food on ethanol plates and left for 14-16 hours to allow them to generate eggs. These animals were then moved to non-ethanol containing NGM plates seeded with OP50, and allowed to lay eggs for 2-4 hours before

being removed. Eggs were then grown up on plates at 20°C for 2 days until they were L4 larvae before being scored for AWC phenotype. The next day, when they were first day adults, they were used in chemotaxis and odor discrimination assays.

Chronic developmental exposure to ethanol

Plates were incubated with ethanol to a final concentration of 400 – 700 mM as previously described (above). L4 worms were placed on ethanol plates for 14-16 hours to allow them to become adults, then were moved to a second set of 400 – 700 mM ethanol plates and allowed to lay eggs for 2-4 hours. Eggs were grown up on 400 - 700 mM ethanol treated plates at 20°C. Worms were left on 400 – 700 mM ethanol treated plates and closely monitored and scored for AWC phenotype once they reached the L4 larval stage.

Microscopy

For all microscopy, live animals were immobilized on a 5% agar pad containing 80 mM NaN₃ that was fixed on a glass slide and covered with a coverslip.

AWC cell identification was based on characteristic morphology and location of green fluorescent protein (GFP)-positive cell nuclei examined by simultaneous fluorescence and Nomarski differential interference contrast microscopy (DIC).

Fluorescence microscopy was carried out on a Zeiss Axio Imager Upright Microscope.

Most animals were scored under a 20X Plan-Neofluar or a 40X Plan-Neofluar oil immersion objective.

Aldicarb assay

Aldicarb assay plates contained 10 ml of assay agar (2% agar, 5 mM KPO₄, 1 mM MgSO₄, 1 mM CaCl₂) with 0 mM, 0.25 mM or 0.50 mM aldicarb. Plates were stored overnight at 4°C. The next day copper rings were melted into the plates to allow us to test different strains of animals on one plate at the same time. Ten animals were picked per ring and each animal was poked with a platinum pick at 30-minute intervals for 3 hours. Animals were classified as spontaneously moving, not moving but will move if prodded and paralyzed and will not move when prodded 3 times. Hypersensitive animals from this assay were then maintained and *dgk-1* mutants were verified by PCR.

Dil staining

Stock Dil solution in dimethylformamide (DMF, 2 mg/ml) was diluted in M9 (1:100 Dil:M9). Diluted Dil (150 – 250 µl) was pipetted into wells on Borhner slides and live animals were soaked in diluted Dil for 2 hours. Borhner slides were moved to foil covered large Petri plates, and the chambers were hydrated with 2 large spots of 50 µl of ddH₂O. Worms were then immobilized and mounted on 5% agar pads containing 80 mM NaN₃ fixed on a glass slide with a coverslip. A Texas Red filter was used for fluorescence microscopy.

Developmental timing of GFP expression

To evaluate AWC marker expression in the L1, L2, L3, L4, and young adult larval stages, animals were age-matched and scored at each stage. Thirty gravid adult worms were picked to an NGM plate seeded with OP50 and allowed to lay eggs. After 2 hours, adult worms were picked off and eggs were left on plates. Eggs were allowed to hatch

and grow up for 60 hours at 20°C until worms were L4s. 75 – 400 L4 worms were transferred to half seeded plates untreated or treated with various ethanol concentrations (see Chronic developmental exposure to ethanol, above, for preparation of the plates). These animals developed into adults overnight and began to lay eggs on the ethanol-containing plates. The eggs were allowed to hatch, and the animals' postembryonic development occurred on ethanol-containing plates. To compare GFP-expressing neurons in L1, L2, L3, L4, and young adults grown on ethanol, 50 animals were picked for scoring at each time point. L1 animals were scored 9 hours after hatching at 20°C. L2 animals were scored 19.5 hours after hatching at 20°C. L3 animals were scored 30 hours after hatching at 20°C. L4 animals were scored 44 hours after hatching at 20°C. Young adult animals were scored 54 hours after hatching at 20°C.



(b) Chemotaxis Index (CI) =

$$\frac{\text{number at odorant} - \text{number at diluent}}{\text{total number animals in assay}}$$

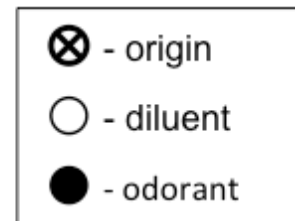


Figure 4. Chemotaxis assay

(a) In a chemotaxis assay, worms move towards a spot of attractive odorant (filled circle). Worms are placed at the origin spot and allowed to freely move for 1 and 2 hours. Worms are immobilized by sodium azide if they reach either the odorant or diluent spot. (b) The chemotaxis index is calculated by counting the number of animals immobilized at the spot of odorant minus the number of animals immobilized at the diluent spot (open circle) divided by the total number of animals in the assay.

		Butanone	Chemotaxis Index
(a)	N2 (1 AWC ^{ON})	-	high
		+	high
(b)	<i>slo-1gf</i> (2 AWC ^{ON})	-	high
		+	low

Figure 5. Chemotaxis and odor discrimination assays

Animals were tested on plates absent of butanone (upper plate, white) or containing butanone in the agar (lower plate, yellow) for chemotaxis to benzaldehyde. (a) Wild-type, N2 animals with 1 AWC^{ON} chemotax toward benzaldehyde. (b) *slo-1gf* animals with 2 AWC^{ON} chemotax toward benzaldehyde and are not able to discriminate benzaldehyde in the presence of butanone in the agar (see Introduction, AWC chemosensory neurons in *C. elegans*).

Chapter 3: Results and Discussion

***kyls140; slo-1(ky389gf)* mutants are deficient in chemotaxis**

Wild-type N2 and *kyls140* have 1 AWC ON (data not shown) and a high CI indicating they are both strongly attracted to benzaldehyde (Figure 6). *kyls140* animals contain the *str-2* promoter fused to GFP on chromosome I in a wild-type N2 background, which allows us to distinguish *str-2* expressing AWC neurons from non-*str-2* expressing AWC neurons and is a marker of AWC asymmetry. Wild-type animals express *str-2::gfp* in 1 AWC neuron, whereas *slo-1(gf)* animals have a loss of asymmetry and express *str-2::gfp* in both AWC neurons. We first scored *kyls140; slo-1(ky389gf)* mutants using fluorescence microscopy and found they have 2 AWC ON (data not shown), as has been previously reported (Troemel et al., 1999). This suggests that *slo-1* plays an important role in AWC cell fate decisions. We then tested wild-type and mutant animals in chemotaxis assays to see if loss of asymmetry affects their chemotaxis behavior.

slo-1 gain-of-function mutants have inappropriately active SLO-1 channels and are slightly locomotor defective. *slo-1* gain-of-function mutants move slower than wild-type N2 animals. Due to the *slo-1* gain-of-function mutants' slower locomotion phenotype, time course assays were done to see if additional time would allow more of these mutants to reach odorant or diluent spots. Control experiments showed that 2 hours was an adequate amount of time for animals to reach odorant spots. We tested

the ability of *slo-1* gain-of-function mutants to chemotax toward benzaldehyde and found that they have a low CI indicating they are not able to chemotax as well as wild-type N2 and *kyIs140* animals (Figure 6). This suggests that loss of AWC asymmetry affects chemotaxis behavior.

