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Lipid Environment Modulates the Development of Acute Tolerance to Ethanol in *Caenorhabditis elegans*

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**Abstract**

The development of tolerance to a drug at the level of the neuron reflects a homeostatic mechanism by which neurons respond to perturbations of their function by external stimuli. Acute functional tolerance (AFT) to ethanol is a fast compensatory response that develops within a single drug session and normalizes neuronal function despite the continued presence of the drug. We performed a genetic screen to identify genes required for the development of acute functional tolerance to ethanol in the nematode *C. elegans*. We identified mutations affecting multiple genes in a genetic pathway known to regulate levels of triacylglycerols (TAGs) via the lipase LIPS-7, indicating that there is an important role for TAGs in the development of tolerance. Genetic manipulation of *lips-7* expression, up or down, produced opposing effects on ethanol sensitivity and on the rate of development of AFT. Further, decreasing cholesterol levels through environmental manipulation mirrored the effects of decreased TAG levels. Finally, we found that genetic alterations in the levels of the TAG lipase LIPS-7 can modify the phenotype of gain-of-function mutations in the ethanol-inducible ion channel SLO-1, the voltage- and calcium-sensitive BK channel. This study demonstrates that the lipid milieu modulates neuronal responses to ethanol that include initial sensitivity and the development of acute tolerance. These results lend new insight into studies of alcohol dependence, and suggest a model in which TAG levels are important for the development of AFT through alterations of the action of ethanol on membrane proteins.


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**Introduction**

Alcohol abuse is a prevalent and serious disorder. Current drug treatments are inadequate, in part because the molecular nature of acute ethanol response is not well understood. A component of the ethanol response in humans that is predictive of the susceptibility of alcohol dependence, and suggest a model in which TAG levels are important for the development of AFT through alterations of the action of ethanol on membrane proteins.

Several proteins and pathways have been described that modify the development of acute functional tolerance in different species. """"GABA<sub>A</sub> receptors isolated from ethanol-treated mice demonstrate acute tolerance *in vitro* [5], and this requires functional Protein Kinase C<sub>e</sub>. In addition, Protein Kinase C<sub>e</sub> knock out mice show defects in AFT [6]. In *C. elegans*, we have previously shown that the Neuropeptide Y Receptor-like protein, NPR-1, acts to negatively regulate the development of acute functional tolerance. This observation extends to the mouse, where animals with loss of function of various components of the brain NPY pathway recover more rapidly from hypnotic doses of ethanol [7,8], a measure of the development of AFT in mice [9].

Much work has focused on the effects of ethanol on protein targets, and several excellent candidates for ethanol effector molecules have been identified [10,11]. As the dominant theory for many years, the lipid fluidity hypothesis of ethanol intoxication proposed an indirect effect of ethanol on membrane proteins via perturbation of membrane lipid structure [12,13]. While this theory has fallen out of favor for several reasons, including the observation of direct actions of ethanol on proteins, the relevance of lipid-protein interactions in modulating protein function and in modulating direct ethanol-protein effects are increasingly a target of study [14–17].
This study is designed to identify the molecular mechanisms for the development of AFT. Here, we report that an unbiased genetic screen for modulators of the development of AFT yielded genes involved in determining levels of triacylglycerols in the animal. Our data support a conclusion that the lipid environment is important in the ability of the animal to modify cell function in the development of AFT to ethanol.

Results

Isolation of Mutants Defective in Acute Functional Tolerance

In order to identify the molecular mechanisms underlying acute functional tolerance (AFT) to ethanol, we performed an unbiased genetic screen for animals that are impaired in the ability to develop AFT. We have previously shown that NPR-1 activity antagonizes the development of AFT; in an npr-1 mutant background, AFT develops more quickly than in wild type, suggesting that NPR-1 function acts to slow the development of AFT [4]. We took advantage of this phenotype of fast and obvious development of AFT in the npr-1 mutant background to sensitize our screen to make it easy to identify animals with diminished AFT. The advantage of using this sensitized background is that we should be able to identify mutations in which AFT is diminished but not completely abolished, which would be difficult in the N2 wild-type background.

