Acute ethanol responses in *Drosophila* are sexually dimorphic

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In mammalian and insect models of ethanol intoxication, low doses of ethanol stimulate locomotor activity whereas high doses induce sedation. Sex differences in acute ethanol responses, which occur in humans, have not been characterized in *Drosophila*. In this study, we find that male flies show increased ethanol hyperactivity and greater resistance to ethanol sedation compared with females. We show that the sex determination gene *transformer* (*tra*) acts in the developing nervous system, likely through regulation of *fruitless* (*fru*), to at least partially mediate the sexual dimorphism in ethanol sedation. Although pharmacokinetic differences may contribute to the increased sedation sensitivity of females, neuronal *tra* expression regulates ethanol sedation independently of ethanol pharmacokinetics. We also show that acute activation of *fru*-expressing neurons affects ethanol sedation, further supporting a role for *fru* in regulating this behavior. Thus, we have characterized previously undescribed sex differences in behavioral responses to ethanol, and implicated fru in mediating a subset of these differences.

Alcohol is one of the most widely used and abused drugs in the world. The acute effects of ethanol are biphasic: at lower internal concentrations, ethanol acts as a stimulant, whereas, at higher concentrations, it acts as a depressant (1). The stimulant effects of ethanol manifest as elevated mood and energy level in humans and as increased locomotor activity in animal models, and are thought to reflect the reinforcing properties of ethanol (2, 3). In contrast, the depressant effects of ethanol manifest in humans as depressed mood, fatigue, and cognitive and motor impairment (2, 4); animal models similarly exhibit motor incoordination and ultimately sedation (1). Several studies have suggested that susceptibility to alcohol use disorders (AUDs) is correlated with increased sensitivity to the stimulant effects of ethanol and decreased sensitivity to its depressant effects (5, 6). Characterizing the mechanisms underlying acute ethanol responses may therefore provide insight into alcohol addiction.

Men and women are differentially affected by acute and long-term ethanol exposure. Men exhibit increased alcohol consumption and a higher incidence of alcohol use disorders compared with women (7, 8). However, women are more susceptible to the negative physical consequences of heavy drinking, such as organ damage and risk of death, and exhibit a faster progression from first use to alcohol abuse and addiction (9, 10). Women are also more strongly affected by acute ethanol intoxication. Part of this effect is pharmacological, as the same ethanol dose (adjusted for body weight) induces a higher blood alcohol content (BAC) in women as a result of differences in body water content (11). However, even when BAC is equalized between the sexes, women exhibit greater ethanol-induced motor impairment and subjective feelings of intoxication than men (4). Thus, there are likely to be sex differences in how ethanol affects the nervous system, but these mechanisms have not yet been identified.

The fruit fly *Drosophila melanogaster* is an established model for studying the genes underlying acute ethanol responses (12). As in humans and rodents, lower doses of ethanol stimulate locomotor activity in flies (13), whereas higher doses induce motor incoordination and sedation (14, 15). Several evolutionarily conserved genes and neuronal signaling pathways regulate ethanol responses in flies and mammals (12). *Drosophila* therefore offers powerful tools for dissecting the molecular and neural pathways regulating ethanol responses.

Despite the number of studies examining acute ethanol responses in flies, sex differences in these behaviors have not been reported. In this study, we find clear sexual dimorphisms in *Drosophila* ethanol responses. We report that male flies show increased ethanol-induced hyperactivity and greater resistance to ethanol sedation compared with females. The sex difference in ethanol sedation is at least partially mediated by neuronal expression of *transformer* (*tra*), which regulates splicing of the neural sex determination gene *fruitless* (*fru*). In addition, acute activation of *fru*-expressing neurons enhances ethanol sedation sensitivity. Thus, we have identified sex differences in ethanol-induced behavior and linked a subset of these differences to *fru*.

Results

Males Show Greater Ethanol-Induced Hyperactivity than Females. Flies exhibit locomotor hyperactivity at low to moderate ethanol doses and sedation at high doses. Ethanol-induced hyperactivity can be assayed by a locomotor tracking system (13), and ethanol sedation can be measured by using a loss-of-righting assay (15). These two responses are uncorrelated and measure distinct aspects of ethanol intoxication (16). Sex differences in ethanol hyperactivity or sedation have not been reported, as most studies have tested males or females but not both (17–19). We therefore asked whether wild-type (WT) males and females show differences in these ethanol responses.

We first tested ethanol-induced hyperactivity in WT males and females. Upon exposure to a moderate dose of ethanol vapor (47% relative vapor concentration), flies exhibit two phases of locomotor activation: an immediate, transient startle response to the smell of ethanol, and a more gradual, sustained hyperactivity response mediated by ethanol intoxication (13) (Fig. 1*A*). We observed that males showed a more rapid onset of ethanol hyperactivity compared with females, as well as an increase in peak hyperactivity (Fig. 1 *A*–*C*). Males also showed a higher olfactory startle response than females, suggesting that females might be physically unable to move as quickly as males. However, when exposed to a mechanical stimulus, males and females showed startle responses of a similar magnitude (Fig. 1*D*), and females achieved a similar speed as males exhibit during peak ethanol hyperactivity (compare with Fig. 1*C*). The decreased ethanol hyperactivity of females is therefore unlikely to be caused by a difference in general locomotion.