Loss of *lips-7* can suppress the chemotaxis defect of *slo-1(ky389gf)*

Recent work in our lab has shown that triacylglycerol (TAG) levels are important in the development of tolerance to alcohol. Importantly, it was shown that genetic manipulation of *lips-7*, which encodes a lipase that regulates TAG levels, can modulate the locomotion phenotype in *slo-1* gain-of-function mutants (Bettinger et al., 2012). Furthermore, *slo-1* gain-of-function mutants show *str-2::gfp* expression in 2 AWC neurons whereas the *lips-7* mutation in a *slo-1* gain-of-function background restores the wild-type phenotype of *str-2::gfp* expression in 1 AWC neuron (Kalyann Kouv and Jill C. Bettinger, personal communication). Therefore, we hypothesized that *lips-7* might suppress *slo-1* gain-of-function chemotaxis defects if increased levels of TAGs downregulate SLO-1 channels in AWC neurons. We assayed *kyIs140;slo-1(ky389gf);lips-7* mutants and found the *slo-1* gain-of-function phenotype is suppressed when the *lips-7* mutation is present (Figure 6). This result suggests that TAG levels can alter the activity of SLO- channels and that that suppression could be significant enough to suppress a behavioral chemotaxis phenotype of the *slo-1* gain-of-function.

***kyIs140;slo-1(ky389gf)* are defective in odor discrimination**

Previously, Wes and Bargmann proposed a model that AWC ON and AWC OFF are both required for the discrimination of benzaldehyde in a field of butanone. They proposed that the AWC OFF neuron detects benzaldehyde and 2,3-pentanedione whereas the AWC ON neuron detects benzaldehyde and butanone (Wes and Bargmann, 2001). Furthermore, in the AWC ON neuron, butanone signaling attenuates benzaldehyde signaling thus animals with 2 AWC ON neurons will not be able to detect benzaldehyde in a field of butanone (Wes and Bargmann, 2001). As previously reported by Wes and Bargmann, we found that *slo-1(ky389gf)* mutants are not able to chemotax toward benzaldehyde in the presence of butanone (Figure 7). We were also able to observe this effect after 1 (data not shown) and 2 hours with increasing concentrations of butanone (Figure 7). We used increasing concentrations of butanone to see a dose-dependent effect that was butanone-specific in *slo-1(ky389gf)* mutant behavior. We hypothesized that, similar to our chemotaxis data, *lips-7* might suppress *slo-1* gain-of-function odor discrimination defects. We found the *lips-7* mutation in a *slo-1(ky389gf)* background suppressed the *slo-1* gain-of-function defective phenotype and restored the high CI toward benzaldehyde in the presence of butanone (Figure 7). This suggests that TAG levels can alter SLO-1 channel activity causing a change in AWC cell fate thereby causing a change in chemotaxis and odor discrimination behaviors.

Dil staining and scoring of *str-2::gfp* expression in neurons

Based on the fact that inappropriately active SLO-1 channels in the gain-of-function mutant could alter AWC asymmetry, and we have previously found that ethanol

activates SLO-1, we asked if ethanol could alter *str-2::gfp* expression. Dil is a lipophilic dye used to stain 6 amphid neurons that can be visualized using fluorescence microscopy. We used Dil staining to score untreated and ethanol treated animals to distinguish GFP fluorescing AWC neurons from other RFP fluorescing amphid neurons. Baseline Dil staining and scoring of AWC ON neurons in untreated *kyIs140*, *kyIs140;slo-1(eg142)*, and *kyIs140;slo-1(ky389)* showed animals expressed 1 or 2 AWC ON neurons, respectively (Table 1). We scored ethanol treated animals when they were L4s and found that ethanol changed *str-2::gfp* expression in AWC neurons of *kyIs140* animals from 1 AWC ON to a second apparent AWC ON (Table 2). These results were similar to previous preliminary data scoring AWC ON neurons using fluorescence microscopy (data not shown).

Ethanol did not alter animals' ability to chemotax

If ethanol transforms *str-2::gfp* expression to 2 AWC ON, then ethanol treated progeny should have functional consequences and show defects in chemotaxis similar to *slo-1* gain-of-function mutants. *kyIs140* animals treated with 600 mM of ethanol were tested in chemotaxis and odor discrimination assays. We expected that ethanol treated animals had a second apparent AWC ON, and similar to *slo-1* gain-of-function, would be able to chemotax toward benzaldehyde in the absence of butanone but would not be able to chemotax toward benzaldehyde in the presence of butanone. We found that *kyIs140* animals exposed to 0 mM and 600 mM ethanol were still able to strongly chemotax toward benzaldehyde (Figure 8). Similarly, *kyIs140* mutants treated with 600

mM ethanol were still able to chemotax toward benzaldehyde in the presence of butanone after 1 (data not shown) and 2 hours (Figure 9). This suggests that ethanol was causing a change in *str-2::gfp* expression during embryogenesis but this change did not have functional consequences. It is possible that the animals who were exposed to ethanol during embryogenesis were developmentally delayed. Both untreated and ethanol treated animals were scored at the same time and it is possible that mid-L4 untreated animals and mid-L3 to early L4 stage ethanol treated animals were scored. This difference in developmental timing could have altered the scoring results and favored a second apparent AWC ON.

Ethanol did not alter *dgk-1* mutants' ability to chemotax

We also tested *kyls140;dgk-1(nu62)* mutants in chemotaxis and odor discrimination. DGK-1 displays diacylglycerol kinase activity and *dgk-1(nu62)* mutants have hyperactive neurotransmission. We were interested in testing if altering neurotransmission would change chemotaxis behavior. We found that *kyls140;dgk-1(nu62)* mutants were able to chemotax strongly toward benzaldehyde (Figure 6) and this suggests that altering neurotransmission does not change chemotaxis behavior. Also ethanol treated *kyls140;dgk-1(nu62)* mutants were still able to chemotax toward benzaldehyde in the presence of butanone after 2 hours (Figure 10). This suggests that ethanol is not changing neurotransmission and altering chemotaxis behavior.

Chronic ethanol altered *str-2::gfp* expression in neurons in *kyls140* and *ctbp-1* mutants

Based on the initial observation that ethanol exposure altered *str-2* expression in AWC neurons, but the surprising observation that there is no defect in chemotaxis, we hypothesized that continuous chronic ethanol exposure during embryogenesis and larval development could alter AWC asymmetry. *kyls140* animals were chronically treated with increasing ethanol concentrations starting from embryogenesis until they were scored as L4s. We found L4 *kyls140* animals chronically treated with ethanol in concentrations of 0 mM, 400 mM, 500 mM, 600 mM, and 700 mM had 1 AWC ON neuron (Figure 11). This suggested that prolonged ethanol exposure does not change *str-2* expression in worms. We were surprised by this result because we saw before that ethanol treatment during embryogenesis caused a second apparent AWC to express *str-2*.

