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Alcohol-induced Aggression in Drosophila

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Abstract

Alcohol-induced aggression is a destructive and widespread phenomenon associated with violence and sexual assault. However, little is understood concerning its mechanistic origin. We have developed a *Drosophila melanogaster* model to genetically dissect and understand the phenomenon of sexually dimorphic alcohol-induced aggression. Males with blood-alcohol levels of 0.04 mg/ml BAC were less aggressive than alcohol-naive males, but when the BAC had dropped to ~0.015 mg/ml the alcohol-treated males showed an increase in aggression toward other males. This aggression-promoting treatment is referred to as the post-ethanol aggression (PEA) treatment. Females do not show increased aggression after the same treatment. PEA-treated males also spend less time courting and attempt to copulate earlier than alcohol-naive flies. PEA-treatment induces expression of the FruM transcription factor (encoded by a male-specific transcript from the *fruitless* gene), whereas sedating doses of alcohol reduce FruM expression and reduce male aggression. Transgenic suppression of FruM induction also prevents alcohol-induced aggression. In male flies, alcohol-induced aggression is dependent on the male isoform of the *fruitless* transcription factor (FruM). Low-dose alcohol induces FruM expression and promotes aggression, whereas higher doses of alcohol suppress FruM and suppress aggression.

Keywords

alcohol-induced aggression; alcohol-use disorder; aggression; Drosophila; *fruitless* ; sexually dimorphic behavior

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Author Contributions

AP and NSA designed the experiments. AP and NSA interpreted the data and wrote the manuscript. AP, TT, LG, CJS, JDYP, GAT, and EAS conducted the experiments. DPS and RAB provided a key resource and assisted with experimental design and data interpretation.

Introduction

Alcohol has long been recognized to induce or uncover aggression in humans [1]. Approximately 40% of violent offenses are committed by people under the influence of alcohol, and at least 50% of sexual assault cases are associated with alcohol consumption [2]. Despite alcohol-induced aggression being costly and pervasive, we do not have a comprehensive understanding of the neurobiological mechanisms that promote aggression after consuming alcohol. In mice, low-dose alcohol and withdrawal from long-term alcohol use have been shown to produce heightened aggression in a subpopulation of animals. Miczek and colleagues suggest that this response involves alcohol's simultaneous effects on serotonergic, GABAergic, glutamatergic (NMDAR) and neuropeptidergic signaling [3]. For *Drosophila melanogaster*, there exists a substantial literature on both male aggression and on the response to alcohol [4; 5]. However, little is known about the intersection between these topics, that is, whether or not alcohol modulates aggression in flies. To help bridge this gap in knowledge, we introduce *Drosophila melanogaster* as a model organism in which the aggressiveness of male flies can be elevated by systemic alcohol. *Throughout this paper, alcohol will be used to refer to only ethanol.*

Flies, like humans, naturally consume alcohol, and they share with humans many of the same alcohol responses. Flies become intoxicated, acquire tolerance, show withdrawal responses, voluntarily drink solutions containing substantial quantities of alcohol, show preference for alcohol-containing solutions, overcome obstacles to seek cues associated with alcohol, and even use alcohol medicinally [6; 7]. The evolutionary conservation of fly genes and conserved responses to alcohol have allowed researchers to reliably translate results from flies to mammals. In mammals, there are many sex-dependent alcohol responses prior to the acquisition of alcohol dependence and after alcohol dependence has arisen. Despite its prevalence, the mechanistic origin of sex-specific responses to alcohol is little understood [8; 9]. *Drosophila melanogaster* also show sexually dimorphic alcohol responses and provide the opportunity to genetically dissect and describe how a sexually dimorphic alcohol response can arise.

The *fruitless* gene has been implicated in a number of sexually dimorphic alcohol behaviors, including sensitivity, tolerance, and preference [10; 11]. Promoter 1–specific *fruitless* transcripts show sex-specific alternative mRNA splicing [reviewed in [12]]. In males, alternate splicing generates transcription factor-encoding fru^{M} transcripts that are exclusively expressed in male neurons, whereas female splicing generates a message that does not encode a protein (here referred to as fru^{F} transcripts) [13–16]. FruM has been implicated in regulating courtship behavior and aggression and in determining patterns of sex-specific neuronal arborization [17–19].

Materials and Methods

Additional methods are presented in Supplementary Information.

Fly husbandry

Flies were raised on cornmeal malt extract food (7.6% CH Guenther & Son Pioneer Corn Meal (Walmart, Inc), 7.6% Karo syrup (Walmart, Inc), 1.8% Brewer's yeast (SAF, Milwaukee WI), 0.9% Gelidium agar (Mooragar, Inc, Rocklin, CA), 0.1% nipagin (Fisher Scientific, Inc.) in 0.5% ethanol, 11.1% #5888 amber malt extract (Austin Homebrew, Austin, Tx) and 0.5% propionic acid (Fisher Scientific, Inc). Solids are weight/volume and liquids are volume/volume. Flies were housed a 12:12 light:dark cycle at ~21°C. Males used in aggression and courtship receptivity behavioral assays were all taken from group housed bottles as pupae and individually raised in vials. Females were group housed (about 10 females per vial). Behavioral tests were performed on flies that were 4–6 days old. For courtship assays males were 4–6 days old and virgin females were 3–4 days old. Flies used in imaging, immunohistochemistry, and qPCR were group housed. Genotypes of flies are in Supplementary Information.