We performed EMS mutagenesis on npr-1(ky13) animals, and subjected their F2 progeny to a behavioral screen. We treated the animals with ethanol, allowed them time to develop AFT, and then identified animals that had not developed tolerance using a locomotion assay in which animals that could not develop tolerance would be too impaired by ethanol to crawl to an attractive odorant. We isolated animals that had not developed tolerance, allowed them to produce self-progeny, and retested their progeny in a quantitative assay of basal activity and ethanol responses that measured both initial sensitivity and the development of AFT. In this secondary assay, we placed worms on plates that were contained 0 mM or 400 mM of ethanol. Movies were recorded of locomotion at 10 and 30 minutes of treatment, and speed was determined by computer analysis of the movies. We define the decrease in speed at 10 minutes of treatment, relative to untreated animals of the same genotype, as the initial sensitivity of animals to ethanol. We have previously shown that wild-type worms move faster at 30 minutes relative to their speed at 10 minutes of ethanol exposure, and we consider the amount of recovery of speed between 10 and 30 minutes to be the degree of acute functional tolerance. We judged animals to be defective in recovery of speed between 10 and 30 minutes to be the degree of minutes of ethanol exposure, and we consider the amount of worms move faster at 30 minutes relative to their speed at 10 minutes of treatment, and that were contained 0 mM or 400 mM of ethanol. Movies were recorded of locomotion at 10 and 30 minutes of treatment, and speed was determined by computer analysis of the movies. We define the decrease in speed at 10 minutes of treatment, relative to untreated animals of the same genotype, as the initial sensitivity of animals to ethanol. We have previously shown that wild-type worms move faster at 30 minutes relative to their speed at 10 minutes of ethanol exposure, and we consider the amount of recovery of speed between 10 and 30 minutes to be the degree of acute functional tolerance. We define the decrease in speed at 10 minutes of treatment, relative to untreated animals of the same genotype, as the initial sensitivity of animals to ethanol. We have previously shown that wild-type worms move faster at 30 minutes relative to their speed at 10 minutes of ethanol exposure, and we consider the amount of recovery of speed between 10 and 30 minutes to be the degree of acute functional tolerance. We judge animals to be defective in recovery of speed between 10 and 30 minutes to be the degree of

Identification of ctpb-1 as a Mediator of AFT

The eg613 mutation was mapped to the left arm of the X chromosome using SNP mapping methods with the polymorphic wild strain, CB4856 [18,19]. Using transformation rescue experiments we identified the gene affected by mutation in the eg613 mutant strain as ctpb-1. We found that the eg613 mutation is a splice site mutation in the last nucleotide of intron 9 in the open reading frame of the gene ctpb-1 that is predicted to generate a premature stop codon that results in a truncated protein. A deletion allele of ctpb-1, ok498, failed to complement eg613 for the AFT phenotype, and homozygous ctpb-1(ok498) animals phenocopied eg613 mutants by suppressing the fast development of AFT of npr-1(ky13), tested in a visual assay of speed on ethanol, indicating that ctpb-1 is disrupted by the eg613 mutation. The CTPB-1 protein contains a DNA-binding THAP domain, and

Figure 1. The transcriptional regulators CTBP-1 and PAG-3 are required for the development of AFT. Animals were treated with 0 mM and 400 mM exogenous ethanol. Relative speeds were calculated as treated over untreated speeds. Animals shown in the same graph were tested simultaneously on the same plates. a) Wild-type N2 animals develop AFT, and npr-1(ky13) animals are significantly faster developers of AFT. Compared with npr-1(ky13), npr-1(ky13) ctbp-1(eg613) animals have decreased initial sensitivity to ethanol; the decrease in development of AFT was not significantly different from npr-1(ky13). ctbp-1(eg613) animals in an npr-1(ky13) mutant background do not develop AFT, and when tested in the sensitized background, pag-3(n3098) animals do not develop AFT, and when tested in the sensitized background, pag-3(n3098) animals have decreased initial sensitivity and are slow developers of AFT compared with npr-1(ky13) (n=13). Note that the large decrease in speed in pag-3(n3098) animals at 30 minutes of ethanol exposure is largely driven by an increase in their untreated speed at 30 minutes, and may not reflect an actual increase in sensitivity to ethanol during that interval. We have not observed this kind of increase in untreated speed in other strains of animals tested, including in the npr-1(ky13) pag-3(n3098) double mutant. pag-3(n3098) animals do not increase their speed on ethanol over the course of 30 minutes, indicating that they are defective in AFT (at 400 mM: 10 minutes = 32.4 ± 2.6 m/sec, 30 minutes = 33.7 ± 1.3 m/sec; not significantly different 140 = 0.42, P=0.67). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, P < 0.05; **, P < 0.01; *** P < 0.001; for comparison to N2 at 10 minutes: ###, P < 0.001; for comparison to npr-1(ky13) at 10 minutes: ††, P < 0.05; †††, P < 0.01; ††††, P < 0.001.

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CTBP-1 has been shown to act as a negative regulator of transcription [20].

In order to determine if ctbp-1(eg613) is dependent on a loss of npr-1 function to modify AFT, we crossed the mutation away from the npr-1(fy13) mutation and tested its ability to develop acute tolerance in the presence of wild-type npr-1. When we examined the development of AFT in animals carrying the ctbp-1(eg613) mutation alone, we found that they had reduced AFT compared with N2 animals (Figure 1a), demonstrating that ctbp-1 is a component that is generally required for the development of AFT, independent of the genetic background.