In parallel, we also treated and scored *ctbp-1* mutants. *ctbp-1* is a transcriptional co-repressor that represses the transcription of the *lips-7* gene. Chen et al. showed that *ctbp-1* mutants have increased LIPS-7, a TAG lipase, causing a decrease in TAG levels. Due to having decreased TAG levels, *ctbp-1* mutants provide a sensitized background to test ethanol's effects on *str-2* expression because more of their SLO-1 protein should be accessible to activation by ethanol. We found that, similar to *kyls140* L4s, *ctbp-1* mutants chronically treated with 0 mM, 400 mM, 500 mM, and 600 mM of ethanol had 1 AWC ON neuron (Figure 12). However, about half of the *ctbp-1* mutants treated with 700 mM ethanol had 0 AWC ON while half had 1 AWC ON (Figure 12).

This suggests that chronic exposure to a high concentration of ethanol can change *str-2*

expression to 0 AWC ON. This could be occurring by ethanol is interfering with signaling between the two AWC neurons. An AWC neuron requires a signal from the other AWC neuron in order to express *str-2::gfp* (Troemel et al., 1999). When either the left or right AWC precursor neuron was laser ablated, the other neuron always became AWC OFF (Troemel et al., 1999). Thus, disrupting the cross communication between AWC neurons could cause both neurons to become AWC OFF.

***kyIs140* animals express GFP in cells**

To address the developmental timing issue of scoring ethanol treated progeny, a time course experiment was conducted. All previous AWC scoring experiments were performed on L4 worms. To verify that this was an ideal larval stage to score animals, a time course scoring experiment was performed on various larval stages of *kyIs140* animals exposed to 0 mM – 600 mM ethanol. A total of 50 individual worms were examined at each larval stage. Based on a previous study, we hypothesized that in untreated animals *str-2* expression would be fixed after the L1 stage when expression is upregulated in AWC ON (Troemel et al., 1999). In contrast, we expected to see a change in *str-2* expression in ethanol treated animals, especially in animals during the L4 stage, as we previously observed. Interestingly, we found that L1 animals had a range of 2 – 9 cells expressing GFP (Figure 13). L2 animals had 2 – 6 cells expressing GFP while L3 animals had a 1 – 4 cells expressing GFP (Figures 14 and 15). Additionally L4 animals had 0 – 3 cells expressing GFP with most animals having 1 or 2 cells expressing GFP whereas young adult *kyIs140* animals had either 1 or 2 cells

expressing GFP with the majority of animals having only 1 cell expressing GFP (Figures 16 and 17). This suggests that as *kyls140* animals progress through larval development from L1 to young adults, the number of cells expressing GFP decreases from 9 cells to 1 cell expressing GFP (Figures 13 – 17). Importantly, this showed that developmental timing of scoring ethanol treated animals affects the number of cells expressing GFP. Therefore, cells expressing GFP and not AWC neurons of ethanol treated animals were originally scored. This was verified when characteristic AWC morphology and gonadal development were studied closely, and we observed that ethanol treated worms had 1 AWC ON (data not shown). This suggests that ethanol is not causing a change in AWC cell fate decisions.

Discussion

Recent work in our lab studying ethanol's physiological effects has suggested that lipids are an important factor in the development of tolerance. Triacylglycerol levels have emerged as a contributor to AFT and initial sensitivity to ethanol (Bettinger et al., 2012). Previous work identified the SLO-1/BK channel as an ethanol target which acts to hyperpolarize neurons and depress neuronal excitation (Davies et al., 2003). It was also shown that lipid bilayer thickness can modulate basal activity and ethanol activation of the BK channel (Treisman and Martin, 2009; Yuan et al., 2007, 2008). Additionally it was shown that neuronal excitability effects AWC ON expression (Troemel et al., 1999). Taken together, based on previous findings, our proposed model was that lipids play an important role during ethanol exposure to modulate basal activity by the BK channel in

AWC chemosensory neurons. Our hypothesis was that modulation of the lipid membrane composition changes the activity of BK channels. An increase in lipids, such as TAGs, would create larger or more lipid microdomains which sequesters more BK channels. The sequestered BK channels are not able to be activated by ethanol and would not hyperpolarize AWC neurons thereby increasing neuronal excitability and favoring an AWC OFF phenotype.

We predicted that ethanol exposure during embryogenesis would activate BK channels that would decrease neuronal excitability and alter AWC cell fate to 2 AWC ON. This would subsequently cause a change in chemotaxis behavior similar to mutants with 2 AWC ON, such as *slo-1(ky389gf)*, who are not able to odor discriminate. Based on preliminary scoring data, we initially thought that ethanol was inducing a cell fate change in AWC neurons to 2 AWC ON, which could then cause a functional change in chemotaxis behavior. From baseline chemotaxis and odor discrimination assays, we found that *kyls140;slo-1(ky389gf)* animals are not able to chemotax towards benzaldehyde in the presence of butanone. However, the *lips-7* mutation was able to suppress this phenotype and *kyls140;slo-1(ky389gf);lips-7* mutants were able to strongly chemotax toward benzaldehyde in the presence of butanone. *lips-7* is a lipase that has been shown to regulate TAG levels and alters tolerance to ethanol (Bettinger et al., 2012; Chen et al., 2009). So we tested if ethanol treated animals, who presumably have an altered AWC phenotype, show changes in their chemotaxis behavior.

When *kyIs140* animals were exposed to ethanol during embryogenesis their chemotaxis behaviors did not change from baseline behaviors. Both untreated and 600 mM ethanol treated *kyIs140* animals were able to chemotax toward benzaldehyde in the absence and presence of butanone. These results did not correlate with our working model or hypothesis. We thought that it was possible that animals exposed to ethanol during embryogenesis were developmentally delayed and subsequently, we were scoring animals during the wrong stage of larval development. The difference in developmental timing between untreated and ethanol treated animals could have altered scoring results. From our preliminary scoring results of ethanol causing an apparent change in a second AWC ON neuron and the results of no changes in chemotaxis behavior in ethanol treated animals, we thought that chronic ethanol exposure would alter AWC asymmetry.

After chronic ethanol exposure, we found that AWC cell fate was not changed in *kyIs140* animals but was altered in *ctbp-1* mutants. About half of ethanol treated *ctbp-1* mutants had 0 AWC ON while the other half had 1 AWC ON. *ctbp-1* is a transcriptional co-repressor that has been shown to increase lifespan in *C. elegans* (Chen et al., 2009). Importantly, when CTBP-1 is inhibited, LIPS-7 is over expressed leading to a decrease in TAG and suggested that *ctbp-1* is upstream of *lips-7*. Therefore, *ctbp-1* mutants provide a sensitized background in which to test AWC cell fate changes. Our results indicate that chronic ethanol exposure may cause an increase in neuronal excitability and favor an AWC OFF phenotype. Ethanol could be acting to disrupt the cross

signaling between AWC neurons thereby causing both neurons to become AWC OFF. A study by Troemel et al. showed that the basal state of AWC neurons is OFF and when signaling is disrupted neurons become AWC OFF.

To next address if developmental timing of scoring animals affected the results, we conducted an extensive time course assay with *kyls140* animals treated with a range of ethanol concentrations from 0 mM to 600 mM ethanol. All previous scoring of animals was done during the L4 stage and chemotaxis and odor discrimination assays were done on first day adults. From our time course ethanol experiment we found that as animals age, the number of cells expressing GFP decreases from 9 cells during the L1 stage to eventually 1 in young adult animals. We also found that the majority of animals expressed 1 AWC ON during the L4 stage. Consequently, we then went back and reassessed our scoring and found that we were scoring the cells that were expressing GFP and not AWC ON neurons. We used gonadal development to indicate the specific L4 larval stage and closely observed AWC characteristic morphology. We found that ethanol did not affect AWC cell fate decisions but instead ethanol was affecting the developmental rates of animals. This shows that ethanol exposure during embryogenesis causes developmental delays, which are not apparent in chemotaxis behavior.