Ethanol Treatments

Ethanol vapor was administered using a glass and teflon "inebriator" [20]. ***PEA treatment.*** Flies were exposed to the vapor produced by bubbling air at 2.5 L/min through a 30% ethanol by volume in ddH_20 solution (5-minute exposure). Flies were immediately transferred to a fly food vial. After one hour they were aggression tested. ***Low-dose alcohol treatment.*** Flies are treated as for PEA but tested immediately after ethanol-vapor exposure. ***Alcohol sedation treatment.*** Flies were exposed to the vapor produced by bubbling air at 2.5 L/min through through a 100% ethanol solution (15-minute exposure). Following sedation flies were transferred to food vials and allowed to recover for 24 h. In all vapor treated animals, the control animals (ethanol-naive animals) were vapor treated in the same apparatus, in the same way, and for the same amount of time as the experimental animals except that the control animals were exposed to an airstream containing only water vapor whereas the experimental animals were exposed to an airstream containing both water and ethanol vapor. ***Alcohol administration in food.*** Flies were housed for three days on twenty percent ethanol food. This food was made by melting standard fly food in a microwave. After cooling to ~45° C, 100% ethanol was added to reach 20% w/v ethanol. Flies were placed into fresh 20% ethanol food vials each day. Control animals were treated in the same way except that no ethanol was added to the food.

Aggression and Dominance

Aggression assays were performed in a chamber described by Mundiyanapurath et al. [21] with slight modifications (Supplementary Information). A virgin female was placed on her back with her wings lightly pressed into the food to prevent her from standing up and walking. Males were loaded into the chamber by gentle aspiration, allowed to acclimate for 5 minutes, and a video camera used to record the behavior from a slightly elevated side view. Aggression assays were conducted between the hours of 9 a.m. - 4 p.m. Animals were allowed to acclimate for 5 minutes and then a 30-minute recording was scored by at least two observers and averaged.

Male dominance was assayed as described by Chouhan et al. [22] using the chambers described for the aggression assay. The assay proceeds in the same manner as the aggression

assay except that two males are added to the chamber—one of which has experienced ethanol treatment. A small white dot (Sherwin Williams interior acrylic latex paint, 650428204)) was painted on the upper thorax of one of the males on the day of eclosion so that the two can be distinguished in the video recording. The dot was alternated between ethanol-treated and ethanol-naive flies. The video cameras were placed above the chambers —the aerial view made it easier to determine the identity of the flies. We recorded for 30 minutes and videos were scored for the number of lunges made by each male and the identity of the male who retreated from the first bout.

Courtship

Courtship behavior was assayed essentially as described by Manoli et al. [23] using a laser cut 1.5 cm diameter by 0.5 cm deep plexiglass chamber in a 3×4 array. Flies were loaded by gentle aspiration and the behavior video recorded flies for 3 minutes and manually scored for unilateral wing extensions (time of occurrence and duration) and attempts to copulate (time of occurrence). We tested alcohol-treated males and ethanol-naive males in parallel. Lunge index = (number of lunges by PEA-treated male - number lunges by control-treated male) / total number of lunges. Retreat index = (number of retreats by control - number of retreats by PEA) / total of retreats.

Immunohistochemistry and Imaging

Brains were dissected from CO_2-sedated flies in 1X PBS and fixed with 4% paraformaldehyde at 4° C for 1 hour. An aliquot of the FruM antibody [24] was generously provided by Daisuke Yamamoto. For FruM antibody staining the brains were washed in 0.2% PBT (1X PBS and 0.2% Triton X-100) three times for 15 minutes each and blocked with 10% Normal Goat Serum (NGS) for at least 3 hours. The brains were then incubated in guinea pig anti-FruM primary antibody in PBTN (0.2% PBT in 10% NGS) for at least 10 hours, washed in PBT three times for 15 minutes each, and stained with Life Technologies Alex Fluor 488 goat anti-guinea pig IgG (Eugene Oregon). Secondary antibody was diluted in PBTN at 1:200. Brains were incubated at least 3 hours with the secondary antibody at room temperature. Brains were then washed in twice in PBT for 15 minutes and dehydrated in 3 one-hour steps (10%, 50%, and 80% glycerol in PBS). Brains were mounted anterior side up on frosted Poly-L-lysine slides with Vectashield with DAPI (Vectorlabs, Burlingame, CA). Brains were imaged with a Zeiss LSM 780 (Jena, Germany). We sampled at an interval of 1.81 μm at 20X through the whole volume of the brain.

When comparing staining between two samples, a single staining solution was prepared and aliquots used to stain both samples at the same time in adjacent wells. When quantifying samples to be compared, the gain and laser power was kept constant across all samples for the same experiment and a manually set thresholded integrated gray signal was measured and kept constant across all samples. Finally, all quantifications were performed from the first scan to avoid bleaching.

Results

PEA treatment increases male aggression and dominance.

A 5-minute exposure to the vapor from a 30% alcohol solution, which produces a 0.047± 0.002 mg/mL (~ 1 mM) blood alcohol concentration (Fig. 1A and B), suppresses the incidence of aggressive interactions (Fig. S1B and C). However, one hour later, blood alcohol dropped to 0.0145 ± 0.009 mg/mL (~ 0.31 mM, Fig. 1B), and we observed a rise in the aggressive interactions between males (high fencing, shoving, and lunging; Fig. 1C–H; Movie S1 and S2). Less-common forms of male–male aggressive behavior did not show a significant increase (low fencing, tussling, and boxing; Fig. 1I–K). The 5-minute treatment followed by 1-hour recovery paradigm will be referred to as the Post-Ethanol Aggression (PEA) paradigm. This alcohol treatment does not produce noticeable changes in locomotion or appear to reduce the capacity to feel pain (Fig. S2). The PEA treatment does not alter the incidence of aggressive acts by females (Fig. S1D and E).

