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An analysis of fatty acid metabolism's role in the development of acute functional tolerance to ethanol in Caenorhabditis elegans

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An analysis of fatty acid metabolism's role in the development of acute functional tolerance to ethanol in *Caenorhabditis elegans*

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

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3 List of Abbreviations

2-AG – 2-arachidonyl-glycerol

5-HT – serotonin

AA – arachidonic acid (also used once for alcohol preferring rats)

AC – adenylyl cyclase

ACC – acetyl-CoA carboxylase

ACh – acetylcholine

AChE – acetylcholinesterase

AFT – acute functional tolerance

ANA – alcohol avoiding

AUD – Alcohol abuse disorders

BK – Big Potassium

C1P – ceramide-1-phosphate

ChAT – choline acetyltransferase

COX – cyclooxygenase

DA – dopamine

DHA - Docosahexaenoic acid

DHEA – Dehydroepiandrosterone

DR – direct repeats

EPA – eicosapentaenoic acid

ER – everted repeats

EtOH – ethanol

FARS – Fatal Analysis Reporting System

FAS – fatty acid synthase

FFAR – free fatty acid receptor

FLP – FMRF-like peptides (FMRF = Phe-Met-Arg-Phe)

GABA – gamma-aminobutyric acid

GIRK – G protein-coupled inwardly-rectifying potassium channel

Glu – glutamate

GluCl – glutamate-gated chloride channels

GPCR – G protein-coupled receptor

GxE – gene-environment interactions

HAD – high alcohol drinking

HNF – hepatic nuclear factor

iGluR – ionotropic glutamate receptor

IR – inverted repeats

IS – initial sensitivity

LA – Linoleic acid

LAD – low alcohol drinking

LC-PUFA – long-chain polyunsaturated fatty acid

LORR – loss of righting reflex

LOX – lipoxygenase

LPA – lysophosphatidic acid

LR – Level of Response (to ethanol)

LT - Leukotriene

LXR – lever X receptor

MCoD – Multiple Cause of Death

NAC – nucleus accumbens

NAE – n-acetylethanolamines

nAChR – nicotinic acetylcholine receptors

NLP – neuropeptide-like protein

NMDA – N-methyl-D-aspartate

O3AA – omega-3 archidonic acid

PAF – platelet-activating factor

PG - Prostaglandin

PKA – protein kinase A

PPAR – peroxisome proliferator-activated receptors

PPRE – peroxisome proliferator response element

PtdIns(4,5)P_2 – phosphatidylinositol 4,5-bisphosphate

PUFA – polyunsaturated fatty acid

RXR – retinoid X receptor

S1P – sphingosine-1-phosphate

SRE – sterol response element

SREBP – sterol response element-binding protein

TAG – triacylglyceride

TM – Transmembrane

VTA – ventral tegmental area

4 Abstract

AN ANALYSIS OF FATTY ACID METABOLISM'S ROLE IN THE DEVELOPMENT OF ACUTE FUNCTIONAL TOLERANCE TO ETHANOL IN *CAENORHABDITIS ELEGANS*

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An individual's naïve level of response (LR) to ethanol is predictive of their lifetime likelihood to abuse alcohol. LR is heavily genetically influenced, suggesting that the genes responsible for LR may also be central to the development of abuse disorders. Our laboratory uses the model organism *C. elegans* to investigate the genetic influences on responses to acute ethanol exposure. We recently found that changes in TAG levels can alter LR. From this result we investigated the role of long-chain polyunsaturated fatty acids (LC-PUFAs) as well enzymes involved in lipid modifications of proteins. We found that LC-PUFAs are necessary for acute functional tolerance and that supplementation of eicosapentaenoic acid is able to rescue AFT. We also identified mutations in several palmitoyltransferases, a thioesterase, and elongases that alter AFT. These novel results highlight the importance of fatty acids in the response to ethanol and suggest exciting new potential therapeutic targets.

5 Introduction and Background

5.1 Alcohol use and abuse

Alcohol abuse disorders (AUD) affect approximately eighteen million Americans and are estimated to cost the United States approximately 2% of its GDP each year [1, 2]. Data collected by the World Health Organization indicates that in the US 5.48% of males and 1.92% of females 15 years or older suffer from an AUD. Considering that this amounts to roughly 1 in 20 males and 1 in 50 females that are directly affected by alcohol it may not be surprising that 43% of US adults, 76 million people when this study was conducted and 135 million today, have been exposed to alcoholism in their family [3]. A recent study found that the involvement of alcohol in motor vehicle deaths is profoundly underreported [4]. National mortality statistics often use the cause of death on death certificates as a way of tracking the number of alcohol related traffic deaths. The cause of death listed in the Multiple Cause of Death (MCoD) database, run by the National Center for Health Statistics, is often listed prior to toxicological analysis of blood work or only considers the traffic accident as the cause of death. The Fatal Analysis Reporting System (FARS), maintained by the National Highway Traffic Safety Administration, lists multiple influencing factors that lead to the motor vehicle related death including blood alcohol concentrations of 0.08% and higher. Analysis of the MCoD data found that 3.3% of the motor vehicle traffic deaths from 1999-2009 were alcohol related while analysis of the more complete FARS data found that in 21.1% of motor vehicle traffic deaths the deceased had a BAC of 0.08% or higher. The study's authors further hypothesized that underreporting is likely occurring for other alcohol-related fatalities [4].

Despite the seriousness of AUD, current pharmacological interventions are inadequate. This is due, in part, to the complexity of alcohol's molecular effects. The following introduction will summarize some of the known ethanol responsive proteins, the signaling pathways that are affected, as well a brief summary of the genetics underlying AUD.

5.2 Molecular targets of ethanol

Ethanol is a two carbon, polar, organic molecule with a single hydroxyl group that allows for hydrogen bonding. It is the simplicity of this molecule that allows it so many interactions *in vivo*. It has been hypothesized that ethanol may compete with water molecules or lipids in binding domains of target proteins [5]. This sheer number of possible interactions makes targeted pharmacological intervention difficult. This section will summarize some of the data that supports direct interactions between ethanol and proteins. A later section, Ethanol and Neurotransmission, will tie-in the effects of ethanol interacting with these proteins in neurotransmission.

5.2.1 N-methyl-D-Aspartate (NMDA) receptors

In 1989 David Lovinger found that NMDA function in hippocampal neurons was dose-dependently inhibited by biologically relevant doses of ethanol [6]. In support of a direct effect-interaction, single-channel recordings were consistent with ethanol acting as an allosteric modulator of NMDA [7]. Site directed mutagenesis identified several key

residues for ethanol's inhibitory actions between transmembrane (TM) domains 3 and 4 [8-11]. The identification of these residues strongly supported the hypothesis that ethanol acted directly on the channel. In further support of ethanol in interacting with NMDA receptors, non-competitive NDMA antagonists substituted for ethanol in drug discrimination in mice [12]. The inhibition of NMDA by ethanol likely results in the anesthetic effects of ethanol.

5.2.2 Ionotropic γ-aminobutyric acid ($GABA_A$) receptors

A very large body of work has been dedicated to understanding GABA's role in intoxication, especially at low doses. $GABA_A$ is a pentameric, ligand-gated ion channel that is permeable to chloride ions. $GABA_A$ channels can be composed of various combinations of six different α subunits, three β subunits, three γ subunits, one δ, one ε, one π, one θ, and three ρ subunits. The minimal requirement for channel formation is the presence of at least one α and one β subunit [13]. Activation of the channel causes hyperpolarization of the neuron and inhibits firing. $GABA_A$ activity has been shown to be enhanced by ethanol. Two specific amino-acid residues in TM domains 2 and 3 are critical for modulation of the channel by ethanol [14]. These data suggested that a binding pocket exists between the α-helices [15]. Work in the TM domains of glycine receptors found a similar binding pocket. Mutating these residues to cysteines revealed that disulfide bonds could form and thus the residues were in a close enough proximity to accommodate ethanol [15]. Application of propyl methanethiosulfonate, which binds irreversibly to cysteine residues, produced irreversible changes in the receptor function which further supported the hypothesis that these residues were accessible to ethanol

and were involved in the response it elicited [16]. The numerous combination of $GABA_A$ subunits combined with the challenge in expressing specific combinations *in vitro* led to controversy over which subunits were sensitive to ethanol and at what ethanol concentrations [17, 18]. There is now a general consensus that channels containing β3 and δ subunits are sensitive to low doses of ethanol and are hypothesized to be involved in the activating response to ethanol [17] which may be involved in the alcohol's disinhibiting effects [19].

5.2.3 Glycine receptors

Like $GABA_A$ channels, the opening of glycine channels results in an influx of chloride ions and results in inhibition. Likewise, their activity is also enhanced by ethanol [14, 20]. Glycine receptors are also pentameric, ligand-gated ion channels that are composed of four different α subunits and one β subunit. Similarity in the α-helical TM domains of $GABA_A$ and glycine receptors suggests that ethanol binds between these domains [14, 15]. Glycine receptors are also pentameric ions channels with variations in subunit composition modulating the response to ethanol. Specifically, channels containing the α_1 and α_2 subunits have been shown to be more sensitive to the potentiating effects of ethanol [21]. Activation of glycine receptors by ethanol may be involved in dopamine release and the subsequent rewarding effects [22, 23] however the localization of the glycine receptors likely matters as inhibition of glycine transporters can reduce ethanol intake and preference in rats [24].

5.2.4 Nicotinic acetylcholine receptors

Another of the pentameric, ligand-gated, ion channels that ethanol interacts with are the nicotinic acetylcholine receptors (nAChRs). Electrophysiological studies of human neuronal nAChRs expressed in *Xenopus* oocytes showed that short-chain alcohols, including ethanol, potentiate the channel activity for a variety of subunit combinations [25]. High doses of ethanol, greater than 100 mM, have been shown to potentiate neuronal nAChRs [26] while doses below this only potentiate $\alpha_2\beta_4$*, $\alpha_4\beta_4$*, $\alpha_2\beta_2$*, $\alpha_4\beta_2$* receptors[27]. $\alpha_3\beta_2$* and $\alpha_3\beta_4$* have been shown to be unaffected by ethanol while the homomeric α_7 receptor is inhibited [27]. Activation of dopamine-stimulating nAChRs in the VTA have been shown to promote ethanol-seeking behavior [28].

5.2.5 Serotonin receptors (5-HT$_3$)

Another of the ligand-gated ion channels that are potentiated by ethanol is the 5-HT_3 receptor [29, 30]. These channels are permeable to Na^+, K^+, and Ca^{2+} ions and are excitatory. Ethanol potentiation of 5-HT_3 has been shown in numerous *in vitro* preparations [31]. Mutation of amino acids in TM1 and TM2 domains have been shown to alter the potentiating effects of ethanol, suggesting that an ethanol binding site may exist between these domains [32, 33] much the same as in $GABA_A$ and glycine receptors. Ethanol increases the open probability of 5-HT_3 without altering agonist affinity for the channels [34] which suggests that activation of 5-HT_3 in the presence of ethanol may result in its rewarding effects.

5.2.6 L-type calcium channels

Voltage-gated L-type calcium channels are inhibited by ethanol and data from single channel recordings is consistent with a model of a single drug molecule acting on a single target site [35]. Voltage-gated L-type channel opening results in the influx of mainly Ca^{2+} ions and, depending on the cell type it is expressed, leads to neuronal firing. The inhibition of this channel by ethanol would be predicted to decrease synaptic transmission.

5.2.7 G protein-coupled inwardly-rectifying potassium (GIRK) channels

GIRK channel activity is enhanced by ethanol and is dependent on a region of 43 amino acids in the carboxy terminus [36, 37]. GIRK channels are opened *in vivo* by the βγ subunits of activated G-protein coupled receptors (GPCRs) and this causes an outward flow of K^+ ions and hyperpolarization of the neuron [38]. This suggests that activation of GIRK channels by ethanol may result in hyperpolarization of neurons and a decrease in synaptic signaling.

5.2.8 Big potassium (BK) channels

BK channels, also known as SLO-1 channels in *C. elegans* and *Drosophila melanogaster*, are voltage-gated ion channels permeable to potassium and their activation is necessary for repolarizing neurons. Electrophysiological work in various membranes, including artificial membranes where lipid species and proteins can be selected, supported a direct interaction between the channel and ethanol. The activation

of the channel was also shown to be modulated by the lipid environment surrounding the channel [39-45]. This channel was first identified as a possible target of ethanol in *C. elegans* by a forward genetic screen for mutants that were resistant to the locomotor decreasing effects of ethanol [39]. The continued activation of the BK channel by ethanol is predicted to cause hyperpolarization of neurons and a decrease in synaptic firing.

5.3 Ethanol and neurotransmission

Drug substitution studies can be used to assess if a drug with a known mechanism of action functions in a similar way as one with an unknown mechanism. Briefly, a mouse or rat is trained to press a lever in a response to an injection of vehicle or drug. Once a the rodent can discriminate between the drug and the vehicle, a researcher can then give a different drug and allow the rodent to press either lever. By doing this the researchers can ask if the novel drug “feels” like the training drug to the rodent. These studies have shown that GABA-mimetic drugs completely substitute for ethanol. Both competitive and noncompetitive NMDA receptor antagonists are capable of substituting for ethanol. Some antagonists of strychnine-insensitive glycine modulatory sites among the NMDA receptor complex dose-dependently substitute for the ethanol discriminative stimulus in trained mice [46]. These studies help to support previous studies that found interactions between $GABA_A$, NMDA, and glycine receptors and support pathways that ethanol affects.

Perhaps the most studied system in drug reinforcement is the dopaminergic (DAergic) system. The release of dopamine (DA) activates various DA receptors. D1-like receptors, which include D1 and D5 receptors, enhance the activity of adenylyl cyclase (AC) through their coupling to the stimulatory $G_{\alpha s}$ containing GPCRs. D2-like receptors, D2 through D4, inhibit AC through inhibitory $G_{i/o}$ GPCRs. D1-like receptor activation results in an increase in the concentration of cAMP and the activation of cAMP-dependent protein kinase A (PKA) signaling, which then leads to substrate phosphorylation [47]. This signaling cascade can lead to numerous changes in protein function and gene expression.

Alcohol enhances DAergic activity in the nucleus accumbens (NAC) shell region which has been shown to be involved in both the acute rewarding [48] and chronic neuroadaptive changes elicited by ethanol [49]. Dopamine, GABA, and 5-HT are the major neurotransmitters in the NAC shell. Low systemic doses of ethanol dose-dependently increase the firing rate of DAergic neurons [50]. This increased firing stimulates DA transmission in the mesolimbic pathway [48]. The mesolimbic pathway is a DAergic pathway that starts in the ventral tegmental area (VTA) and projects to the NAC, amygdala, hippocampus, and prefrontal cortex. Ethanol may decrease the activity of GABAergic interneurons in the mesolimbic pathway which would disinhibit the mesolimbic DA neurons to further increase DA release in the NAC shell [51]. 5-HT modulates DAergic activity in the VTA and NAC. Blockade of $5\text{-}HT_3$ with ICS 205-930 in rats prevents ethanol-induced increases of DA in the VTA [52] and NAC [53]. This suggests that ethanol increases DA release in the mesolimbic pathway by activating $5\text{-}HT_3$. Furthermore, a nAChR antagonist, mecamylamine, blocks DA release in the NAC

when ethanol trained rats are presented with an ethanol-associated conditioned stimulus [28]. Antagonism of glycine receptors also decreases extracellular levels of DA in the NAC while agonism has the opposite effect [22]. Strychnine, an antagonist at both glycine and acetylcholine receptors, is capable of blocking the release of DA in the NAC caused by ethanol in male Wistar rats [23].

While dopamine levels are increased by ethanol administration, glutamate levels in the NAC have been shown to increase in response to a low dose of ethanol (0.5 g/kg) and decrease in response to a high dose (2.0 g/kg) of ethanol [54] suggesting that concentration dependent interactions between neuronal proteins and ethanol alter the response.

There are many points of access for ethanol to affect DAergic signaling. Most of these access points have been shown to be direct targets of ethanol. A great deal of research supports the hypothesis that ethanol's effects on DAergic signaling are important in its rewarding aspects and that this reward reinforcement is central to the development of dependence. For example, Wistar rats have been shown to self-administer ethanol into the posterior VTA and this effect is blocked by quinpirole, a D2 agonist. Further self-administration occurred when a D2 antagonist, sulpiride, was administered [55]. The results of these experiments strongly suggest that ethanol acting at D1-like receptors activates DAergic neurons in the VTA and this is necessary for the reinforcing effects. A particularly interesting study measured DA release in the NAC in high and low alcohol drinking (HAD/LAD) as well as alcohol preferring (AA) and alcohol avoiding (ANA) rats during their first exposure to ethanol. The researchers then monitored voluntary ethanol drinking for each rat over the course of 30 days and found

that increased DA release during the first exposure to ethanol was predictive of a higher preference for ethanol regardless of the strain of rat [56]. Taken together, it is clear that DAergic signaling is altered by ethanol's actions at $GABA_A$ and 5-HT_3 receptors and that this signaling is central to ethanol's effects.

5.4 Pharmacological and genetic modulation of ethanol responding

From what is known about the targets of ethanol we can begin to understand how these interactions may effect neurotransmission. This section highlights pharmacological agents and knockout (KO) strains that alter ethanol responding and thus suggest a role for the target protein in the ethanol response.

First, inverse agonists of $GABA_A$, Ro 15-4513 and Ro 19-4603, reduce alcohol consumption in alcohol-preferring rats [57] which supports the hypothesis that $GABA_A$'s disinhibition of DAergic neurons is required for the reinforcing effects of ethanol. Furthermore, KOs of $GABA_A$ subunits α_1, α_2, α_5, and δ reduce alcohol consumption in mice [58]. This result not only supports the role of $GABA_A$ activation in ethanol reinforcement but also suggests the subunit composition of the pentameric channel that may be important in ethanol's effects. The distribution of the subunits differ in the brain [59] and this may help to identify brain regions that are be affected by ethanol.

Second, deletion of 5-HT transporters or blockade of 5-HT transporters by fluoxetine (which would increase synaptic 5-HT_3) reduces alcohol self-administration in mice [60]. In agreement with 5-HT_3 signaling having an inverse correlation with self-

administration, overexpression of 5-HT_3 receptors in mice has been shown to reduce alcohol self-administration [61].

Third, cholinergic signaling has been shown to be involved in voluntary alcohol consumption in both rats and mice. Mecamylamine, a nAChR antagonist, administered into the VTA reduced voluntary alcohol consumption in rats [62] while antagonism of $\alpha_3\beta_2^*$, β_3^*, and α_6^* containing nAChRs with α-conotoxin delivered to the VTA reduced voluntary alcohol consumption in both rats and mice [63]. Furthermore, crosstalk with the DAergic system was supported by the result that pretreatment with α-conotoxin reduced the release of DA in the NAC of mice [63].

Fourth, increase of extracellular glycine decreases both alcohol preference and intake in male Wistar rats. The glycine reuptake inhibitor Org 25935, which acts on glycine transporter 1, increases extracellular glycine which activates inhibitory strychnine-sensitive glycine receptors [24]. Systemic administration of Org 25935 elevates NAC DA levels and prevents further increases in DA levels with ethanol administration [64]. Org 25935 blockage of ethanol-increased DA release may explain why, in the presence of elevated DA (due to Org 25935), voluntary ethanol consumption is decreased.

These studies highlight the importance of cholinergic and glycinergic input onto DAergic signaling in the mesolimbic pathway in alcohol reinforcement.

5.5 Neurotransmitters in *C. elegans*

Many neurotransmitters, enzymes, transporters and receptors involved in *C. elegans* neurotransmission have been identified. This section briefly reviews some of the major neurotransmission pathways in *C. elegans*.

5.5.1 Acetylcholine

Acetylcholine (ACh) was first found in *Ascaris lumbricoides* in 1957 [65] and was shown to be an excitatory neurotransmitter at NMJ by del Castillo *et al.* in 1963 [66]. Sydney Brenner, in his canonical *C. elegans* paper [67], reported the identification of mutants resistant to lannate, an acetylcholinesterase (AChE) inhibitor, and tetramisole, an AChR agonist. ACh in worms is synthesized by *cha-1,* the mammalian homolog of choline acetyltransferase (ChAT), from choline and acetyl-CoA [68]. *C. elegans* have at least 27 nAChR subunits [69] compared to 17 nAChR subunits in mammals. Similar to mammals, cessation of cholinergic signaling is accomplished by the metabolism of ACh to choline and acetate by any of several cholinesterases [70]. Cholinergic systems can be probed pharmacologically by the addition of aldicarb, an AChE inhibitor, or levamisole, an AChR agonist. Aldicarb inhibits the breakdown of acetylcholine in the synapse which in turn increases the signaling duration. Levamisole acts directly on the AChRs to activate the channel. Both of these compounds can be used to induce tonic paralysis caused by increased cholinergic signaling [71]. Strains that release more acetylcholine or have a higher expression of acetylcholine receptors will paralyze faster than wildtype when exposed to aldicarb or levamisole, respectively.

5.5.2 Dopamine

Dopamine (DA) was first identified as a neurotransmitter in *C. elegans* by Sulston *et al.* in 1975 [72]. It was found to be important in modulating locomotion in response to mechanosensory input, including the slowing response to food [73]. For example, *cat-2* mutants cannot synthesize DA and do not slow in response to a lawn of *E. coli* [73]. DA has also been linked to state-dependent learning. Wild-type animals adapted to an odorant in the presence of ethanol exhibited adaptation to the odorant only if ethanol was again present during reexposure. If adapted animals were instead reexposed to the odorant in the absence of ethanol they responded to the odorant as if it were novel. *cat-1* and *cat*-2 mutants adapted to an odorant in the presence of ethanol showed adaptation regardless of whether ethanol was present during reexposure [74]. This demonstrated the necessity for DA in state-dependent learning. Four dopamine receptors have been found in *C. elegans*, DOP-1 through DOP-4. DOP-3 is a D2-like receptor that antagonizes the activity of the D1-like DOP-1 receptor [75]. Disinhibition of behaviors inhibited by ethanol are partially dependent on the D1-like DOP-4 receptor [19].

5.5.3 Serotonin

Serotonin was first identified by Horvitz *et al.* in 1982 in *C. elegans* [76]. Like DA, it was also shown to be involved in the slowing response to food [73]. Worms stop in response to a bacterial lawn in order to eat. This stopping response requires 5-HT because mutations that disrupted serotonin signaling inhibited the full stop response of food deprived worms when they encountered a food source. Serotonin has also been

shown to modulate pharyngeal pumping rates [77]. Four serotonin receptors have been found; three metabotropic, SER-1, SER-4, and SER-7, and one serotonin-gated chloride channel, MOD-1 [78-81]. Beyond responses to food, serotonin has been shown to both stimulate and inhibit egg laying via the HSN neurons which has been hypothesized to cause the bursts of egg laying followed by a timeout period [82].