While we found that ethanol was not able to transform AWC cell fate in *C. elegans*, there could be a protective mechanism during maturation that adjusts neuronal excitability. Animals might be able to utilize lipids both in their diet and by ramping up

their own *de novo* synthesis of fatty acids. This increase in lipids would then protect their neurons from ethanol damage. Furthermore, they might be exploiting large lipids such as sphingomyelin and cholesterol to increase the thickness of their cell membranes thereby sequestering more SLO-1/BK channels and protecting their AWC neurons.

Future Experiments

To test if worms have a protective mechanism against alcohol damage during larval development by utilizing their lipids, lipid levels could be measured during various larval stages. It has been shown that TAG levels are increased in animals where *lips-7* is inactivated by RNA interference whereas *ctbp-1* mutants have increased *lips-7*, a lipase that negatively regulates TAG levels, and decreased TAG levels (Chen et al., 2009). So we could quantify TAGS levels in various larval stages of ethanol treated animals along with *lips-7* mutants as a positive control and *ctbp-1* mutants as a negative control. TAG levels could be quantified using a colorimetric glycerol based assay. This assay measures the concentration of TAGs by the absorbance of a blue pigment and takes into account free glycerol levels. Previously this experiment has been performed but the results were confounded by sample size and low absorbance outputs. More specifically, gas chromatography-mass spectroscopy (GC-MS) can be used to find candidate fatty acids important in the protective role of lipids from ethanol. Gas chromatography-mass spectrometry (GC-MS) has become the superior method for analyzing complex mixtures of fatty acids. GC-MS first separates volatile constituents in

a mixture and then analyzes and characterizes each fatty acid. We are currently investigating methods to extract lipids from animals for samples for GC-MS.

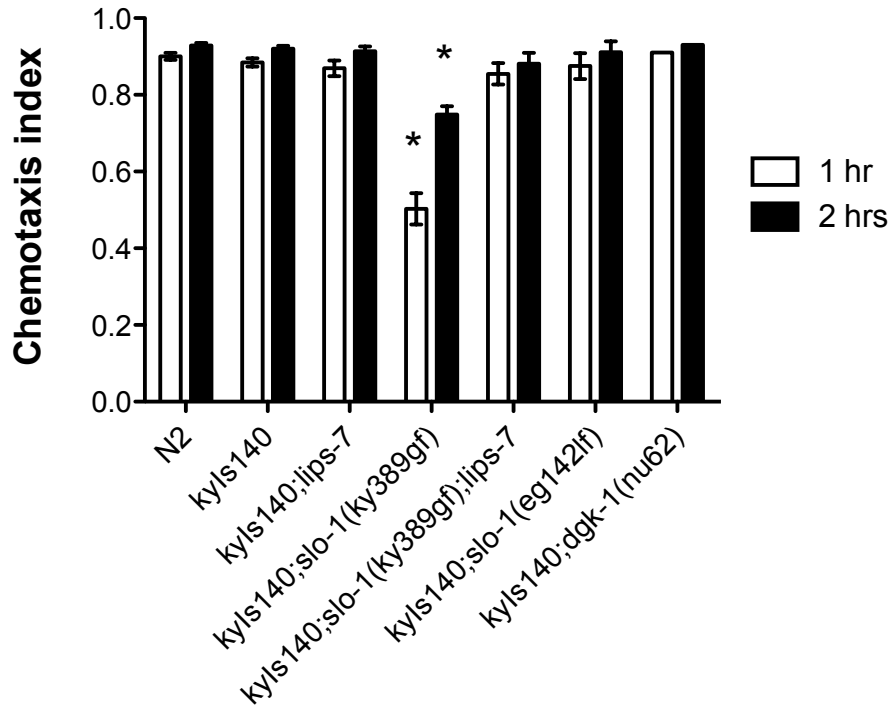


Figure 6. *slo-1* gain-of-function mutants are deficient in chemotaxis, and *lips-7* can suppress this defect

N2, *kyls140*, *kyls140;lips-7*, *kyls140;slo-1(ky389gf)*, *kyls140;slo-1(ky389gf);lips-7*, *kyls140;slo-1(eg142lf)*, and *kyls140;dgk-1(nu62)* all chemotax towards benzaldehyde after 1 and 2 hours. Mutant *kyls140;slo-1(ky389gf)* does not significantly chemotax toward benzaldehyde after 1 and 2 hours relative to *kyls140*.

One-way ANOVA

p-value<0.05

post-hoc – Bonferroni

*relative to *kyls140* within time point

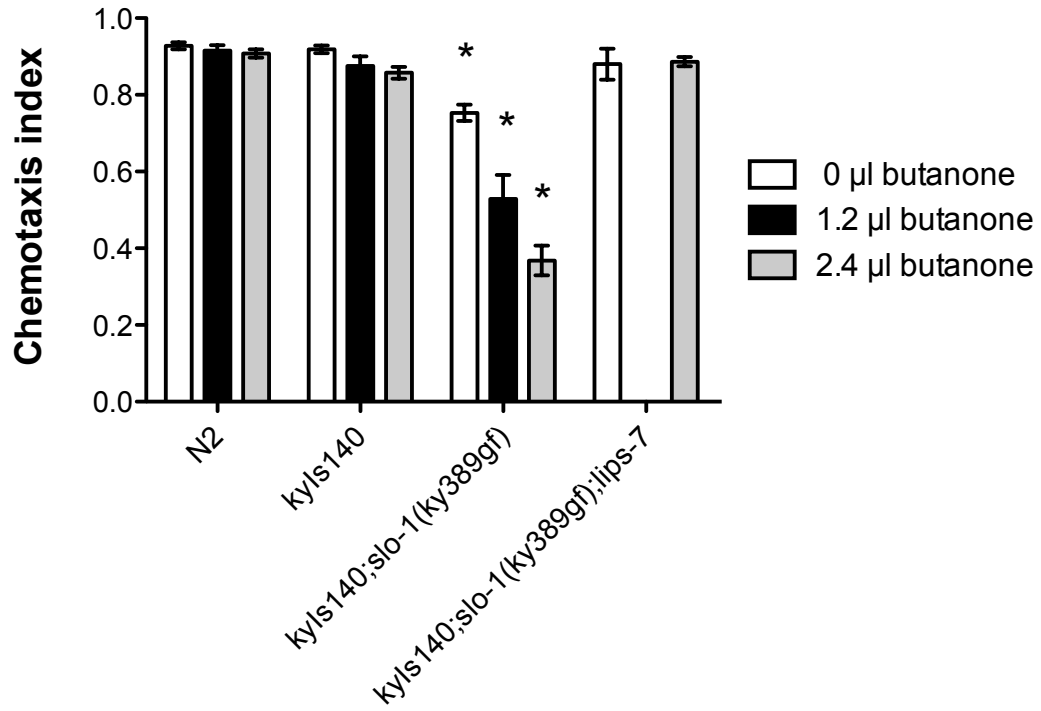


Figure 7. *slo-1* gain-of-function defects in odor discrimination are suppressed by *lips-7*

N2 and *kyls140* animals strongly chemotax toward benzaldehyde in the absence and presence of butanone. *kyls140;slo-1(ky389gf)* mutants are defective in chemotaxis toward benzaldehyde in the presence of butanone but *lips-7* suppresses this phenotype.