Males Show Greater Resistance to Ethanol Sedation than Females. We next tested the sedation sensitivity of WT males and females exposed to a high ethanol vapor concentration (67%). Sedation

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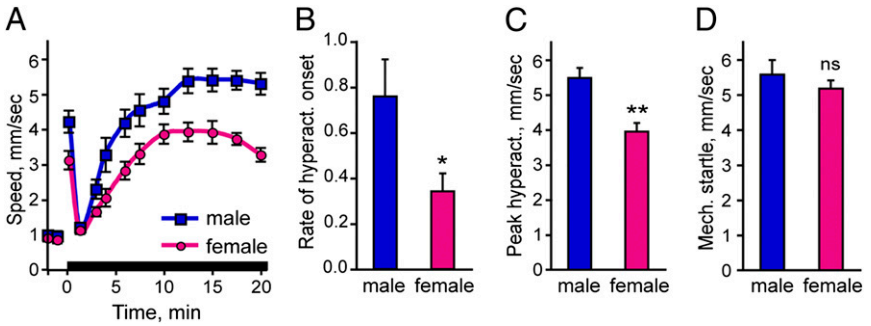

Fig. 1. Males show greater ethanol hyperactivity than females. (*A*) Tracking profiles of male and female flies exposed to 47% ethanol vapor ($n = 8$). The black bar represents the duration of ethanol exposure. A time of 0 is defined as the onset of ethanol exposure and is preceded by 2 min of baseline recordings. Upon ethanol exposure, flies exhibit an immediate, transient startle response followed by a more gradual hyperactivity response. (*B*) Males showed an increased rate of ethanol hyperactivity onset compared with females ($n = 8$). (*C*) Males exhibited greater peak ethanol hyperactivity than females ($n = 8$). (*D*) Males and females did not differ in the startle response induced by a mechanical stimulus (vigorously agitating the fly vials; $P > 0.05$, $n = 11$). $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired *t* tests.

sensitivity can be quantified as the time required for 50% of flies to sedate (ST50). Males sedated more slowly than females, showing a higher ST50: the ST50 of males was 26.5 ± 2.1 min, whereas the ST50 of females was 14.0 ± 0.6 min (Fig. 2 *A* and *B*). This difference appeared even stronger at a lower concentration of 50% ethanol vapor, at which females showed an ST50 of 29.4 ± 1.4 min whereas very few males sedated at all (18% sedated at the end of the 45-min experiment; *SI Appendix*, Fig. S1*A*).

Some studies have also characterized the speed of recovery from ethanol sedation as a measure of sensitivity to the sedative effects of ethanol (18, 20). Following a 45-min exposure to 67% ethanol vapor, which caused most flies to become sedated, males recovered more quickly than females (Fig. 2 *C* and *D*). Because a slightly higher percentage of males than females failed to sedate during the ethanol exposure, we also calculated sedation recovery curves after excluding the flies that never sedated. Using this method, males still showed a more rapid recovery than females (*SI Appendix*, Fig. S1 *B* and *C*). Thus, males are generally more resistant than females to the sedative effects of ethanol, as they sedate more slowly and recover more quickly.

At very high ethanol concentrations, sedation is followed by death (21). We expected that, because males are more resistant to ethanol sedation than females, males might also be more resistant to ethanol-induced lethality. Surprisingly, males were instead more sensitive to ethanol lethality than females. When exposed to 100% ethanol for 60 min, 82% of males and 16% of females died (Fig. 2*E*). These results indicate that the processes mediating ethanol-induced sedation and lethality are dissociable, and both are sexually dimorphic: males are more resistant to ethanol sedation, but more sensitive to ethanol lethality, compared with females.

Males and Females Show Differences in Ethanol Pharmacokinetics. We have described sex differences in ethanol-induced hyperactivity and sedation. To determine whether these effects could be a result of a difference in ethanol pharmacokinetics, we measured the internal ethanol concentration in males and females. During multiple ethanol exposure protocols, females contained ~20% more ethanol than males (Fig. 3*A*). To confirm that this difference also occurs in the brain, where ethanol is likely to mediate its behavioral effects, we measured the ethanol concentration in the heads of males and females. Female heads contained a higher ethanol concentration than male heads, and the magnitude of this difference was similar to the difference within the decapitated bodies and the whole flies (*SI Appendix*, Fig. S2; compare with Fig. 3*A*). The increased ethanol concentration in females likely contributes to their increased sedation sensitivity. In contrast, the pharmacokinetic difference is not likely to explain the sex difference in ethanol hyperactivity, as increased ethanol levels would be expected to shift the hyperactivity curve to the left, in contrast to the delay and overall decrease in hyperactivity exhibited by females.

The increased ethanol concentration in females could result from an increased rate of ethanol absorption or a decreased rate of ethanol metabolism. To quantify the rate of ethanol metabolism, we measured the decrease in internal ethanol concentration during recovery from ethanol exposure. Females showed decreased ethanol metabolism compared with males (Fig. 3 *B* and *C*). Males and females may also differ in ethanol absorption; however, ethanol absorption cannot be measured separately from ethanol metabolism, as both processes occur simultaneously during ethanol exposure. Overall, our results indicate that females metabolize ethanol more slowly than males, leading to increased internal ethanol levels that likely contribute to their greater sedation sensitivity.

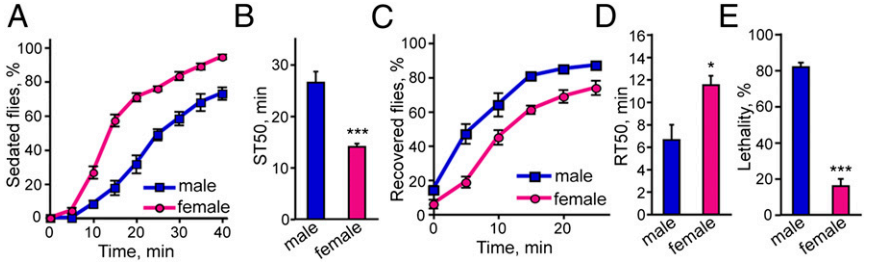

Fig. 2. Sex differences in ethanol-induced sedation and lethality. (*A*) Sedation curves of males and females exposed to 67% ethanol vapor ($n = 8$). (*B*) ST50 values for the experiment shown in *A*. Males had a greater ST50 than females, representing increased resistance to ethanol sedation ($n = 8$). (*C*) Sedation recovery curves following 45 min exposure to 67% ethanol vapor ($n = 5$–6). (*D*) RT50 values for the experiment shown in *C*. Males had a lower RT50 than females, representing faster recovery from sedation ($n = 5$–6). (*E*) Lethality induced by 60 min exposure to 100% ethanol vapor ($n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$, unpaired *t* tests.

