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Genetic and Neural Mechanisms Underlying Ethanol-Related Behaviors in Drosophila melanogaster

by

Anita V. Devineni

Dissertation

Submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

in the
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Author Contributions

Some of the text of Chapters 1 and 5 of this thesis is a reprint of the material that originally appeared in *Human Genetics*. Anita Devineni, Karla Kaun, and Ulrike Heberlein co-wrote this review article.


*These authors contributed equally.

The text of Chapter 2 of this thesis is a reprint of the material as it originally appeared in *Current Biology* (with minor formatting changes for consistency). Ulrike Heberlein, the co-author, directed and supervised the research. Anita Devineni and Ulrike Heberlein co-wrote the paper.


The text of Chapter 3 of this thesis is a reprint of the material as it originally appeared in *Fly* (with minor formatting changes for consistency). Anita Devineni performed all behavioral experiments. Ulrike Heberlein, the senior author, supervised the research. The other co-authors, Kimberly McClure, Douglas Guarnieri, Ammon Corl, Fred Wolf, and Mark Eddison, originally isolated the mutants used in the paper, mapped the mutations, and characterized many of the expression patterns. Anita Devineni and Ulrike Heberlein co-wrote the paper.


The text of Chapter 4 of this thesis is a draft of a manuscript under review at Neuron at the time that this thesis was completed. Anita Devineni performed all experiments. Ulrike Heberlein, the co-author, directed and supervised the research. Anita Devineni and Ulrike Heberlein co-wrote the paper.
GENETIC AND NEURAL MECHANISMS UNDERLYING ETHANOL-RELATED BEHAVIORS IN DROSOPHILA MELANOGASTER

by Anita V. Devineni

ABSTRACT

Ethanol is one of the most widely used and abused drugs in the world. Ethanol consumption produces short-term changes in behavior as well as long-term adaptations that can lead to addiction. The mechanisms underlying both acute and chronic responses to ethanol are still not fully understood. Human and rodent studies have suggested that acute ethanol sensitivity may be related to risk of alcohol abuse, and that the same genes often regulate both types of behavior. In this thesis I have used the fruit fly Drosophila melanogaster as a model to study the genetic and neural mechanisms underlying ethanol-induced behavior.

In Chapter 2, I show that flies prefer to consume food containing ethanol and that this ethanol preference may represent a new model for studying addiction-related behavior. In Chapter 3, I examine the relationships between acute ethanol sensitivity, ethanol tolerance, and ethanol consumption preference by measuring these behaviors in a set of Drosophila mutants. I found that ethanol tolerance and preference were positively correlated, suggesting that the development of tolerance might be one factor contributing to the increase in ethanol consumption that we observe over time. Finally, in Chapter 4 I characterize sex differences in acute ethanol responses in Drosophila, which also occur in humans. I identify two genes, fruitless and tank, that regulate ethanol responses in a sexually dimorphic manner, and I show that these genes define distinct sets of neurons that interact to regulate ethanol sensitivity. Overall, this work has helped to expand the repertoire of behavioral assays available to study ethanol-related behavior.
in flies, shed light on the relationships between different ethanol-induced behaviors, and identified two new genes and corresponding sets of neurons that regulate acute ethanol responses.
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CHAPTER 1

Introduction:

*Drosophila melanogaster* as a Model to Study Ethanol-Related Behavior

Abstract

Animal studies have been instrumental in providing knowledge about the molecular and neural mechanisms underlying ethanol addiction. The fruit fly *Drosophila melanogaster* has become a valuable system to model the acute stimulating and sedating effects of ethanol as well as its more complex rewarding properties. In this Introduction, I will describe the advantages of using the fly to study ethanol-related behavior, provide a brief overview of the behavioral assays used, and review the molecular mechanisms and neural circuits that have been shown to mediate ethanol-induced behavior in flies. Many of these mechanisms have been validated in mammals, suggesting that the fly is a useful model to understand the mechanisms underlying ethanol-related behavior and potentially addiction.