Two-way ANOVA

p-value<0.05

post-hoc – Bonferroni

*relative to *kyls140* within dose

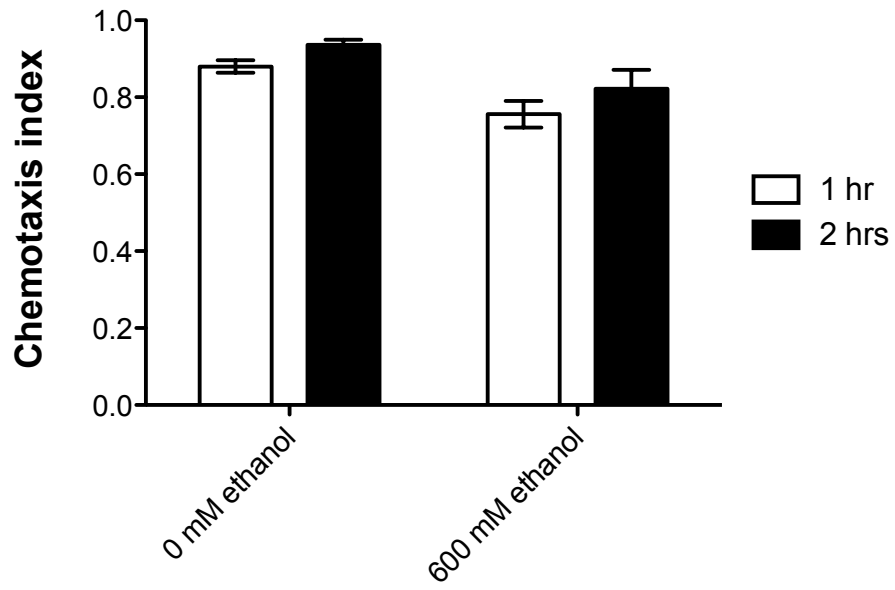


Figure 8. Ethanol treated animals do not show defects in chemotaxis

kyIs140 animals treated with 0 mM and 600 mM ethanol were able to chemotax toward benzaldehyde. There were no statistically significant differences in their chemotaxis indices.

One-way ANOVA

p-value<0.05

post-hoc – Bonferroni

*relative to *kyIs140* within time point

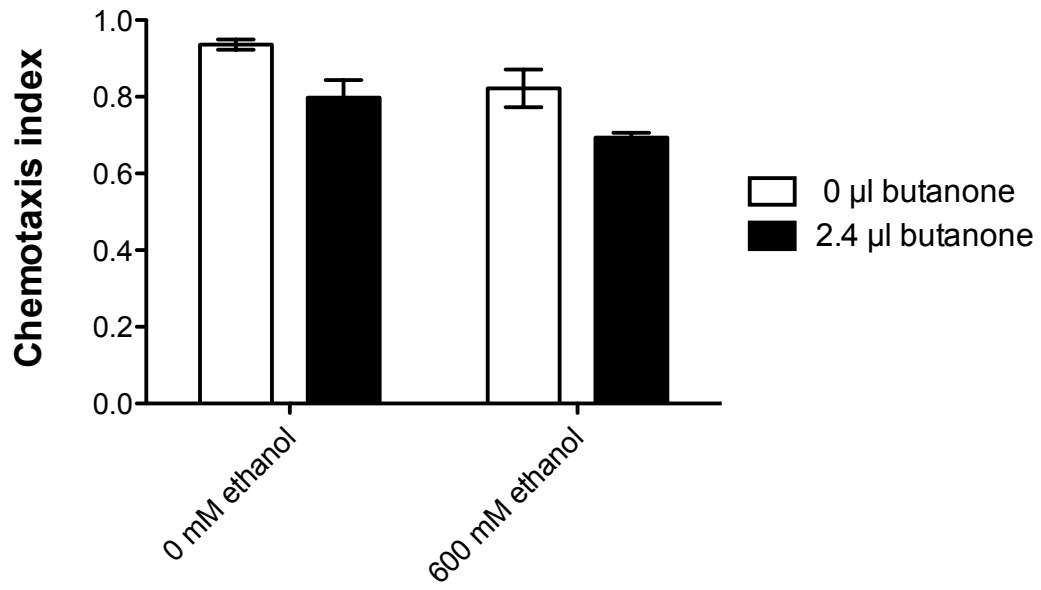


Figure 9. Ethanol treated animals do not show defects in odor discrimination

kyIs140 animals exposed to 600 mM ethanol in the presence of butanone were able to chemotax toward benzaldehyde. There were no statistically significant differences in chemotaxis indices between untreated and ethanol treated animals in the absence or presence of butanone.

Two-way ANOVA

p-value<0.05

post hoc – Bonferroni

*relative to *kyIs140* within dose

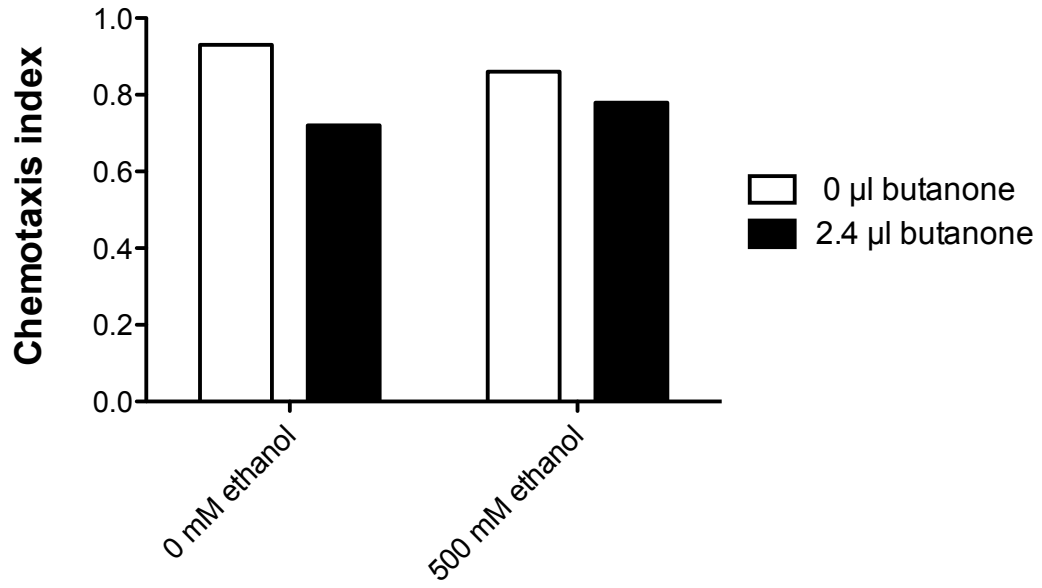


Figure 10. Ethanol treated *dgk-1(nu62)* mutants do not show defects in odor discrimination

dgk-1(nu62) animals exposed to 500 mM ethanol in the presence of butanone were able to chemotax toward benzaldehyde. There were no differences in chemotaxis indices between untreated and ethanol treated *dgk-1(nu62)* animals in the absence or presence of butanone.