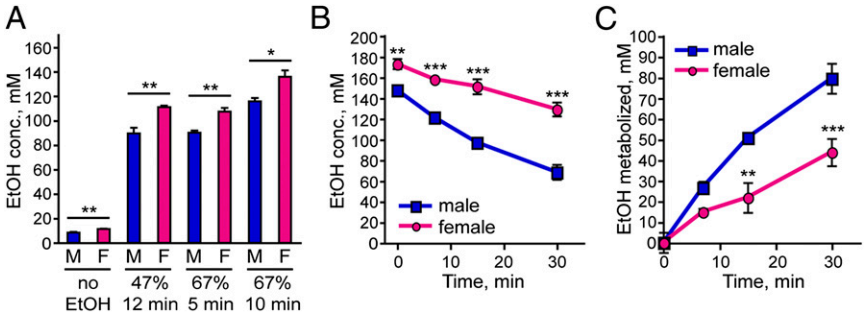

Fig. 3. Sex differences in ethanol pharmacokinetics. (*A*) Females (marked "F") contained a higher internal ethanol concentration than males ("M") using various ethanol exposure protocols. The ethanol vapor concentration and duration of exposure is specified. Unexposed females also contained a slightly higher baseline ethanol level than males, but this difference was not large enough to account for the sex difference in any of the ethanol-exposed groups ($n = 4$). (*B*) Ethanol concentration in males and females during recovery from a 30-min exposure to 67% ethanol vapor. A time of 0 indicates the end of ethanol exposure. Two-way ANOVA revealed a significant effect of both sex and time ($P < 0.001$), as well as a significant interaction ($P < 0.01$), indicating that the slopes of the recovery curves are significantly different ($n = 4$). (*C*) The amount of ethanol metabolized was quantified from the data shown in *B* as the decrease in ethanol concentration compared with the initial concentration present at time 0. Females showed overall decreased ethanol metabolism compared with males ($P < 0.001$, $n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, unpaired *t* tests in *A*, two-way ANOVA followed by Bonferroni posttests in *B* and *C*.

Several *Drosophila* Strains Show Sexually Dimorphic Ethanol Responses. To determine whether the sex differences in ethanol hyperactivity and sedation were specific to our fly strain (*w* Berlin) or are general within *D. melanogaster*, we tested the behavior of several other WT strains (strains commonly used as genetic background controls, although some carry eye color mutations; *SI Appendix*, *SI Methods*). We initially tested ethanol hyperactivity and sedation in four WT strains (Canton S, Oregon R, 2202U, and *w* iso). However, two strains (Oregon R and 2202U) showed very low ethanol hyperactivity in both sexes (peak hyperactivity less than 2 mm/s) and were therefore excluded from further hyperactivity analysis. We then selected two additional strains (*rosy* and Dahomey) to specifically test for ethanol hyperactivity to ensure that we analyzed four strains for each behavior.

In three of the four strains analyzed for ethanol hyperactivity, males showed increased hyperactivity compared with females (Fig. 4*A*), similar to *w* Berlin. In only one of these three strains did males also show an increased ethanol startle response (*w* iso; Fig. 4*B*). This dissociation between the startle and hyperactivity responses again suggests that decreased ethanol hyperactivity in females is not attributable to a general locomotor difference. Similar to ethanol hyperactivity, in three of the four strains analyzed for ethanol sedation, males were more resistant to sedation than females (Fig. 4*C*), again resembling *w* Berlin. Two of these three strains also showed a sex difference in internal ethanol concentration, as in *w* Berlin, with females containing more ethanol than males (Fig. 4*D*). However, in one strain (Oregon R), males and females contained a similar ethanol concentration despite showing a robust difference in sedation sensitivity (Fig. 4 *C* and *D*). This result indicates that sexually dimorphic sedation sensitivity can occur independently of a difference in ethanol levels, suggesting that ethanol may have a sexually dimorphic effect within the nervous system. Interestingly, neither of the two strains tested for ethanol hyperactivity as well as sedation showed sex differences in both behaviors: *w* iso showed sexually dimorphic hyperactivity but not sedation sensitivity, whereas the reverse was true for Canton S (Fig. 4 *A* and *C*). Thus, sexual dimorphisms in ethanol hyperactivity and sedation are dissociable, with both dimorphisms occurring together in *w* Berlin but not in Canton S or *w* iso. Overall, the data suggest that these two dimorphisms each occur in several *Drosophila* strains but can be modified or suppressed by genetic background, highlighting the importance of genes in regulating these behavioral differences.

Tra Acts in Developing Nervous System, Likely Through Regulation of *fru*, to Regulate Ethanol Sedation Sensitivity. Given that sex differences within the nervous system may contribute to the sexual dimorphisms in ethanol-induced behavior, we asked whether the gene *fru* might play a role. *fru* regulates the sexual differentiation of the nervous system and mediates sexually dimorphic behaviors such as courtship and aggression (22–24). *fru* encodes a set of male-specific putative transcription factors collectively termed FruM. The female-specific splicing factor Transformer (Tra), along with Transformer 2 (Tra2), enables sex-specific splicing of *fru*, ensuring that FruM is expressed in males but not females (25). We hypothesized that this pathway might generate the sex differences in ethanol-induced behavior; specifically, FruM would act in males to promote greater ethanol hyperactivity and sedation resistance, whereas Tra/Tra2 repression of FruM in females would prevent these effects.