The transcriptional regulator sir-2.1 acts genetically upstream to repress function of ctbp-1 [21]; we predicted that in a sir-2.1 mutant strain, loss of negative regulation of ctbp-1 might result in fast development of AFT. We found that sir-2.1 mutant animals were resistant to ethanol relative to N2, and, while there was a trend toward fast development of AFT, there was not a statistical difference in rate of AFT from N2 (Figure 2). Together, these results suggest that ctbp-1 is a regulator of AFT.

pag-3 is Required for AFT and pag-3 was Isolated in the Screen

The transcription factor CTBP-1 has been shown to bind to and act with the zinc-finger transcription factors PAG-3 and ZAG-1 to repress transcription of target genes [20]. We reasoned that if ctbp-1 mutant animals are defective in AFT because of loss of transcriptional regulation of some effector genes that are cooperatively regulated by these co-repressors, then animals defective in either of the other two co-repressors should also be defective in AFT. Animals mutant in either pag-3 or zag-1 have locomotion defects; we were unable to test zag-1 mutants in our behavioral assays because they are too uncoordinated to allow us to distinguish between locomotion defects induced by ethanol and their baseline uncoordination. The more extreme phenotype of the zag-1 and pag-3 mutants compared with ctbp-1 mutants suggests that those genes have functions in addition to their cooperative role with ctbp-1. While pag-3 mutant animals are also uncoordinated, their locomotion defect is less severe, and we were able to assess them for the development of AFT. We found that the loss-of-function allele pag-3(n3090) did not develop AFT, and it also suppressed the fast development of AFT of npr-1(fy13) (Figure 1b), suggesting that ctbp-1(eg613) is defective in AFT because it fails to regulate transcription, and that this function of ctbp-1 requires pag-3. Since a mutation in pag-3 was able to suppress the npr-1-mediated fast development of AFT, we reasoned that our genetic screen may have identified an allele of pag-3. Another mutation isolated in our screen, bet16, mapped to the genetic interval containing pag-3 and failed to complement four alleles of pag-3 for the AFT phenotype, as tested in visual assays of speed on ethanol. These results indicate that this behavioral screen identified multiple members of a molecular pathway that is involved in the development of AFT.

Dysregulation of ida-1’s Control of Neurosecretion is not Responsible for the AFT Phenotypes of ctbp-1

We were interested in determining which genes that are regulated by ctbp-1 and pag-3 are responsible for the defect in AFT that we observed in those animals. To date, in C. elegans, the only specific regulatory target of pag-3 that has been reported is the gene ida-1, which encodes a protein involved in dense core vesicle release. The loss of negative regulation of ida-1 in pag-3 mutants causes increased neurosecretion [22]. We would expect any gene that is important for the shared AFT phenotype in pag-3 and ctbp-1 to also be misregulated in the ctbp-1 mutant. Chen et al. [21] performed microarray analysis of a ctbp-1 mutant, and reported genes with expression differences of 2-fold or more from wild type, and while expression of ida-1 was not reported to be altered in this study, it remained possible that a more subtle transcriptional dysregulation, which did not meet the criteria for inclusion in that report, may be functionally relevant in the development of AFT. Therefore, we tested whether altering ida-1 function could be an explanation for the altered rate of development of AFT in ctbp-1 and pag-3. We found that the response of the strong loss-of-function ida-1(ok409) mutant animals to ethanol was indistinguishable from that of N2 (Table 1), suggesting that a change in the rate of neurosecretion is not the explanation for the AFT phenotype of animals carrying mutations in these transcriptional regulators. As noted above, we have further observed that pag-3 mutant animals are mildly uncoordinated, while ctbp-1 mutants are not, suggesting that this transcriptional repressor may regulate expression of a larger group of genes than those that overlap with ctbp-1, and ida-1 may be in that class of differentially regulated genes.

cmb-1 Regulates AFT through Repression of lips-7

Expression

CTBP-1 has been demonstrated to regulate the transcription of approximately 200 genes [21]. Chen et al [21] reported that cmb-1(ok498) mutants have an extended lifespan, and that the loss of regulation of the lips-7 triacylglycerol (TAG) lipase in the cmb-1 mutant could explain this aspect of the cmb-1 mutant phenotype. In cmb-1(ok498) mutant animals, lips-7 message is increased, and the resulting lipase overexpression caused animals to accumulate 16.8% less TAGs, and animals in which lips-7 had been inactivated by RNA interference had dramatically increased levels of TAGs [21]. We hypothesized that if the effect of loss of function of cmb-1 on ethanol response, like its effect on lifespan, is due to its

Figure 2. The ctbp-1-regulated gene lips-7 modulates the rate of the development of AFT. Animals were tested on 0 mM and 400 mM exogenous ethanol. Relative speeds were calculated as treated over untreated speeds. All animals were tested simultaneously on the same plates. a) lips-7(ok3110) animals are resistant to ethanol at 10 minutes of exposure relative to wild-type N2, and they develop significantly more AFT than wild-type N2 animals (n = 13). b) The transcription factor sir-2.1 is a negative regulator that acts genetically upstream of ctbp-1 [21]; in a sir-2.1 mutant strain, loss of negative regulation of ctbp-1 should result in fast development of AFT. Compared with N2, sir-2.1(ok434) mutant animals were resistant to ethanol at 10 minutes of exposure but their apparent increase in development of AFT did not reach statistical significance (n = 13). Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, P < 0.05; **, P < 0.001; for comparison to N2 at 10 minutes: #, P < 0.001, doi:10.1371/journal.pone.0035192.g002
Table 1. Mutants tested for initial sensitivity and development of acute functional tolerance to ethanol.