5.5.4 GABA

GABA is an amino acid neurotransmitter synthesized via the decarboxylation of glutamate by glutamic acid decarboxylase. It was found to be active in *C. elegans* by Hedgecock in 1976 [83]. Twenty-six GABAergic neurons were identified by immunostaining for GABA [83]. The functions of these neurons were determined by laser ablation experiments in which each neuron was destroyed in turn using a precise laser and then phenotypes were assessed [84]. The ventral D-type GABAergic neurons were found to inhibit contraction of the dorsal and ventral body wall muscles allowing for sinusoidal movement. Mutants lacking D-type GABAergic neurons shrank when poked. The shrinking reaction was due to loss of contractile inhibition, or relaxation, leaving only the contraction [84]. Surprisingly, mutants lacking AVL and DVB lost the ability to stimulate the enteric muscles during defecation [84]. Thus the role of GABA to either stimulate or inhibit is based on the system it is acting in.

5.5.5 Glutamate (Glu)

Excluding neuromuscular transmission, most rapid excitatory synaptic transmission is mediated by glutamatergic signaling. At least 10 putative ionotropic Glu

receptor (iGluR) subunits are expressed in *C. elegans* [85, 86]. iGluRs are expressed in command interneurons that are responsible for forward and backward movement as well as nose poke reversals [85], RIA interneurons which are involved in thermotaxis [85, 87], and in the pharyngeal nervous system [85]. Genes encoding glutamate-gated chloride channels (GluCl) were identified in *C. elegans* by Cully *et al.* in 1994. These heteromeric channels are the channels that confer sensitivity to the anti-parasitic drug avermectin [88].

5.5.6 Neuropeptides

Beyond the classical neurotransmitters there are numerous small peptides that are involved in signaling in both mammals and *C. elegans*. Based on genomic analysis and some functional testing the number of predicted neuropeptides in *C. elegans* is well over one hundred [89]. A single gene encodes a pro-peptide that can release multiple peptides. Approximately 113 neuropeptide genes in *C. elegans* are expected to account for over 250 unique neuropeptides [90]. These peptides can be divided into two large families: insulin-like peptides and FMRF-like peptides (FLPs), the remaining non-insulin, non-FLP peptides are classified as neuropeptide-like proteins (NLPs). Neuropeptides are located in dense core vesicles which are derived from the trans-Golgi network. This is different from classical neurotransmitters that are in small vesicles clustered in synaptic zones. The processing of pro-peptides start in the endoplasmic reticulum with their cleavage into peptides and continues in the Golgi complex where the peptides are packaged into dense core vesicles and then transported to nerve terminals [90]. Neuropeptides in *C. elegans* can also act as hormones as shown by Sieburth *et al*. The group of researchers took advantage of the scavenger activity of the coelomocytes and

monitored release of GFP-tagged neuropeptide precursors into the pseudocoelom by the appearance of GFP in the coelomocytes [91]. Confirming a role for neuropeptide signaling in ethanol responding, mutation of NPR-1, the homolog to the mammalian neuropeptide Y receptor, increases the development of AFT to ethanol [92].

5.6 Level of response and AUDs

While the propensity to develop alcoholism has been shown to be strongly genetically influenced [93], causative genes for AUD have also remained largely elusive. A large body of work spanning multiple decades has identified a low level of response (LR) to ethanol as a predictive phenotype for increased likelihood of abuse [94]. Specifically, Marc Schuckit measured both the subjective high and the degree of sway in the response to alcohol in a group of 20 year old men. He found a high degree of variation in both responses. A decade later he grouped the men into those who developed alcoholism and those who did not. He found that the men who developed alcoholism reported lower subject highs and swayed less than the men who did not develop alcoholism. This work identified that LR was predictive of alcoholic outcomes [94]. Furthermore, a low LR at age 20 has been shown to increase the likelihood of alcoholism fourfold in both the sons of alcoholics and control groups [94]. Like AUDs, LR has also been shown to be heritable [95-98]. Taken together, these data suggest that the genes that are responsible for LR may also be central to the development of AUD, making them attractive targets for research.

5.7 Behavior as related to ethanol

C. elegans exhibit numerous behaviors that are altered by intoxication and these aid in identification of ethanol responsive genes in forward genetic screens. Exogenous doses of ethanol ranging from 100-500 mM, of which approximately 5-10% accumulates [99], dose-dependently and reversibly decrease body bends, speed, and egg-laying of N2 worms on agar [39] while doses around 1 M reversibly inhibit locomotion in liquid [100]. These measures can be used to quantify the degree of intoxication, called the initial sensitivity, of treated strains compared to wild-type. Additionally, *C. elegans* develop acute functional tolerance (AFT) to ethanol over a 30 minute single exposure to ethanol. AFT is observed as an increase in speed from 10 minutes, the time point of maximum locomotor depression used to assess initial sensitivity, to 30 minutes. Furthermore, this has been shown to not be an effect of ethanol metabolism as internal ethanol concentrations increase slightly from 10 to 30 minutes [101]. Some behaviors observed on agar, such as foraging and reversals from touch, are inhibited when the worm is moved to a liquid environment. Recently, a 500 mM dose of ethanol was shown to disinhibit these behaviors in liquid [19]. These results show that both decreases and increases in behavior can be used to assess the response to ethanol in *C. elegans*.

5.8 Alcohol and worms

Given that *C. elegans* become visibly intoxicated and develop tolerance to ethanol, genetic screens have been able to identified several mutations that alter this response.

Intoxication in *C. elegans* results in decreases in both locomotion and egglaying. Davies *et al.* found that mutations in *slo-1*, the gene encoding the nematode BK potassium channel, conferred resistance to ethanol induced decreases in both of these measures. Loss of BK channel activity resulted in hyperactive neurotransmission. The hyperactive neurotransmission mutants *dgk-1* and *goa-1* were not resistant to ethanol which confirmed that the resistance in *slo-1* was not due to increased neurotransmission [39]. Rescuing constructs showed that neuronal SLO-1 was responsible for the resistance phenotype. Electrophysiological testing suggested that SLO-1 itself is a target of ethanol that is activated and leads to hyperpolarization of the neuron. This activation was also shown to be modulated by the lipid environment surrounding mouse SLO-1 in cell culture [39-41, 43, 45].

Clic4, an intracellular chloride channel, was identified as an ethanol regulated gene in microarray studies in mice and humans [102]. *C. elegans* has two *Clic* homologs, *exl-1* and *exc-4*. Mutation of *exl-1* conferred resistance and increased AFT, while mutation of exc-4 abolished AFT. Studies confirmed that changes in expression of *Clic4* homologs also altered acute responses in mice and flies [102] suggesting the role of *Clic4* in intoxication was conserved through multiple species. A mechanism of action for *Clic4* in response to ethanol was not determined.

RAB-3 is a neuronally expressed G protein that interacts with synaptic vesicles to modulate neurotransmitter release [103, 104]. RAB-3 mutants are resistant to aldicarb indicating that they release less acetylcholine than wild-type worms [104]. AEX-3 is a GDP exchange factor that interacts with RAB-3 [105]. RAB-3 mutants displayed resistance to 400 mM ethanol in both dispersal and locomotion assays. AEX-3 mutants

phenocopied RAB-3 indicating that RAB-3's role in synaptic vesicle release was central to the resistance phenotype. These findings extended to a mouse model where *Rab3A-/-* and *Rab3A+/-* mice had significant reductions in recovery time in LORR assays. *Rab3A+/-* mice also voluntary drank more of both 14% and 20% EtOH solutions. [106]

npr-1 encodes a GPCR in the neuropeptide Y receptor family. Differences in NPR-1 activity in wild-type strains, N2 and CB4856, were shown to account for variation in social and feeding behavior [107]. Variation in ethanol response was also shown to correlate with NPR-1 activity. NPR-1 acts to antagonize the development of AFT. In CB4856 (carrying the low activity allele) and N2 carrying null-alleles of *npr-1*, AFT develops to a much higher degree than N2 (carrying the high activity allele) [101]. These results also extend to neuropeptide Y levels and mouse models of AFT in that signaling through the neuropeptide Y pathway opposes the development of AFT [108, 109].

The mutations mentioned to this point were primarily in neuronal genes (SLO-1 is also expressed in muscle though its neuronal expression was found to be more important for intoxication). In a forward genetic screen, Bettinger *et al.* identified a mutation in the transcription factor *ctbp-1* that resulted in decreased development of AFT. Further work identified *lips-7*, a triacylglyceride lipase under transcriptional regulation by *ctbp-1*, as having a central role in the modulation of AFT. Specifically, loss of *lips-7* resulted in increased development of AFT and decreased initial sensitivity [110].

Loss of LIPS-7 was found to increase the levels of TAGs [111].Other mutations that altered fat storage and metabolism were found to also alter AFT. However, levels of fat storage did not simply correlate with changes in AFT. Investigation of membrane components found that cholesterol was necessary for the development of AFT [110]. These data suggested that the composition of the membrane altered the ethanol response. In support of this idea, a loss of *lips-7* was able to rescue the locomotion of a *slo-1* gain-of-function mutant suggesting that *lips-7* interacts with SLO-1 and can alter its activity probably through changes in the lipid environment surrounding the channel. This result is in good agreement with findings that the lipid environment around various mammalian SLO-1 channels modulate their gating both basally and in response to ethanol [40, 41, 45]. An alternative or parallel hypothesis is that the loss of free fatty acids cleaved by *lips*-7 may alter the ethanol phenotype. However, the targets of LIPS-7 are currently unknown and total lipid analysis has not consistently identified altered free fatty acid (FFA) levels. Of particular interest was the result that mutations in two desaturase genes (*fat-7; fat-5*) resulted in faster development of AFT. This suggested that the desaturation state of the lipids present in the worm played a crucial role in the development of AFT.

KLF-3, a Kruppel-like transcription factor, that regulates fatty acid metabolism and lipid profile [112], is also involved in the development of AFT. Mutation of *klf-3* results in altered expression of numerous desaturase and elongase genes as well as increases in stearic and linolenic acids and a decrease in palmitic acid. Unfortunately, the lipid profile spectra did not adequately resolve polyunsaturated fatty acid (PUFA)

levels. [112]. A *klf-3* mutant is also unable to develop AFT (citation in preparation). This supports another link between fatty acid metabolism and ethanol response.

5.9 GxE interactions in alcohol abuse

Research over the past decade has elucidated environmental risk factors that alter alcohol abuse liability through interactions with genetic predispositions. These gene-environmental interactions (GxE) are found when the expression of a gene or genotype differs across environments [113]. These studies are typically conducted using mono- or dizygotic twins that have been adopted into different households allowing researchers to separate the genetic and environmental factors that can lead to AUD. Alcohol-related outcomes, peers, positive alcohol expectancies, and drinking to cope with stress have all been shown to be mediators that interact with LR to influence abuse liability [96]. A large body of work exists on the GxE that influence AUD (for a review see [113]) and while these studies consider a large variety of psychological factors that influence behavior they do not consider diet as a factor that may modulate the development of AUD.

It has been shown that lipids directly interact with and modulate known targets of ethanol, including the BK channels, and furthermore that the effects of ethanol on these targets can be modulated by the surrounding lipid environment [39-45, 110, 114-117]. Diet is an attractive target for research as it can be assessed and altered with minimal effort. Numerous studies have shown that lipid levels are altered by diet and that these changes can also have positive health outcomes for patients [118-126]. Recent work from our lab confirmed that altering triacylglyceride levels in *C. elegans* modulates the development of acute functional tolerance (AFT) [110].

5.10 Fatty acids

5.10.1 Metabolism

Much is known about fatty acid metabolism in *C. elegans* (Figure 1). 471 genes that are potentially involved in 16 lipid metabolic pathways have been identified [127]. Of these 471 genes, 419 have human orthologs, which covers approximately 79% of the lipid metabolic genes in humans. This high degree of conservation in lipid metabolism makes *C. elegans* a good model organism to test the effects of FA metabolism on the response to ethanol [127]. Genes involved in the elongation and desaturation of fatty acids have been identified in worms (Figure 1). Furthermore, the lipid profiles of wild-type and mutant worms have been determined by thin-layer chromatography and gas-chromatography/mass spectrometry to better understand how mutations in the pathway alter the lipid profile [128-131]. Like mammals, *C. elegans* contain a wide array of saturated, monounsaturated, and polyunsaturated (sometimes further broken down into poly- and highly unsaturated) fatty acids. While most of the elongases and desaturases in *C. elegans* are conserved in mammals there are a few notable exceptions: (1) While mammals can synthesize low levels of EPA and DHA from ALA, *C. elegans* is able to efficiently synthesize omega-3 fatty acids from omega-6 precursors by the action of the omega-3 desaturase FAT-1 which mammals do not have, and (2) *C. elegans* do not synthesize detectable levels of docosahexaenoic acid (DHA) or any fatty acids with carbon chains greater than 20 [132]. Regardless of these changes, dietary supplementation of fatty acids as well as polymorphisms in lipogenic

genes have been shown to affect the fatty acid profiles of both mammals and *C. elegans* [131, 133, 134].

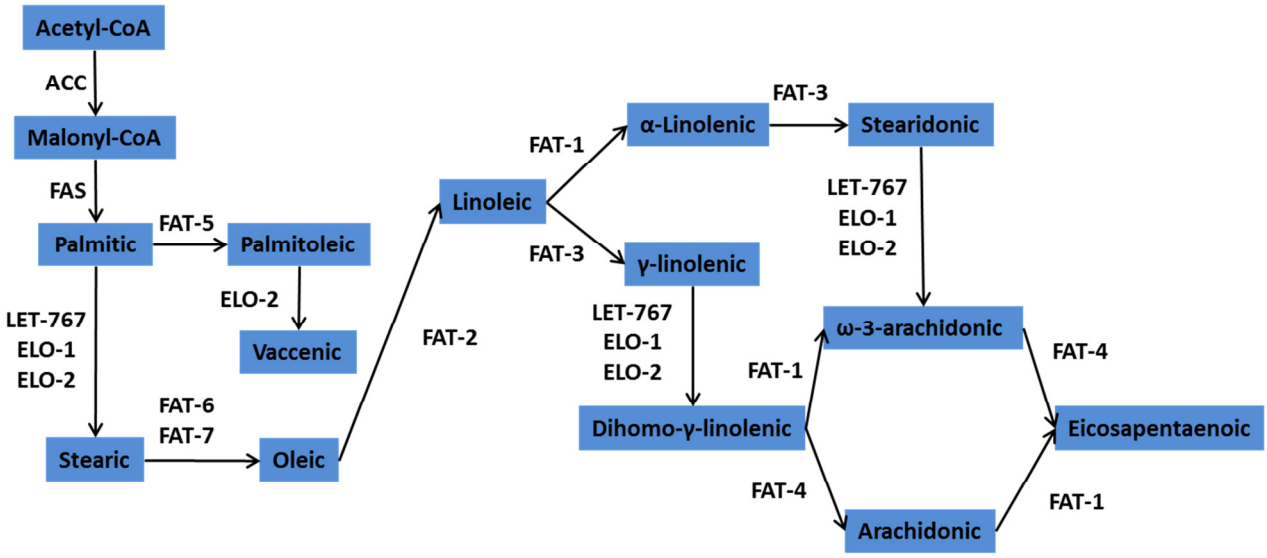

Figure 1. Fatty acid metabolism in *C. elegans*. Elongases and desaturases, in the blue boxes, catalyze the conversion of fatty acid species per the arrow they are next.

5.10.2 Roles of fatty acids

Fatty acids have numerous roles beyond energy storage in TAGs. Fatty acids compose the lipid bilayer of each cell, interact with membrane bound proteins to modulate their function (e.g. in neurotransmission), can act as signaling molecules or precursors to signaling molecules, or as regulators of gene transcription. For the work presented in the next chapters, three of these roles are of particular interest: (1) modulation of synaptic vesicle release, (2) direct and indirect signaling (both as free fatty acids and as precursor molecules), and (3) gene regulation.

5.10.2.1 Synaptic vesicle release

Some of the first mutants identified by Sydney Brenner had mutations in cholinergic genes that altered the sensitivities to acetylcholine receptor agonists and acetylcholinesterase inhibitors [67]. While the pharmacological agents in popular use have changed since then, assessing cholinergic activity by means of agonists and acetylcholinesterase inhibitors is still in use [71]. Given that long acyl chain PUFAs are often highly enriched in synaptic membranes [135, 136] it is not surprising that mutations of genes in the PUFA pathway can cause neuronal and behavioral disorders in humans and *C. elegans* [137, 138]. Watts and Browse found a mutation in the FAT-3 desaturase gene that resulted in a complete loss of long chain PUFAs (LC-PUFAs) [130]. Work done shortly afterwards by Lesa *et al*. determined that these mutants were slower, laid fewer eggs, and were resistant to the paralyzing effects of aldicarb which suggested that they were deficient in cholinergic neurotransmission. Furthermore, they showed that these phenotypes were rescued by exogenous treatment with arachidonic acid (AA) and docosahexaenoic acid (DHA). Electrophysiological studies showed that *fat-3* mutants were releasing fewer synaptic vesicles than wild-type worms and presynaptic sites were depleted of synaptic vesicles suggesting a deficiency in vesicle formation or recycling [117]. Follow-up work by Marza *et al.* determined that synaptojanin localization was dependent on LC-PUFAs.

It is important to note that the rescue of *fat-3* phenotypes by DHA may not have been directly attributable to DHA or any of the signaling molecules it could be a precursor for. While Lesa *et al.* did not observe an increase in EPA levels in *fat-3* worms treated with DHA, in a separate fatty acid supplementation experiment Hillyard and

German did see a small but significant increase in EPA levels when these worms were treated with DHA [132]. While the conversion of DHA to EPA has not been shown, but beta oxidation would be capable of shortening DHA to 20 carbons (DHA is 22 carbons and EPA is 20 carbons). Likewise, saturation of PUFAs has been noted before as EPA treatment in *fat-3* has been shown to cause a slight increase in omega-3 arachidonic acid (O3AA). This saturation pathway is probably not observed in wild-type animals due to the presence of both substrate (e.g. AA) and product keeping the reaction at equilibrium (e.g. EPA). Only when EPA is abundant and its precursor O3AA is absent does the reverse reaction occur in normal lab conditions. This suggests that small amounts of EPA may have been responsible for the rescue observed in the Lesa *et al.* paper. An alternative hypothesis is that DHA is structurally substituting for a PUFA that is normally present in the worm that allows for the modulation of membrane bound proteins. If EPA is acting as a precursor to a signaling molecule then the possibility exists that DHA may also be a precursor to a similar or the same signaling molecule. While unlikely, the DHA preparation could have been contaminated with low but sufficient levels of EPA.

Once synaptic vesicles have fused with the synaptic membrane and released their cargo of neurotransmitters the lipids and proteins that made up the vesicle diffuse into the synaptic membrane. These lipids and proteins are recycled to form new synaptic vesicles [139]. Synaptojanin is responsible for the dephosphorylation of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], a phosphoinositide that is anchored to the membrane by a LC-PUFA, which decreases the affinity of clathrin adaptor proteins for the membrane and is an essential step for endocytosis of synaptic

vesicle membrane and proteins [140]. LC-PUFAs are also enriched in synaptic vesicles and are partially responsible for their high degree of curvature [141]. Marza *et al*. confirmed that LC-PUFAs were necessary for proper localization of synaptojanin at release sites in *C. elegans*. Loss of LC-PUFAs also resulted in an accumulation of PtdIns(4,5)P_2 in the presynaptic membrane, a reduction in synaptic vesicles, and an increase in the size of the synaptic vesicle (due to a reduction in the curvature of the vesicle). Dietary supplementation of LC-PUFAs was also shown to be sufficient to rescue synaptojanin localization and the signaling deficiencies [142].

5.10.2.2 Lipid signaling

Lipids are involved in signaling both directly as signaling molecules and as precursors to signaling molecules. These molecules include eicosanoids, lysophospholipids, sphingolipids, and endocannabinoids. Lipid signaling pathways are recognized as major components of many cellular processes, most notably for their role in immune and injury responses. Their role in drug addiction is less well understood but has been demonstrated [143, 144]. Lipid transmitter signaling has already been suggested as a target of research for the alcohol and cocaine communities [143] as well as being a possible target to treat cocaine addiction [144].

In mammals, lipid transmitters fall into three major classes: (1) AA-derived eicosanoids, (2) lysophospholipids and their derivatives, and (3) newly discovered lipid transmitters that are derived from omega-3 fatty acids [145]. These general classes help

to simplify the very complicated pathways that exist in the creation and signaling of lipids.

5.10.2.2.1 Class 1: AA-derived eicosanoids

Many of the most well-known lipid transmitters fall into the first class. These include prostaglandins, leukotrienes and their relatives. After its release from the membrane, AA is converted to PGG_2 and then to PGH_2 by cyclooxygenases (COX) and then further converted to five major signaling molecules (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, TXA_2 PGE_2) by prostaglandin synthases [145]. PGE_2 is the most studied of the group for its roles in inflammation, fever, pain, and cancer through its receptors EP1 to EP4 [146]. Eicosanoids synthesized by COX have been a major focus for the treatment of pain through the use of non-steroidal anti-inflammatory drugs (NSAIDs) [147].

Leukotriene (LT) synthesis is similar to PG synthesis. After release from the membrane, AA is converted to the unstable intermediate LTA_4 by 5-LOX and then converted to either LTC_4 or LTB_4 by LTA_4 hydrolase or LTC_4 synthase, respectively [145]. LTs are central to the asthmatic response and mice deficient in LT synthesis are resistant to asthmatic challenge [148].

5.10.2.2.2 Class 2: Lysophospholipids and derivatives

Class 2 lipid transmitters include platelet-activating factor (PAF), lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), and the endocannabinoids. PAF was the first of the lysophospholipid derived

transmitters that was discovered. Knockout studies of the PAF receptor in mice have suggested that PAF is involved in inflammation and allergy response [149]. LPA has been shown to have a role in both brain and cardiovascular development in mice and humans [150] indicating that these lipid transmitters are involved in both acute response and developmental pathways. S1P and C1P were found to have unique roles in the lipid signaling pathways. S1P induces COX-2 [151] and C1P activates PLA_2 in the Golgi membrane [151]. This highlights the crosstalk between various lipid signaling pathways and further complicates targeting of the pathways by pharmacological interventions. The endocannabinoids are nearly a class unto themselves but due to structurally similarity fall under class 2. The *N*-acetylethanolamine (NAE) anandamide and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids. They have been shown to mediate anti-nociceptive and anti-inflammatory effects through interactions with CB1 and CB2 receptors [152, 153]. Inhibition of monoacylglycerol lipase and fatty acid amide hydrolase has been used to increase levels of 2-AG and anadamide, respectively, and have been of particular interest in the treatment of chronic pain after many exogenous cannabinoids showed abuse liability [154].

5.10.2.2.3

5.10.2.2.4 Class 3: Omega-3 derived lipid mediators

The newest class of lipid transmitters, studied most extensively in mammals, are the resolvins, protectins, maersins, and lipoxins that are all involved in resolving inflammation. Lipoxin, although it falls into this class based on its involvement in inflammation, is actually derived from AA. The remaining lipid transmitters are derived from omega-3 fatty acids via conversion by 12/15-LOX and other unidentified enzymes.