We initially investigated the role of this pathway in regulating ethanol sedation sensitivity. First, we generated males lacking FruM by using the *Gal4*/*UAS* system to pan-neuronally express Tra

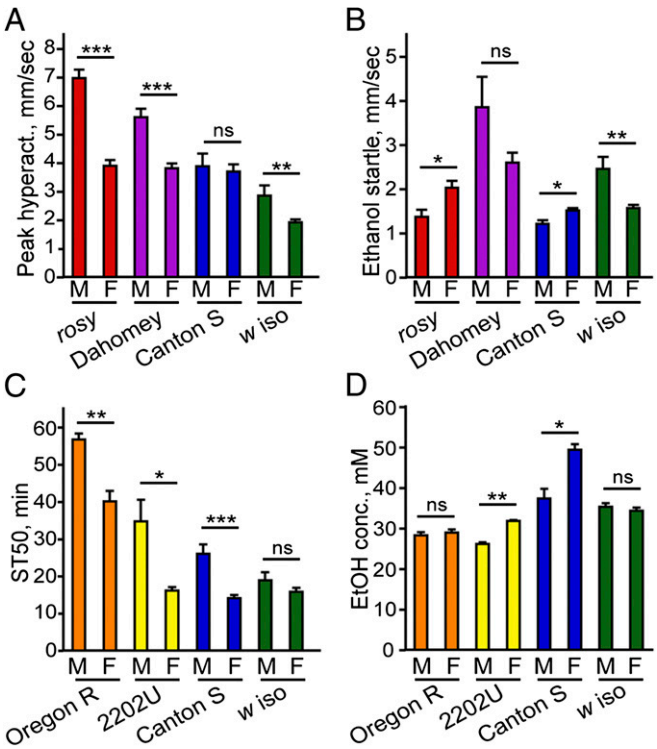

Fig. 4. Several WT *Drosophila* strains show sexually dimorphic ethanol responses. (*A*) In three of four WT strains, males (marked "M") showed greater peak ethanol hyperactivity than females ("F"; $n = 8$–12). (*B*) Ethanol startle responses of WT strains shown in *A* ($n = 8$–12). Sex differences in the startle response were not correlated with sex differences in peak ethanol hyperactivity. (*C*) In three of four WT strains, males showed greater resistance to ethanol sedation than females ($n = 8$–12). (*D*) Internal ethanol concentration in WT strains shown in *C* after 12 min exposure to 47% ethanol. Two of four strains showed a sex difference in internal ethanol concentration ($n = 4$–8). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, paired or unpaired *t* tests.

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($elav^{Gal4}$/*UAS-tra*). These males showed increased ethanol sensitivity, indicative of feminized behavior (Fig. 5*A*). Next, we generated females expressing FruM by using RNAi to down-regulate Tra2 in *fru*-expressing cells (fru^{Gal4}/*UAS-tra2*^{RNAi}). These females showed increased sedation resistance, representing masculinized behavior (Fig. 5*B*). The relatively weak phenotype of FruM-expressing females may be a result of incomplete knockdown of *tra2*, or it might indicate that FruM is not entirely sufficient to mediate the sex difference in sedation sensitivity. It should be noted that the Tra/Tra2 pathway also regulates sex-specific splicing of *doublesex* (*dsx*), which primarily controls somatic sexual differentiation, but also shows some neuronal expression and can affect behavior (26–28). It is therefore possible that the increased ethanol sensitivity of Tra-expressing males is caused by *dsx* rather than *fru*. However, our use of fru^{Gal4} to selectively down-regulate *tra2* in *fru*-expressing cells of females suggests that their increased ethanol resistance is likely attributable to *fru*. Interestingly, neither males in which FruM was down-regulated nor females expressing FruM showed a significant alteration in internal ethanol concentration (Fig. 5 *C* and *D*). Thus, although differences in internal ethanol concentration may contribute to sexually dimorphic ethanol sensitivity in WT flies, the Tra/Tra2 pathway acts within the nervous system, likely through regulation of *fru*, to modulate sedation sensitivity without affecting ethanol pharmacokinetics.