<table>
<thead>
<tr>
<th>GENE</th>
<th>PROTEIN</th>
<th>PHENOTYPE of LOSS-OF-FUNCTION</th>
<th>INITIAL SENSITIVITY*</th>
<th>DEVELOPMENT OF AFTb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ida-1(ok409) [22]</td>
<td>Protein tyrosine</td>
<td>Increase in neurosecretiona</td>
<td>Not different from N2</td>
<td>Not different from N2</td>
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<tr>
<td></td>
<td>phosphatase-like receptor</td>
<td></td>
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<td></td>
<td></td>
<td>N2 (10'): 28.1 ± 2.4%</td>
<td>N2 (30'): 36.4 ± 2.3%</td>
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<tr>
<td></td>
<td></td>
<td>ok409 (10'): 32.1 ± 2.1%</td>
<td>recovery (30'-10'): 8.3 ± 2.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>t = 1.66, P = 0.15</td>
<td>ok409 (30'): 41.8 ± 1.9%</td>
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<td></td>
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<td></td>
<td>recovery (30'-10'): 9.7 ± 1.9%</td>
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<td></td>
<td></td>
<td></td>
<td>t = 0.49, P = 0.64</td>
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<tr>
<td>nhr-49(ok2165) and</td>
<td>Nuclear hormone</td>
<td>Increase in fat storaged</td>
<td>Not different from N2</td>
<td>No development of AFT</td>
</tr>
<tr>
<td>nhr-49(gk405) [40]</td>
<td>receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2 (10'): 30.3 ± 1.4%</td>
<td>N2 (30'): 34.8 ± 3.3%</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ok2165 (10'): 30.3 ± 3.0%</td>
<td>recovery (30'-10'): 4.5 ± 3.7%</td>
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<tr>
<td></td>
<td></td>
<td>t = 0.002, P = 0.99</td>
<td>ok2165 (30'): 28.0 ± 3.2%</td>
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<tr>
<td></td>
<td></td>
<td>N2 (10'): 34.9 ± 2.6%</td>
<td>recovery (30'-10'): 2.3 ± 1.4%</td>
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<tr>
<td></td>
<td></td>
<td>gk405 (10'): 38.8 ± 3.7%</td>
<td>N2 (30'): 47 ± 4.0%</td>
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<tr>
<td></td>
<td></td>
<td>t = 0.97, P = 0.37</td>
<td>recovery (30'-10'): 11.7 ± 2.5%</td>
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<td></td>
<td></td>
<td>gk405 (30'): 38.6 ± 3.8%</td>
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<td></td>
<td></td>
<td>recovery (30'-10'): -0.2 ± 3.1%</td>
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<tr>
<td>bbs-1(ok1111) [41]</td>
<td>Ortholog to human BBS1</td>
<td>Increase in fat storaged</td>
<td>More sensitive than N2</td>
<td>Faster development of AFT relative to N2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2 (10'): 31.4 ± 3.4%</td>
<td>N2 (30'): 43.1 ± 2.1%</td>
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<tr>
<td></td>
<td></td>
<td>ok1111 (10'): 15.2 ± 0.95%</td>
<td>recovery (30'-10'): 11.7 ± 2.5%</td>
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<td></td>
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<td>t = 4.5, P &lt; 0.01</td>
<td>ok1111 (30'): 32.0 ± 2.1%</td>
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<td>recovery (30'-10'): 16.7 ± 2.0%</td>
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<td></td>
<td></td>
<td>t = 2.9, P &lt; 0.05</td>
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<tr>
<td>tub-1(ok1972) [42]</td>
<td>tubby</td>
<td>Increase in fat storaged</td>
<td>Not different from N2</td>
<td>Not different from N2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2 (10'): 34.9 ± 3.5%</td>
<td>N2 (30'): 48.9 ± 2.9%</td>
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<td></td>
<td></td>
<td>ok1972 (10'): 28.3 ± 2.5%</td>
<td>recovery (30'-10'): 13.8 ± 1.9%</td>
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<tr>
<td></td>
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<td>t = 1.4, P = 0.21</td>
<td>ok1972 (30'): 46.6 ± 4.9%</td>
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<td></td>
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<td></td>
<td>recovery (30'-10'): 15.1 ± 2.0%</td>
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<td></td>
<td></td>
<td>t = 0.47, P = 0.65</td>
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<tr>
<td>fat-7[wa36]; fat-5(tm420) [43]</td>
<td>Fatty acid desaturases</td>
<td>Decrease in fat storaged</td>
<td>Not different from N2</td>
<td>Faster development of AFT relative to N2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2 (10'): 32.2 ± 1.6%</td>
<td>N2 (30'): 35.3 ± 3.2%</td>
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<tr>
<td></td>
<td></td>
<td>wa26;tm420 (10'): 30.3 ± 2.8%</td>
<td>recovery (30'-10'): 6.5 ± 1.6%</td>
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<tr>
<td></td>
<td></td>
<td>t11 = 0.80, P = 0.44</td>
<td>wa26;tm420 (30'): 44.6 ± 2.5%</td>
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<td>recovery (30'-10'): 14.2 ± 1.7%</td>
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<td></td>
<td></td>
<td>t11 = 6.7, P &lt; 0.0001</td>
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<tr>
<td>sbp-1(ep79) [44]</td>
<td>Homolog to mammalian SREBP</td>
<td>Decrease in fat storaged</td>
<td>Less sensitive than N2</td>
<td>No development of AFT</td>
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<tr>
<td></td>
<td></td>
<td>N2 (10'): 32.4 ± 2.6%</td>
<td>N2 (30'): 43.6 ± 2.8%</td>
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<tr>
<td></td>
<td></td>
<td>ep79 (10'): 45.9 ± 4.2%</td>
<td>recovery (30'-10'): 11.2 ± 2.4%</td>
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<tr>
<td></td>
<td></td>
<td>t = 3.0, P &lt; 0.05</td>
<td>ep79 (30'): 37.9 ± 5.5%</td>
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<td></td>
<td></td>
<td></td>
<td>recovery (30'-10'): -8.0 ± 8.5%</td>
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</table>