Introduction to Drug Addiction

Drug addiction is a disorder characterized by excessive use of a drug to the point of compulsive drug seeking and consumption. The American Psychiatric Association (DMS-IV) differentiates between substance abuse, considered an earlier stage of addiction, and substance dependence. Substance abuse is defined as continued drug use despite interpersonal problems, legal problems, failure to fulfill obligations, or physically hazardous situations. The criteria for substance dependence include physical symptoms, such as tolerance and withdrawal, as well as signs of uncontrolled use, which include giving up normal activities and continued use despite knowledge of self-
harm and the desire to stop. These definitions highlight the fact that addiction is an exclusively human phenomenon. However, animal models have been used to study specific aspects of addiction, and have proved invaluable in understanding the underlying neural and molecular mechanisms.

Animal models allow the experimenter to focus on distinct components of the addiction process, ranging from simple, acute drug responses to more complex behaviors such as drug seeking, self-administration, and relapse. Each behavioral model has advantages and disadvantages. Whereas the more complex models likely have greater relevance to the human condition, assays for acute drug responses are simpler to perform and thus provide the potential for high-throughput analysis, facilitating the identification of the underlying mechanisms.

Although rodent models have provided crucial insights into the mechanisms underlying drug-related behaviors, they are not ideal for unbiased, forward genetic approaches aimed at identifying novel and unsuspected mechanisms. This is due primarily to the expense and time required for animal maintenance, breeding, and behavioral analyses. In contrast, the fruit fly Drosophila melanogaster is one of the most genetically and experimentally accessible model organisms in biology. While for many years flies were used primarily to identify the molecular and neural mechanisms regulating acute drug responses, the recent development of assays that measure drug preference and reward has allowed the analysis of these more complex behaviors.

Drosophila as a Model System to Study Behavior

Drosophila has been used to gain insight into molecular, cellular, developmental, and disease processes that are conserved in mammals, including humans, as most of these fundamental biological mechanisms are shared throughout the animal kingdom. Although mammals have two to three times as many genes as flies, they have
approximately the same number of gene families (Holland, 2003). About 75% of human
disease genes have related sequences in Drosophila, suggesting that flies can serve as
an effective model to study the function of a wide array of genes involved in human
disease (Adams et al., 2000; Reiter et al., 2001). The nervous system of the fly
comprises approximately 300,000 neurons including a brain, ventral nerve cord (the
equivalent of the spinal cord), and peripheral nervous system. Despite their relatively
small number of neurons in comparison with mammals, flies exhibit many complex
behaviors such as associative learning, sensorimotor integration, and social behaviors
(Quinn et al., 1974; Pick and Strauss, 2005; Greenspan and Ferveur, 2000; Chen et al.,
2002).

The classical advantages of using Drosophila include factors such as cost, size, fecundity, and timescale. First, flies are easy and inexpensive to rear in the laboratory using small vials or bottles and a yeast-based food medium. Due to their small size, thousands of genotypes of flies can be maintained in a typical laboratory. Second, due to their high fecundity, hundreds of flies can be obtained from a single female. Third, flies have a rapid life cycle, requiring only 10 days at 25ºC to develop from egg to mature adult.

For these reasons, flies have long represented an ideal organism to conduct mutagenesis screens to isolate genes regulating a particular biological process of interest (“forward genetics”, i.e. going from phenotype to gene). The advent of genetic transformation in the 1980s also allowed for “reverse genetics” (i.e. going from gene to phenotype) by allowing researchers to introduce specific genes of interest into a fly (Rubin and Spradling, 1982). The subsequent sequencing and annotation of the Drosophila genome has greatly facilitated both of these approaches (Adams et al., 2000).
In recent years, the generation of large collections of publicly available mutants and other transgenic tools has allowed for the functional study of nearly any fly gene of interest. The traditional use of X-ray or chemical mutagenesis is becoming gradually supplanted by insertional mutagenesis, in which a transposable genetic element creates a mutation by inserting into a random genomic site, and the gene affected can be easily identified by sequencing the flanking DNA (Bingham et al., 1981). Several groups have now generated large mutant collections for which the insertion site in each mutant has been sequenced (Bellen et al., 2004; Thibault et al., 2004; Schuldiner et al., 2008). In addition, an RNA interference (RNAi) library has been generated in which each fly line contains an inducible RNAi construct for silencing a single fly gene, with nearly 90% of the fly genome represented (Dietzl et al., 2007).