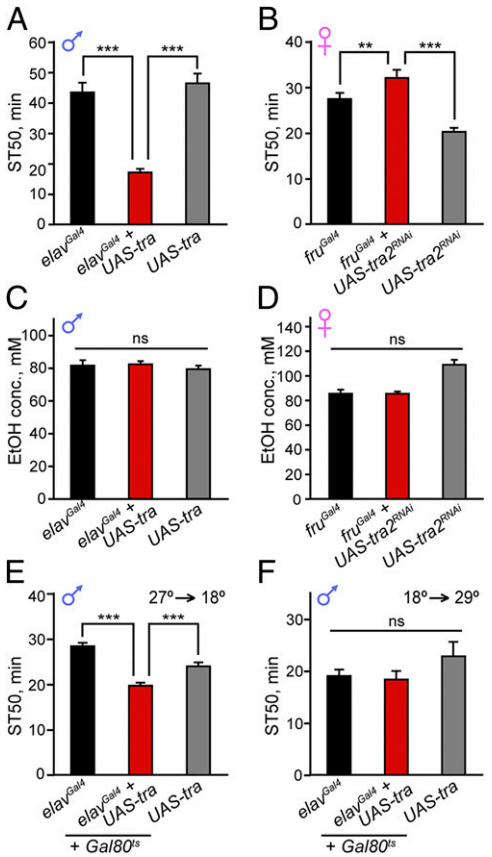

Fig. 5. *tra* acts in the nervous system to regulate ethanol sedation without affecting ethanol pharmacokinetics. (*A*) Males expressing *tra* pan-neuronally ($elav^{Gal4}$/*UAS-tra*) showed increased sedation sensitivity compared with control males (n = 7). (*B*) Females expressing *tra2*^{RNAi} (fru^{Gal4}/*UAS-tra2*^{RNAi}) showed increased sedation resistance compared with control females (n = 16). (*C* and *D*) After 12 min exposure to 47% ethanol, males expressing *tra* ($elav^{Gal4}$/*UAS-tra*; *C*) and females expressing *tra2*^{RNAi} (fru^{Gal4}/*UAS-tra2*^{RNAi}; *D*) did not show altered internal ethanol levels compared with their respective controls ($P > 0.05$, $n = 4$). (*E*) Developmental expression of *tra* ($elav^{Gal4}$;$Gal80^{ts}$/*UAS-tra*) increased sedation sensitivity in males (n = 8). *tra* expression was restricted to development by growing flies at 27° ($Gal80^{ts}$ inactive) and shifting them to 18° within 24 h after eclosion ($Gal80^{ts}$ active). (*F*) Adult-specific expression of *tra* ($elav^{Gal4}$;$Gal80^{ts}$/*UAS-tra*) did not affect ethanol sedation in males (n = 8–10). Expression of *tra* was restricted to adulthood by growing flies at 18° ($Gal80^{ts}$ active) and shifting them to 29° within 24 h after eclosion ($Gal80^{ts}$ inactive). $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA followed by Newman–Keuls posttests; repeated-measures ANOVA used in *B* and *E*.

fru is well known for its developmental role in regulating neuronal survival and differentiation (29), leading to the generation of sex-specific neurons and neurons with sexually dimorphic morphology (30–32). To examine whether *fru* is required during development for normal sedation resistance in males, we used Tra expression to down-regulate FruM in a temporally specific manner by using Gal80^{ts}, a temperature-sensitive Gal4 repressor that inhibits Gal4 function at 18° but not at temperatures higher than 27° (33). Pan-neuronal expression of Tra exclusively during development increased ethanol sedation sensitivity in males, whereas Tra expression during adulthood had no effect (Fig. 5 *E* and *F*). These results indicate that Tra acts in the developing nervous system to regulate sedation sensitivity, and suggest that FruM is required developmentally for normal sedation resistance in males.

In contrast to ethanol sedation, manipulating FruM expression by targeting Tra/Tra2 did not produce a clear effect on ethanol hyperactivity. Reducing FruM expression in males by pan-neuronal expression of Tra ($elav^{Gal4}$/*UAS-tra*) had no significant effect on peak hyperactivity (*SI Appendix*, Fig. S3*A*). These males did show a slower rate of hyperactivity onset, but this effect may be a result of male–male chaining exhibited by these flies, which likely affects locomotor speed. Similarly, expression of FruM in females by down-regulation of Tra2 (fru^{Gal4}/*UAS-tra2*^{RNAi}) did not affect ethanol hyperactivity (*SI Appendix*, Fig. S3*B*). These results suggest that the sexual dimorphism in ethanol hyperactivity is attributable to factors other than FruM, and may be mediated by Tra/Tra2 function outside the nervous system. In contrast, our data indicate that the Tra/Tra2 pathway acts in the developing nervous system, likely via regulation of *fru*, to mediate the sex difference in ethanol sedation sensitivity.

Activity of *fru*-Expressing Neurons Regulates Ethanol Sedation Sensitivity. We have shown that males and females show differences in ethanol hyperactivity and sedation, and that the sex difference in sedation sensitivity is likely mediated by *fru*. Approximately 1% of neurons express *fru*, and the activity of these neurons is necessary and sufficient for male courtship behavior (23, 34–36). We asked whether the activity of *fru*-expressing neurons also regulates ethanol sedation in males. First, we acutely activated *fru* neurons using the heat-activated cation channel TrpA1, which causes neuronal depolarization (37). Activation of *fru* neurons in males at 26° increased ethanol sedation sensitivity, but did not induce sedation in the absence of ethanol (Fig. 6*A*). Next, we silenced *fru* neurons using *Shi*^{ts}, a temperature-sensitive dynamin allele that depletes synaptic vesicles (38). Silencing *fru* neurons in males had no effect on ethanol sedation (Fig. 6*B*). Thus, although activating *fru* neurons is sufficient to enhance sensitivity to ethanol sedation, *fru* neuron activity is not necessary for normal sedation behavior. These results suggest that *fru* neurons may not represent an essential component of the neural circuitry mediating ethanol sedation, but they may be capable of modulating this circuit.

Discussion

In this study, we characterize sex differences in acute ethanol responses in *Drosophila*. Males show increased ethanol hyperactivity and sedation resistance compared with females. Thus, males appear to be more sensitive to the stimulant effect of ethanol, whereas females are more sensitive to its sedative effect. Expression of Tra in the developing nervous system contributes to the sex difference in ethanol sedation, whereas the sex difference in ethanol hyperactivity may arise from Tra function outside the nervous system. Although pharmacokinetic differences likely contribute to the sex difference in ethanol sedation, neuronal Tra expression regulates ethanol sedation without affecting ethanol pharmacokinetics. By inhibiting Tra2 function in *fru*-expressing

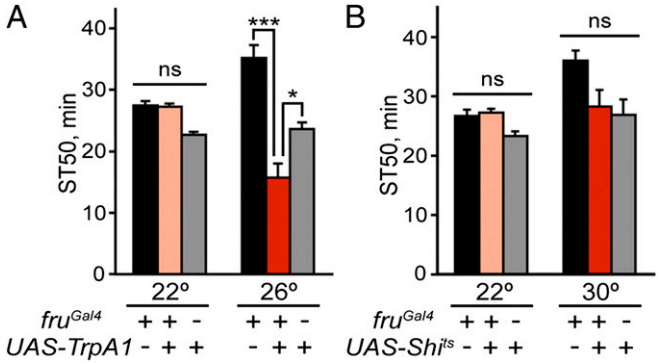

Fig. 6. Activation of *fru*-expressing neurons alters ethanol sedation sensitivity. (*A*) Activation of *fru* neurons in males (*fru*^{Gal4}/*UAS-TrpA1*) at 26° increased sedation sensitivity ($n = 6$); no effect was observed at 22° when TrpA1 was inactive ($P > 0.05$, $n = 8$). (*B*) Silencing *fru* neurons in males (*fru*^{Gal4}/*UAS-Shi*^{ts}) at 30° did not affect sedation sensitivity, nor was an effect observed at 22° when Shi^{ts} was inactive ($P > 0.05$, $n = 8$). *$P < 0.05$, ***$P < 0.001$, one-way ANOVA followed by Newman–Keuls posttests; repeated-measures ANOVA was used for all comparisons except for the 30° experiment in *B*.