aThe degree of initial sensitivity was determined as the percent of untreated treated after 10 minutes of ethanol exposure. Significance was determined relative to the effect of ethanol on wild-type N2 animals treated on the same plates using paired two-tailed t-tests. *The development of acute functional tolerance (AFT) was determined by comparing the rate of locomotion at 30 minutes vs. 10 minutes of ethanol exposure. If the mutant strain developed significant tolerance, we compared its rate of development of tolerance to the rate of wild-type N2 animals treated on the same plates using unpaired two-tailed t-tests. t = 1.66, P = 0.15, the response of the strong loss of function ida-1(ok409) mutant animals to ethanol was indistinguishable from that of N2, suggesting that a change in neurosecretion is not the explanation for the AFT phenotype of cebp-1 and pag-3. *Genetic manipulation of fat stores by mutations in the genes nhr-49, bbs-1, tub-1, fat-7 and fat-5, and sbp-1 did not alter ethanol sensitivity or AFT in a consistent manner, suggesting that fat levels themselves do not influence the rate of development of AFT. It is possible that mutations in these genes cause basal changes in membrane composition. This is consistent with the finding that the increase in fat storage and decrease in the rate of development of AFT for the mutant. For this reason, the magnitude and interpretation of the ethanol responses of this strain may be subject to a floor effect.

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failure to down-regulate lips-7, then loss of lips-7 should have the opposite phenotype to loss of ctbp-1; that is, lips-7 mutants should display ethanol resistance and/or fast development of AFT. We found that lips-7(ok3110) mutant animals are both resistant to ethanol and fast developers of AFT, indicating that regulation of TAG levels is important for the development of AFT (Figure 2a). These results support the notion that the effect on lipase regulation via lips-7 is a mechanism of ctbp-1’s effect on AFT (Figure 2b).

Fat Stores do not Regulate AFT
Regulation of the lipase gene lips-7 has a significant effect on the development of AFT. In one model of how this lipase function affects AFT, fat levels in the animals could regulate ethanol effects because TAGs are the main stored fat in C. elegans. To test this hypothesis, we altered fat levels in the animals using genetic manipulations, and tested the effect on ethanol sensitivity and AFT development in our locomotion assay. If the changes in the level of stored fat in ctbp-1 and lips-7 mutants is the cause of the defect in AFT, then decreasing fat levels through genetic manipulation should cause a decrease in AFT (mimicking ctbp-1(eg613) mutants), while increasing fat levels should cause animals to be resistant to ethanol or fast developers of AFT (similar to lips-7 mutant animals). We tested several genes involved in regulating the levels of stored fat; mutations in these genes either yield increases or decreases in stored fat (Table 1). While several changes in fat levels did not cause an AFT phenotype, for those that did alter AFT, there was no consistent correlation between fat levels and the ability to develop AFT (Table 1). These data suggest that, by themselves, levels of stored fat do not predict the ability of the animal to develop AFT.

The Development of AFT is Cholesterol Dependent
In addition to their role in fat storage, TAGs are also components of the lipid membrane. To analyze the involvement of membrane environment in the development of AFT, we manipulated the plasma membrane by depleting the worms of cholesterol, a component of membrane lipid rafts [23]. C. elegans are cholesterol auxotrophs; all of their cholesterol is captured through their diet, and acute effects of cholesterol depletion include a suppression of clathrin-independent endocytosis in neurons, a lipid raft-mediated process [24], suggesting that cholesterol is involved in the development or maintenance of particular membrane structures. If AFT requires specific membrane microdomain structures that include cholesterol, then altering the amount of cholesterol available to the animals may modify their ability to develop tolerance. We found that N2 and npr-1(ky13) animals reared on cholesterol-depleted media demonstrated a substantial suppression of the development of AFT (Figure 3a and 3b). In contrast, adding additional cholesterol to the medium did not affect the ability of animals to develop AFT (not shown). Together, these data suggest that the structure of the lipid bilayer is essential for the development of tolerance.