One would expect the more aggressive PEA males to dominate alcohol-naive control males. To test this idea, we pitted a PEA male against an alcohol-naive male, counted the number of lunges made by each, and recorded which male retreated from the first aggressive interaction. The positive retreat index in Fig. 2A indicates that PEA males were less likely to retreat than the alcohol-naive control males. Fig. 2C more clearly shows the relationship between the frequency of lunging and the retreat index. Quadrant 2 (Q2) contains PEA flies that lunged more often and retreated less and were thus the winners of most fights. Q3 contains a smaller number of PEA flies that lunged less and were also the losers of their bout. These two categories are as expected—males that lunged more usually dominate their opponent. However, the data points in Q4 were unexpected. These represent bouts in which PEA males did not retreat even though they lunged less than their competitor, suggesting that PEA dominance involves not only the act of being aggressive but also the response to aggressive actions.

Because alcohol can impair memory and because submission may require remembering that one has lost [25], we asked whether PEA flies were more dominant because of a deficit in short-term memory. To assess whether flies had impaired short-term learning or memory, we used the learned-suppression-of-phototaxis assay [26]. In this assay, flies in a T maze choose between an illuminated arm or a dark arm. Untrained animals almost always choose the lit arm. PEA and control males showed indistinguishable baseline phototaxis. To evaluate the capacity for associative memory, we paired the illuminated arm with quinine, which flies find aversive. Learning, assessed as the fly's acquired decision to enter the dark arm, was indistinguishable between PEA-treated and control animals (Fig. S3).

Previously, we reported that the odor of alcohol potentiates the response of sensory neurons that respond to the aggression-promoting pheromone cVA [27]. We thought it unlikely that PEA treatment would also cause this effect because following the alcohol exposure, the flies had been isolated for an hour in an alcohol-free vial—a period of time expected to eliminate the odor of alcohol from the fly. However, to determine whether the PEA treatment also potentiated the response to cVA, we compared extracellular recordings of the cVA response of a T1 sensilla neuron from alcohol-naive and PEA-treated flies. The PEA treatment clearly

did not potentiate the response to cVA pheromone (Fig. S4), unlike what was observed when the alcohol odor was presented during the recording (*c.f.* Fig. 2F in Park et al., 2020).

Because sexual and aggressive behaviors are often intertwined on a neurobiological level [28], we examined whether PEA treatment influenced male courtship of a virgin female. We visually scored unilateral wing extensions (UWEs) by which the male generates its courtship song. PEA males spent less time performing UWEs compared to controls (Fig. 2D), had a shorter average UWE bout duration than controls (Fig. S5A), and were more likely than control males to attempt copulation with the female carcass (Movie S3 and Fig. S5B).

Alcohol-increased male aggression is dependent on the FruM transcription factor.

FruM is a transcription factor that underlies many male-specific behaviors. Transformer F (TraF) is a splicing regulator that suppresses splicing events that generate the fru^{M} isoform [29] (Fig. 2F). Expression of a transgenic TraF suppresses FruM production and prevents alcohol-induced increases in male:male aggression (Fig. 2G). TraF also alters splicing of *doublesex* (*dsx*) transcripts, causing expression of a female-specific *dsx* isoform known as Dsx^{F}. However, overexpression of Dsx^{F} from a transgene did not alter alcohol-induced male:male interaction, indicating that Dsx^{F} does not modulate alcohol-induced aggression.

PEA treatment stimulates fru^{M} expression.

To determine if alcohol affects FruM expression, we measured changes in transcript and protein abundance in fly heads. PEA produces a slight drop in fru^{M} mRNA abundance compared to controls (Fig. 2H). We measured FruM protein levels in the whole brain using a FruM antibody [24]. PEA flies had a ~3-fold increase in FruM protein in the adult brain (Fig. 2I, J).

A sedating dose of alcohol depresses fru^{M} expression.

Using primers specific for the male exon [30] we used qPCR to determine fru^{M} transcript abundance after alcohol-vapor sedation (Fig. 3A,B). We found that 6 and 24 hours following alcohol sedation, the abundance of the fru^{M} splice variant was substantially depressed in males (Fig. 3C). Furthermore, 24 hours following alcohol sedation, male aggression was nearly completely suppressed (Fig. S1B and C). We also observed that males housed for 3 days on 20% alcohol food had a significant reduction in fru^{M} transcript, suggesting that high alcohol exposure leads to decreases in fru^{M} regardless of administration route (Fig. 3D).

To confirm that the FruM protein levels were reduced by the sedating dose of alcohol, we used a FruM-antibody [24] to quantify protein-level changes in the whole brain 24 and 48 hours after a 15-minute exposure to a sedating dose of alcohol vapor. After 48 hours, there was a significant reduction in FruM expression in the whole brain (Fig. 4H and I).

The *fru* gene also expresses a female variant, called fru^{F}, and common variant that is expressed in both sexes, called fru^{COM} (Fig. 3A). To determine if fru^{F} or fru^{COM} were alcohol responsive, we measured their transcript levels 24 hours following alcohol-vapor sedation. This time point was chosen because it was the time point during which fru^{M} had

the largest reduction. Neither *fru*^{F} nor *fru*^{COM} changed expression following this alcohol treatment (Fig. 3E, F).