Although the enzymes have not been identified, EPA and DHA are the known precursors for resolvins and proctectins, respectively [155, 156]. Whereas before omega-3 fatty acids were thought to be anti-inflammatory through their competition with AA for COX conversion to pro-inflammatory eicosanoids, now data exists that these LOX derived signaling molecules also act to resolve inflammation rather than only interfere with its formation [156].

Work over the past decade has found that numerous orphan mammalian GPCRs respond to various chain length fatty acids. These receptors have been deemed free fatty acid receptors (FFARs). FFARs act as physiological sensors for the intake of dietary free fatty acids and regulate energy metabolism through the modulation of insulin secretion. This means that free fatty acids by themselves can also be considered as a class of lipid transmitters. FFARs are now the target of investigations pertaining to the treatment of type 2 diabetes and offer an intriguing new view for free fatty acids in signaling [157].

Discoveries of lipid signaling pathways have been noticeably slower than protein based signaling molecules. This is in part due to signaling fatty acids being masked by the large steady-state mass of structural lipids in membranes [145]. While some of the enzymes that convert free fatty acids to signaling molecules have been identified and can be found in the genome, free fatty acids that require no conversion have no direct genome encoding proteins to alter their function. This further increases the difficulty in targeting these pathways for study and pharmacological intervention. However the receptor or the fatty acid itself can still be targeted.

5.10.2.3 Gene regulation by lipids

In 1992 Gottlicher *et al.* established the existence of nuclear receptors that bound fatty acids [158]. Nuclear receptors bind to nuclear response elements within the promoter regions of genes to affect their transcription. These response elements are encoded by direct, everted or inverted repeats (DR, ER, and IR, respectively) with a variable number of nucleic acid "spacers" between the repeats. For example, AATTGG-ATG-GGTTAA would be an inverted repeat with three nucleic acids between the repeats and its shorthand would be IR3.

PUFAs have been shown to regulate numerous lipogenic genes both in mammals [159] and in cell culture [160]. Furthermore, this effect has been shown to work within hours of feeding PUFAs through observed decreases in S14 gene transcription and abundance of mRNA in hepatic tissue [161]. Regulation can occur in a tissue specific manner which could be advantageous for targeted therapies. In general, PUFAs increase genes responsible for β-oxidation and decrease expression of lipogenic genes [162]. PUFAs have been demonstrated to interact with at least three nuclear receptors: hepatic nuclear factors (HNF)-4α, liver X receptors (LXRs) α and β, and peroxisome proliferator-activated receptors (PPARs) α, β, and γ [163].

Fatty acyl CoA thioesters bind to HNF-4α and can either activate or inhibit the receptor depending on the chain length of the lipid [164]. When activated, HNF-4α binds to DR1 sequences as homodimers [165]. It's believed that PUFAs are inhibitory to HNF-4α and disrupt its ability to bind to its recognition site [166].

Peroxisome proliferator-activated receptors (PPARs) dimerize with retinoid X receptors (RXRs) and bind DR1 peroxisome proliferator response elements (PPREs) [167]. To test their ability to bind PUFAs, Gottlicher *et al.* used a chimeric construct consisting of the ligand-binding domain of rat PPAR and the DNA-binding domains of the human glucocorticoid receptor. This construct was expressed in CHO cells with a gene for alkaline phosphatase under the control of the mouse mammary tumor virus promoter as a reporter for receptor activation. Phosphatase activity was assessed spectrophotometrically. Phosphatase activity was inducible by linoleic and arachidonic acids but not by steroid hormones or sterols (DHEA, cholesterol or 25-OH-cholesterol). Furthermore, the activation by linoleic and arachidonic acids were dose-dependent suggesting that the regulation of transcription can be fine-tuned based on dietary intake of these free fatty acids [158].

Cellular cholesterol and fatty acid metabolism in mammals is controlled by a family of transcription factors called sterol regulatory element-binding proteins (SREBP). In sterol-deficient cells, cleavage of SREBP from the Golgi membrane results in its transit to the nucleus, binding to sterol response elements (SRE), and the transcription of lipogenic genes to increase cholesterol, triglyceride, and fatty acid biosynthesis [168]. SREBP1c gene expression is induced by cholesterol and repressed by PUFAs. In a high cholesterol and high PUFA diet, cholesterol was still able to increase SREBP1c mRNA and protein levels, however levels of the mature, cleaved protein were repressed as were the genes normally activated by SREBP1c binding. These results suggest that PUFAs regulate SREBP1c activity through opposing the maturation of the protein and not its transcription or translation [169].

LXRs bind oxysterols and heterodimerize with retinoid X receptor (RXR)α. They then bind DR4 sequences (known as LXR response elements [LXREs]) [170], and regulate fatty acid and cholesterol transport and metabolism [171]. Regulation by LXRs is accomplished through activating the SREBP1c gene promoter [172]. Genes regulated in this manner include fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) [173] which are the first two enzymes in fatty acid elongation in *C. elegans* (Figure 1) [174]. Although the mechanism is not known, PUFAs block LXR gene regulation possibly by inhibiting the binding of LXR to RXR [175].

5.10.2.4 Lipid modification of proteins

Palmitoylation, or S-acylation, is the reversible addition of an activated fatty acid, usually palmitic acid, to a cysteine residue of a protein by a thioester bond. This linkage

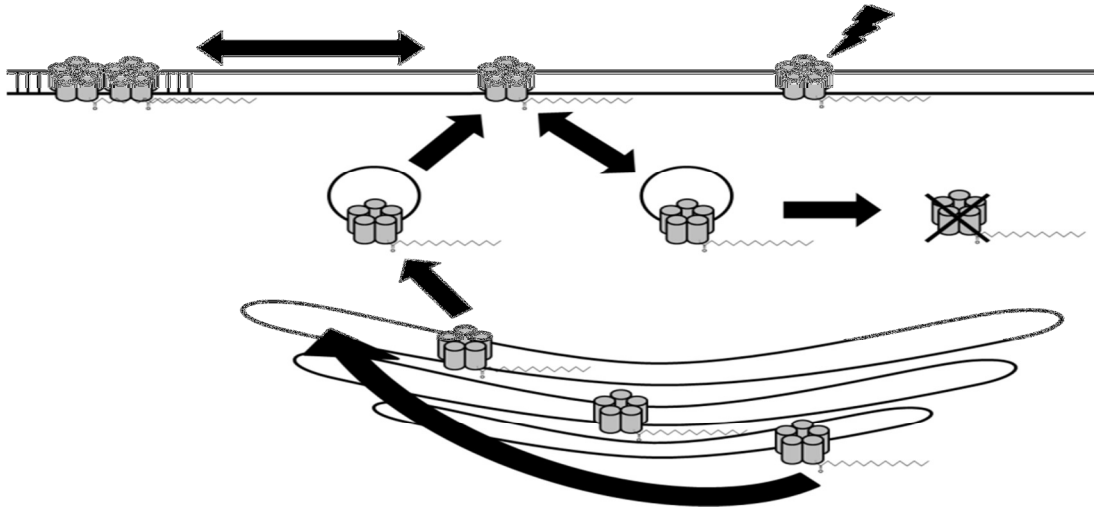

Figure 2. Palmitoylation has been shown to affect protein trafficking through the Golgi network, trafficking to the membrane, localization within the membrane, activity, internalization and subsequent recycling or degradation.

is achieved by the action of a palmitoyltransferase and can be reversed by a thioesterase. While not much is known about the specificity of palmitoyltransferases for their targets, many ion channels have been shown to be palmitoylated. Of particular interest are $GABA_A$, NMDA, and BK channels, all known targets of ethanol, all of which are palmitoylated [176-181]. Palmitoylation has been shown to affect trafficking of channels between the endoplasmic reticulum, Golgi, and the plasma membrane as well as the localization within the membrane, the activity of some channels, as well as modulating the internalization and subsequent recycling or degradation (Figure 2) [182]. Most proteins that have been shown to be palmitoylated contain multiple palmitoylation sites with each modulating an individual process.

5.11 *C. elegans* eicosanoid production

In mammals, CYP-dependent eicosanoids such as EETs and 20-HETEs modulate the activity of various ion channels including BK channels [183, 184], L-type calcium channels [185], and TRPV channels [186]. 17(R),18(S)-EETeTR acts as a highly potent activator or vascular smooth muscle BK channels [184].

Eicosanoid signaling in *C. elegans* is less well understood than in mammals. However, it has been shown that AA and EPA are both metabolized to epoxy and hydroxy derivatives that are known to be signaling molecules in mammals. Specifically, CYP-29A3 and CYP-33E2 are the major P450 enzymes responsible for the metabolism of EPA and AA [187]. With 75 cytochrome P450 genes in the genome of *C. elegans*, it is possible that these additional enzymes also act in lipid signaling pathways [187]. CYP-35A2, CYP-35A3, CYP-35A4, CYP-35A5, and CYP-35B1 have been shown to alter fat storage pathways in the nematode [188, 189]. Given the numerous possible

roles for lipids (e.g., signaling, structural, gene regulation) these P450s may also play an indirect role in signaling. *C. elegans* has no obvious orthologs of mammalian COX and LOX enzymes as well as no obvious PG or LT receptors [117]. This suggests that while lipid signaling may occur through P450 dependent pathways more work will need to be done to identify the enzymes, molecules and receptors. Additionally, eicosanoid signaling in *C. elegans* may rely more heavily on EPA as it is the predominant lipid species in the worm [130] as compared to AA in humans [134]. The lack of longer chain fatty acids in *C. elegans* also precludes the use of DHA as a signaling molecule and precursor. Given that different PUFAs are enriched in synaptic membranes between *C. elegans* (EPA) and humans (DHA) their roles may be similar but accomplished through different pathways. To this end, Kulas *et al.* noted that while the mammalian homologs of CYP-29A3 and CYP-33E can be distinguished as AA hydroxylases and AA epoxygenases that the *C. elegans* isoforms have the ability to catalyze both reaction types when converting EPA [187].

Given the diverging functions of these homologs, Kulas *et al.* tested the ability to block EPA and AA metabolism with 17-ODYA, PPOH, and CO. These inhibitors of mammalian P450 enzymes potently inhibited *C. elegans* metabolism of EPA and AA in a microsomal preparation. Also similar to mammalian eicosanoid signaling, the derivatives were increased by treatment with the PPARα activator, fenofibrate. A homology search for human homologs of CYP-29A3 and CYP-33E2 found that CYP-29A3 was similar to the family of CYP4 enzymes and CYP-33E2 was similar to the CYP2 family of enzymes. While no function was assigned to any of the lipid derivatives

detected in their work, Kulas *et al.* did conclude that the metabolites are endogenous constituents of *C. elegans* with functions to be determined.

6 Eicosapentaenoic Acid Necessary for the Development of AFT

6.1 Introduction

6.1.1 Alcohol use and abuse

Alcohol abuse disorders affect approximately eighteen million Americans and are estimated to cost the United States $223.5 billion, or 2% of its GDP each year [1, 2]. Current pharmacological interventions are inadequate, which is due, in part, to the complexity of alcohol's molecular effects. The propensity to develop alcoholism is strongly genetically influenced [93]. However, due to these complex molecular effects and despite intense study, few candidate genes for alcoholism have been identified.

6.1.2 Genetic and environmental influences on alcoholism

While causative genes for alcohol abuse disorders (AUD) have remained largely elusive, a large body of work spanning multiple decades has identified a low level of response (LR) to ethanol as an phenotype of alcohol use disorders [94]. A person's level of response has been typically assessed by body sway, subjective high, and cognitive testing within a single drinking session. A low LR at age 20 has been shown to increase the likelihood of alcoholism fourfold in both the sons of alcoholics and control groups [94]. Furthermore, LR has been shown to be heritable [95-98]. Taken together these data suggest that the genes that are responsible for LR may also be central to the development of AUD, making them particularly attractive targets for research. Additionally, work over the past decade has elucidated environmental risk factors that alter alcohol abuse liability through interactions with genetic predispositions. These

gene-environmental interactions (GxE) are found when the expression of a gene or genotype differs across environments [113]. These studies are typically conducted using mono- or dizygotic twins that have been adopted into different households. This allows the researchers to separate the genetic and environmental factors that can lead to alcohol abuse disorders. Alcohol-related outcomes, peers, positive alcohol expectancies, and drinking to cope with stress have all been shown to be mediators that interact with LR to influence abuse liability [96]. A large body of work exists on the GxE that influence AUD (for a review see [113]) and while these studies consider a large variety of psychological factors that influence behavior they do not consider the possible role of diet modulating the development of AUD. It has been shown that lipids directly interact with and modulate known targets of ethanol, including the BK and TRPV channels, and furthermore that the effects of ethanol on these targets can be modulated by the surrounding lipid environment [39-45, 110, 114-117]. Diet is an attractive target for research as it can be assessed and altered with minimal effort. Numerous studies have shown that not only can lipid levels be altered by diet but that these changes can also have positive health outcomes for the patients [118-126]. Recent work from our lab confirmed that altering triacylglyceride levels in *C. elegans* modulates the development of acute functional tolerance (AFT) [110]. With this in mind, we have assessed the role of long-chain polyunsaturated fatty acids (LC-PUFA) on initial sensitivity and development of AFT in *C. elegans*.

6.1.3 C. elegans as a model organism

Caenorhabditis elegans provides a simple system to study the genetic and molecular effects of ethanol intoxication and LR. Intoxication in *C. elegans* occurs at doses that cause intoxication in other organisms [99, 190, 191]. Genes identified in the worm that alter ethanol response, when they have been tested, also modify ethanol responses in rodents [192]. These observations suggest a conserved mechanism for ethanol responses between *C. elegans* and mammals. This initial response to ethanol is composed of at least two components [191] that can be assessed experimentally in *C. elegans*: initial sensitivity and AFT. Initial sensitivity corresponds to the magnitude of intoxication at its maximum effect and is observed in *C. elegans* as the maximum loss of speed at ten minutes of ethanol exposure. AFT is predicted to be the summation of homeostatic neuronal mechanisms that adapt to the intoxicating effects of a single dose of ethanol, and in humans is observed as the recovery in behavioral impairment over the course of a single drinking bout at the same or higher blood alcohol concentrations [94]. In *C. elegans*, AFT is measured as a recovery in locomotion between the ten and thirty minute time points of an ethanol exposure assay. Over this time period the worms' speed increases as does the tissue concentration of ethanol [99], demonstrating that AFT is neither due to metabolism nor a change in the pharmacokinetics of ethanol over the course of the assay. Previous work has shown that the effects of ethanol in *C. elegans* are largely neuronal in nature and that initial sensitivity and AFT are dependent on proper neuronal expression of ethanol targets [39].

Mutations that affect fatty acid metabolism in humans have been shown to have neurological consequences. Specifically, mutations in ELOVL4 and FACL4 can cause macular degeneration and nonspecific X-linked mental retardation, respectively [137, 138]. This is perhaps not surprising given the enrichment of long chain polyunsaturated fatty acids (LC-PUFA) in the brain and retina [193, 194]. Their importance is further stressed by their near absence in adipose tissue [195]. Mutations that alter fatty acid metabolism in worms have also been shown to negatively affect synaptic vesicle recycling and neurotransmitter release [117, 142]. These same mutations, as well as many others in the fatty acid metabolic pathway, have been well characterized both in their effects on lipid profiles and behavioral phenotypes [116, 117, 128-130, 174, 196, 197]. By using these mutations in various desaturases and elongases, specific fatty acids can be altered to ascertain their roles in the response to acute ethanol.

Mutants that lacked polyunsaturated fatty acids (PUFAs) were unable to develop AFT and were more sensitive to the intoxicating effects of ethanol. Furthermore, we identified eicosapentaenoic acid (EPA) as the main fatty acid that is necessary for the development of AFT. Additionally arachidonic acid (AA) is capable of rescuing AFT only if *fat-1* is wild-type and can convert AA to EPA. This rescue was shown to be possible in as little as 19 hours of treatment. Paralysis assays with an acetylcholinesterase inhibitor suggested that the rescue of AFT was not correlated with the rescue of cholinergic signaling. These results indicate a yet undefined role for EPA.

6.2 Material and methods

6.2.1 Culture

Unless otherwise noted, strains were maintained on nematode growth media (NGM) plates at 20℃ . Before use, NGM plates were seeded with OP50 *E. coli* and a lawn of bacteria was allowed to grow overnight at room temperature.

6.2.2 Strains

The wild type strain used was N2 (var Bristol). Mutant strains used were: BX24 *fat-1(wa9)*, BX17 *fat-4(wa14)*, BX30 *fat-3(wa22)*.

6.2.3 Locomotion tracking

Speed was analyzed as described previously [101] with minor changes: Assay plates were dried for one hour at 37℃ with the lids removed. Four copper rings were heated and melted into the surface of the plates. Ice-cold 100% ethanol was added to a final concentration in agar of 0 mM or 400 mM. Plates were immediately sealed with Parafilm and the ethanol was left to absorb into the plate for two hours at room temperature. Age-matched first day adults were acclimated by moving them to unseeded plates with copper rings for thirty minutes. At thirty minutes ten worms of each strain were then moved from acclimation plates to the corresponding copper ring on the assay plate. The first ten minutes of exposure as well as the two minutes following the thirty minute time point were recorded for each plate. Movies were made on an Leica MZ6 stereo microscope with a 0.5x objective and 0.8x magnification using a

Retiga 4000R camera (QImaging) and ImagePro Plus (6.2) (MediaCybernetics) software. Recordings were made at one frame per second and the speed of each worm was tracked using ImagePro Plus software. The average speed for each group was calculated and treated as a single trial. All strains were tested a minimum of five times.

6.2.4 Fatty acid supplementation

Fatty acid salts (Nu-Chek Prep, Elysian, MN) were diluted to 20 mM in dH2O. NGM solutions were prepared with the addition of 0.1% NP-40 (Sigma) and autoclaved. The NGM solutions were held in a 60°C water bath for one hour. EPA or AA was added to a final concentration of 160 µM, control plates were supplemented with an equal volume of water. Plates were poured immediately and dried overnight at room temperature in a dark box. Plates were seeded the following day and stored at 20°C for 48 hours before use [116].

6.2.5 Age synchronization

Egg laying: Adult worms were allowed to lay eggs for two hours on seeded NGM or fatty acid supplemented plates. The adults were then removed. The eggs were allowed to hatch and develop to first day adults before assaying.

6.2.6 Assessment of ethanol response

A relative speed for each time point is calculated by dividing the treated speed by the untreated speed within a genotype. We assess initial sensitivity by comparing the

ten minute relative speeds between strains. A statistical difference in the speeds reflects a significant change in the initial sensitivity. We next compare the ten and thirty minute relative speeds within a strain to determine if AFT has developed. A statistically significant increase in the relative speed at thirty minutes is AFT. Lastly, we compare the degree of AFT between strains. AFT is quantified as percent recovery of speed and is calculated by subtracting the ten minute relative speed from the thirty minute relative speed. A statistically significant difference between strains' percent recovery reflects a significant difference in AFT.

6.2.7 Statistics

Animals that were compared to each other were treated on the same plate at the same time under identical conditions. Data were converted to relative speeds to account for basal speed differences between strains. Statistics were performed using Prism 5.0 (GraphPad). For comparisons of a single strain or treatment to a control, two-tail paired t-tests were performed. When multiple comparisons were necessary, as in the fatty acid supplementation assays, one-way ANOVA with a Dunnett's post hoc test was used. Aldicarb assays were analyzed by two-way ANOVA with a Bonferroni post-hoc test.

6.2.8 Aldicarb assay

Plates containing 1 mM aldicarb (Sigma) were prepared fresh for each set of paralysis assays as described previously [71]. Twenty adult animals were scored per experiment and three independent experiments were carried out [198]. Worms were counted as paralyzed if they were unable to respond to any of three pokes with a

platinum wire on the head and tail. Statistical analysis was performed using Prism 5.0 (GraphPad).

6.3 Results

6.3.1 LC-PUFAs are necessary for the development of AFT

We began by asking if LC-PUFAs had a role in the development of AFT. To test this we exposed *fat-3(wa22)* mutants, which lack all LC-PUFAs, to 400 mM ethanol and assessed their locomotion over time. We found that *fat-3(wa22)* mutants were unable to develop AFT and were more sensitive to the intoxicating effects of acute ethanol (Figure 3A). These data suggested that one or more of the LC-PUFAs were necessary for the molecular mechanisms that compensate for acute intoxication. We next asked which of the LC-PUFAs were necessary for the development of AFT. To test this we repeated our 400 mM ethanol assay with *fat-1(wa9)* and *fat-4(wa14)* mutants which lack two LC-PUFAs each, EPA and omega-3 arachidonic acid (O3AA) in *fat-1* and EPA and AA in *fat-4*. Our results showed that both mutants displayed near wild-type initial sensitivity and that both were unable to develop AFT (Figure 3B). Taken together with the *fat-3* result these data suggest that initial sensitivity is likely affected by linoleic acid (LA), and dihomo-γ-linolenic acid (DGLA) and furthermore either that O3AA and AA must both be present for the development of AFT or that EPA, the LC-PUFA lost in both mutants, is necessary for AFT.

6.3.2 Fatty acid supplementation is capable of rescuing AFT

Dietary supplementation of PUFAs is capable of rescuing affected levels of PUFAs as well as phenotypes associated with their loss [116, 117, 131, 142]. Consistent with these reports, we found that basal speeds of *fat-1(wa9), fat-4(wa14),* and *fat-3(wa22)* could be rescued by fatty acid supplementation (Figure 6). In order to confirm that supplementation is capable of rescuing the AFT phenotype we first supplemented AA into the diet of *fat-4(wa14)* worms in order to restore AA and EPA (through the desaturation of AA to EPA by FAT-1). Raising *fat-4(wa14)* worms on NGM supplemented with 160 µM AA resulted in recovery of AFT to wild-type levels. AA also did not have a significant effect on the development of AFT in wild-type (Figure 4A). These results suggested that PUFA supplementation was capable of rescuing AFT and further suggested that AA and EPA were sufficient for the development of AFT. This result allowed us to ask if EPA would be sufficient to rescue AFT in *fat-4(wa14)* or, conversely, if AA was necessary in the presence of EPA. EPA supplementation of *fat-4(wa14)* resulted in the robust development of AFT to wild-type levels (Figure 4B). While EPA did not have a statistically significant effect on wild-type in these assays it did tend to increase the development of AFT. However, EPA supplemented *fat-4(wa14)* AFT was statistically not different from EPA supplemented wild-type AFT. These results suggested that AA was not necessary for the development of AFT in the *fat-4(wa14)* background and that EPA alone was sufficient.