Some of the genetic tools developed in *Drosophila* have particular relevance to studying the relationship between genes, the brain, and behavior. For example, genetic tools in flies allow one to manipulate the nervous system independently of other tissues in the body. Furthermore, because different neural circuits may have distinct and perhaps opposing roles in behavior, one would ideally like to target specific sets of neurons within the brain. This cellular specificity can be accomplished by the bipartite Gal4/UAS system, in which the transcriptional activator Gal4 is expressed in a spatially restricted pattern and activates any gene placed downstream of the upstream activating sequence (UAS) (Brand and Perrimon, 1993; Fig. 1A). The generation and characterization of thousands of Gal4 lines expressed in various patterns allows for manipulation of specific brain regions or neuronal types (Pfeiffer et al., 2008). This technique allows one to ask in which neurons a particular gene functions to regulate a behavioral response. These patterns can be further spatially refined to very small subsets of neurons using the “split Gal4 system” in which the DNA-binding and transcriptional-activation domains of Gal4 are targeted to different neuronal subsets.
using different promoters; transcriptional activation of target genes occurs only in
neurons expressing both domains (Luan et al., 2006). Temporal specificity can be
achieved by using a temperature-sensitive Gal4 repressor called Gal80<sup>ts</sup> and shifting the
flies from the permissive to the restrictive temperature during a particular time period
(McGuire et al., 2003; Fig. 1B).

In addition to studying the function of genes within the nervous system, the
Gal4/UAS system is well suited to studying neural circuit function. UAS lines are
available in which proteins that inducibly control neuronal activity can be expressed,
allowing one to activate or silence a particular set of neurons during a specific behavioral
task. For example, neurons can be silenced using Shibire<sup>ts</sup>, a temperature-sensitive
dynamin allele that blocks synaptic vesicle recycling (Fig. 1C), and neurons can be
activated using TrpA1, a temperature-sensitive cation channel that causes neuronal
depolarization (Fig. 1D) (Kitamoto, 2001; Hamada et al., 2008). Furthermore, the
development of a second binary system in addition to the Gal4/UAS system, the
LexA/LexAop system (Lai and Lee, 2006), allows for the independent manipulation of
multiple neural circuits, such as activating some neurons while inhibiting others. Thus,
flies have now become a leading model organism for studying not only the molecular
mechanisms but also the neural circuits that underlie behavior.

Models to Study Ethanol-Related Behaviors in Flies

Ethanol is the drug that has been by far the most intensively studied in
<em>Drosophila</em>. Ethanol is a commonly abused psychoactive drug that can produce both
short-term behavioral impairment as well as long-term addiction. Unlike other drugs such
as cocaine and nicotine, ethanol does not act on a single molecular target but instead is
thought to affect a variety of molecules, including multiple ion channels (Koob, 2004).
Fruit flies encounter ethanol in their natural environment since one of the main
metabolites of fermenting fruit is ethanol. Ethanol can act as a long-distance signal to draw flies to rotting fruit, as flies are attracted to low concentrations of ethanol vapor (Dudley, 2002; Hoffmann and Parsons, 1984). Female flies prefer to lay their eggs in media containing up to 5% ethanol (McKenzie and Parsons, 1972), and larvae efficiently metabolize ethanol and use it as a food source (Geer et al., 1993).

Despite this long-standing relationship between *Drosophila* and ethanol, the molecular underpinnings of the effects of ethanol on fly behavior were not investigated until relatively recently. Several types of ethanol-related behaviors have now been characterized in flies, with the goal of using the abundant genetic tools in *Drosophila* to understand the underlying mechanisms. These behaviors range from simple to complex: (1) acute locomotor responses to ethanol, (2) ethanol tolerance following an initial exposure, and (3) conditioned ethanol preference. Voluntary ethanol consumption in *Drosophila* will be described in Chapter 2 of this thesis.

**Acute Ethanol Sensitivity**

Flies exhibit acute responses to ethanol exposure that are quite similar to those of mammals, including humans (Morean and Corbin, 2010). There is evidence in humans as well as mammalian models that sensitivity to acute ethanol-induced motor impairment correlates inversely with ethanol consumption and risk of abuse, and that the same genes can influence both types of behavior (Schuckit, 1994; Morean and Corbin, 2010; Kurtz et al., 1996; Thiele et al., 1998; Hodge et al., 1999). Studying these simpler ethanol responses, which are often easier to test in the laboratory, is therefore likely to provide insight into the mechanisms regulating more complex addiction-related behaviors as well.