neurons, we show that the Tra/Tra2 pathway likely regulates ethanol sedation by regulating the splicing of *fru*, as opposed to *dsx*. In support of a role for *fru*, activation of *fru*-expressing neurons enhances ethanol sedation sensitivity. In summary, we have characterized sex differences in ethanol-induced behaviors and linked some of these differences to the action of *fru* as well as *fru*-expressing neurons.

Sexually Dimorphic Ethanol Responses in Flies and Humans. Women are more sensitive than men to ethanol-induced motor impairment. This difference partly results from differences in BAC, but women also exhibit greater impairment when BACs are equalized (4). We have discovered a similar sexual dimorphism in *Drosophila*. Female flies are more sensitive to ethanol sedation than males, and this difference is attributable partly to a difference in ethanol pharmacokinetics and partly to sex differences within the nervous system. A previous study did not observe a sex difference in *Drosophila* ethanol sensitivity using a negative geotaxis assay (39). This discrepancy may be a result of the behavioral assay used, as the loss of negative geotaxis is not a direct correlate of sedation and may also be affected by the stimulant effect of ethanol. The increased ethanol concentration in females at least partly results from a decreased rate of ethanol metabolism, which in turn may be caused by decreased activity or expression of alcohol dehydrogenase, the enzyme that metabolizes ethanol. Previous studies have in fact reported decreased alcohol dehydrogenase activity in females compared with males (40, 41), but not all studies have observed this difference (42).

It will be interesting to determine whether similar mechanisms underlie the sex differences in ethanol sensitivity observed in flies and humans. A mammalian homologue of *fru* has not been identified, but it is possible that a different human gene may play a functional role similar to *fru*, or that the mechanisms downstream of *fru* are conserved. The neural factors mediating the human sex difference are unknown, although differences in dopaminergic transmission and GABA receptor function have been proposed to play a role (10, 43). These processes may be good candidates for potential mechanisms operating downstream of *fru* to promote sedation resistance in male flies.

In addition to the sex difference in ethanol-induced motor impairment, women are also more sensitive than men to physical harm induced by ethanol, such as organ damage and risk of death (9, 10). In contrast, we found that female flies are more resistant than males to ethanol toxicity, as assessed by lethality. This sex difference has been previously reported, although the effect varies greatly depending on fly strain (44, 45). It is interesting that, in our experiments, males were more sensitive than females to lethality despite showing greater resistance to ethanol sedation. This dissociation between ethanol-induced sedation and lethality in *Drosophila* suggests that these effects are mediated by distinct pathways, both of which are sexually dimorphic.

Some have questioned why sexual dimorphism in ethanol sensitivity should exist at all. An evolutionary explanation has been proposed in humans (9). The crux of the argument is that ethanol consumption by women induces more physical harm than in men, and, in the case of pregnancy, also harms the offspring, which may have led to greater selective pressure against ethanol consumption in women. Because ethanol consumption is inversely correlated with sensitivity to the depressant effects of ethanol, increased ethanol sensitivity in women may have been evolutionarily favored. This explanation may not apply to *Drosophila*, as we have shown that, in contrast to humans, female flies are more resistant to ethanol toxicity than males. However, it is possible that female flies are more sensitive than males to other effects of ethanol that impact reproductive fitness, such as egg/sperm production. Because flies encounter ethanol in fermenting fruit within their natural environment, and sex differences in ethanol hyperactivity and sedation were each observed in multiple *Drosophila* strains, it seems likely that these differences have been evolutionarily selected (46).

Regulation of Ethanol Sedation Sensitivity by FruM. Our manipulations of Tra/Tra2 suggest that male-specific FruM acts during development to contribute to the sex difference in ethanol sedation. A developmental requirement for *fru* is consistent with its known role in regulating neuronal survival and differentiation (30, 31, 47). Both FruM and *fru* neuron activity promote courtship behavior: evidence suggests that FruM directs the male-specific development of neural circuits whose activation acutely elicits courtship behavior during adulthood (22, 30, 34, 36). In contrast, our data suggest that FruM and *fru* neurons have opposing roles in regulating ethanol sedation: although FruM may act developmentally to promote sedation resistance, acutely activating *fru* neurons promotes sedation sensitivity, suggesting a different relationship between the gene and the neurons than has been shown for courtship behavior.

The fact that silencing *fru* neurons did not affect sedation sensitivity suggests that these neurons are not an essential component of the circuitry mediating ethanol sedation. This result raises the possibility that *fru* neuron activation, which elicits courtship behavior such as wing extension and abdominal bending, might indirectly promote ethanol sedation (e.g., by inducing fatigue). In addition, *fru* is expressed in ~1,500 neurons distributed throughout the nervous system (31), so activation of this large set of neurons may stimulate the release of many different neurotransmitters and neuropeptides. Future studies should therefore focus on activating smaller subsets of *fru* neurons to identify cells with a direct role in promoting ethanol sedation. One candidate set of neurons resides in the pars intercerebralis, a major locus of neurosecretory cells, as expression of Tra in a cluster of pars intercerebralis neurons regulates sexually dimorphic locomotor patterns (48, 49). Although the neuronal loci and downstream mechanisms of *fru* function remain to be elucidated, in this work we have characterized sexual dimorphisms in ethanol-induced behavior and shown evidence that *fru* mediates a subset of these differences.