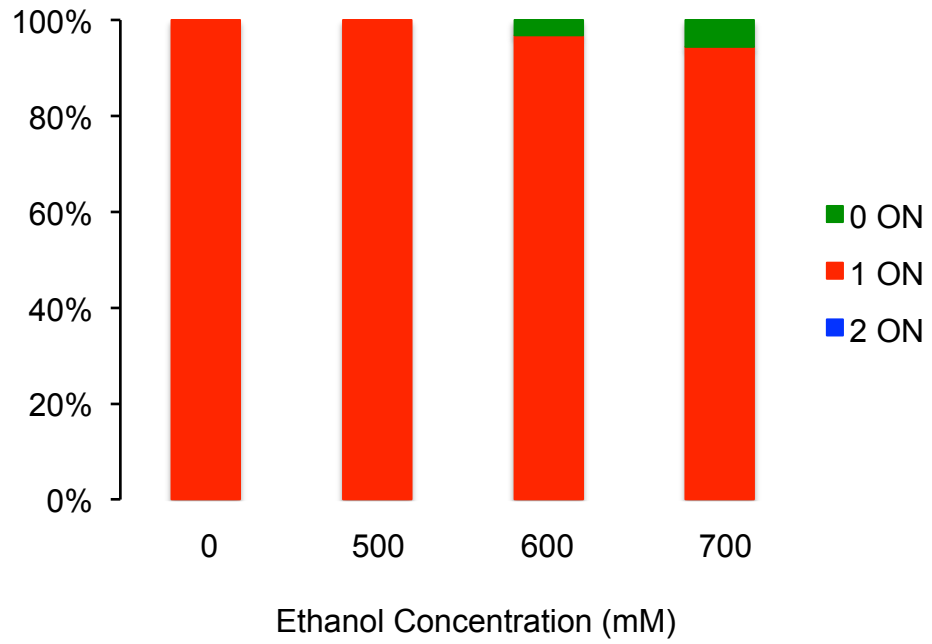


Figure 11. Chronic ethanol exposure does not alter *str-2::gfp* expression in neurons in *kyls140* animals

kyls140 animals exposed to 0 mM, 500 mM, 600 mM, and 700 mM had 1 AWC ON.

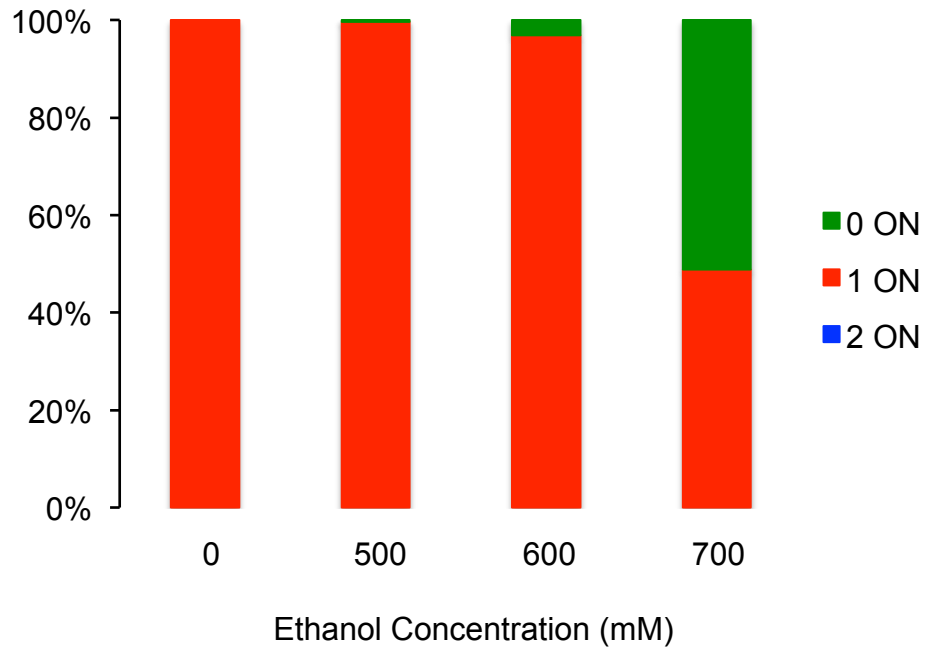


Figure 12. Chronic ethanol exposure alters *str-2::gfp* expression in neurons in *ctbp-1* mutants

ctbp-1 mutants exposed to 0 mM, 500 mM, and 600 mM of ethanol had 1 AWC ON. About half of the *ctbp-1* mutants exposed to 700 mM had 0 or 1 AWC ON.

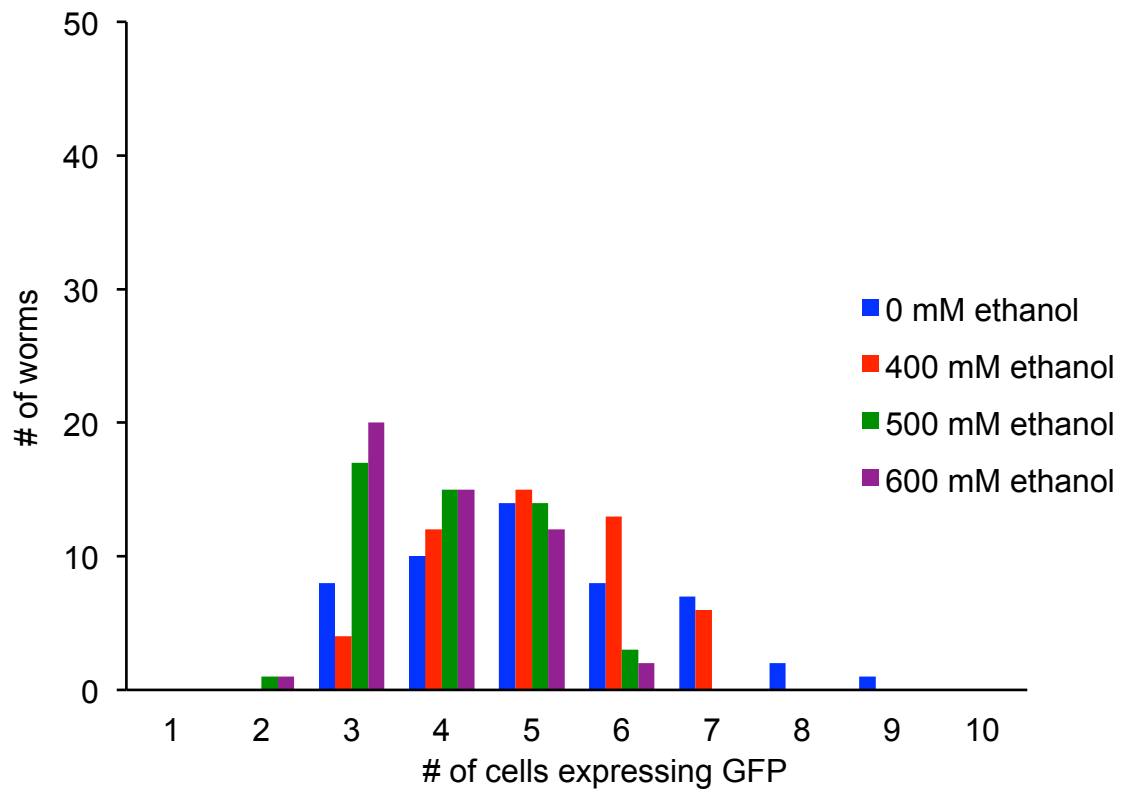


Figure 13. Cells expressing GFP in L1 worms exposed to increasing concentrations of ethanol

kyls140 L1 animals raised on 0 mM, 400 mM, 500 mM, and 600 mM ethanol had 2 – 9 cells expressing GFP. Most animals had 3 – 7 cells expressing GFP.