6.3.3 EPA and not AA is necessary and sufficient for the development of AFT

In order to further differentiate between the roles of AA and EPA we supplemented each LC-PUFA into *fat-1(wa9)*, which is unable to convert AA to EPA and DGLA to O3AA and thus lacks both EPA and O3AA. Supplementation of AA into the *fat-1(wa9)* background resulted in no significant development of AFT (Figure 4C). This suggested that AA was only capable of rescuing AFT when it could be further converted to EPA. EPA supplementation of *fat-1(wa9)* rescued AFT back to wild-type levels (Figure 4D), ensuring that EPA was still sufficient to rescue AFT in this background. These results also suggest that O3AA is not necessary and increased levels of AA do not result in a loss of AFT.

To further test sufficiency of EPA in the development of tolerance we supplemented it into the diets of *fat-3(wa22)* worms. This supplementation has been shown to result in the significant rescue of EPA levels while not affecting the loss of GLA, DGLA, and AA, and O3AA [116]. EPA alone was able to rescue the development of AFT but not restore it to wild-type levels (Figure 4F). This suggested that EPA in conjunction with upstream fatty acids are responsible for robust development of AFT or that the loss of LC-PUFAs in *fat-3* results in further defects that affect our assay and are unable to be rescued by EPA alone. Supplementation of AA into the diets of *fat-3(wa22)* worms, increasing both AA and EPA levels, resulted in wild-type levels of AFT developing (Figure 4E). Taken together these results suggest that EPA is necessary for the development of AFT but that it also works in conjunction with other upstream LC-PUFAs.

6.3.4 Wild-type AFT can be modulated by fatty acid supplementation

The increased development of AFT in N2 from both AA and EPA supplementation was never significantly different from N2 within a set of experiments. When the data was compiled a small but significant increase in initial sensitivity was observed from the AA supplementation and a significant increase in AFT was observed for both LC-PUFAs (Figure 5). These results suggested that the intact molecular pathways involved in the development of AFT via LC-PUFAs could be further modulated through dietary supplementation.

6.3.5 Rescue of basal speeds and AFT by EPA are separable

fat-1(wa9), fat-4(wa14), and *fat-3(wa22)* are significantly slower than N2 and their speeds are rescued by rearing the worms on 160 µM EPA (Figure 6). In determining the time course of supplementation necessary for the rescue of AFT we found that 19 hours of EPA supplementation was sufficient to rescue AFT but not basal speeds in *fat-1(wa9)* (Figure 7). This suggested that the reduction in basal speed was a defect that required supplementation likely throughout development while the loss of AFT could be rescued by supplementation only during the L4 to adult timeframe. The separation of basal speed and AFT rescue also suggests that differences in basal speed did not account for the loss of AFT.

6.3.6 Cholinergic signaling does not correlate with AFT rescue

While these data show that EPA is both necessary and sufficient for the development of tolerance to acute ethanol, they do not elucidate a mechanism by which this might be happening. It has been shown that PUFAs are central to CNS signaling as components central to neurotransmitter release. Specifically PUFAs are enriched in

synaptic membranes [135] and their loss results in a decrease of cholinergic neurotransmission through deficient recycling of vesicular proteins, which can be rescued by PUFA supplementation [117, 142]. In order to assess the possible role of cholinergic neurotransmission in the development of AFT we used the acetylcholinesterase inhibitor aldicarb. Aldicarb inhibits the degradation of acetylcholine to acetate and choline. The subsequent buildup of acetylcholine causes a tonic paralysis. The time necessary for paralysis is indicative of the amount of acetylcholine being released. Specifically, a mutant that release more acetylcholine will paralyze sooner while a mutant that releases less acetylcholine will paralyze later. We predicted that if AFT is dependent on cholinergic signaling that mutants unable to develop tolerance will be resistant to aldicarb, and furthermore, fatty acids that are able to rescue AFT will also rescue aldicarb resistance. In agreement with our hypothesis *fat-1(wa9)* and *fat-4wa14)* were both resistant to aldicarb compared to N2 (Figure 8A) and supplementation of EPA was able to rescue aldicarb resistance in both mutant backgrounds (Figure 8C,D). Not in agreement with our hypothesis, AA was also able to rescue aldicarb resistance in *fat-4(wa14)* and *fat-1(wa9)* (Figure 8C,D) suggesting that, unlike in our AFT assays, AA was able to rescue cholinergic neurotransmission without being converted to EPA. These results suggested that while *fat-1(wa9)* and *fat-4(wa14)* mutants are deficient in cholinergic signaling, its rescue by EPA and AA supplementation does not correlate with the rescue of AFT that we observed.

6.4 Discussion

Based on the knowledge that the lipid microenvironment around ethanol responsive proteins can alter their function and response to ethanol we asked if specific species of fatty acids may be responsible for this response. This worked aimed to determine the role of LC-PUFAs in the response to acute ethanol. We determined that LC-PUFAs modulate the initial sensitivity to ethanol and that EPA is the main LC-PUFA that modulates the development of AFT. Furthermore, dietary supplementation of EPA is able to rescue genotypes that are unable to synthesis the fatty acid and this supplementation does not need to occur throughout development. LC-PUFA supplementation has been shown to rescue cholinergic signaling possibly through interactions with synaptojanin [142]. Intriguingly, AA and EPA both rescue cholinergic signaling in a *fat-1* mutant whereas only EPA rescues the development of AFT. This suggests that the mechanism for AFT in these supplemented strains is not cholinergic in nature.

Given that our results suggest a non-cholinergic mechanism for EPA's rescue of AFT, alternative hypotheses are necessary. One hypothesis is that EPA alters lipid raft composition and this has functional consequences for interacting proteins that are involved in AFT. It has been shown that dietary n-3 fatty acids, such as DHA and EPA, alter both the lipid raft structure and the signaling of proteins known to reside in lipid rafts [199]. Numerous papers have shown modulation of BK channel activity by lipids [40, 41, 43, 45, 179-181]. These results agree with the hypothesis of Bettinger *et al.* that SLO-1, the *C. elegans* BK channel and known target of ethanol, is trafficked to lipid rafts in order to modulate its activity in response to acute ethanol [110]. We may be

observing a similar effect with loss and supplementation of EPA. If EPA either interacts directly with SLO-1 or aids in altering its localization, then in mutant backgrounds that lack EPA we'd expect to observe intoxication without the development of AFT. In agreement with this theory, this is what we observed. Further testing involving crosses and monitoring of SLO-1 activity will be necessary to test this hypothesis.

There has been increasing interest in treating neurological disorders with omega-3 fatty acids. Maes *et al.* demonstrated abnormal omega-3 fatty acid metabolism in depressed patients and showed that this was not reversed in traditional antidepressant therapy in some patients [200]. Peet *et al.* determined that omega-3 fatty acid concentrations are lower in red blood cell membrane, thought to relate directly to the neuron membrane concentration, in depressed patients compared with controls [201]. Major depressive disorder and alcohol abuse disorders (AUD) have been shown to be co-morbid although the low number of patients treated for AUDs decreases the power of these studies [2, 202]. Decreases in omega-3 fatty acids also correlate with deficiencies in dopaminergic signaling [203], a pathway that has been shown to respond profoundly to both acute and chronic ethanol [24, 28, 48, 50, 51, 204]. Furthermore, EPA/DHA supplementation for three weeks was found to significantly reduce stress and cortisol levels in abstinent alcoholics [205] suggesting a role for omega-3 supplementation in AUDs beyond the involvement of ethanol. The data presented in this paper support a role for EPA in the ethanol response but does not test the possible role of dopamine. Dopamine has been shown to alter state-dependent recognition of an odorant when paired with ethanol [74] and is necessary for disinhibition of feeding behavior by ethanol [19]. Future work with EPA should assess its ability to modulate dopaminergic signaling

especially in response to ethanol. This line of investigation could identify possible dopaminergic targets for EPA thus furthering our understanding of the mechanisms that underlie AFT.

Microarray studies have consistently identified lipid metabolism genes as being altered in alcoholics [206, 207]. These include genes involved in elongation and desaturation of fatty acids as well as their transport and synthesis of their derivatives. Of particular interest was the finding that FADS1 and FADS2 were misregulated in alcoholic brain tissue. Polymorphisms in these same genes have been shown to alter lipid profiles in humans [133]. Additionally, a study by Corella *et al.* found a novel interaction between MC4R and FTO polymorphism, known to affect obesity, and a decrease in drinking behavior [208] although this study did not look at possible differences in lipid profiles as a result of the polymorphisms. Taken together these studies suggest that lipid profile, metabolism, and ethanol interact and provide strong evidence for diet to be considered as a possible environmental factor in alcoholic gene interactions. Future studies that assess naïve response, like the work of Schuckit [209], should consider determining the lipid profile of the subjects. Additionally, work in mice or rats could alter dietary intake of omega-3s and test for a correlation between their levels and ethanol responding. It would be particularly interesting if omega-3s modulated dopamine and ethanol responding as the magnitude of dopamine release during the first exposure to ethanol was shown to be predicative of voluntary drinking in rats [56].

6.5 Figures

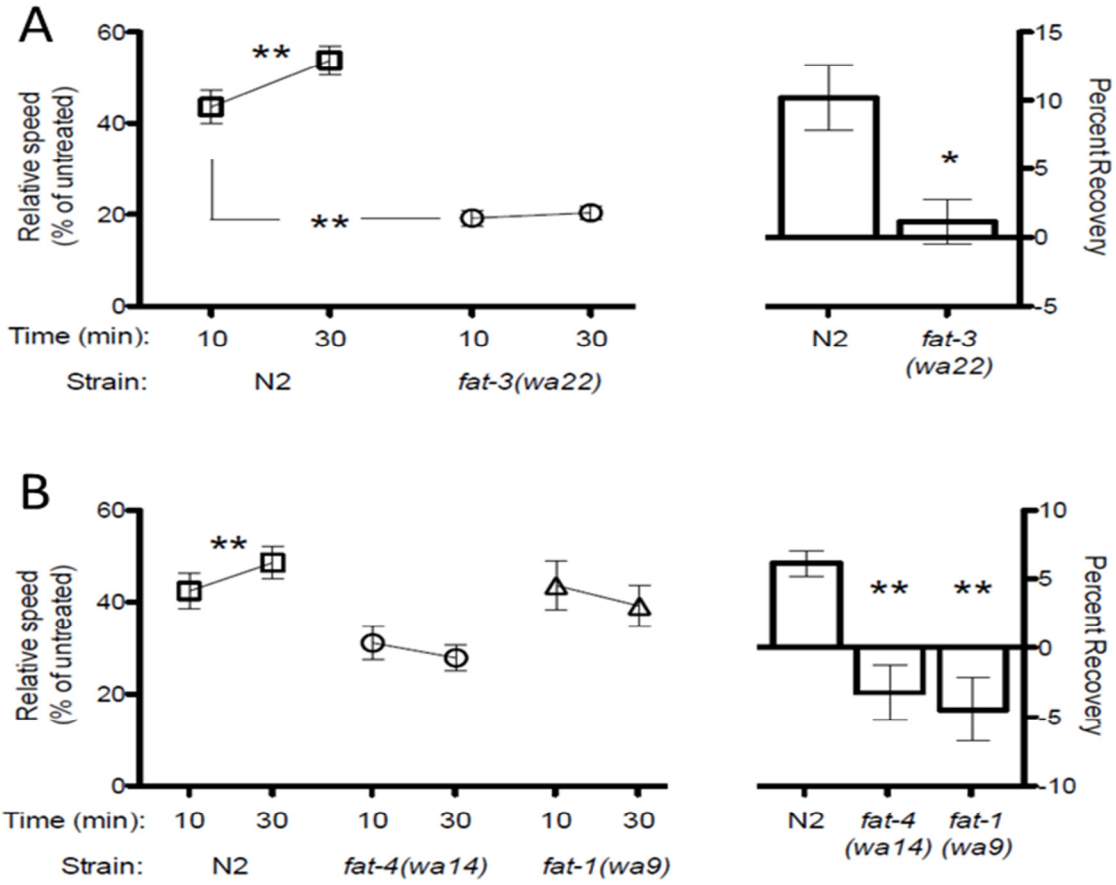

Figure 3. *fat-3(wa22), fat-4(wa14), and fat-1(wa9)* all fail to develop AFT. Additionally *fat-3(wa22)* is sensitive to the intoxicating effects of ethanol at ten minutes.

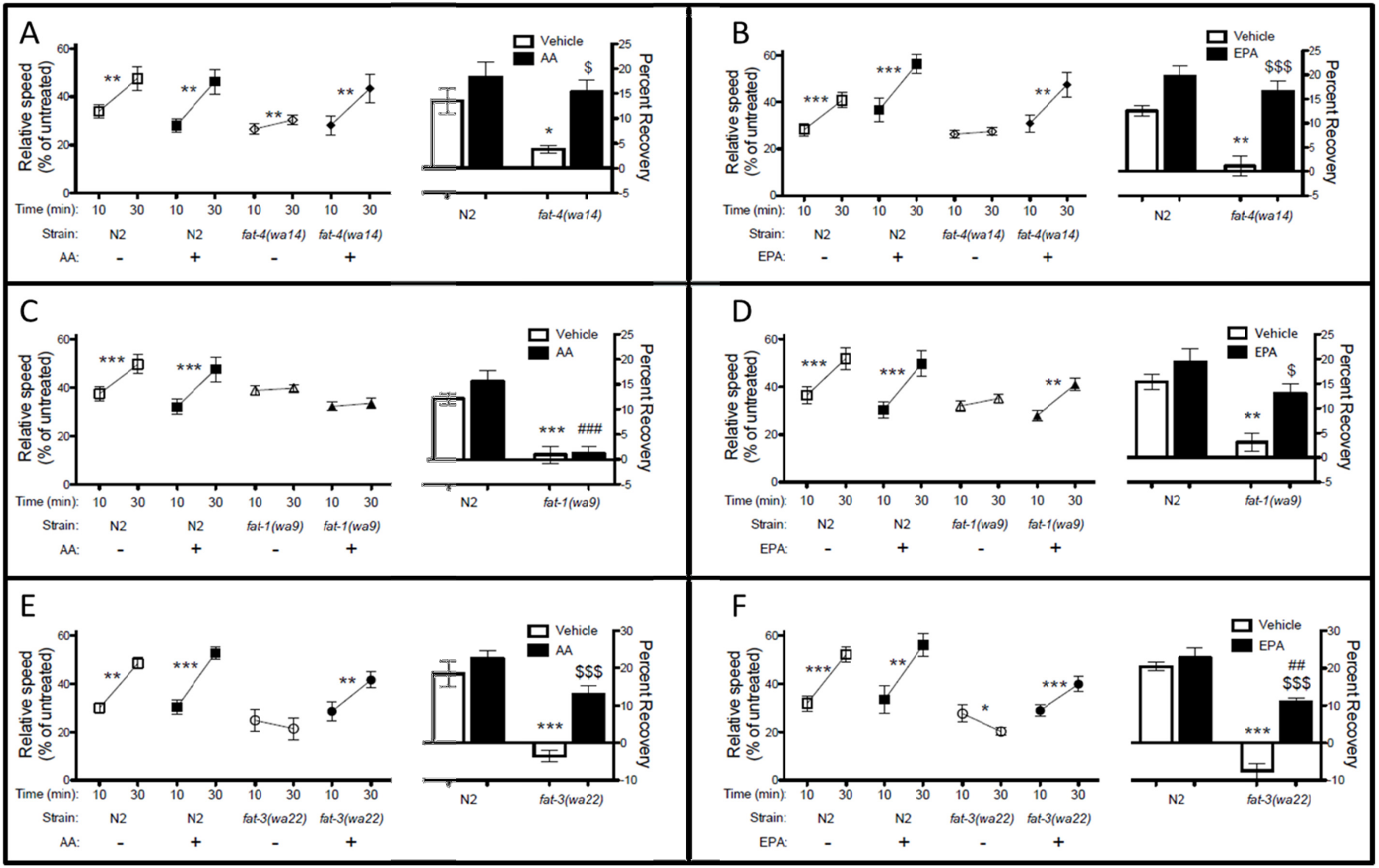

Figure 4. AFT in *fat-4(wa14)* is rescued by AA (A) and EPA (B) supplementation. AFT in *fat-1wa9)* is not rescued by AA (C) but is rescued by EPA (D) supplementation. The development of AFT in *fat-3(wa22)* is rescued by AA (E) and EPA (F) .supplementation.

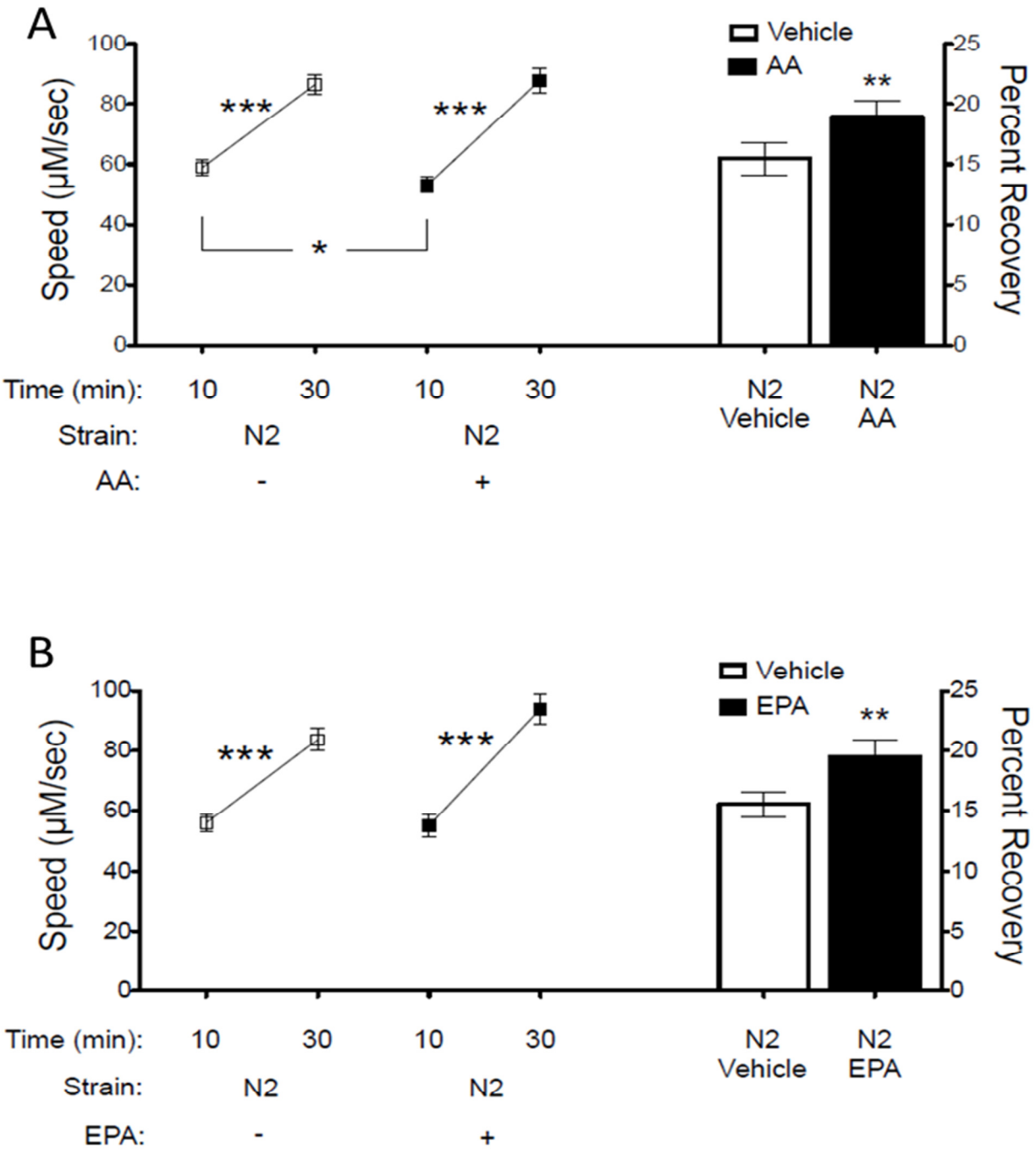

Figure 5. Supplementation with AA (A) or EPA (B) resulted in increased development of AFT in N2. AA supplementation also increased the initial sensitivity in N2 (A).

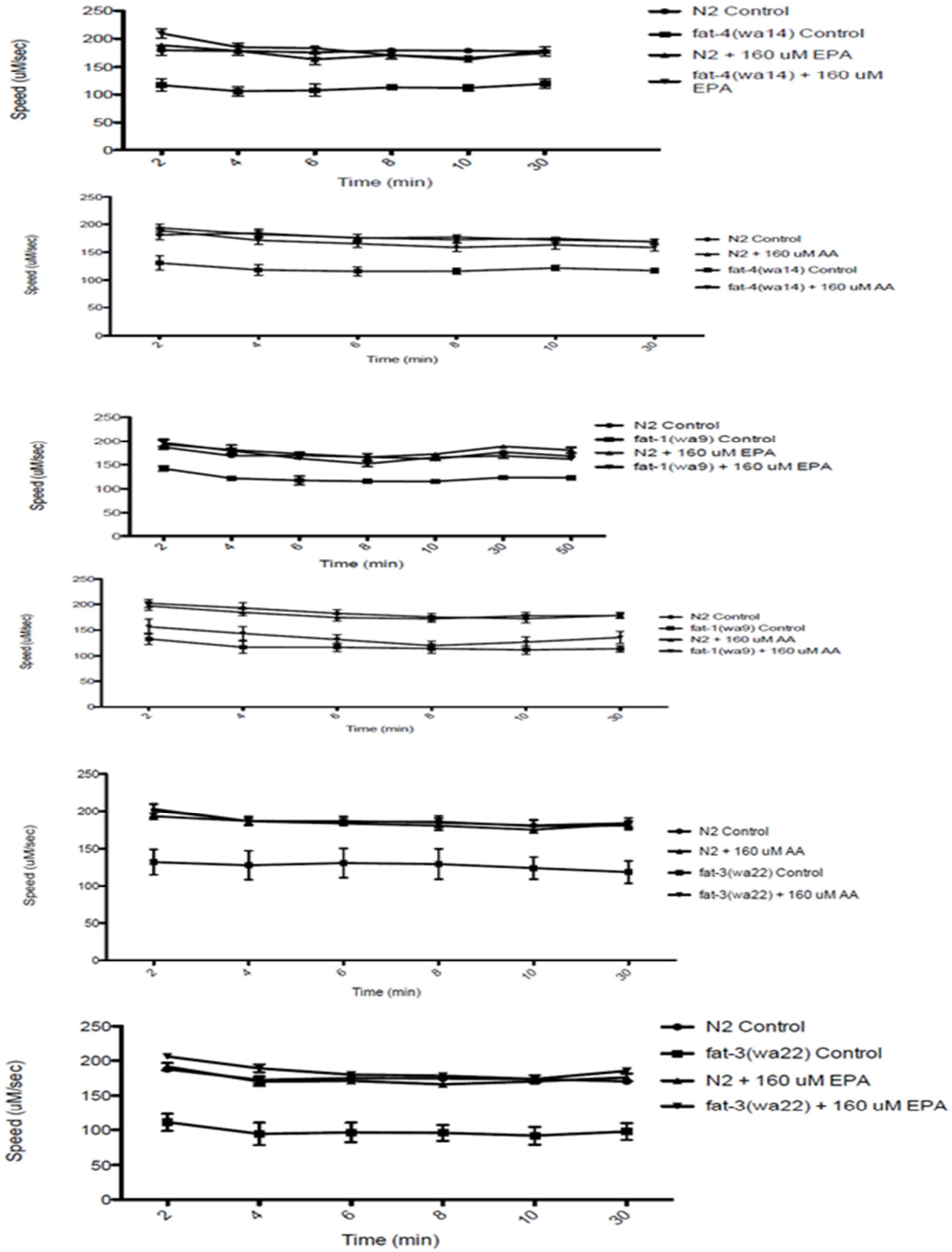

Figure 6. Basal speeds for all strains treated with vehicle, AA, or EPA. EPA rescued all basal speeds. AA rescued *fat-4(wa14)* and *fat-3(wa22)* but not *fat-1(wa9)* basal speeds.