Materials and Methods

Fly Stocks and Maintenance. *w* Berlin was used as the control strain. All transgenic stocks were outcrossed into this background for at least five generations or crossed into *w* Berlin using balancers if they lacked a phenotypic marker. Assays were performed on 3- to 5-d-old males or females that were generally nonvirgin. Flies were generally reared on standard cornmeal/molasses food at 25 °C and 70% relative humidity. Flies for TrpA1 or Shi^{ts} experiments were reared at 18° or 22 °C, respectively. Flies for Gal80^{ts} experiments were reared at the specified temperatures; for developmental expression of *tra*, these flies appeared unhealthy when reared at 29° and were instead reared at 27°, which still inhibits Gal80^{ts} function. *SI Appendix, SI Methods*, provides details regarding fly strains.

Behavior and Lethality Assays. Ethanol hyperactivity and sedation were assayed in the booz-o-mat, an eight-chambered apparatus that delivers a specific concentration of ethanol vapor by mixing pure ethanol vapor with humidified air at a specified ratio (13). Twenty flies per vial were assayed, and *n* refers to the number of vials tested. Assays were conducted at ~25°, except for TrpA1 and Shi^{ts} experiments, which were conducted at the specified temperatures. Flies for TrpA1 and Shi^{ts} experiments were allowed to equilibrate to the higher temperature for ~15 min before the assay. Ethanol hyperactivity was measured by video tracking of flies as described previously (13) using 47% ethanol vapor. Ethanol sedation was assayed manually as described previously (19) using 67% ethanol vapor unless otherwise specified. Sedation recovery was measured by transferring flies to normal vials after ethanol exposure and counting the number of flies that were walking or standing upright at 5-min intervals. ST50 and time to 50% recovery (RT50) values were determined by linear interpolation. Ethanol lethality was assayed by exposing flies to 100% ethanol for 60 min, then counting the number of dead flies following overnight recovery. *SI Appendix, SI Methods*, provides details regarding these assays.

Measurement of Internal Ethanol Concentration. Internal ethanol concentration was assayed in fly extracts as previously described (16). Ethanol concentration within flies was calculated based on their water content (18). Water content was measured by subtracting dry weight from wet weight, and was 0.60 μL for *w* Berlin males and 0.93 μL for *w* Berlin females. The water content of fly heads was estimated based on their wet weight (assuming a similar density as whole flies).

Statistical Analyses. Statistical analyses were performed using GraphPad Prism, version 4. Statistical tests are specified in the figure legends. Experimental and control flies were always tested simultaneously; experiments in which each run contained one sample of each genotype were considered to be paired experiments, whereas unpaired tests were used for all other experiments. All graphs represent mean ± SEM. For *Gal4/UAS* experiments, experimental lines were statistically compared with both the *Gal4/+* and *UAS/+* controls; only experimental lines that differed from both controls were considered to have a phenotype.