Because TraF directly depresses *fru*^{M} splice variant abundance, which can reduce FruM protein production [29], we asked whether alcohol effects a decrease in FruM by ectopically activating TraF in males. Active TraF protein also regulates the splicing of *doublesex* (*dsx*) transcripts, causing the production of the *dsx*^{F} splice variant. To determine if reductions in FruM were a result of alcohol-induced increased TraF activity, we measured levels of *dsx*^{F} in male flies. We found that *dsx*^{F} did not change in levels of expression 6 or 24 hours following alcohol exposure, suggesting there is an alternative mechanism by which *fru*^{M} is suppressed by alcohol (Fig. 3G).

Ethanol sedation decreases *fru*^{M} expression in a region-specific manner.

To determine if *fru*^{M} was decreasing in specific brain regions after alcohol exposure, we used FruM>EGFP flies in which a Gal4 under the control of the endogenous *fru*^{M} promoter constitutively drives expression of a UAS-EGFP reporter. We measured the intensity of the GFP signal in brains of animals that had been sedated with ethanol vapor (Fig. 4A, B and C). We found that there was a significant reduction in GFP levels in the whole brain as well as in specific brain regions. One obvious change was complete loss of *fru*^{M} expression in the neurons that innervate the antennal lobes in alcohol-treated animals (circled in Fig. 4B). The FruM>EGFP staining pattern differs from the FruM antibody pattern because the former is cytosolic and the latter is nuclear. We also found a slight reduction in the mushroom bodies but no change in the optic lobes (Fig. 4D). The antennal lobe GFP intensity in vapor-treated animals was not quantified because, after the alcohol-sedation treatment, the GFP signal was below the level of detection. However, we did quantify a drop in GFP intensity in the antennal lobe DA1 and Va1v projection neurons in animals fed alcohol for three days (Fig. 4E). To confirm that the activity of GFP reporters are not depressed by alcohol exposure in general, we used the *Syb*-Gal4 transgene (R57C10-Gal4) to drive the UAS-EGFP. With this combination, we found no substantial changes in GFP expression in the brain (Fig. 4F).

The *fru*^{M}-expressing antennal lobes consist of glomeruli that integrate axonal processes from the primary olfactory sensory neurons (OSNs) and dendritic arbors of the projection neurons (PNs). The OSNs are housed in the fly antennae and are also *fru*^{M} expressing. We sought to determine if the antennal OSNs also had reductions in *fru*^{M}, especially since antennal OSNs are directly exposed to alcohol vapor and are known to be affected by alcohol [31]. We exposed *fru*$^{Gal4>GFP}$ flies to alcohol, and then dissected and imaged their antennae (Fig. 4G). The cuticle of the antennae is thin enough to image through without removal. We found that the OSNs also showed significant reductions in the *fru*^{M} GFP intensity following alcohol exposure.

Alcohol vapor can cause cell death [31], and this cell death could be responsible for the drop in GFP abundance. To determine if alcohol was causing cell death at this alcohol dose, we stained whole brains and antennae with acridine orange. Acridine orange staining is sensitive to both apoptotic and necrotic cell death, with the green signal being proportional to genomic DNA accessibility and the red signal reflecting RNA abundance [32; 33]. There

were no significant changes in red or green channel fluorescence between control and alcohol treated in whole brain or in antennae (Fig. S6).

Discussion

The FruM protein is a transcription factor that is a primary regulatory switch responsible for many male-specific behaviors, despite the fact that it is expressed in only ~2% of neurons in the male brain [34]. One hour after a low dose of alcohol (~0.05 mg/mL BAC; PEA treatment), FruM protein abundance had increased, and the males had become more aggressive and tended to dominate alcohol-naive males in the fighting arena. (An ethologically interesting aside is that *D. melanogaster* seek fermenting foods as a reproductive niche, and our data indicate that the consumption of this alcohol food could change male behavior in ways that make male flies compete more vigorously for use of the reproductive site.) On the other hand, a higher dose of alcohol had the opposite effect. Twenty-four hours after a sedating dose of alcohol, FruM protein abundance dropped throughout the brain, including in the mushroom body and antennal lobes, and the males were much less aggressive. The drop in abundance in the antennal lobe Da1 and Va1v structures was so profound as to be below the level of detection. This is not the first time FruM has been connected with an alcohol-vapor-induced social behavior. Lee et al. [35] found that exposing male flies to multiple sedating doses of alcohol vapor increased male–male courtship. Male–male courtship is a phenotype associated with mutations that reduce FruM expression [36; 37]. These results could be explained by our observation that sedating doses of alcohol reduce FruM expression, and FruM is important for normal courtship behaviors. Interestingly, PEA, which enhances FruM expression, reduces the time spent courting.