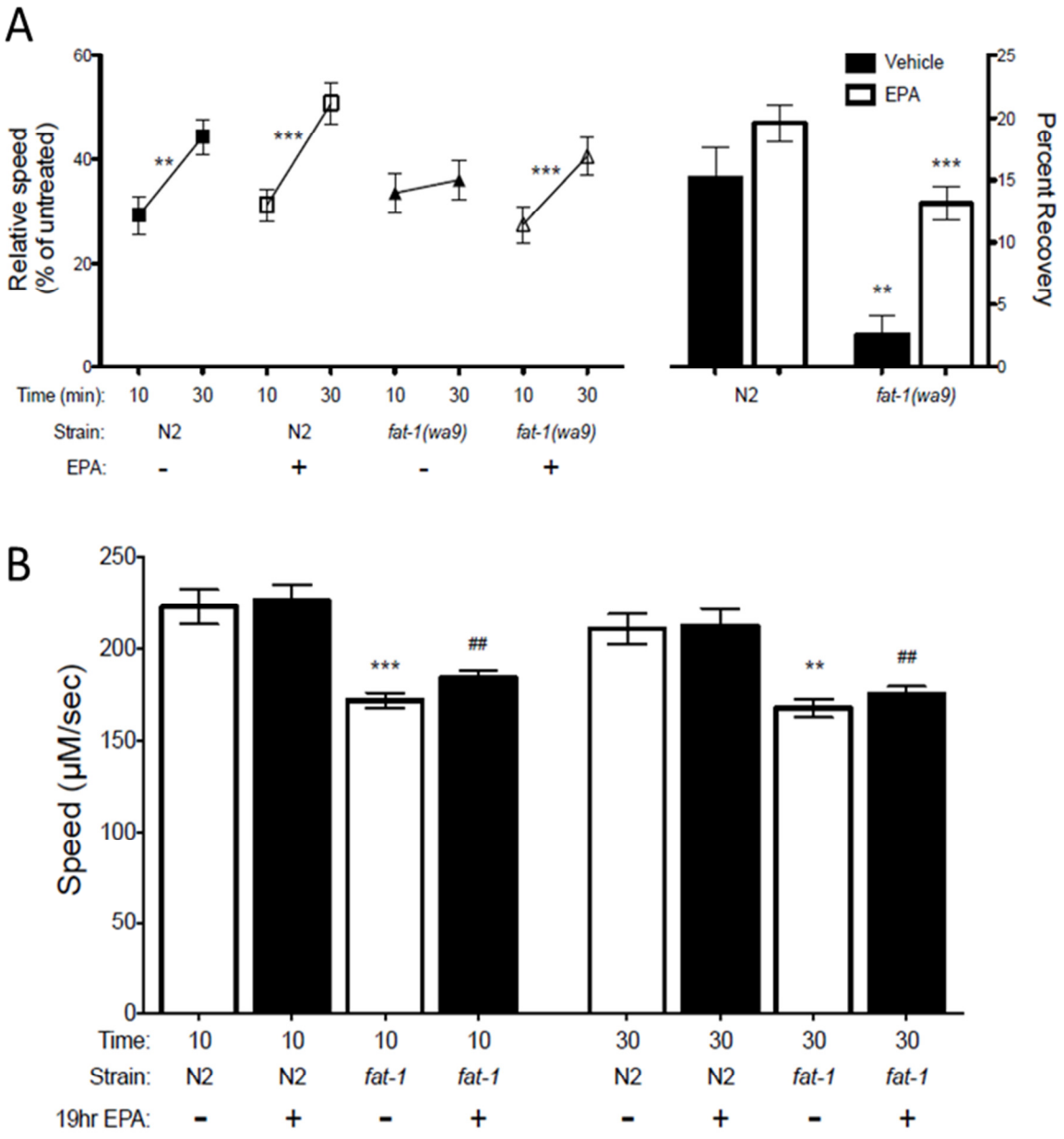

Figure 7. Nineteen hour treatment of *fat-1(wa9)* with EPA was sufficient to rescue AFT (A) but not basal speeds (B).

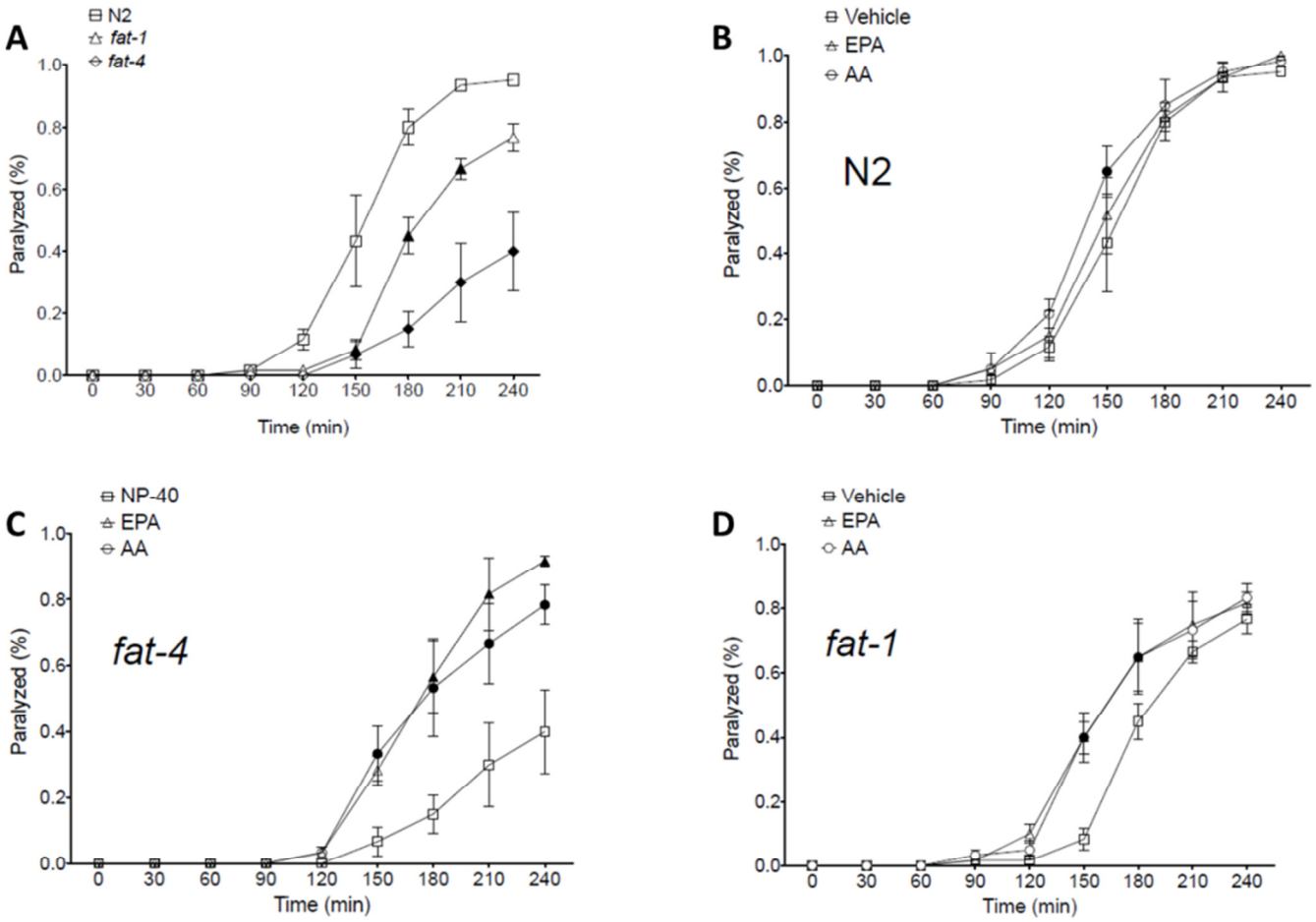

Figure 8. *fat-1(wa9)* and *fat-4(wa14)* are resistant to the paralyzing affects of aldicarb (A). EPA and AA supplementation do not alter aldicarb sensitivity in N2 (B). *fat-4(wa14)* aldicarb resistance is rescued by EPA and AA (C). *fat-1(wa9)* aldicarb resistance is also rescued by EPA and AA (D).

7 The Role of Palmitoylation in the Level of Response to Ethanol

Abstract

An individual's naïve level of response (LR) to ethanol is predictive of his or her lifetime likelihood to abuse alcohol. LR is heavily genetically influenced, suggesting that the genes responsible for LR may also be central to the development of abuse disorders. Our laboratory uses the model organism *C. elegans* to investigate the genetic influences on responses to acute ethanol exposure. We have focused on two components of LR: initial sensitivity, measured as the change in mean velocity over the first ten minutes of treatment, and the development of acute functional tolerance (AFT), measured as an increase in the mean velocity at thirty minutes versus ten minutes of exposure. We have previously found that SLO-1, the large-conductance potassium (BK) channel, is a target of ethanol. *slo-1* mutant animals are profoundly resistant to ethanol, and are defective in AFT. Recently, we used a forward genetic screen for mutations that disrupt the ability of worms to develop AFT to identify *ctbp-1*, a transcriptional repressor that affects AFT through its negative regulation of a triacylglyceride lipase, *lips-7*. *lips-7* mutants have the opposite phenotype; enhanced development of AFT. Preliminary analysis of the lipid profile of the *lips-7* mutant versus wild type has suggested that *lips-7* mutants may have an increase in palmitic acid. This led us to ask if and how palmitic acid may be involved in AFT. Palmitoylation, the reversible addition of a palmitic acid to a protein, can alter ion channel activity both directly and indirectly. Mouse mSLO-1 has multiple palmitoylation sites that affect localization, internalization, activity, and trafficking. This suggests one model for AFT in which the effects of ethanol on targets such as SLO-1 are modulated by palmitoylation. We have tested the ethanol responses

of animals with loss-of-function of various genes related to the synthesis, degradation, or use of palmitic acid, and have identified several of these genes that alter both initial sensitivity and AFT. These results strongly support the model that palmitoylation is required for AFT. Our goal is to better understand how the palmitoylation machinery modifies acute responses to ethanol.

7.1 Introduction

We have chosen to study the genetic influences on LR using the model organism *Caenorhabditis elegans. C. elegans* provides a simple system to understand the genetic and molecular effects of ethanol intoxication. We observe intoxication in *C. elegans* at doses that cause intoxication in mammals, and genes that have been identified in worms that modify ethanol responses, when they have been tested in rodents, also modify ethanol responses [41, 102, 106, 108, 109]. These observations suggest a conserved mechanism for intoxication between *C. elegans* and mammals. This initial response to ethanol is comprised of at least two components that are conserved and can be assessed experimentally in *C. elegans*: initial sensitivity and the development of acute functional tolerance (AFT). Initial sensitivity corresponds to the magnitude of intoxication at its maximum effect, and in *C. elegans* we observe initial sensitivity as the maximum loss of speed at ten minutes of ethanol exposure. AFT is the summation of homeostatic mechanisms utilized by the nervous system to adapt to the intoxicating effects of a single dose of ethanol, and in humans is observed as the recovery in behavioral impairment over the course of a single drinking bout at the same or higher blood alcohol concentrations. In *C. elegans*, AFT is measured as a recovery in locomotion between the ten and thirty minute time points of an ethanol exposure assay.

Over this time period the worms' speed increases, and this increase does not reflect a decrease in the tissue concentration; previous work in the laboratory has demonstrated that there is an increase in the internal ethanol concentration over the time course of this assay, demonstrating that AFT is neither due to altered metabolism nor a change in the pharmacokinetics or ethanol [99].

We have developed a model of the development of AFT that involves lipid modulation of protein activity via palmitoylation during exposure to ethanol. This model is based on several converging lines of data. First, a forward genetic screen for mutants that were unable to develop AFT identified a mutation in *ctbp-1* [110]. Chen *et al.* showed that CTBP-1 is a negative regulator of *lips-7*, a gene encoding a triacylglyceride (TAG) lipase and that TAG levels in *ctbp-1* mutant animals were 16.8% lower than in wild type [111]. Our laboratory found that a loss of *lips-7* resulted in initial resistance and exaggerated development of AFT, indicating that altered lipid levels affect the initial response to ethanol *in vivo* [110]. We have begun to examine the change in lipid profile in the *lips-7* mutant animals relative to wild-type. A GC/MS lipid analysis of *lips-7(ok3110)* mutant animals showed a potential increase in palmitic acid (unpublished results, Figure 9), which led us to hypothesize that palmitic acid may be modulating the initial response to ethanol in *C. elegans* via palmitoylation of ethanol-affected proteins. In further support of our model, our laboratory has previously identified SLO-1, a large conductance potassium channel, as a major target of ethanol. *slo-1* null mutant animals exhibit resistance at ten minutes and a marked reduction in AFT [39, 110]. In both *C. elegans* and mouse, SLO-1 is inappropriately activated by ethanol and this likely causes hyperpolarization of the neuron [39]. Importantly, the lipid microenvironment around

SLO-1 modulates ethanol's effects *in vitro* [41]. Furthermore, our group has demonstrated that a *lips-7* mutation suppresses a *slo-1(gf)* phenotype, indicating that the altered lipid levels in the *lips-7* mutant background have effects on SLO-1 channel activity [110]. This suggests that lipids have a direct role in the channel's activity and that the lipid microenvironment may either provide fatty acid substrate for palmitoylation or be the targeted destination of palmitoylated proteins.

Palmitoylation is the reversible addition of an activated palmitic acid to a cysteine residue of a protein through a thioester bond. This linkage is achieved by the action of a palmitoyltransferase and can be reversed by a thioesterase (Figure 10). While not much is known about the specificity of palmitoyltransferases for their targets, many ion channels have been shown to be palmitoylated. Of particular interest to our lab are GABA-A, NMDA, and SLO-1 channels (also known as BK channels), all known targets of ethanol, all of which are palmitoylated [176, 178-181, 204]. Palmitoylation has been shown to affect trafficking of channels between the endoplasmic reticulum, Golgi, and the plasma membrane as well as the localization within the membrane, the activity of some channels, as well as modulating the internalization and subsequent recycling or degradation (as reviewed by Shipston MJ in [182]). Most proteins that have been shown to be palmitoylated contain multiple palmitoylation sites with each modulating an individual process. Any of these effects may contribute to a change in the activity of an ethanol response protein, and we hypothesize that one or more may account for the very fast development of AFT that is observed in *C. elegans* and in mammals. This has led us to develop the following Specific Aims:

Aim 1: Determine if the loss of palmitoyltransferases can affect the initial response to ethanol.

Aim 2: Determine if altered lipid metabolism can affect the initial response to ethanol.

Aim 3: Use site directed mutagenesis of cysteine residues on the known ethanol target SLO-1 to determine if it is palmitoylated and if palmitoylation is necessary for the development of AFT.

Aim 4: Identify the targets of palmitoyltransferases that cause altered initial sensitivity and AFT.

7.2 Methods

7.2.1 Worm husbandry

C. elegans strains were maintained on nematode growth medium (NGM) plates with *E. coli* as a food source [67]. Strains used in this study were N2 (var. Bristol), VC168 *ppt-1(gk134)*, VC771 *dhhc-14(gk330)*.

7.2.2 RNAi

RNAi induction was performed as previously described [210]. Cultures of RNAi vector containing bacteria from the RNAi feeding library generated by J. Ahringer at the University of Cambridge (Geneservice, Cambridge, UK) were grown in LB with 50 µg/ml

ampicillin for 12 to 18 hours at 37°C while shaking. RNAi containing bacteria were seeded on to NGM plates supplemented with 1 mM IPTG and 25 µg/ml ampicillin (RNAi plates) and incubated at 20°C for 24 hours. Ten L4 stage N2 worms were placed on each seeded plate and allowed to develop to adulthood for 24 hours. Gravid adults were moved to new RNAi plates and allowed to lay eggs for one hour. Eggs were incubated at 20°C and allowed to develop to first day adults. These first day adults were used for behavioral assays.

7.2.3 Locomotion tracking

Speed was analyzed as described previously [101] with minor changes: Assay plates were dried for one hour at 37°C with the lids removed. Copper rings were heated and melted into the surface of the plates. Ice-cold 100% ethanol was added to a final concentration in agar of 0 mM or 400 mM. Plates were immediately sealed with Parafilm and the ethanol was left to soak into the plate for two hours at room temperature. Age-matched first day adults were acclimated by moving them to unseeded plates with copper rings for thirty minutes. Ten worms of each strain were then moved into the corresponding copper ring on the assay plates. Movies of their movements were recorded at ten and thirty minutes of exposure. Movies were made on an Leica MZ6 stereo microscope with a 0.5x objective and 0.8x magnification using a Retiga 4000R camera (QImaging) and ImagePro Plus (6.2) (MediaCybernetics) software. Two-minute recordings were made at one frame per second and the speed of each worm was tracked using ImagePro Plus software. The average speed for each group was calculated and treated as a single trial. All strains were tested a minimum of three times.

7.2.4 Assessment of ethanol response

A relative speed for each time point is calculated by dividing the treated speed by the untreated speed within a genotype. We assess initial sensitivity by comparing the ten minute relative speeds between strains. A statistical difference in the speeds reflects a significant change in the initial sensitivity. We next compare the ten and thirty minute relative speeds within a strain to determine if AFT has developed. A statistically significant increase in the relative speed at thirty minutes is AFT. Lastly, we compare the degree of AFT between strains. AFT is quantified as percent recovery of speed and is calculated by subtracting the ten minute relative speed from the thirty minute relative speed. A statistically significant difference between strains' percent recovery reflects a significant difference in AFT.

7.2.5 Statistics

Animals that were compared were treated one the same plate with speeds determined at the same time under identical conditions. Data were converted to relative speeds to account for basal speed differences between strains and for ease of viewing the results between experiments. Statistics were performed using Prism 5.0 (GraphPad). All comparisons were made to the control under the same condition and were calculated using paired two-tailed t-tests.

7.2.6 Lipid isolation

Lipids were extracted from whole worms as previously described [130] with slight modifications. Several thousand worms were washed from sixty 10 cm plates with water

and placed into 1.5 mL Eppendorf tubes. Worms were pelleted by centrifugation at 1,000 x g for 1 minute. As much water as possible was removed and 1 mL of 2.5% H_2SO_4 in methanol was added to the tube. The contents were transferred to glass scintillation vials and sonicated for 5 minutes in a Branson 2510 water bath sonicator at room temperature. The homogenate was transferred to a 1.5mL Eppendorf tube and incubated at 80℃ for one hour. The samples were then transferred to a glass vial and 0.2 mL of hexane and 1.5 mL of water were added. Fatty acid methyl esters were extracted into the hexane layer by vigorous shaking and centrifugation at 1,000 x g for one minute. The hexane layer was removed and given to Justin Poklis for analysis at the Department of Pharmacology and Toxicology GC/MS Core.

7.3 Results

7.3.1 Aim 1: Determine if RNAi knockdown and loss-of-function of suspected palmitoyltransferases results in altered ethanol response phenotypes.

To ascertain the role of palmitoylation in initial sensitivity and AFT, we took the approach of assessing the ethanol response of mutant animals lacking the activity of an individual palmitoyltransferase. We predicted that loss of certain palmitoyltransferases would alter the initial sensitivity and/or the development of AFT. *C. elegans* has at least fifteen suspected palmitoyltransferases, most of which are named for the conserved sequence of aspartate, histidine, histidine, and cysteine (DHHC).

Available loss-of-function alleles for each palmitoyltransferase were ordered from the *Caenorhabditis* Genetic Center. If mutant alleles were not available, we generated RNAi knockdown of target genes by feeding the worms *E. coli* expressing double-

stranded RNA against the gene of interest. All RNAi expressing bacterial strains were sequenced to ensure the proper gene was being targeted. Additionally, sequences were subjected to a BLAST search to find any possible off-target genes that may be knocked down. To date, we have tested eight of the 15 candidate palmitoyltransferases, and three altered the ethanol response phenotype.

RNAi knockdown of the suspected palmitoyltransferase *dhhc-6* resulted in a minor decrease in initial sensitivity and a significant reduction in AFT compared to worms fed an empty vector plasmid (Figure 11). This combination of initial resistance with a subsequent reduction in AFT phenocopied *slo-1* mutant worms. One possible interpretation of this result is that palmitoylation by DHHC-6 is necessary for the membrane expression of an ethanol target, perhaps SLO-1, either through trafficking or inhibition of internalization, and its loss both causes resistance and removes the chance for modulation of the target leading to a reduction in AFT.

A loss of function of *dhhc-14* resulted in an altered ethanol response phenotype from that of *dhhc-6*. *dhhc-14(gk330)* mutant animals trended toward increased sensitivity to ethanol compared to wild-type worms at ten minutes and, like *dhhc-6* knockdowns, these animals developed significantly less AFT (Figure 12), indicating that DHHC-14 activity is essential for the complete development of AFT. This result was of particular interest as it could be explained by a model where palmitoylation by DHHC-14 is not necessary for membrane expression of the ethanol target but is necessary for adaptation to ethanol activation via internalization or movement into a protective membrane microdomain.

RNAi knockdown of the palmitoyltransferase *hhat-2* resulted in a significant decrease in initial sensitivity with near wild-type AFT (Figure 13). While the initial resistance could be explained by a loss of an ethanol-target at the plasma membrane, the unaffected AFT suggests that *hhat-2* is not involved in the development of tolerance. Furthermore, this result could be interpreted as palmitoylation resulting in sensitivity to ethanol and suggests that the balance between palmitoylation and depalmitoylation may modulate the homeostatic mechanisms triggered by ethanol.