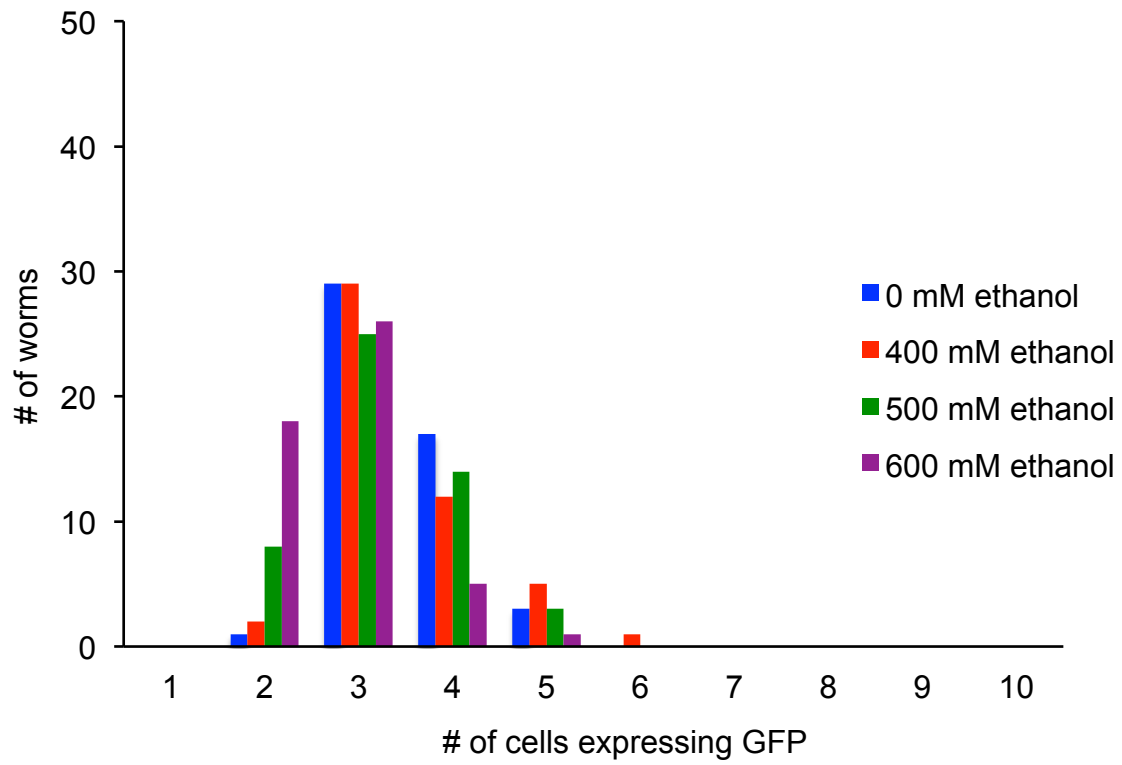


Figure 14. Cells expressing GFP in L2 worms exposed to increasing concentrations of ethanol

kyls140 L2 animals raised on 0 mM, 400 mM, 500 mM, and 600 mM ethanol had 2 – 6 cells expressing GFP. Most worms had 2 – 4 cells expressing GFP.

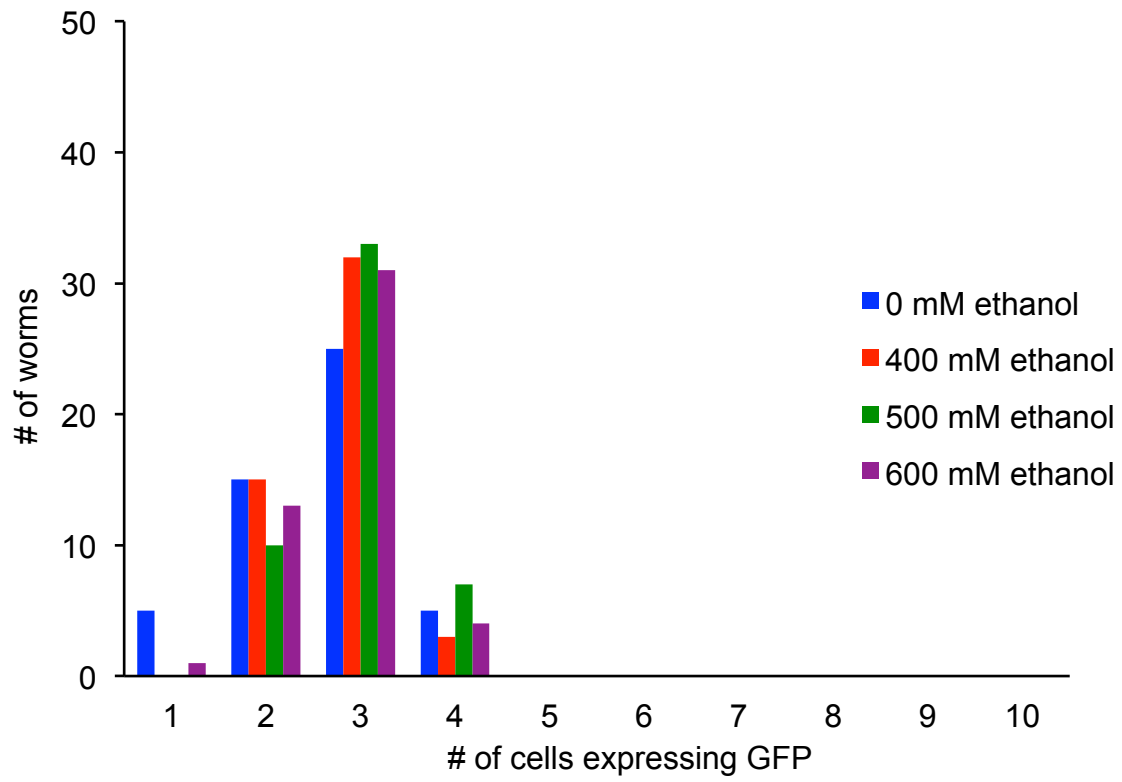


Figure 15. Cells expressing GFP in L3 worms exposed to increasing concentrations of ethanol

kyls140 L3 animals raised on 0 mM, 400 mM, 500 mM, and 600 mM ethanol had 1 – 4 cells expressing GFP. Most animals had 2 -3 cells expressing GFP.