We compared the putative transcriptional targets of FruM identified using DamID [19] with functionally validated alcohol genes (genes whose expression alters one-or-more alcohol responses) [38; reviewed in 5] and observed that almost half of the alcohol genes were targets of FruM binding (Fig. 5). Four genes in the intersection between alcohol-responsive genes and FruM-bound genes have also been shown to alter aggression when mutant, although the mechanisms or relevant cell types have not been described [39]. These genes are *Bacc* (molecular function unknown but negatively regulates conversion of tyramine to octopamine), *NMDAR1* (NMDA type ligand-gated receptor channel), *Bx* (LIM-only protein with roles in alcohol and cocaine responses), and *pxb* (molecular function unknown but involved in long-term memory) [40]. Perhaps, these genes contribute to molecular changes involved in producing alcohol-induced aggression. In addition, the Tachykinin/Substance P (Tk) signaling pathway, which promotes aggression in flies and mammals, also has substantial circumstantial evidence implicating it in the production of alcohol-associated aggression. In flies, the Tk-producing neurons are also FruM positive [41], which offers the possibility of FruM influencing the activity of these neurons. Furthermore, both the *Tk* gene and the tachykinin-receptor gene, *Takr99D,* have been shown to have nearby FruM binding sites [19], and *Takr99D* was previously identified in a search for ethanol-responsive genes based on increased histone acetylation of the gene following ethanol sedation [42]. This hypothesis that PEA treatment increases aggression by acting on genes and neurons the directly produce male aggressive behavior is appealing. However, an alternative hypothesis is that the increase in aggressive acts is a product of a decrease in impulse control in the

male. Certainly, it seems reasonable that in humans that the increase in aggressive acts associated with alcohol consumption could arise from a suppression of impulse control.

An intriguing but tenuous connection can be made between FruM and its closest mammalian orthologues—ZBTB45, 1, 39, and 24 (determined via DIOPT [43]). The ZBTB (Zn finger and BTB protein-binding domain) family of transcription factors are known to regulate differentiation of immune cells, glia, neurons, and oligodendrocytes [44; 45]. The presence of a ZBTB transcription-factor binding site in the *SRY* gene may mean that ZBTB activity directly modulates SRY expression. In the mammalian brain, a ZBTB also changes expression in response to alcohol [46; 47]. It is possible that a ZBTB transcription factor has a similar role in regulating alcohol-related sex-specific gene expression in mammals.

While it is well known that alcohol responses are sexually dimorphic, this has historically been viewed as a consequence of hormonal differences and not as the consequence of a sexually dimorphic transcription factor. We believe that this is the first report of a gene, expressed in only one sex, as an underlying agent in the production of sexually dimorphic response to alcohol. Understanding how the downstream transcriptional targets of FruM promote male behaviors is important for uncovering how alcohol coordinates these changes in a sexually dimorphic manner.

Data sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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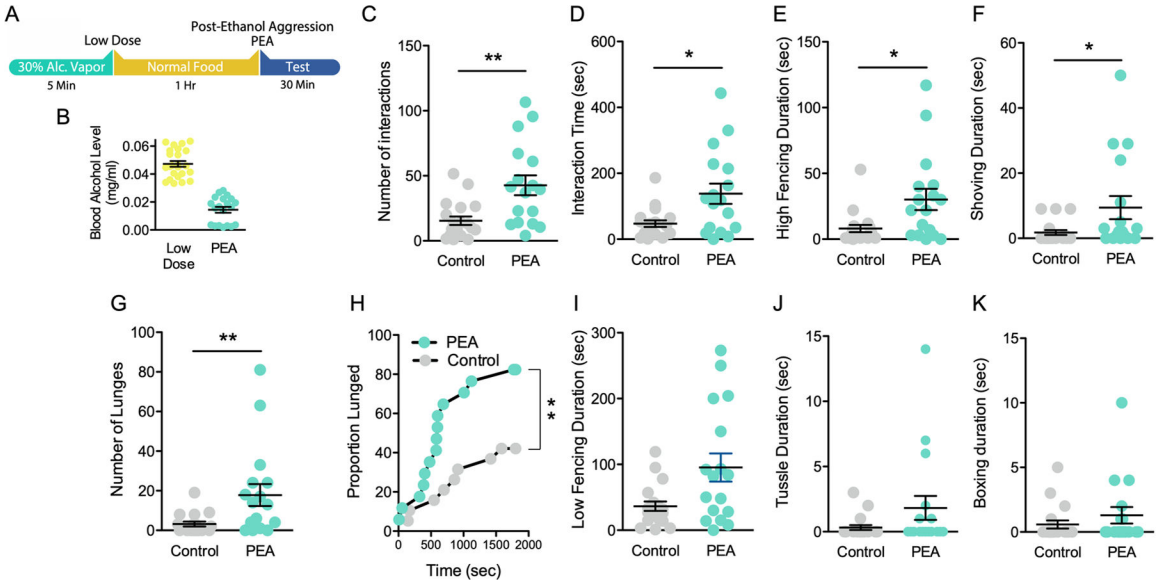


Figure 1. Low dose of ethanol (PEA paradigm) enhances aspects of male aggression.

(A) Alcohol-treatment protocol. **(B)** Blood alcohol levels measured immediately after alcohol exposure (Low Dose Mean +/− SEM; 0.047 +/− 0.002 mg/mL, n = 24) and one hour after alcohol exposure (PEA; Mean +/− SEM; 0.0144 +/− 0.002 mg/mL, n = 20). **(C)** PEA treatment increases the number of male-male encounters (p = 0.0029). **(D)** PEA treatment increases the time spent in male:male encounters (p = 0.0276), **(E)** the time spent high fencing (p = 0.0196), **(F)** the time spent shoving (p = 0.0292,), **(G)** the number of lunges (p = 0.0086), and **(H)** the fraction of flies that lunged (p = 0.0068). However, the period of time spent **(I)** low fencing (p = 0.0572), **(J)** tussling (p = 0.1625), or **(K)** boxing (p = 0.3557) were not increased by PEA treatment. For **(C)** through **(K)**, N = 17 or 19. In (B)-(G) and (I)-(K) the middle line is the mean and outer brackets are SEM and the Mann-Whitney test was used to evaluate significance. For H, the Mantel-Cox log-rank test was used to evaluate significance.