To further investigate a role for palmitoylation state in ethanol responses, we tested a *ppt-1* loss of function allele. *ppt-1* encodes a thioesterase that is predicted to function in the removal of palmitic acid from palmitoylated proteins. In this mutant we would expect the dynamics of palmitoylation to be affected. *ppt-1(gk134*) mutant animals displayed wild-type initial sensitivity to ethanol with a complete loss of AFT (Figure 14). These results would agree with a model where the mechanism of AFT is the degradation of palmitoylated proteins. Furthermore, this is the fourth distinct phenotype that we have identified from our examination of four palmitoylation-related enzymes which suggests that each enzyme may act on distinct palmitoylation sites either within a single protein or, more likely, between many proteins. While the current results do not yet allow for the development of a cohesive model, they do show that palmitoylation is a complicated mechanism that is, in part, responsible for the level of response to ethanol in *C. elegans*.

7.3.2 Aim 2: Determine if altered levels of fatty acids modulate ethanol response in C. elegans.

We next tested the importance of palmitoylation in the dynamic response to ethanol by altering the metabolism of palmitic acid. Much work has been done elucidating the enzymes responsible for fatty acid metabolism in worms [174]. RNAi knockdown of *elo-2*, the elongase responsible for conversion of palmitic acid to stearic acid, has been demonstrated to result in a marked increase in palmitic acid with subsequent decreases in C18:1n9 and C20:2n6 fatty acids [211]. We found that *elo-2* knockdown animals had wild-type initial sensitivity, but, like *ppt-1(gk134)* animals, completely lost their ability to develop AFT (Figure 15). This result showed that the lipid profile of a worm can dramatically affect its development of tolerance to ethanol. Whether the loss of AFT in *elo*-2 knockdown animals is due to the increase in palmitic acid, the reduction of other fatty acids, or some combination of the two is currently unclear. Many mechanisms involving lipid interactions may exist that are necessary for the development of AFT which do not involve palmitoylation. With this in mind, models investigating the role of specific lipids in AFT should be kept separate from those involving palmitoyltransferases until the data suggests otherwise.

7.4 Discussion

Our data suggest that there is an important role for palmitoylation in the acute response to ethanol. While we are not aware of any direct studies of the role of palmitoylation in the response to ethanol before our work, there are several observations that support a role for palmitoylation in the ethanol response in mammals.

Ethanol administration has been shown to alter hepatic protein palmitoylation and trafficking in mice after one week of chronic administration [212]. Chronic administration of opiates has also been shown to alter the palmitoylation states of G_s alpha and PSD-95, which correlated with in increased adenylyl cyclase activity and increased conditioned place preference to ethanol, respectively [212, 213]. These researchers suggest that the drug they administer misregulates the palmitoylation of their proteins of interest. We hypothesize that changes in palmitoylation states may also be the homeostatic response to a drug and that targeting palmitoylation may alter the molecular responses to a drug and perhaps interrupt cellular changes necessary for behaviorally important responses to ethanol in mammals. The human homologues of *dhhc-6, dhhc-14, ppt-1,* and *elo-2* (ZDHHC6, ZDHHC17, PPT1 and ELOVL4, respectively) are differentially expressed in the brains of alcoholics [207]. We are particularly intrigued by the fact that these are the human homologs of the palmitoylation genes for which we observed ethanol response phenotypes in our studies in worms. In addition, the functions of several known ethanol targets are modified by palmitoylation. We have shown, through genetic manipulations of palmitoylation-related genes, that palmitoylation plays an important role in the initial response to ethanol and the subsequent development of acute functional tolerance. Given that an individual's initial response to alcohol can be predictive of their likelihood to abuse alcohol, these palmitoylation genes represent attractive targets of research for understanding both the molecular responses to ethanol and addiction.

Our future studies will aim to identify the molecular targets of the palmitoyltransferases and to understand their roles in the response to ethanol. We have

identified the BK channel, SLO-1, as a likely candidate for palmitoylation for several reasons. First, there is experimental evidence that the mammalian SLO-1 (mSLO-1) is palmitoylated [179, 180]. Second, mSLO-1 is extremely homologous to *C. elegans* SLO-1. Third, we and others have observed that alterations in lipid profile can affect SLO-1 activity both *in vivo* [110] and *in vitro* [41]. Finally, we have used the CSS-Palm 3.0 software to predict palmitoylation sites in SLO-1. We have identified three cysteine residues on the SLO-1a isoform that are likely sites of palmitoylation. We will use site-directed mutagenesis to change each cysteine residue to an alanine, thereby preventing any palmitoylation at that site, and assess the ethanol response for each mutation. In this way, we may identify specific cysteine residues that are important in the initial response to ethanol.

We anticipate that additional studies will involve the identification of proteins that are palmitoylated during the development of AFT using cysteine accessibility assays and acyl-biotin exchange [214]. By manipulating the chemistry of the thioesterase bond, these techniques would allow us to biotin label proteins that have been palmitoylated, purify them and identify them by mass spectrometry. This global approach may be useful in confirming known channels that are palmitoylated as well as identifying novel targets of palmitoylation specifically during the development of AFT. A subtraction approach could also be used in palmitoyltransferase knockout animals to identify the targets of specific palmitoyltransferases. This technique, combined with the ethanol response assays could yield strong evidence for the role of specific proteins in AFT. Further behavioral experiments with animals in which these proteins are knocked out could confirm their role in ethanol response.

These studies highlight palmitoylation as one homeostatic mechanism that is likely to be required for the development of AFT. Understanding the interactions between ethanol response and palmitoylation will help us identify potential targets for pharmacological intervention for alcoholism in humans.

7.5 Figures

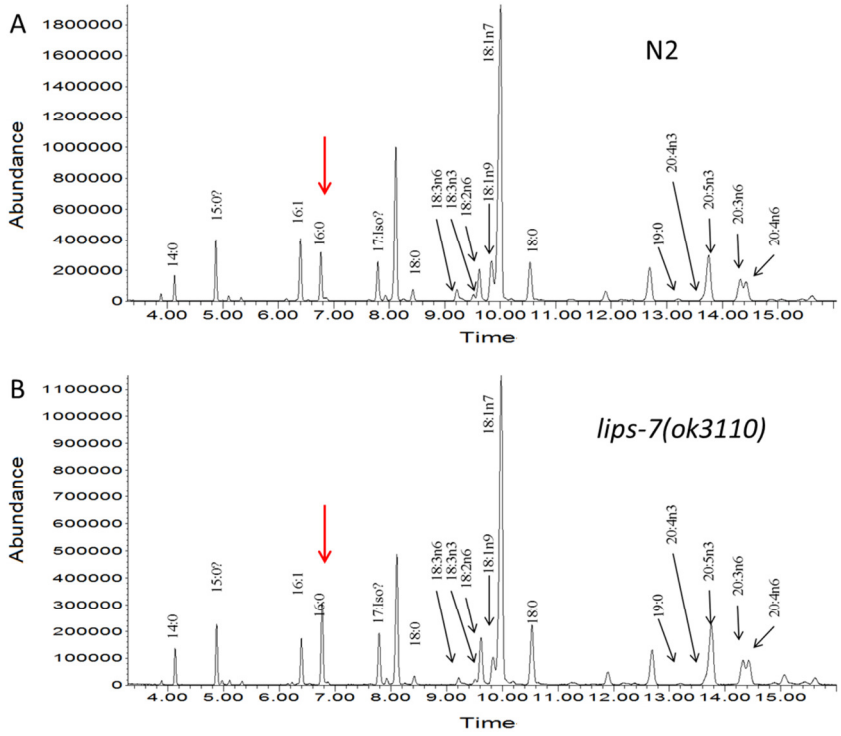

Figure 9. Loss of *lips-7* results in an altered lipid profile. a) GC/MS analysis of wild-type worms. Note the ratio between 16:0 and 16:1 fatty acids (palmitic and palmitoleic acids, respectively). b) GC/MS analysis of *lips-7(ok3110)* shows a possible increase in palmitic acid (16:0, arrow) when compared to palmitoleic acid (16:1) and this ratio appears to be different between the genotypes. This result suggests that palmitic acid may be increased in the *lips-7(ok3110)* background and may be partly responsible for the decrease in initial sensitivity and increased development of AFT. Data collected by Justin Poklis of the GC/MS Core (VCU).

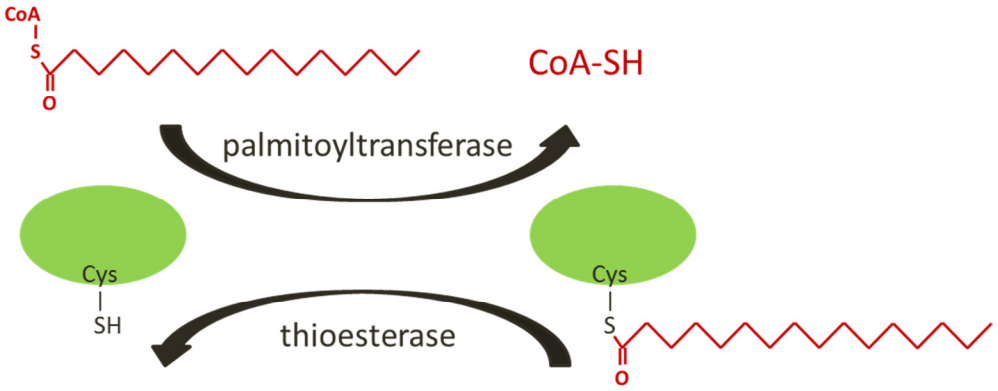

Figure 10. Protein palmitoylation is controlled palmitoyltransferases and thioesterases. Palmitoylation is the reversible addition of an activated palmitic acid to the cysteine residue of a protein by a palmitoyltransferase. This lipid modification can be removed by a thioesterase.

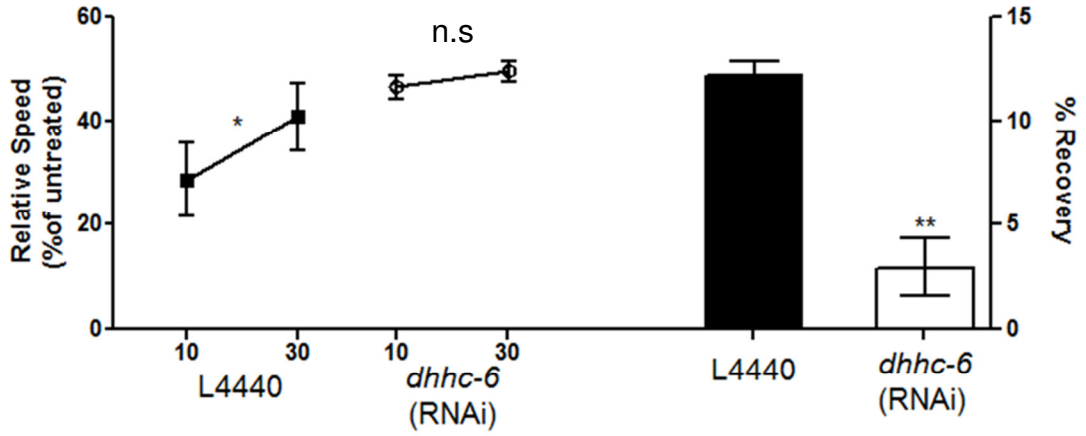

Figure 11. Reduction in DHHC-6 alters initial sensitivity and AFT. RNAi knockdown of the suspected palmitoyltransferase encoded by *dhhc-6* resulted in a phenotype that displayed initial resistance to the intoxicating effects of ethanol and a significant reduction in the development of AFT compared to wild-type worms fed empty plasmid vectors (n = 3). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, $p < 0.05$; **, $p<0.01$.

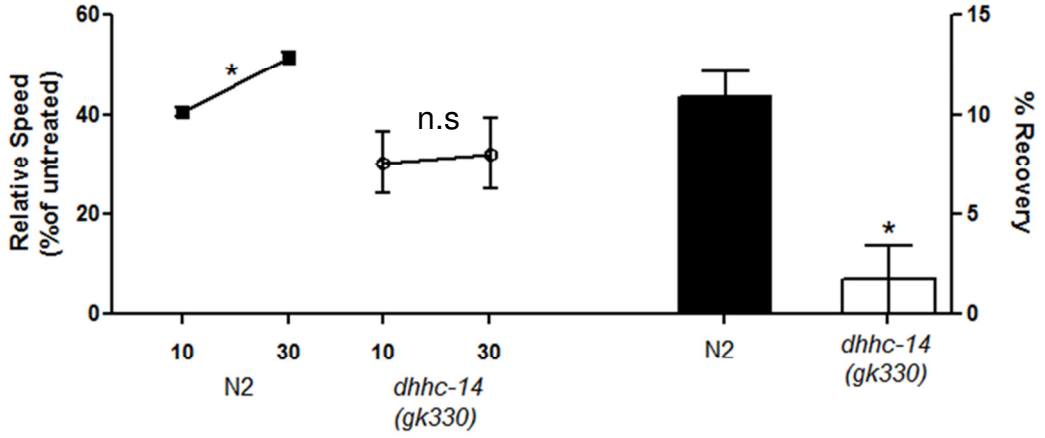

Figure 12. Loss of *dhhc-14* increases initial sensitivity and decreases AFT. *dhhc-14(gk330)* mutant animals trended towards increased initial sensitivity to the intoxicating effects of ethanol and a significant reduction in the development of AFT compared to wild-type worms (n = 3). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, $p < 0.05$.

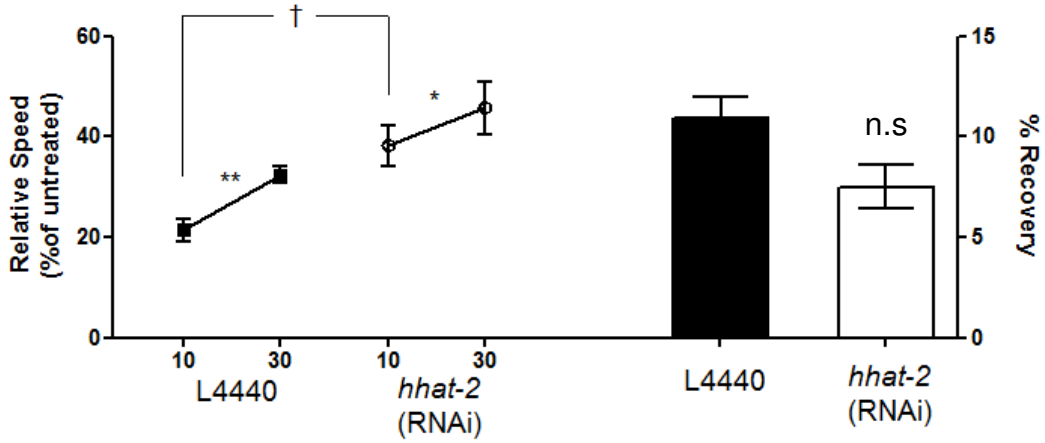

Figure 13. Reduction in HHAT-2 decreases initial sensitivity and does not affect AFT. RNAi knockdown of the suspected palmitoyltransferase encoded by *hhat-2* resulted in a phenotype that displayed initial resistance to the intoxicating effects of ethanol and wild-type development of AFT compared to wild-type worms fed empty plasmid vectors (n = 3). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, $p < 0.05$; **, $p<0.01$; for comparison to wild-type: †, $p < 0.05$.

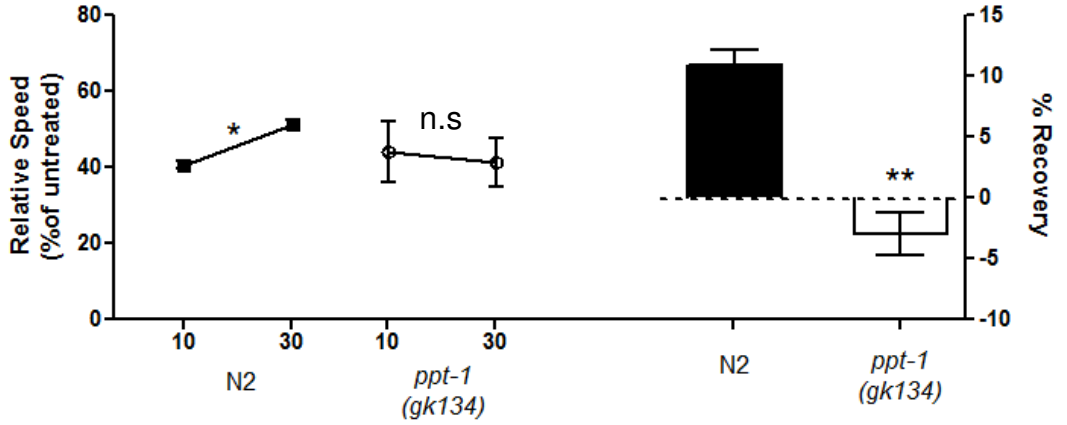

Figure 14. Loss of *ppt-1* does not affect initial sensitivity and abolishes AFT. *ppt-1(gk134)* mutant animals displayed wild-type initial sensitivity to the intoxicating effects of ethanol and a complete loss of AFT compared to wild-type worms (n = 3). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, $p < 0.05$, **, $p < 0.01$.

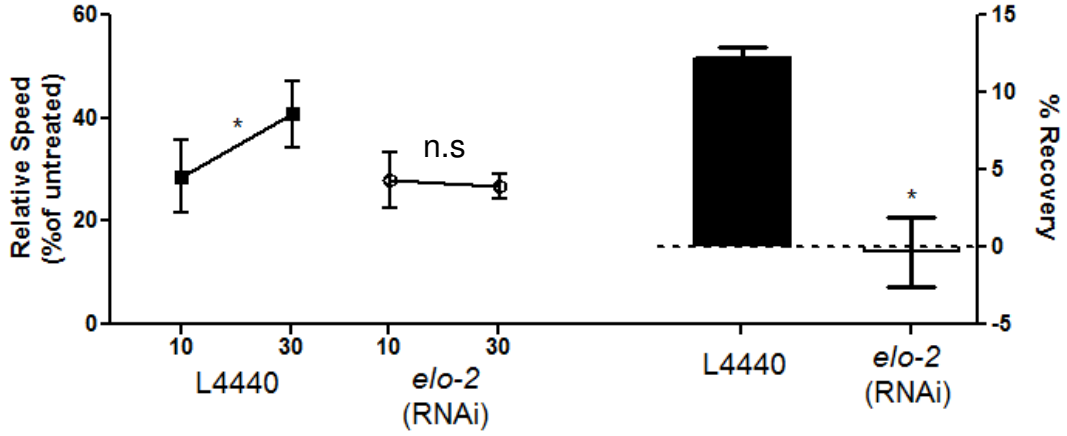

Figure 15. Reduction in ELO-2 does not affect initial sensitivity and abolishes AFT. RNAi knockdown of the elongase responsible for the conversion of palmitic acid to stearic acid resulted in wild-type initial sensitivity to the intoxicating effects of ethanol and complete loss of AFT compared to wild-type worms fed empty plasmid vectors (n = 3). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, $p < 0.05$.

8 Summary of Results

The work displayed in the previous two chapters highlights the involvements of palmitic acid and eicosapentaenoic acid in the level of response to acute ethanol in *C. elegans*.

EPA is necessary and sufficient to rescue AFT in mutant backgrounds that lack some or all LC-PUFAs. The mechanism by which EPA alters ethanol responding is unknown and EPA rescue does not correlate with cholinergic signaling. This leaves many possible roles for EPA in the ethanol response that must be tested. Briefly, EPA may be acting through (1) alterations in membrane fluidity or lipid microdomains, (2) lipid signaling either directly as a free fatty acid or as a precursor to a signaling molecule, or (3) direct protein interactions either on a target of ethanol or a protein necessary in the response to ethanol, or (4) gene regulation by EPA prior to ethanol exposure may alter the expression of ethanol-responsive proteins.

The loss of specific palmitoyltransferases, the enzymes responsible for attaching a palmitate to a protein, alters the acute response to ethanol in *C. elegans.* Palmitoylation of known protein targets has been demonstrated [176-182]. We have developed a hypothesis that the membrane localization of SLO-1 alters its activation by ethanol and that movement of the channel in and out of lipid microdomains allows for the development of AFT. It is also possible that additional targets of ethanol were being affected in our assays as at least three distinct phenotypes emerged. Numerous possible changes could occur in response to protein palmitoylation both directly to the protein (see Figure 2) and indirectly to any complexes the protein interacts with. Palmitoylation of a protein may move it into a protective lipid domain, move it into contact with its partners in a signaling complex or cascade, sequester the protein away

from its signaling cascade, or lead to downregulation of the protein through internalization.

All of the possible mechanisms for the involvement of these fatty acids warrant further investigation. To this end the following section will propose a few experiments or lines of investigation in hopes of elucidating a mechanism of action.

9 Future Directions

9.1 Lipid profiles in alcoholics

The level of omega-3 fatty acids has been shown to be altered in some patients suffering from mood disorders [200, 201, 203, 215-217]. Omega-3 fatty acid levels have also been shown to correlate with dopamine release in rodent models suggesting a possible mechanism for omega-3 involvement in depression [203]. Co-morbidity of mood disorders, alcohol use, and alcohol abuse have also been found [2, 202] . Given that numerous lipid metabolism genes have been found to be either misregulated in alcoholics [206, 207] or alter ethanol responses (e.g., KLF-3 (unpublished result), *fat-1, fat-4, fat-3, elo-2, elo-1, fat-5, fat-6, ctbp-1,* and *lips-7*) I recommend mammalian studies, especially in humans, monitor red blood cell lipid profiles when testing the effects of acute and chronic ethanol [56, 126, 200, 201]. There are two important questions to be asked in these studies: (1) Is lipid profile predicative of level of response to ethanol? And (2) Are lipid profiles altered by chronic ethanol exposure? It is very possible that these two questions are intimately related. If the initial response to ethanol is affected by lipids and the response then alters the lipid profile one could imagine a feedback loop. While I believe the entire lipid profile should be measured, I expect that changes in omega-3 levels (EPA and DHA) will most likely be observed. It is important to note that while *C. elegans* does not have DHA, I expect the roles of EPA in the nematode may mimic the roles of DHA in mammals. This comes from the observation that EPA is enriched in neuronal membranes in *C. elegans* while DHA is enriched in mammalian neuronal membranes [117, 142, 199, 218, 219]. Additionally, changes in palmitic acid may also be expected given our results with palmitoyltransferases.

Changes in the mammalian system may help identify new genes or signaling molecules of interest. For example, if a single fatty acid change is correlated to ethanol response then the metabolites of that fatty acid should be investigated as well as any known receptors or interacting proteins.