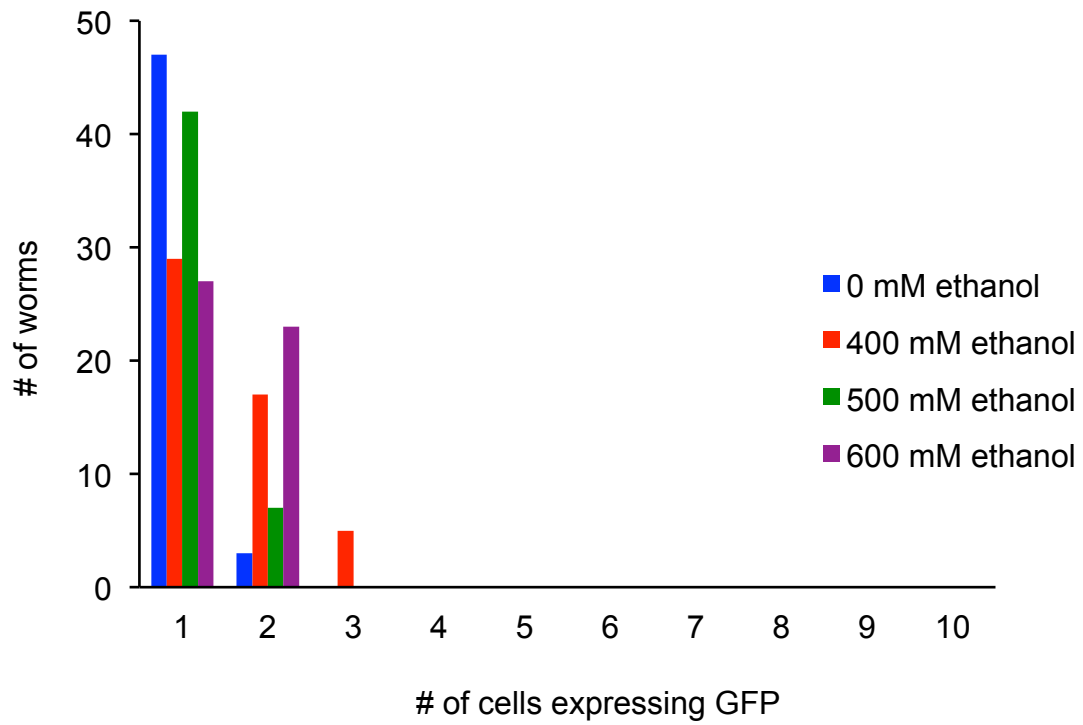


Figure 16. Cells expressing GFP in L4 worms exposed to increasing concentrations of ethanol

kyls140 L4 animals raised on 0 mM, 400 mM, 500 mM, and 600 mM ethanol had 0 – 3 cells expressing GFP. Most animals had 1 or 2 cells expressing GFP.

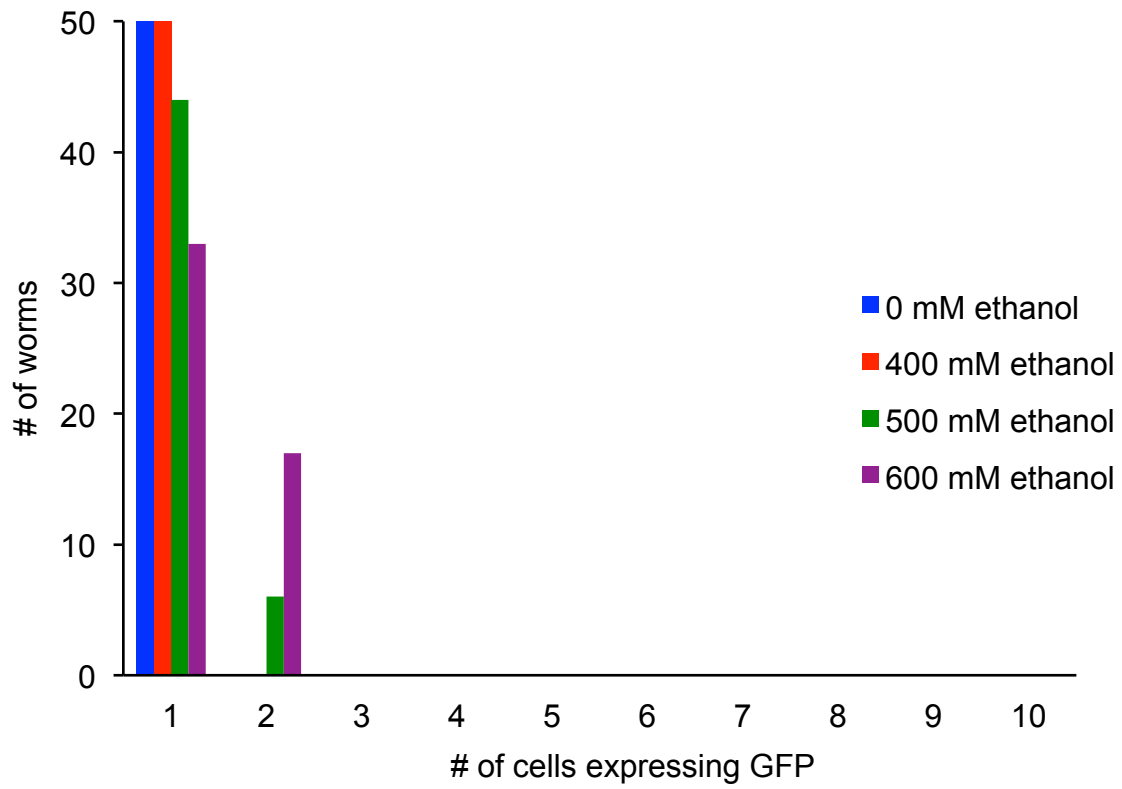


Figure 17. Cells expressing GFP in young adult worms exposed to increasing concentrations of ethanol

kyls140 young adult animals raised on 0 mM, 400 mM, 500 , and 600 mM ethanol had 1 or 2 cells expressing GFP. Most young adult animals had 1 cell expressing GFP.

Table 1. Dil staining of *kyls140*, *kyls140;slo-1(eg142)*, and *kyls140;slo-1(ky389)* shows 0, 1, and 2 AWC ON

All *kyls140* animals have 1 AWC ON. Likewise, almost all *kyls140;slo-1(eg142)* mutants have 1 AWC ON. *kyls140;slo-1(ky389)* mutants have 2 AWC ON.

Strains	AWC ^{ON}		
	(Percentage)		
	0	1	2
<i>kyls140</i>	0 (0%)	100 (100%)	0 (0%)
<i>kyls140; slo-1(eg142)</i>	4 (4%)	96 (96%)	0 (0%)
<i>kyls140;slo-1(ky389)</i>	0 (0%)	1 (1%)	99(99%)

Table 2. Dil staining of ethanol treated animals have changes in *str-2::gfp* expression in neurons

All untreated *kyIs140* animals have 1 AWC ON. About half of *kyIs140* animals treated with 600 mM ethanol have 1 or an apparent second AWC ON.

Strains	Ethanol Concentration (mM)	AWC ^{ON} (Percentage)		
		0	1	2
<i>kyls140</i>	0	0 (0%)	100 (100%)	0 (0%)
	600	0 (0%)	58 (58%)	42 (42%)

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