The Development of AFT Requires SLO-1
Both the manipulation of cholesterol and the lipid constituents of the membrane environment are known to modulate the ethanol effect on the mammalian BK (KCNMA1/Slo1) potassium channel, an ethanol target protein [14,17,25–28]. In worms, ethanol activates SLO-1 in vivo, causing a large efflux of potassium ions, hyperpolarizing the cell and depressing neuronal excitability, which is a major cause of intoxication in C. elegans [29]. The mammalian BK channel is also activated by ethanol, and this effect, as well as the channel’s basal activity, depends on the thickness of the lipid bilayer; channels that reside in thicker membrane are less basally active and are less activated by ethanol [17,28], suggesting that movement of the BK channel into and out of areas of thicker membrane microenvironments may allow for very fast modulation of the effects of ethanol on this channel [28]. We hypothesized that this may be one mechanism by which worms develop AFT to ethanol. If AFT requires modulation of SLO-1 function, then complete loss of SLO-1 should reduce or eliminate the development of tolerance. In an otherwise wild-type background, null slo-1(eg142) mutant animals are quite resistant to the sedative effects of ethanol on locomotion, and, importantly, do not develop significant tolerance to ethanol (Figure 4b). We asked if loss of slo-1 completely eliminated the ability of animals to develop tolerance, even in a genetic background of fast development of tolerance. In the sensitized npr-1(ky13) mutant background, slo-1(eg142) animals, that lack SLO-1, are able to develop a degree of tolerance (Figure 4b), indicating that modulation of the ethanol effect on SLO-1 occurs during AFT but that SLO-1 is not the sole mediator of AFT.
contrast, ctbp-1(eg613) slo-1(eg142) lips-7 that significantly suppresses the slow movement phenotype of both slo-1(ky399gf) mutations increase the open probability or open time of the SLO-1 channel, resulting in a slow locomotion phenotype [29]. Loss of trend for an attenuated response compared with the robust development of AFT by degree of recovered speed of

$$P_{0.05}$$; ***, $$P < 0.001$$; for comparison to N2: #, $$P < 0.05$$; ##, $$P < 0.01$$; ###, $$P < 0.001$$. doi:10.1371/journal.pone.0035192.g004

Figure 4. SLO-1 protein function is modulated by lips-7 function. a) Representative micrographs of crawling C. elegans, when the animal’s head is at the dorsal most point in the propagation of its waveform. Anterior is left, scale bar is 200 µm. Wild-type N2 demonstrates the normal sinusoidal body posture of a crawling worm. slo-1(eg142), lips-7(ok3110), and sir-2.1(ok434) demonstrate a similar exaggerated, loopy, body posture. In contrast, ctbp-1(eg613) has a flattened body posture. b) SLO-1 function is required for normal AFT. Worms were treated with 400 mM exogenous ethanol. slo-1(eg142) null mutant animals do not develop significant AFT, whereas slo-1(eg142); npr-1(ky13) double mutants do develop tolerance. The degree of recovered speed of slo-1(eg142); npr-1(ky13) did not achieve statistical significance compared with npr-1(ky13), although there was a strong trend for an attenuated response compared with the robust development of AFT by npr-1(ky13) ($n = 14$). These data suggest that SLO-1 is not the sole mediator of the development of AFT in the worm. c) Loss of lips-7 can modulate function of slo-1 gain-of-function alleles. slo-1(ky398gf) and slo-1(ky399gf) mutations increase the open probability or open time of the SLO-1 channel, resulting in a slow locomotion phenotype [29]. Loss of lips-7 significantly suppresses the slow movement phenotype of both slo-1(gf) alleles in the absence of ethanol ($n = 6$ for slo-1(ky389gf); $n = 9$ for slo-1(ky399gf)) indicating that the increase in TAGs in lips-7 can cause a change in the function of the membrane channel, SLO-1. These results suggest that lips-7 alters SLO-1 function through a plasma membrane mechanism. Error bars are s.e.m. For indicated comparisons: n.s., not significant; *, $$P < 0.05$$; ***, $$P < 0.001$$; for comparison to N2: #, $$P < 0.05$$; ##, $$P < 0.01$$; ###, $$P < 0.001$$. doi:10.1371/journal.pone.0035192.g004

cctp-1 and lips-7 Modulate the Function of SLO-1

If modulation of SLO-1 activity represents a significant component of AFT then it is possible that the increased TAG levels associated with loss of lips-7 or sir-2.1 function are acting to either protect the SLO-1 protein from ethanol or are reducing overall activity of the SLO-1 protein, or both. If the action of this increase in TAGs is to reduce overall activity of SLO-1 then we predicted that we should be able to observe an inhibitory effect on SLO-1 function in the lips-7 and sir-2.1 mutants in the absence of ethanol. Reduced SLO-1 function in the slo-1 mutant causes an increase in the amplitude of the locomotion waveform (loopy motion phenotype, Figure 4a), and we observed that both lips-7 and sir-2.1 mutants are loopy (Figure 4a). In contrast, we predicted that in a ctbp-1 mutant, in which there is a decrease in TAGs, there should be an increase in SLO-1 activation, which should lead to the opposite phenotype, a flattened body waveform. Consistent with the prediction, we found that ctbp-1 mutants are flat relative to N2 (Figure 4a).