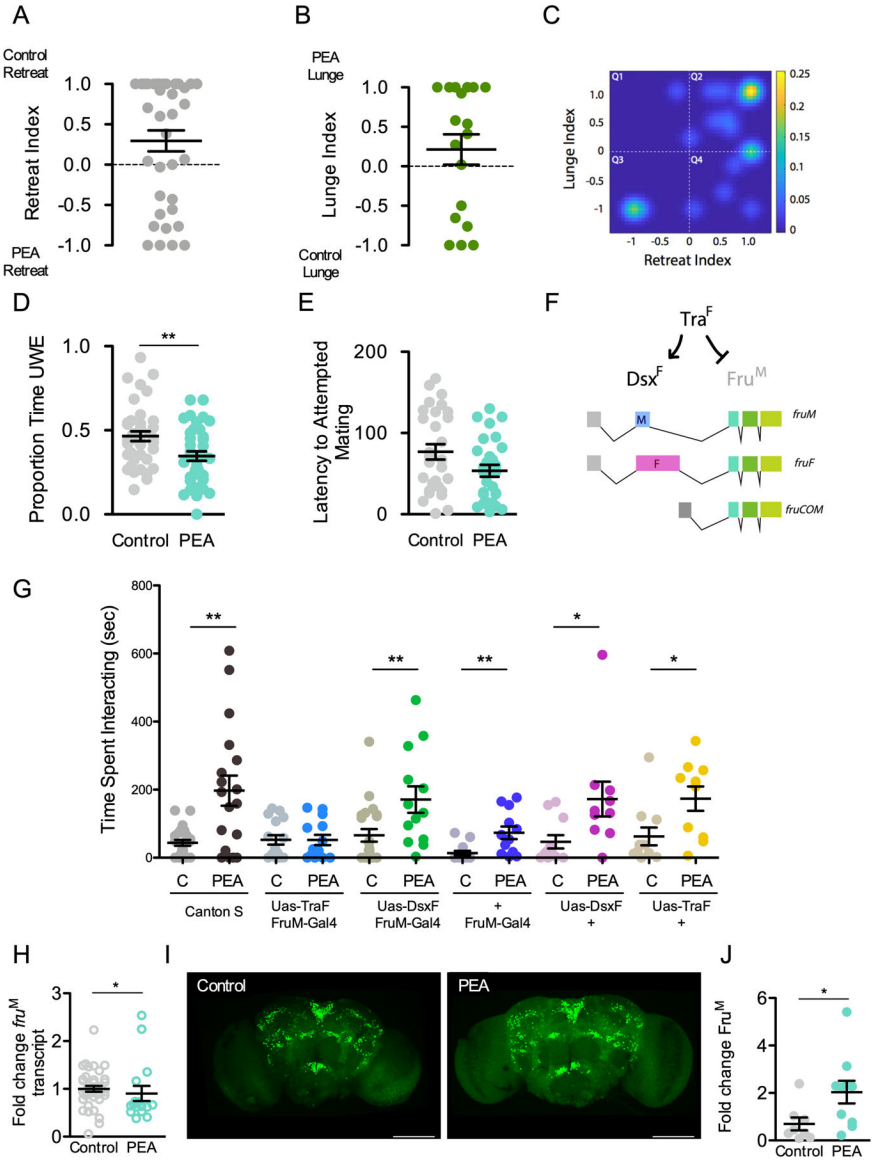


Figure 2. PEA treatment increases dominance but reduces courtship behavior in males.

(A) Positive Retreat Index indicates that alcohol-naive flies are more likely to retreat, and negative values indicate that PEA flies are more likely to retreat (one-sample Wilcoxon Signed Rank test, μo = 0, p = 0.0229, n = 36). **(B)** Positive Lunge Index indicates that PEA flies are more likely to lunge, and vice versa for negative values (one-sample Wilcoxon Signed Rank test, $\mu_o = 0$, n = 18 p = 0.2890). **(C)** Increased lunging and dominance are associated with PEA treatment. Shown is Lunge Index vs. Retreat Index. More positive ordinate values represent greater incidence of lunging by PEA flies and more positive abscissa values represent greater incidence of retreat by control flies. Conversely, more negative ordinate values represent greater incidence of lunging by control flies and more negative abscissa values represent greater incidence of retreat by PEA flies. Heat map represents frequency. Incidence of PEA lunging is correlated with dominance by the PEA individual (Spearman r test, p = 0.0090, N = 27 XY pairs). **(D)** PEA treatment reduces

the time spent performing UWEs (Mann Whitney test p = 0.0107, n = 39, 38) and the **(E)** latency to attempt mating (Mann Whitney test p = 0.0777, N = 29). **(F)** Three categories of *fru* splice variants. Transformer suppresses FruM production and induces DsxF expression. **(G)** Time spent in male:male interaction plotted across genotypes (includes all forms of male:male aggression; + indicates no UAS or Gal4 transgene) and treatment (C = control, PEA = Post-Ethanol Aggression treatment; Mann Whitney test, * $p < 0.05$, ** $p < 0.01$, n = 10–24). **(H)** Fold change fru^{M} transcript abundance calculated using ΔΔCT method (Mann Whitney test, * p=0.0395, n = 40, 16). **(I)** Merged optical stacks across whole-brain volume for Control and PEA flies stained for FruM protein. Scale bar = 100 um. **(J)** Whole-brain relative intensity of FruM antibody stained fly brains. Relative intensity (normalized to control) of FruM signal (Mann Whitney test, * p = 0.0434, n = 8, 10).