9.2 Altering lipid levels may change the response to ethanol

Whether or not lipid profiles correlate with ethanol response or are altered in response to ethanol, a still larger question remains: Can dietary fatty acids alter the response to ethanol? We know that the initial response to ethanol is predicative of abuse liability [94] and that the level of response is composed of at least the initial sensitivity and the development of AFT [191]. The level of dopamine release during the first exposure to ethanol has been shown to be predictive of voluntary drinking in multiple rat strains [56] and omega-3 fatty acids have been shown to alter dopamine release in rodent models [203, 217, 220]. Taken together, a possibility emerges that altering dopamine release through dietary omega-3 fatty acids will alter the response to ethanol. Testing with the no, low, and high omega-3 diets could be performed in the loss-of-righting reflex assay. This would test if diet can alter the initial response to ethanol. Additionally, switching the diets of stably responding mice should be tested to see if it alters ethanol responses. Being able to alter stable responding by switching diet would be a strong argument for omega-3 treatment of AUDs. Further testing of this possibility could include monitoring of dopamine levels, preferably in the NAC, by microdialysis. Both the initial and subsequent dopamine releases should be monitored with each ethanol administration. This may not be possible in a voluntary drinking paradigm so ethanol-induced dopamine release should be monitored at various time

points to assess change over time. Changes in ethanol induced dopamine release caused by EPA supplementation could suggest changes in the rewarding properties of alcohol although this would not be a measure of reward. These experiments would give valuable insight into how dietary fatty acids could alter responding and potentially abuse liability. Omega-3 diets have also been shown to reduce stress in abstinent alcoholics [205] and fatty acid metabolism has been suggested as a target for treating cocaine addiction [143, 144]. It is possible that a diet rich in omega-3 fatty acids should be tested as a treatment for alcohol abuse disorders.

9.3 Site-directed mutagenesis of SLO-1 palmitoylation sites and monitoring of localization

We have identified the BK channel, SLO-1, as a likely candidate for palmitoylation for several reasons. First, there is experimental evidence that the mammalian SLO-1 (mSLO-1) is palmitoylated [179, 180]. Second, mSLO-1 is extremely homologous to *C. elegans* SLO-1. Third, we and others have observed that alterations in lipid profile can affect SLO-1 activity both *in vivo* [110] and *in vitro* [41]. Finally, we have used the CSS-Palm 3.0 software to predict palmitoylation sites in SLO-1. We have identified three cysteine residues, Cys73, Cys74, and Cys659 on the SLO-1a isoform that are likely sites of palmitoylation. We could use site-directed mutagenesis to change each cysteine residue to an alanine, thereby preventing any palmitoylation at that site, and assess first the rescue of the *slo*-1 null phenotype followed by the ethanol response for each rescuing mutant. In this way, we may identify specific cysteine residues that are important in the initial response to ethanol. It will be important to test for rescue of *slo-1* phenotypes with each mutated construct. One of the palmitoylation sites on SLO-1

may be important for its proper trafficking to the membrane. If this is the case, we would predict that a mutation here would phenocopy a SLO-1 null. Because assessing the phenotype may be difficult (e.g., the palmitoylation site involved in ethanol responding also causes the phenotypes observed in SLO-1 null, meaning the channel is appropriately trafficked but the transformed line phenocopies a null allele) I would suggest the addition of a tag to the injected SLO-1. We know we are able to separate lipid raft domains and can use UNC-1::GFP as a marker of this (Figure 16). If we're also able to detect SLO-1 in a Western blot, we'd be more confident in determining if the mutated SLO-1 was properly trafficked to the membrane. Additionally, this construct would allow us to monitor the localization of SLO-1 over time and exposure to ethanol. We've hypothesized that SLO-1's movement in the membrane is necessary for the development of AFT [110] and that this may be modulated by palmitoylation. These experiments may intersect if this hypothesis is correct. If SLO-1 does alter its localization in response to ethanol and it's mediated by palmitoylation, then one of the mutated sites may create a construct that rescues the basal SLO-1 phenotypes but does not rescue the development of AFT. This would be a very exciting result!

The previously tested SLO-1::GFP construct was not reliably detected by antibodies we tested so I recommend a FLAG or HA tagged version be used with an antibody known to work with the tag.

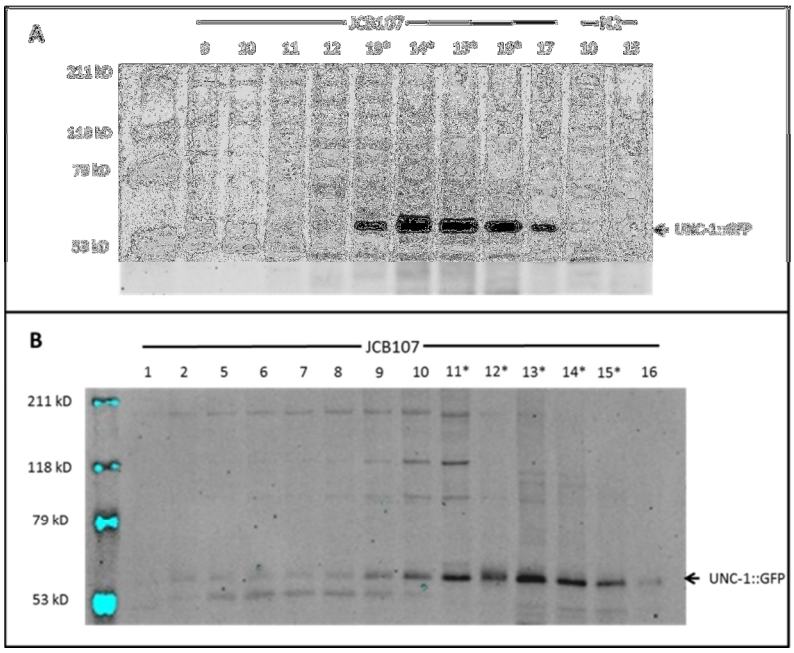

Figure 16. Detergent resistant membrane (lipid rafts) can be isolated from *C. elegans*. UNC-1 is a protein almost exclusively associated with lipid rafts and is thus used as a marker lipid rafts.

9.4 Identification of palmitoylated proteins in *C. elegans*

Further studies may identify additional ethanol responsive proteins that are targets of palmitoylation. I propose the use of acyl-biotin exchange [214] to identify proteins that are palmitoylated in response to ethanol and proteins that are no longer palmitoylated in a palmitoyltransferase mutant. By manipulating the chemistry of the thioesterase bond, this technique would allow us to biotin label proteins that have been palmitoylated, purify them and identify them by mass spectrometry. This global approach may be useful in confirming known channels that are palmitoylated as well as identifying novel targets of palmitoylation specifically during the development of AFT. If a large portion of proteins are palmitoylated, then a subtraction approach could also be used in palmitoyltransferase knockout animals to identify the targets of specific palmitoyltransferases. Results from these experiments would not only identify the

protein involved and the residue that causes the phenotype, but also give the mechanism by which it is occurring. This would allow for very focused experiments in other model organisms and possibly narrow down polymorphism to look for in GWAS studies (i.e. mutations that change a palmitoyltransferase accessible cysteine residue). The techniques necessary for these experiments may take time to develop and learn but could yield multiple proteins involved in AFT and hopefully multiple publications given the highly specific results.

9.5 *klf-3* supplementation with fatty acids

Mutation of *klf-3* decreases palmitic acid levels, increases *fat-1* expression (as well as changing the expression of many other lipid-related genes) [112] and disrupts the development of AFT. Supplementation with EPA or palmitic acid may rescue this phenotype. While on its own this would not elucidate the mechanism by which KLF-3 affects AFT, it would tie into the work presented here and would be another piece of the emerging story of fatty acid involvement in the level of response to acute ethanol. Likewise, no rescue suggests a mechanism that may be separate from those observed in this thesis work which would allow for other lines of investigation.

9.6 Ethanol reponses at lower doses

We often test various exogenous doses of ethanol when assessing new strains of worms. However, we typically only publish the results of 400 and 500 mM exposures. This is mainly due to the observation that AFT only develops when the worms become sufficiently intoxicated. A 200 mM dose of ethanol results in a significant decrease in

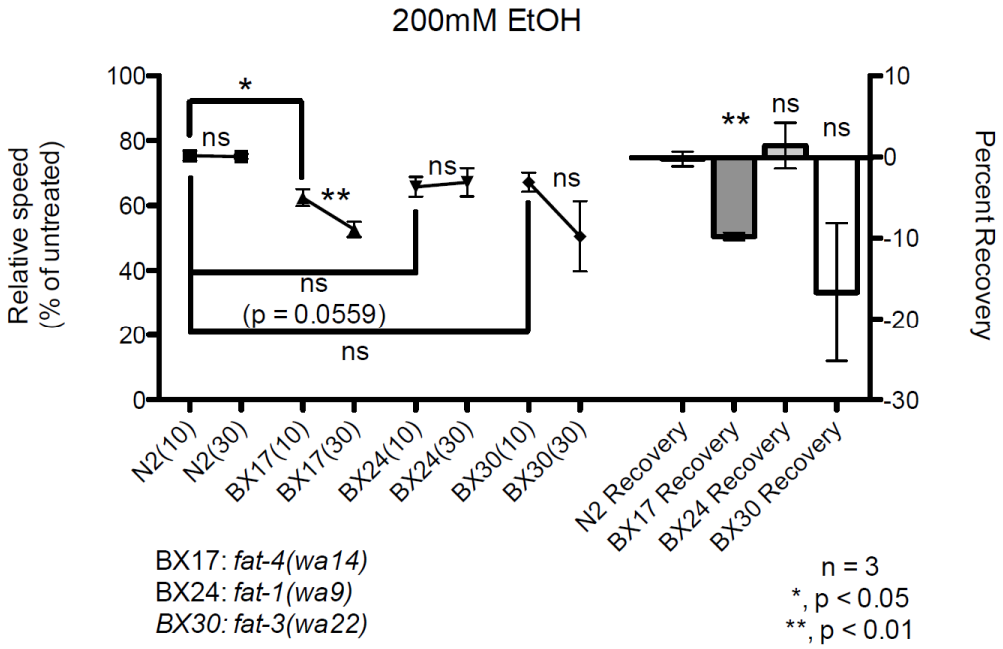

Figure 17. N2, *fat-1, fat-4,* and *fat-3* on 200 mM exogenous ethanol. Note the significant reduction in speed from 10 to 30 minutes observed in *fat-4(wa14)* and the non-significant but obvious decrease in *fat-3(wa22)*.

locomotion at ten minutes with no change in speed at the 30 minute time point. Mutant strains, however, can respond differently than N2 at this dose (Figure 17). These three mutants (*fat-1, fat-4,* and *fat-3*) all fail to develop AFT at 400mM doses of ethanol but have different phenotypes from each other at 200 mM. There may be a fundamental difference in the pathways affected at 200 and 400 mM ethanol that are also affected differently between the *fat* mutants. A complete curve of the speeds over time at 200 mM ethanol may offer better insights into what exactly is occurring. While I do not have

a concrete suggestion for how these phenotypes should be discussed, I think they still warrant further investigation and may expose different ethanol response pathways that were masked by a 400 mM dose. If these responses are separable, the 200 mM response may be confounding some of the 400 mM results.

9.7 Testing of other *fat* phenotypes

Mutations in nearly all the genes involved in fatty acid synthesis were tested for their effects on AFT. Mutations in genes that have little or no effect of EPA levels (Table

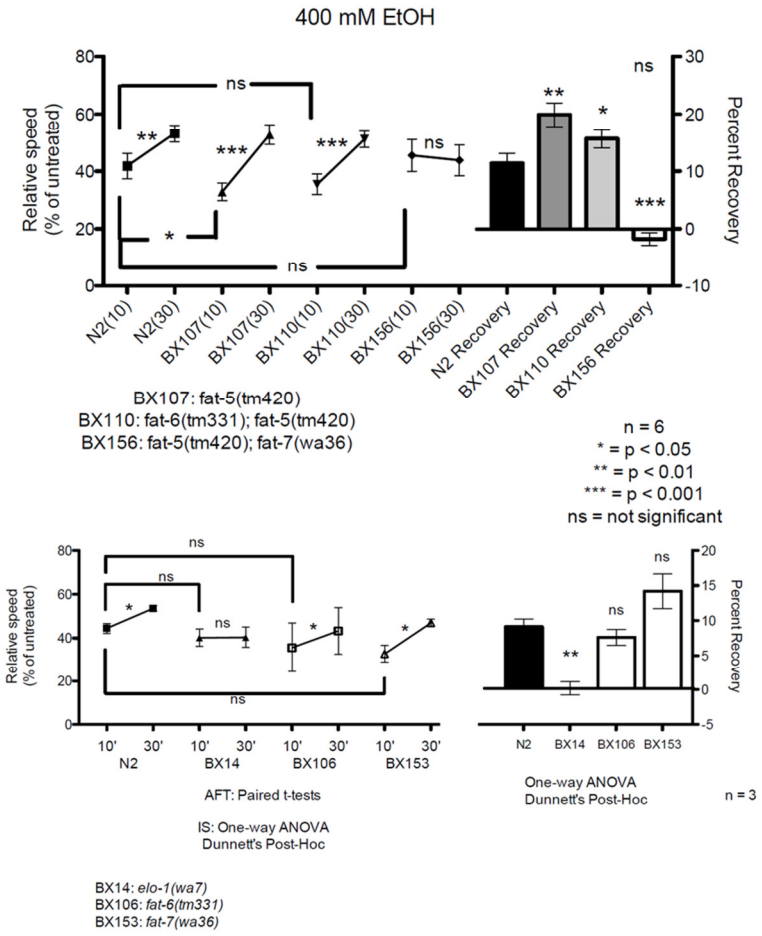

Figure 18. Other mutations in the lipid metabolism pathway alter LR.

1) can still alter AFT. Intriguingly, increases in AFT were observed for strains carrying *fat-5(tm420)*. Investigation of the pathway should assess supplementation with any altered fatty acids, possible metabolites, as well as a search for genes that may be regulated by *fat-5*. A cursory search on WormBase.org shows many genes interact with *fat-5* but a few stand out: three nuclear hormone receptors (*nhr-35*, *nhr-49*, and *nhr-80*) and two transcription factors (*daf-16* and *sbp-1*). Misregulated genes in the *fat-5* background could be identified by microarray analysis and would yield candidates for further testing. A proper lipid profile for *fat-5(tm420*) should also be completed as one does not exist in the literature. Care should be taken in these experiments as loss of any of the *fat-5, fat-6, or fat-7* desaturases causes upregulation and compensation by the remaining two. For this reason different phenotypes can be observed between genetic nulls and RNAi knockdown strains [128]. Additionally, if the loss of other fatty acids needs to be tested during the course of these experiments Table 2 should offer insights into how to target individual fatty acids.

Strain	LA	ALA	GLA	DGLA	O3AA	AA	EPA	IS	AFT
fat-1(wa9)	NC	NC	NC	↑	X	↑	X	NC	X
fat-4(wa14)	NC	NC	NC	↑	↑	X	X	NC	X
fat-1(wa9); fat-4(wa14)	NC	NC	NC	↑	X	X	X	NC	X
fat-3(wa22)	↑	↑	X	X	X	X	X	↑	X
fat-5(tm420); fat-7(wa36)	↑	N.D.	N.D.	NC	NC	NC	NC	N.D.	N.D.
fat-5(tm420); fat-6(tm331)	NC	N.D.	N.D.	NC	NC	NC	NC	NC	↑
fat-6(tm331); fat-7(wa36)	X	N.D.	N.D.	X	X	X	X	NC	X
fat-2(wa17)	NC	X	X	↓	X	X	↓	N.D.	N.D.
fat-5(tm420)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	↑	↑
elo-1(wa7)	NC	NC	↑	↓	↓	X	↓	NC	↓
elo-2 (RNAi)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	NC	X

Table 1. A summary of the approximate change in fatty acid profile and the resulting LR phenotypes. NC = no change, N.D. = not done, X = not detected, ↑ = increase, ↓ = decrease. Data compiled from various works by Jennifer Watts.

Fatty Acid to Remove	Genes to Target	Suspected Fatty Acids Lost	Suspected Fatty Acids Increased	Fatty Acid(s) to Feed
16:0	*fasn-1*^{A}	All downstream	Malonyl-CoA	16:1, 18:0
16:1	*fat-5*^{AVS}	16:1, 18:1(n-7)	16:0	18:1(n-7)
18:1(n-7)	*elo-2*^{AV}	18:0 and downstream	16:0, 16:1	18:0, 20:3(n-6), 20:4(n-3)
18:0	*let-767*^{AV}, *elo-1*^{AVS}, *elo-2*^{A}	18:0 and downstream	16:0, 16:1	18:1(n-9), 20:3(n-6), 20:4(n-3)
18:1(n-9)	*fat-6*^{AS}, *fat-7*^{AVS}	18:1(n-9) and downstream	18:0	18:2(n-6)
18:2(n-6)	*fat-2*^{AS}	18:2(n-6) and downstream	18:1(n-9)	18:3(n-3), 18:3(n-6)
18:3(n-3)	*fat-1*^{S}	18:3(n-3), 18:4(n-3), 20:4(n3), 20:5(n-3)	20:4(n-6)	18:4(n-3)
18:3(n-6)	*fat-3*^{AVS}	18:3(n-6), 20:3(n-6), 20:4(n-6), 18:4(n-3), 20:4(n-3), 20:5(n-3)	18:3(n-3)	18:4(n-3), 20:3(n-6)
20:3(n-6)	*let-767*^{AV}, *elo-1*^{AVS}, *elo-2*^{A}	18:0 and downstream	16:0, 16:1	18:0, 18:1(n-7), 20:4(n-3), 20:4(n-6)
18:4(n-3)	*fat-3*^{AVS}	18:4(n-3), 20:4(n-3), 20:5(n-3), 18:3(n-6), 20:3(n-6), 20:4(n-6)	18:2(n-6), 18:3(n-3)	18:3(n-6)
20:4(n-3)	*let-767*^{AV}, *elo-1*^{AVS}, *elo-2*^{A}, *fat-1*^{S}	18:0 and downstream	16:0, 16:1	18:0, 18:1(n-7), 18:3(n-3), 20:3(n-6)
20:4(n-6)	*fat-4*^{AVS}	20:4(n-6), 20:5(n-3)	20:3(n-6), 20:4(n-3)	20:5(n-3)
20:5(n-3)	*fat-1*^{S}, *fat-4*^{AVS}	20:4(n-6), 20:5(n-3)	20:3(n-6), 20:4(n-3)	20:4(n-6)

Table 2. Proposed targeting of lipid metabolism enzymes to remove individual fatty acids. Superscript next to gene names denote the following: A, Ahringer RNAi available; V, Vidal RNAi available; S, strain available from the CGC.

9.8 Lipid signaling molecules derived from EPA

EPA may be acting as a precursor to signaling molecules that are necessary for the development of AFT. Future studies should target the enzymes responsible for the metabolism/conversion of EPA. A method similar to the one used by Kulas *et al* with pools of RNAi may be the quickest way to test the 75 P450 genes [187]. Identification of a necessary P450 gene could then lead to identification of the EPA-derived signaling molecule by GC/MS. If they are stable, the identified molecules could then be supplemented into a mutant background deficient in EPA to test the sufficiency of the metabolite in the development of AFT. Likewise, upregulation of lipid signaling molecules is possible through treatment with fibrates. Results from RNAi knockdown of CYP-33E2 and fenofibrate treatment were inconclusive (data not shown) and warrant additional trials.

9.9 EPA gene regulation

While transcription is not necessary for the development of AFT, supplementation with EPA may be rescuing AFT by regulating genes that are necessary for its development. An 8 hour treatment with EPA was not sufficient to rescue AFT (data not shown) while a 19 hour treatment was. This could be due to the 19 hour exposure occurring during the L4 to adult molt or due to insufficient time for EPA-regulated genes to be adequately transcribed and translated. Microarray analysis comparing gene regulation of *fat-1(wa9)* with and without 19 hour EPA supplementation should yield candidate genes. If null

alleles of the EPA regulated genes phenocopy *fat-1(wa9)* then EPA may be rescuing AFT through the expression of that gene. Supplementation of EPA of a strain carrying the null allele would test this possibility. Likewise a double mutant of the EPA-regulated gene with *fat-1(wa9)* should yield a mutant that cannot be rescued by EPA supplementation. This would refine the role EPA is having in rescuing AFT.

10 References

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11 Appendix I - Protocols

Title: Aldicarb Plates and Assays

Purpose:

This protocol describes how to pour NGM plates that are supplemented with aldicarb. Aldicarb is an acetylcholinesterase inhibitor that causes tonic paralysis in *C. elegans*. The time it takes for the worms to paralyze is directly proportional to the amount of acetylcholine they release. Strains that release more neurotransmitter will paralyze sooner than those that release less.

Resources:

Mahoney, T.R., S. Luo, and M.L. Nonet, Analysis of synaptic transmission in Caenorhabditis elegans using an aldicarb-sensitivity assay. Nat Protoc, 2006. 1(4): p. 1772-7.

Safety:

Wear gloves and eye protection when working with aldicarb. Work under the hood whenever possible. If possible do not pour aldicarb plates in the plate pouring room. Either move the pump or pour the plates using a graduated pipette. Inhaled aldicarb can cause paralysis.

Pouring Aldicarb Plates:

1. Determine the volume of agar required based on the number of aldicarb plates required for the assay. Smaller plates are better for this assay.

_____	x	_____	=	_____	+	10 mL	=	_____
Number of plates		Volume per plate (typically 5 mL)		Exact volume required		Extra volume		Volume of agar to make

2. Label an autoclavable bottle that can hold a volume equal to approximately twice the volume of agar being made.

3. Calculate the mass of aldicarb that will be required. For strains that are expected to be resistant use 1 mM aldicarb. For sensitive strains use 0.5 mM aldicarb.

_____	x	_____	x	_____	÷	1,000	=	_____
Concentration (mM)		MW of aldicarb (g/mol)		Volume of Agar (L)				Mass of aldicarb (mg)

4. Calculate the amount of water necessary to dissolve the aldicarb to 50 mM:

_____	÷	_____	÷	_____	x	1,000	=	_____
Mass of aldicarb (mg)		MW (g/mol)		Concentration (mM)				Volume of dH_2O (mL)

5. Dissolve the mass of aldicarb, calculated in Step 3, in the volume of sterile dH_2O, calculated in Step 4. Leave shaking while autoclaving the agar. If the aldicarb is not fully dissolved when the agar is ready, put the tube at 37C for 15 - 30 minutes.

6. Calculate the mass each component required for the agar:

Reagent	Amount per Liter		Volume of Agar (L)		Amount Required
NaCl	3 g	x	_____	=	_____ g
Bacto Agar	16 g	x	_____	=	_____ g
Bacto Peptone	2.5 g	x	_____	=	_____ g
1M K_3PO_4	25 mL	x	_____	=	_____ mL
1M $MgSO_4$	1 mL	x	_____	=	_____ mL
1M $CaCl_2$	1 mL	x	_____	=	_____ mL
5 mg/mL Cholesterol	1 mL	x	_____	=	_____ mL

7. Add the NaCl, Bacto Agar, and Bacto Peptone to the autoclavable container and QS to the volume required minus the volume of water needed to dissolve the aldicarb in Step 4. Swirl the container.

8. Either loosely screw the cap on the bottle or cover the top with tin foil and then add a piece of autoclave tape.

9. Turn on the water bath in the plate pouring room and set it to 60C.

10. Autoclave for the recommended time based on the autoclave specifications.

11. Place the agar in the 60C water bath for 0.5 – 1 hour.

12. Add the K_3PO_4, $MgSO_4$, $CaCl_2$, cholesterol, and aldicarb solution. This can be done in or out of the water bath.