We were able to test more directly whether or not loss of lips-7 is able to modulate the function of SLO-1 in the absence of ethanol, using special alleles of slo-1. Two gain-of-function alleles of slo-1 that enhance the open probability and open time of the channel have been described [29]. These animals have a slow locomotion phenotype, which shares characteristics with the phenotype of ethanol intoxication. We hypothesized that lips-7 might suppress these gain-of-function defects if the increase in levels of TAGs reduces the activity of the channel. We tested the locomotion of slo-1(ky389gf); lips-7(ok3110) and slo-1(ky399gf); lips-7(ok3110) animals and found that loss of lips-7 was able to significantly rescue the locomotion defect of both slo-1 gain-of-function mutations (Figure 4c). These data strongly suggest that changes in TAG levels resulting from increased or decreased LIPS-7 activity are able to influence the activity of a transmembrane protein (SLO-1) both in its basal state and in the presence of ethanol.

Discussion

We have used a genetic screen for mutations that result in defects in the development of AFT to identify two transcriptional co-repressors, ctbp-1 and pag-3, that regulate the ability of animals to develop AFT. Transcriptional repression of the lips-7 TAG lipase is required for normal development of AFT, and loss of lips-7 enhances the rate and degree of AFT, indicating that action of
this lipase inhibits the development of AFT. One component of the normal development of AFT appears to involve modulation of the ethanol effect on the transmembrane ethanol target protein SLO-1, and the function of SLO-1 gain-of-function mutations is modulated by the action of lips-7, suggesting that there is a role for TAGs in the regulation of SLO-1 function. Depletion of cholesterol impaired the development of AFT and, while cholesterol is used both as a component of the plasma membrane and as a precursor to steroid synthesis in the worm, taken together, these data suggest a role for membrane microdomain structure in AFT.

These results lead us to propose a model for a mechanism for fast adaptations associated with the development of AFT: the effects of ethanol on a membrane protein, such as SLO-1, are countered by moving the protein into or out of specific membrane microdomains to achieve a more normal function of the neuron in the presence of ethanol. In this model, cholesterol and the TAGs on which LIP7 acts would constitute components of these microdomains that reduce the ethanol effect on the target protein, either by protecting the protein from ethanol or by changing the protein’s activity in opposition to the ethanol effect. An increase in the levels of these TAGs might lead to an increase in the size or quantity of these protective microdomains, thereby increasing the potential for minimizing ethanol’s effects on target proteins; decreases in the level of these TAGs would have the opposite effect on AFT. This model accounts for the altered rates and development of AFT in mutants for the ctbp-1, pag-3 and lips-7 genes, which regulate levels of these TAGs in different directions.

While we favor a model of a direct effect of lipid microdomains in modulation of SLO-1 activity, as is seen for mammalian SLO-1/BK channels (reviewed by Treistman and Martin [30]), the data presented here are also compatible with a model in which lipid modulation of activity of neuronal function occurs independently of SLO-1 function. For example, altering the level of TAGs might increase the excitability of cholineric motor neurons or decrease the excitability of GABAreic motor neurons, both effects might lead to a decreased ethanol response. Loss of lips-7 function, which increases TAG levels, results in a loopy locomotion phenotype, acute ethanol resistance and a more rapid development of AFT. These phenotypes are consistent with an increase in the overall excitability of the locomotor cholineric circuitry. However, we have shown previously with mutants that enhance the activity of the cholineric neurons, that the degree of neuronal excitability and the degree of ethanol sensitivity are not well correlated [29]. Effects by TAGs in a SLO-1-dependent manner and general neuronal excitability effects are not mutually exclusive models, and in fact these two postulated lipid effects may act in concert to modify ethanol response at the behavioral level.

While our results do not directly address the question of the causative microdomains in the membrane, one obvious candidate is the lipid raft, a cholesterol- and sphingomyelin-rich microdomain in the plasma membrane. We show that cholesterol depletion results in a significant reduction in the development of AFT. Mammalian SLO-1/BK function is known to be modulated by the cholesterol environment in which it resides in the membrane [14,25–27], and the mammalian BK channel is found in lipid rafts [31]. Recent studies suggest that the modulation of channel function by cholesterol is through a specific interaction with a protein surface on the SLO-1/BK channel itself rather than a secondary effect of cholesterol’s effect on lipid packing [25,27]. There is increasing awareness of the modulation of function of membrane proteins by their interaction with lipids. This model of proteins moving between different lipid microenvironments during AFT might include the effects of ethanol on multiple ethanol-responsive proteins in addition to SLO-1. For instance, ethanol treatment inhibits localization of the proteins Lck, ZAP70, LAT and PLCγ1 to lipid rafts in T lymphocytes [15]. Another intriguing candidate for a protein whose function is likely to be modulated by its location in rafts is H-ras, which is known to be involved in the dynamic response of NMDA receptors to ethanol [32]. In its inactive form, H-ras resides in lipid rafts, and when activated, it must move out of rafts to efficiently activate its target Raf [33].