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1. Pohorecky LA (1977) Biphasic action of ethanol. *Biobehav Rev* 1:231–240.
2. Babor TF, Berglas S, Mendelson JH, Ellingboe J, Miller K (1983) Alcohol, affect, and the disinhibition of verbal behavior. *Psychopharmacology (Berl)* 80(1):53–60.
3. Phillips TJ, Shen EH (1996) Neurochemical bases of locomotion and ethanol stimulant effects. *Int Rev Neurobiol* 39:243–282.
4. Miller MA, Weafer J, Fillmore MT (2009) Gender differences in alcohol impairment of simulated driving performance and driving-related skills. *Alcohol Alcohol* 44(6):586–593.
5. Schuckit MA (1994) Low level of response to alcohol as a predictor of future alcoholism. *Am J Psychiatry* 151(2):184–189.
6. Morean ME, Corbin WR (2010) Subjective response to alcohol: A critical review of the literature. *Alcohol Clin Exp Res* 34(3):385–395.
7. Substance Abuse and Mental Health Services Administration (2011) *Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings* (Substance Abuse and Mental Health Services Administration, Rockville, MD), NSDUH Series H-41, HHS Publication No. (SMA) 11-4658.
8. Grant BF, et al. (2004) The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991-1992 and 2001-2002. *Drug Alcohol Depend* 74(3):223–234.
9. Nolen-Hoeksema S, Hilt L (2006) Possible contributors to the gender differences in alcohol use and problems. *J Gen Psychol* 133(4):357–374.
10. Ceylan-Isik AF, McBride SM, Ren J (2010) Sex difference in alcoholism: Who is at a greater risk for development of alcoholic complication? *Life Sci* 87(5-6):133–138.
11. Mumenthaler MS, Taylor JL, O'Hara R, Yesavage JA (1999) Gender differences in moderate drinking effects. *Alcohol Res Health* 23(1):55–64.
12. Kaun KR, Devineni AV, Heberlein U (2012) Drosophila melanogaster as a model to study drug addiction. *Hum Genet* 131(6):959–975.
13. Wolf FW, Rodan AR, Tsai LT-Y, Heberlein U (2002) High-resolution analysis of ethanol-induced locomotor stimulation in *Drosophila*. *J Neurosci* 22(24):11035–11044.
14. Moore MS, et al. (1998) Ethanol intoxication in *Drosophila*: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell* 93(6):997–1007.
15. Rothenfluh A, et al. (2006) Distinct behavioral responses to ethanol are regulated by alternate RhoGAP18B isoforms. *Cell* 127(1):199–211.
16. Devineni AV, et al. (2011) The genetic relationships between ethanol preference, acute ethanol sensitivity, and ethanol tolerance in Drosophila melanogaster. *Fly (Austin)* 5(3):191–199.
17. Kong EC, et al. (2010) Ethanol-regulated genes that contribute to ethanol sensitivity and rapid tolerance in Drosophila. *Alcohol Clin Exp Res* 34(2):302–316.
18. Cowmeadow RB, Krishnan HR, Atkinson NS (2005) The slowpoke gene is necessary for rapid ethanol tolerance in Drosophila. *Alcohol Clin Exp Res* 29(10):1777–1786.
19. Corl AB, et al. (2009) Happyhour, a Ste20 family kinase, implicates EGFR signaling in ethanol-induced behaviors. *Cell* 137(5):949–960.
20. Berger KH, Heberlein U, Moore MS (2004) Rapid and chronic: Two distinct forms of ethanol tolerance in *Drosophila*. *Alcohol Clin Exp Res* 28(10):1469–1480.
21. Scholz H, Ramond J, Singh CM, Heberlein U (2000) Functional ethanol tolerance in *Drosophila*. *Neuron* 28(1):261–271.
22. Demir E, Dickson BJ (2005) fruitless splicing specifies male courtship behavior in Drosophila. *Cell* 121(5):785–794.
23. Manoli DS, et al. (2005) Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. *Nature* 436(7049):395–400.
24. Vrontou E, Nilsen SP, Demir E, Kravitz EA, Dickson BJ (2006) fruitless regulates aggression and dominance in Drosophila. *Nat Neurosci* 9(12):1469–1471.
25. Ryner LC, et al. (1996) Control of male sexual behavior and sexual orientation in Drosophila by the fruitless gene. *Cell* 87(6):1079–1089.
26. Nagoshi R, McKeown M, Burtis KC, Belote JM, Baker BS (1988) The control of alternative splicing at genes regulating sexual differentiation in D. melanogaster. *Cell* 22:229–236.
27. Robinett CC, Vaughan AG, Knapp JM, Baker BS (2010) Sex and the single cell. II. There is a time and place for sex. *PLoS Biol* 8(5):e1000365.
28. Shirangi TR, Taylor BJ, McKeown M (2006) A double-switch system regulates male courtship behavior in male and female Drosophila melanogaster. *Nat Genet* 38(12):1435–1439.
29. Kimura K-I (2011) Role of cell death in the formation of sexual dimorphism in the Drosophila central nervous system. *Dev Growth Differ* 53(2):236–244.
30. Cachero S, Ostrovsky AD, Yu JY, Dickson BJ, Jefferis GS (2010) Sexual dimorphism in the fly brain. *Curr Biol* 20(18):1589–1601.
31. Yu JY, Kanai MI, Demir E, Jefferis GS, Dickson BJ (2010) Cellular organization of the neural circuit that drives Drosophila courtship behavior. *Curr Biol* 20(18):1602–1614.
32. Kimura KI, Ote M, Tazawa T, Yamamoto D (2005) Fruitless specifies sexually dimorphic neural circuitry in the Drosophila brain. *Nature* 438(7065):229–233.
33. McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL (2003) Spatiotemporal rescue of memory dysfunction in Drosophila. *Science* 302(5651):1765–1768.
34. Stockinger P, Kvitsiani D, Rotkopf S, Tirián L, Dickson BJ (2005) Neural circuitry that governs Drosophila male courtship behavior. *Cell* 121(5):795–807.
35. Clyne JD, Miesenböck G (2008) Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. *Cell* 133(2):354–363.
36. Kohatsu S, Koganezawa M, Yamamoto D (2011) Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in Drosophila. *Neuron* 69(3):498–508.
37. Hamada FN, et al. (2008) An internal thermal sensor controlling temperature preference in Drosophila. *Nature* 454(7201):217–220.
38. Kitamoto T (2001) Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J Neurobiol* 47(2):81–92.
39. Bhandari P, Kendler KS, Bettinger JC, Davies AG, Grotewiel M (2009) An assay for evoked locomotor behavior in Drosophila reveals a role for integrins in ethanol sensitivity and rapid ethanol tolerance. *Alcohol Clin Exp Res* 33(10):1794–1805.
40. Pipkin SB, Hewitt NE (1972) Variation of alcohol dehydrogenase levels in Drosophila species hybrids. *J Hered* 63(5):267–270.
41. Geer BW, et al. (1988) Induction of alcohol dehydrogenase by ethanol in Drosophila melanogaster. *J Nutr* 118(3):398–407.
42. Barbancho M, Sánchez-Cañete FJ, Dorado G, Pineda M (1987) Relation between tolerance to ethanol and alcohol dehydrogenase (ADH) activity in Drosophila melanogaster: Selection, genotype and sex effects. *Heredity (Edinb)* 58(pt 3):443–450.
43. Grobin AC, Matthews DB, Devaud LL, Morrow AL (1998) The role of GABA(A) receptors in the acute and chronic effects of ethanol. *Psychopharmacology (Berl)* 139(1-2):2–19.
44. Kamping A, van Delden W (1978) Alcohol dehydrogenase polymorphism in populations of Drosophila melanogaster. II. Relation between ADH activity and adult mortality. *Biochem Genet* 16(5-6):541–551.
45. Malherbe Y, Kamping A, van Delden W, van de Zande L (2005) ADH enzyme activity and Adh gene expression in Drosophila melanogaster lines differentially selected for increased alcohol tolerance. *J Evol Biol* 18(4):811–819.
46. Guarnieri DJ, Heberlein U (2003) Drosophila melanogaster, a genetic model system for alcohol research. *Int Rev Neurobiol* 54:199–228.
47. Ito H, et al. (2012) Fruitless recruits two antagonistic chromatin factors to establish single-neuron sexual dimorphism. *Cell* 149(6):1327–1338.
48. Gatti S, Ferveur JF, Martin JR (2000) Genetic identification of neurons controlling a sexually dimorphic behaviour. *Curr Biol* 10(11):667–670.
49. Belgacem YH, Martin JR (2002) Neuroendocrine control of a sexually dimorphic behavior by a few neurons of the pars intercerebralis in Drosophila. *Proc Natl Acad Sci USA* 99(23):15154–15158.