13. Swirl the containers.

14. To the control agar add a volume of dH_2O equal to the volume used to dissolve the aldicarb in Step 4.

15. Ensure the aldicarb is dissolved in the dH_2O. If not, warm the solution to 37C and vortex. Repeat this until the aldicarb is in solution. Then add the aldicarb solution to the aldicarb agar.

16. Swirl both containers.

17. Leave the aldicarb agar in the 60C water bath while pouring the control agar. This will allow you to use the same tubing to pour both containers. Alternatively, graduated pipettes can be used to dispense smaller batches.

18. Dispense the aldicarb agar using the same settings. If the same tubing is being used, consider discarding the first plate poured as it may have less aldicarb from residual control plate agar.

19. Label the plates (color coding works well as long as you have a legend with the plates).

20. Store plates in the dark at room temperature overnight to solidify.

21. The next day, move plates to a light protected container (e.g., a box wrapped in tin foil) and store at either 4 or 20C.

Expiration:

Plates have been used for over a month, however the potency of the aldicarb decreases over time. All assays being compared should be performed on the same day.

Aldicarb Assay:

The timing for the assay will depend on the aldicarb concentration and the strains being used. Typically paralysis is assessed every 15 to 30 minutes until complete paralysis is observed in all strains.

Assays are completed in at least triplicate with ten to thirty worms per plate. Use fewer worms on assays that require shorter intervals and more plates.

Two control strains are used to ensure the aldicarb plates are still good, the sensitive strain *dgk-1(nu62)* and the resistant strain *unc-10(e102)*. Only one plate of each strain needs to be used and complete paralysis does not need to occur in unc-10(e102).

1. Transfer age matched adult worms (15 is a good starting point) to aldicarb plates in triplicate. Do not parafilm the plates.
2. Write the time on each plate to ensure plates are observed at the correct times.
3. You decide how to assess paralysis. The important thing is that you're consistent. See the Nature Protocols paper for suggestions. Previous assays in the lab have assessed paralysis by first tapping the plate to find worms that move freely, then by poking each worm three times in the head and three times in the tail to elicit a movement response. Any movement (but not a contraction) is counted as a worm that is not paralyzed.
4. Assess paralysis in all worms at the determined time points.
5. Continue assessing paralysis until all worms are paralyzed (except *unc-10(e102)*).

6. Calculate the percentage of worms paralyzed at each time point for each trial.

7. Average the percentages across each trial at the same time point.

8. Depending on the number of strains being compared, use a statistical test (e.g. an unpaired t-test or one-way ANOVA) to compare strains at relevant time points. Alternatively, the time required for half of the worms to become paralyzed could be calculated similar to calculating an ED50 or LD50.

Title: Age Synchronization by Bleaching

Purpose:

The purpose of this protocol is to obtain a population of worms that are the same age. This is accomplished by breaking apart worms with a bleaching solution that leaves eggs intact. The eggs are then allowed to hatch in the absence of food to arrest all worms at the L1 stage.

Resources:

Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., Cerón, J. Basic *Caenorhabditis elegans* Methods: Synchronization and Observation. *J. Vis. Exp.* (64), e4019, doi:10.3791/4019 (2012). Link: http://www.jove.com/video/4019/basic-caenorhabditis-elegans-methods-synchronization-and-observation

Method:

1. Make the bleaching solution fresh by adding 2 mL of sodium hypochlorite (bleach) and 1 mL of 5M NaOH to 7 mL of dH_2O.
2. Wash gravid adults from plates with M9 and pipette them into a 15 mL conical. Eggs can also be collected by scraping the plate with a flexible piece of plastic.
3. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the worms and eggs. This speed can be adjusted. Try to avoid pelleting the bacteria.
4. Remove the supernatant.
5. Add 10 mL of M9.
6. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the worms and eggs.

7. Remove the supernatant. (Repeat the rinsing until the M9 is mostly clear. This will mean that most of the bacteria has been removed.)
8. Add 1 – 2 mL (or enough to suspend the worms) of bleaching solution to the worm pellet.
9. Using the dissecting scope to monitor, shake the conical until all worms have burst.
10. Immediately fill the conical with M9.
11. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the eggs. This speed can be adjusted.
12. Remove the supernatant.
13. Add 10 mL of M9.
14. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the eggs. This speed can be adjusted.
15. Remove the supernatant.
16. Add 10 mL of M9.
17. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the eggs. This speed can be adjusted.
18. Remove the supernatant.
19. Using a glass pipette transfer the egg pellet to a new conical with 10 mL of M9.
20. Parafilm the lid clockwise to ensure the cap doesn't come off or leak.
21. Leave the conicals on the shaker overnight at room temperature (or at 20C if possible).

22. The next day, centrifuge the conicals for 30 seconds at 1,000 x g to pellet the L1s
23. Using a glass pipette, transfer the pelleted worms to OP50 seeded NGM plates.

Use:

This protocol can be used either to bleach contaminated plates or to synchronize a large number of worms for assays. Ensure that controls are also bleached and age-matched in this manner. Ideally egg laying should be used to age match worms if possible. This protocol should be used when worms need to be age matched most closely.

Title: Membrane Extraction for GC/MS Analysis

Purpose:

The purpose of this protocol is to describe how to extract the total lipids from *Caenorhabditis elegans* for analysis by gas chromatography/mass spectrometry. This will allow for individual fatty acid species to be identified and quantified (absolute or relative). This protocol will aid in the detection of altered fatty acid metabolism and also confirm the intake of dietary fatty acids.

Resources:

Watts, J. L., & Browse, J. (2002). Genetic dissection of polyunsaturated fatty acid synthesis in Caenorhabditis elegans. Proc Natl Acad Sci U S A, 99(9), 5854-5859. doi: 10.1073/pnas.092064799

Methods:

1. Turn on a hot plate or water bath to 80℃.
2. Wash five plates of age synchronized, adult worms into 15 mL conicals using M9.
3. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the worms. This speed can be adjusted. Try to avoid pelleting the bacteria.
4. Remove the supernatant.
5. Add 10 mL of M9.

6. Centrifuge the conicals for 30 seconds at 1,000 x g to pellet the worms again.
7. Using a glass pipette, transfer the worm pellet to a glass vial with a screw on cap. Glass scintillation vials work well.
8. Add 1 mL of 2.5% H_2SO_4 in methanol to each glass vial.
9. Make sure the cap is securely fastened. It is recommended to tape the cap to the glass vial to ensure it stays on during the next step.
10. Sonicate each capped vial in an ice-cold water bath sonicator for 3-5 minutes or until worms are completely broken apart. The sample will appear milky. (Sonication may also be accomplished using a probe sonicator, but this has not been tested).
11. Incubate the capped vials at 80℃ for 1 hour.
12. Add 0.2 mL hexane and 1.5 mL dH2O.
13. Shake the vials vigorously by hand and vortex.
14. Allow the vials to sit for 5 minutes. The organic and inorganic layers should separate.
15. Transfer the top, hexane layer to a new glass vial with a glass pipette. Ensure that you don't get any of the debris in the interface between the layers. It is more important that good sample is collected than a large volume of sample.
16. Ensure the vial is capped.

Storage:

Store sample at -80℃ until they are ready for GC/MS analysis by Justin Poklis.

GC/MS Analysis:

From the reference above, for GC/MS analysis: Samples (1µl) of the organic phase were analyzed by GC using an Agilent 6890 series gas chromatographer equipped with a 30 x 0.25-mm SP-2380 column (Supelco), helium as the carrier gas at 1.4 ml min, and a flame ionization detector. The gas chromatograph was programmed for an initial temperature of 120℃ for 1 min followed by an increase of 10℃ min to 190℃ followed by an increase of 2℃ min to 200℃.

Title: Lipid Raft Isolation

Purpose:

This protocol describes how to isolate lipid raft fractions from *Caenorhabditis elegans* membrane. This protocol can be used to assess the location/proportion of lipid raft interacting proteins during the exposure to ethanol.

Worm Preparation for Lipid Raft Isolation

Five L4 worms were picked to each of ten 5 cm plates, seeded with OP50. The subsequent F1 population was allowed to develop to gravid adults. At this stage, the ten plates were washed with M9 to collect worms. (See Age Synchronization by Bleaching) The worms were pelleted and resuspended in a bleach solution (20% v/v bleach with 0.5 M NaOH in dH_2O) and shaken vigorously for approximately five minutes. M9 was added after worms had lysed but before eggs were damaged. The eggs were spun down and washed twice with M9. After the final wash, the eggs were transferred to a 15 mL conical with 10 mL of M9. The conical was parafilmed and allowed to shake overnight and hatch to synchronize the F2 population at the L1 stage. Each conical was divided, via pipetting, between sixty OP50-seeded 10 cm plates. The F2 population was allowed to develop to first day adults before testing.

Sixty 10 cm plates seeded in this manner will not exhaust the OP50 before the F2s reached adulthood. This protocol will yield approximately 1 – 2 mL of worms depending on strain (size, egg laying, etc).

For seeding 10 cm plates it is suggested to use a shaken culture of OP50 or to allow the lawn to grow for two days prior to use.

Membrane Isolation Method:

1. Ensure that the S-Basal, homogenization buffer, and TNE with 1% Triton X-100 are on ice (see recipes below).
2. After EtOH exposure, wash worms from the large assay plates to 15 mL conicals using 5 mL of S-Basal per plate. Pool identical plates if possible.
3. Pellet worms by immediately spinning conicals at 3,000 x g for 1 minute.
4. Remove the supernatant.
5. Add a volume of homogenization buffer with protease inhibitors that is sufficient to cover the worms. (Excess buffer will make sonication more difficult).
6. Using a glass pipet, transfer the worms to glass scintillation vials.
7. Sonicate the scintillation vials in a water bath sonicator with ice for approximately 2 minutes.
8. Ensure lysis of worms by observing the scintillation vials under the microscope (the solution will also become very cloudy).
9. Transfer homogenate to 1.5 mL eppendorf tubes.
10. Pellet cellular debris by spinning the eppendorf tubes at 1,000 x g for 5 minutes.

11. Transfer the supernatants from each eppendorf tube to a Beckman ultracentrifuge tube and spin at 60,000 x g spin for 30 minutes to pellet the membranes. (Balance with homogenization buffer).
12. Resuspend the membrane pellet in the desired buffer – or – see below for lipid raft isolation.

Lipid Raft Isolation Method:

1. Pour a sucrose gradient by carefully layering 3.5 mL of 40% sucrose, 7 mL of 35% sucrose, and 3.5 mL of 5% sucrose in a Beckman ultracentrifuge tube. Store the gradient at 4C for at least 15 minutes prior to use.
2. Remove the supernatant from pelleted membrane.
3. Resuspend each membrane pellet in 2 mL of ice-cold TNE with 1% (v/v) Triton X-100. (If multiple tubes were being used for a single condition, they need to be pooled into 2 mL of TNE).
4. Carefully load the 2 mL of TNE with membrane on top of the sucrose gradient.
5. Spin the sucrose gradient with membrane at 100,000 x g for 20 hours.
6. After the spin, puncture the bottom of the tube with a hypodermic needle and collect one milliliter fractions in eppendorf tubes. The lipid raft fraction will appear as a cloudy band.
7. Each fraction can be assessed by Bradford assay for protein content.
8. UNC-1 can be used as a marker of lipid rafts.

Recipes

M9 (1 L)

- 6 g Na_2HPO_4
- 3 g KH_2PO_4
- 5 g NaCl
- 1 mL of 1M $MgSO_4$
- QS to 1 L with dH_2O
- Autoclave
- Store at RT

S Basal (1 L)

- 20 mL of 5M NaCl
- 50 mL of 1M KPO_4 (see recipe)
- QS to 1 L with dH_2O
- Autoclave
- 1 mL of 5 mg/mL cholesterol in 95% EtOH
- Store at 4C

KPO_4 (1 L)

- 110.26 g KH_2PO_4
- 33.06 g K_2HPO_4
- QS to 1 L with dH_2O
- Autoclave
- Store at RT

Bleaching Solution (10 mL)

- 7 mL of dH_2O
- 2 mL Clorox bleach
- 1 mL 5M NaOH
- Make fresh
- Volume to use will depend on the size of the worm pellet (2 mL is typically adequate to bleach ten 5 cm plates worth of worms).

Homogenization Buffer (50 mL)

- 1 mL of 1M Tris, pH 7.4
- 1 mL of 1M HEPES
- 3 mL of 0.5M mannitol
- 0.5 mL of 1M $CaCl_2$
- 1 mL of 0.5 mg/mL leupeptin (or equivalent protease inhibitor cocktail)
- 1 mL of 0.5 mg/mL pepstatin (or equivalent protease inhibitor cocktail)
- 1 mL of 0.5 mg/mL PMSF inhibitor (or equivalent protease inhibitor cocktail)
- QS to 50 mL with dH_2O
- Make fresh
- Keep on ice

TNE with 1% Triton X-100 (10 mL)

- 0.5 mL of 1M Tris, pH 8.0
- 1.3 mL of 1M NaCl
- 0.1 mL of 0.5M EDTA
- 0.1 mL Triton X-100
- QS to 10 mL with dH_2O

Title: Fatty Acid Supplemented NGM Plates

Purpose:

This protocol describes how to pour NGM plates that are supplemented with fatty acid (FA) sodium salts for the purpose of dietary supplementation. These plates are usual for replacing missing fatty acids in *fat* or *elo* mutants or to increase the levels of a fatty acid in N2.

Resources:

Fatty acids can be ordered from Nu-Chek (http://www.nu-chekprep.com).

The catalog can be found http://www.nu-chekprep.com/10_11_catalog.pdf and the “Soaps of Fatty Acids” on Page 30 are used in this protocol.

Molecular weights can be found on the Certificate of Analysis included with the order.

Watts, J. L., Phillips, E., Griffing, K. R., & Browse, J. (2003). Deficiencies in C20 polyunsaturated fatty acids cause behavioral and developmental defects in Caenorhabditis elegans fat-3 mutants. Genetics, 163(2), 581-589.

Deline, M. L., Vrablik, T. L., Watts, J. L. Dietary Supplementation of Polyunsaturated Fatty Acids in *Caenorhabditis elegans*. *J. Vis. Exp.* (81), e50879, doi:10.3791/50879 (2013). Link: http://www.jove.com/video/50879/dietary-supplementation-polyunsaturated-fatty-acids-caenorhabditis

Abbreviations:

FA – Fatty Acid

Method:

1. Determine the volume of agar required based on the number of supplemented plates required for the assay. Repeat this step for each batch or fatty acid.

_____	x	_____	=	_____	+	10 mL	=	_____
Number of plates		Volume per plate (typically 10 mL)		Exact volume required		Extra volume		Volume of agar to make

2. Total the volume of agar being poured. You'll need this much agar for the NP-40 control plates. If only one fatty acid is being used, then simply use a volume of agar equal to the volume determined in step 1.

_____	+	_____	+	_____	=	_____
First Batch		Second Batch		Third Batch		Volume of Agar for Control Plates

3. Label an autoclavable bottle for each batch being poured with the fatty acid included and the concentration (e.g., Eicosapentaenoic Acid, 160 µM). Each bottle should hold a volume equal to approximately twice the volume of agar being made.

4. Calculate the mass of the fatty acid that will be required.

_____	_____	x	_____	x	_____	÷	1,000	=	_____
Name of FA	Concentration (µM)		MW of FA (g/mol)		Volume of Agar (L)				Mass of FA (mg)

5. Calculate the amount of water necessary to dissolve the FA to 10 mM:

_____	÷	_____	÷	10	x	1,000	=	_____
Mass of FA (mg)		MW (g/mol)		Concentration (mM)				Volume of dH_2O (mL)

6. Dissolve the mass of FA, calculated in Step 4, in the volume of sterile dH_2O, calculated in Step 5. Leave shaking while autoclaving the agar. If the FA is

not fully dissolved when the agar is ready, put the tube at 37C for 15 - 30 minutes.

7. Calculate the mass each component required for the agar:

Reagent	Amount per Liter		Volume of Agar (L)		Amount Required
NaCl	3 g	x	_____	=	_____ g
Bacto Agar	16 g	x	_____	=	_____ g
Bacto Peptone	2.5 g	x	_____	=	_____ g
1M K_3PO_4	25 mL	x	_____	=	_____ mL
1M $MgSO_4$	1 mL	x	_____	=	_____ mL
1M $CaCl_2$	1 mL	x	_____	=	_____ mL
5 mg/mL Cholesterol	1 mL	x	_____	=	_____ mL
NP-40	1 mL	x	_____	=	_____ mL

8. Add the NaCl, Bacto Agar, and Bacto Peptone to the autoclavable container and QS to the volume required minus the volume of water needed to dissolve the FA in Step 5. Swirl the container.

9. Either loosely screw the cap on the bottle or cover the top with tin foil and then add a piece of autoclave tape.

10. Turn on the water bath in the plate pouring room and set it to 60C.

11. Autoclave for the recommended time based on the autoclave specifications.

12. Place the agar in the 60C water bath for 0.5 – 1 hour.

13. Add the K_3PO_4, $MgSO_4$, $CaCl_2$, cholesterol, and NP-40. This can be done in or out of the water bath.

14. Swirl the containers to ensure the NP-40 is dissolved.

15. To the “Control Plates Agar” add a volume of dH2O equal to the volume used to dissolve the FA in Step 5.

16. Ensure the FA is dissolved in the dH_2O. If not, warm the solution to 37C and vortex. Repeat this until the FA is in solution. Then add the 10mM FA solution to the “FA Agar”.

17. Swirl both containers.

18. Leave the FA agar in the 60C water bath while pouring the “Control Plate Agar”. This will allow you to use the same tubing to pour both containers. DO NOT USE THE SAME TUBING FOR DIFFERENT FATTY ACIDS. Alternatively, graduated pipettes can be used to dispense smaller batches.

19. Dispense the “FA Agar” using the same settings. If the same tubing is being used, consider discarding the first plate poured as it may have less FA from residual control plate agar.

20. Label the plates (color coding works well as long as you have a legend with the plates).

21. Store plates in the dark at room temperature overnight to solidify.

22. The next day, move plates to a light protected container (e.g., a box wrapped in tin foil) and store at either 4 or 20C.

Expiration:

Plates have been tested out to 6 weeks stored at 20C and rescue phenotypes associated with loss of fatty acids. Fatty acids oxidize over time which will reduce the amount of usable fatty acids in the plates. This should be taken into account and assays should be done using the same age plates. Fatty acids should be stored per the recommendations of the provider. This generally means storage at -20C, in the dark, under an inert gas. Once opened, if stored under the proper conditions, the fatty acids will last 1 – 3 months. Unopened the fatty acids will last indefinitely.

Use:

Seed plates at least two days before use. Fatty acids are taken up by the *E. coli* and then are eaten by *C. elegans*. *E. coli* grows a little slower on these plates than on NGM plates, presumably due to the NP-40. The NP-40 will also allow worms to crawl between the agar and the wall of the plate. Put down extra worms for assays and if egglaying, recognize that not all the adults may be recoverable.

For most of the previous assays, L4s were put on FA supplemented plates and allowed to develop to egglaying adults overnight. These adults were then moved to new FA supplemented plates and allowed to lay eggs for 1 – 2 hours before being removed. For assays that require a larger number of worms, synchronized

L1s (from bleached adults) can be pipetted onto FA supplemented and control plates. Note that development is affected by the loss of many fatty acids.

For many *fat* mutants the basal speeds can be assessed to ensure rescue. Alternatively, changes in aldicarb sensitivity and egglaying can also be used. GC/MS should be used to measure the amount of FA present in the treated worms. Excess worms can be washed from plates and frozen at -80C in dH_2O for lipid extraction at a later time.

Fatty acid supplemented plates can also be used to maintain mutants that would otherwise be lethal (e.g., *fat-5;fat-6;fat-7*) or improve the mating and egglaying of affected strains.

12 Appendix II – Clarification of Contributions

We are like dwarves perched on the shoulders of giants, and thus we are able to see more and farther than the latter. And this is not at all because of the acuteness of our sight or the stature of our body, but because we are carried aloft and elevated by the magnitude of the giants.

Bernard of Chartres - February 15, 1676

The original idea for the LC-PUFA experiments came from a meta-analysis of locomotion data collected by Kapo Leung, Lindsay Kondo, and Mia Bolling under the direction of Dr. Jill Bettinger. Their data is not presented in this thesis, but was instrumental in the development of the project.

All GC/MS analyses, as seen in Figure 9, were completed by Justin Poklis of the Pharmacology and Toxicology Mass Spectrometry Core.

Dr. Scott Bowers provided equipment and instruction necessary for Western blot analysis. The data presented is my own, but would not have been possible without his efforts. Additionally, SLO-1 antibodies (data not shown) were kindly provided by Dr. Hongkyun Kim of the Rosalind Franklin University.

Gina Blackwell scored *dgk-1(nu62)* and *unc-10(e102)* controls for the aldicarb assays (data not shown).

Dr. Greg Hawkins and Linsday Kondo aided in the sequencing of the *fat* mutants (data not shown).

13 Vita

Richard Raabe was born on January 3, 1985 in Scarborough, Maine. He graduated from Scarborough High School in 2003 and went to attend the University of Richmond. He graduated from UR in 2007 with a Bachelor of Science in Biochemistry and Molecular Biology. He stayed at UR for a year as a research technician and published some of his work on anti-depressants interacting with a NMDA receptor subunit. He then went to work at Alere, Inc. as a research scientist. His work was focused on developing a rapid diagnostic immunoassay for influenza. He joined Dr. Jill Bettinger's laboratory in the summer of 2011.

Conferences

19th International *C. elegans* Meeting
June 23-26, 2013
University of California
Los Angeles, CA
The Potential Role of Palmitoylation in the Response to Acute Ethanol
Richard C. Raabe, Andrew G. Davies, and Jill C. Bettinger

Neuroscience Poster Day presented by Central Virginia Chapter of the Society for Neuroscience
December 14, 2012
Virginia Commonwealth University
Richmond, VA
Determining if SLO-1 localization in the membrane affects development of acute functional tolerance
Richard C. Raabe, Andrew G. Davies, and Jill C. Bettinger

Central Virginia Chapter of the Society for Neuroscience
March 16th, 2012
Virginia Commonwealth University
Richmond, VA

Ethanol-Induced Cell Fate Alterations in *C. elegans*
Lindsay Kondo, Kalyann Kauv, Richard Raabe, Mia Bolling, Andrew Davies and Jill Bettinger

John C. Forbes Graduate Student Honors Colloquium
May 7-8, 2013
Virginia Commonwealth University
Richmond, VA
The Role of Palmitoylation in the Level of Response to Ethanol
Richard Raabe, Andrew Davies, and Jill Bettinger

Watts Poster Symposium
October 16, 2013
Virginia Commonwealth University
Richmond, VA
Fatty Acid Regulation Plays a Central Role in the Development of Acute Functional Tolerance to Ethanol
Richard Raabe and Jill Bettinger