Importantly, this mechanism of modulation of protein function may reflect a more general process used in maintaining homeostasis in neurons. The mechanisms of development of AFT to ethanol are likely to represent processes that neurons use to modulate their function in response to depressive stimuli in general.

An individual’s level of response to ethanol is dependent on the AFT process. We hypothesize that subtle alterations in plasma membrane structure, perhaps through natural genetic variation in the lipid metabolism machinery, could lead to differences in rates of development of AFT between individuals, which may contribute to predisposition to alcohol dependence (AD) [1]. Additional support for this hypothesis comes from the observation that membranes isolated from the brains of rats selectively bred for high and low ethanol sensitivity showed differences in their ethanol responsive phenotypes as measured by both membrane protein mobility and membrane thickness [34].

Recently, a human genome-wide association study of AD in an Australian population identified a SNP in the human homolog of ctbp-1, called CTBP2, as the marker with the most significant P value for association with AD [35]. Significantly, the mammalian CTBP2 protein also acts as a transcriptional repressor, and has recently been shown to play a role in regulating genes with roles in lipid metabolism in adipocytes [36]. Variation in the function of this gene may have more general effects on lipid membrane composition and could impact responses to alcohol. We suggest that it may be fruitful to further examine genes involved in cholesterol and lipid biosynthesis in vertebrate models and human genetic association studies of alcohol dependence.

Materials and Methods

Worm Husbandry

C. elegans strains were maintained as described [37]. Strains used in this study were: N2 (var. Bristol), AX201 npr-1(ky13), BZ613 cebp-1(eg613) (4×outcrossed), RB733 cebp-1(ak498), BZ846 cebp-1(ak498); npr-1(ky13), MT3887 pag-3(n3098), JCB40 pag-3(n3098); npr-1(ky13), JCB25 pag-3( bet16); npr-1(ky13) (1×outcrossed), VC226 ucb-1(ak109), RB2287 lips-7(ok3110), VC199 sir-2.1(ok343), RB1716 nhr-49(ak2165), VC870 nhr-49(gk405), VC837 bbs-1(ak1111), RB1600 tab-1(ak1972), BX110 fat-7(ua36), fat-5(m420), CE541 cebp-1(pq79), RB142 slo-1(eg142), JCB95 slo-1(£389) (1×outcrossed), JCB96 lips-7(ok3110); slo-1(£389), JCB97 slo-1(£389) (1×outcrossed), and JCB98 lips-7(£3110); slo-1(£389).

Genetic Screen

npr-1(ky13) animals were mutagenized in 47 mM ethyl methanesulphonate (EMS) for 4 hours. Second generation progeny were screened for failure to develop AFT; Worms were incubated for 90 minutes in 300 mM ethanol in M9, then were placed on one side of a 10 cm Petri plate filled with assay agar [38] and 300 mM ethanol. Worms were enticed to move across the plate to a spot of chemoattractant (1 μL of 1:200 benzaldehyde:ethanol). In these conditions, all npr-1(ky13) animals are able to develop sufficient
AFT to be able to move to the spot of chemoattractant. After 90 minutes, worms that had not reached the chemoattractant were picked individually and allowed to generate self-progeny. These progeny were tested in a secondary screen for the development of AFT; animals that failed to develop AFT were kept.

**Analysis of Speed**

Speed was analyzed as described previously [4], with minor modifications: Briefly, assay plates were dried for two hours at 37°C with lids off on the day of the assay. Copper rings were melted into the surface of the plates, and two hours before the beginning of the assay, ice-cold 100% ethanol was added to plates to a final concentration in the agar of 0 mM or 400 mM. Age-matched first day adult animals were acclimated to the lack of food in the assay for a period of 30 minutes by moving them to unseeded dried plates. Ten worms of each test strain were moved into a copper ring on an assay plate and movies were recorded of their movement at 10 and 30 minutes of exposure. Only strains that were tested in the same assay on the same plates were compared to each other. Movies were made on an Olympus SZX-7 stereo microscope (magnification of 0.8x with a 0.5x objective to make a large enough field of view) using a Retiga 4000R camera (QImaging) and ImagePro Plus (6.2) (MediaCybernetics) software. 2-minute recordings (1 frame per second) were captured, and the speed of each worm was tracked using ImagePro plus software and an average speed for each group of 10 animals was calculated.

**Assessment of Ethanol Response**

We assess ethanol response in three ways:

1. **Initial Sensitivity:** We compare the degree of depression of speed (relative speed) at 10 minutes to that of the control strain. A statistical difference at this timepoint reflects a change in initial sensitivity.

2. **Development of Acute Functional Tolerance:** We compare the speed of a strain at 30 minutes versus 10 minutes to determine if the strain had developed AFT. A statistically significant increase in speed at 30 minutes vs. 10 minutes is AFT.

3. **Comparison of AFT with the control strain:** We compare the amount of speed recovered versus the control strain. Statistically significant differences in the amount of recovered speed reflects a difference in the degree of AFT.

**References**