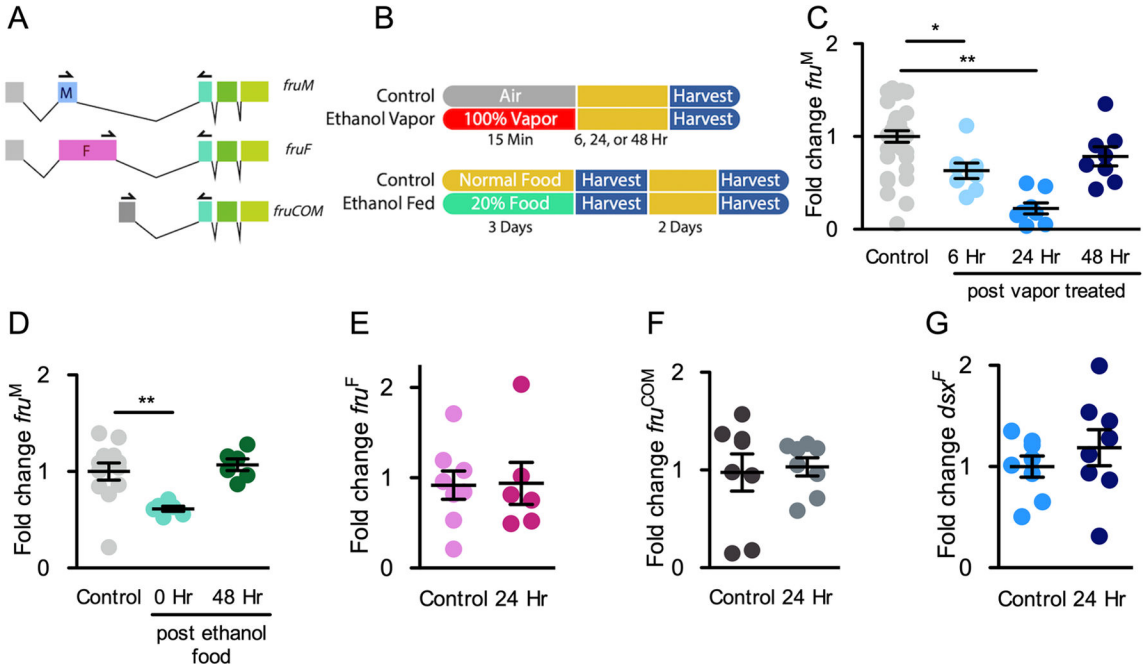


Figure 3. A sedating-dose of alcohol reduces *fru*^{M} expression.

(A) Position of primers used to target each *fru* splice variant. **(B)** Treatment paradigms for alcohol vapor sedation (top) and alcohol feeding (bottom). **(C)** There is a significant reduction in *fru*^{M} 6h and 24h after alcohol vapor sedation (One-way ANOVA p = 1.8e-6 with Dunnett post hoc test; 6h * p = 0.0213; 24h ** p < 0.0001). **(D)** Feeding on 20% alcohol food for 3 days also reduces *fru*^{M} levels (One-way ANOVA p = 0.0053 with Dunnett post hoc test; 0h ** p = 0.00769; 48h p = 0.80023). **(E)** Expression of *fru*^{F} (measured in females) and **(F)** *fru*^{COM} (measured in males) are not changed 24 hours post alcohol vapor sedation. **(G)** Expression of *dsx*^{F} in male flies remains unchanged 24 hours after alcohol vapor sedation (two-tailed unpaired Student's *t*-test).

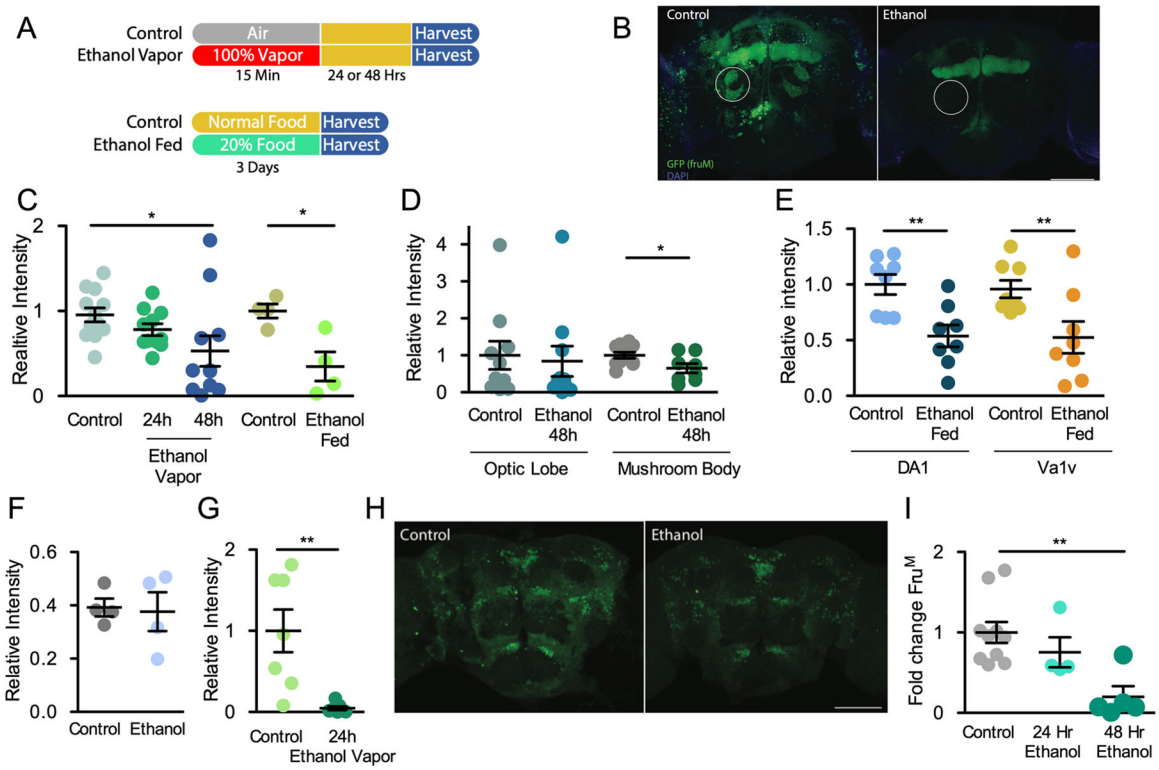
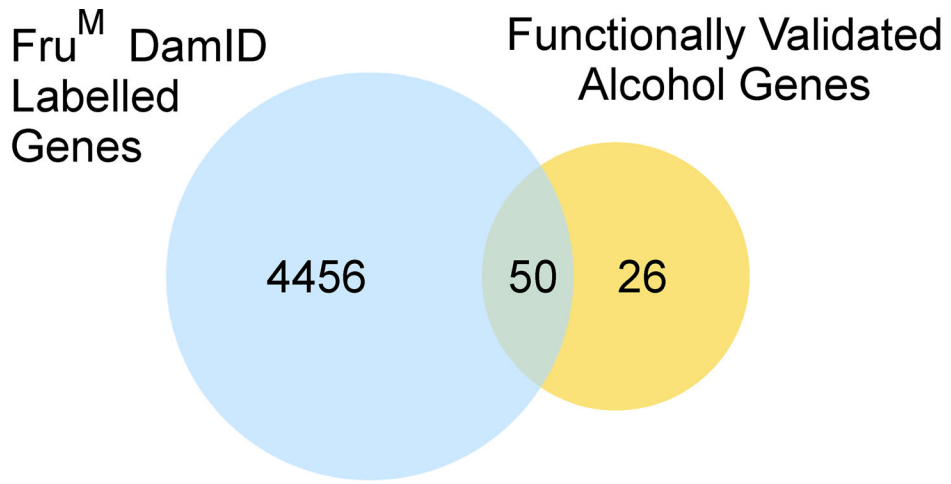


Figure 4. Alcohol vapor sedation suppresses *fru*^{M} expression in a region-specific manner.

(A) Paradigms for alcohol vapor sedation and alcohol feeding. **(B)** GFP fluorescence from dissected fruGal4>eGFP adult brains stained with DAPI to visualize nuclei. Alcohol-naive brain on left and a brain from an animal sedated with alcohol vapor on the right. Scale bar = 100 μm. **(C)** Quantification of whole brain fruGal4>eGFP signal following alcohol vapor sedation (Kruskal-Wallis test p = 0.02 with Dunn's Multiple Comparison Test, 24h post treatment not significant, 48h post treatment * $p < 0.05$, n = 12, 11, 11) and following feeding on 20% alcohol food for 3 days (Student's *t*-test alcohol, p = 0.01399, n = 4). **(D)** Alcohol vapor sedation did not alter fruGal4>eGFP expression in optic lobes whereas mushroom body accumulation was depressed (Mann Whitney test p = 0.0352, n = 10). **(E)** Relative intensity of fruGal4>eGFP signal in animals fed food containing 20% alcohol for 3 days. Expression in the antennal lobe glomeruli DA1 and Va1v were depressed. (DA1: Mann Whitney p = 0.0047; Va1v Mann Whitney p = 0.0379, n = 10). **(F)** Neural-specific R57C10-Gal4>eGRP was not depressed by the alcohol sedation (Mann Whitney p = 0.8857, n = 4). **(G)** fruGal4>GFP signal is depressed in antenna 24 h after alcohol-vapor sedation (Mann Whitney p = 0.0012, n = 7). **(H)** Representative brains stained with FruM antibody control and 48 h after the animals were sedated alcohol vapor. Scale bar = 100 um. **(I)** Whole-brain relative intensity of FruM antibody stained fly brains 24 h and 48 h following alcohol vapor sedation (normalized to control; Kruskal-Wallis test p = 0.0103 with Dunn's Multiple Comparison Test, 24h not significant; 48h * $p < 0.05$, n = 10, 4, 5).



Alk, amn, Arf51F, aru, Bacc, Bx, cact, CASK, cher, Clic, CrebB, dally, Dif, dlg1,dlp, Drat,Egfr, Fas2, form3, hang, htl, InR, KCNQ, klg, moody, Myd88, mys, Nmdar1, Pka-C1, Pka-R2, Pkc53E, ple, pum, pxb, Rac1, Rel, Rho1, RhoGAP18B, rut, S, sca, scb, shi, slo, Syn, Syx1A, tim, Tl, unc-13, Notch

Figure 5. Venn Diagram of FruM DamID identified transcriptional targets and functionally validated alcohol genes.

There is significant overlap (Hypergeometric distribution p = 0.3.8e-11) between FruM target genes [19] and genes functionally validated to modulate alcohol behaviors [5]. The list of genes are those in the region of intersection.