To measure acute ethanol responses in flies, ethanol is typically administered in the form of pure ethanol vapor mixed with air at a specified ratio, allowing one to control
the ethanol concentration that the flies receive (Wolf et al., 2002). Ethanol can also be administered to flies by injection, though few studies have employed this technique (Dzitoyeva et al., 2003). Low to moderate concentrations of ethanol induce locomotor hyperactivity, which can be measured by filming the flies and using tracking software to identify the flies and calculate their locomotor speed (Wolf et al., 2002).

In contrast, high concentrations of ethanol elicit loss of postural control and eventually sedation (Moore et al., 1998; Rothenfluh et al., 2006; Corl et al., 2009). Loss of postural control was initially assayed in the inebriometer, a vertical column containing mesh baffles (Weber, 1988; Cohan and Graf, 1985; Moore et al., 1998; Fig. 2A). Flies naturally exhibit negative geotaxis and therefore tend to remain at the top of the column, but as they lose postural control they gradually fall from one baffle to the next. Ethanol sensitivity can therefore be measured as the time required for the flies to reach the bottom of the column. Negative geotaxis has also been directly assayed as a measure of ethanol sensitivity by quantifying the vertical distance that flies climb after being knocked to the bottom of a vial (Bhandari et al., 2009). More recently, ethanol-induced loss of postural control (referred to more simply as “sedation”) has been assayed manually using a loss-of-righting reflex assay, in which one counts the number of flies that fail to regain upright posture after being knocked over (Fig. 2B) (Rothenfluh et al., 2006; Corl et al., 2009).

**Ethanol Tolerance**

In flies, as in mammals, repeated exposure to ethanol induces tolerance, which is defined as an acquired resistance to the effects of the drug. Tolerance is one of the DSM-IV criteria for alcohol dependence (American Psychiatric Association, 1994) and has been correlated with heavy drinking and alcohol abuse (Schuckit et al., 2008). In flies, ethanol tolerance is assayed by measuring the decrease in sensitivity to ethanol-
induced motor impairment after ethanol pre-exposure. This change in sensitivity can be assayed using the inebriometer (Scholz et al., 2000), the sedation assay (Berger et al., 2004), or negative geotaxis (Bhandari et al., 2009), and can be measured as a change in either the proportion of impaired flies (Urizar et al., 2007), latency or duration of impairment (Dzitoyeva et al., 2003; Devineni et al., 2011), or the recovery time after sedation (Berger et al., 2004; Cowmeadow et al., 2005). Ethanol tolerance appears to be robust to variations in the tolerance protocol, as the studies cited above vary significantly in the timing and concentration of ethanol exposure.

Two types of ethanol tolerance have been characterized in flies, termed rapid and chronic. Rapid tolerance is induced by relatively brief exposure to a sedating concentration of ethanol, while chronic tolerance is induced by prolonged (~24 hr) exposure to a low ethanol concentration that does not produce overt intoxication (Berger et al., 2004). Rapid and chronic tolerance are mediated by distinct mechanisms; for example, only chronic tolerance is dependent on protein synthesis (Berger et al., 2004).

**Conditioned Ethanol Preference**

To test whether intoxicating doses of ethanol are rewarding to flies, a conditioned ethanol preference assay has been recently developed (Kaun et al., 2011; Fig. 2C). In this assay, flies are initially exposed to two neutral odor cues, one of which is paired with an intoxicating exposure to ethanol vapor. Flies are later offered a choice between the two odors, and preference for the ethanol-associated odor is measured. Similar to mammalian conditioned place preference (CPP) models, this assay uses conditioned preference to assess the rewarding properties of ethanol intoxication. This assay has some advantages over a simpler ethanol consumption assay: 1) the ethanol concentration that the flies experience can be controlled by the experimenter, and 2) the
ethanol stimulus is removed during the test, allowing measurement of the rewarding value of the drug rather than immediate preference for the drug.

When flies have been trained to associate an odor cue with ethanol intoxication, they show initial aversion to the cue, which, within 12-15 hours, transforms into a long-lasting preference (Kaun et al., 2011). The development of conditioned preference is dependent on the ethanol concentration: preference is induced only by exposure to moderate ethanol doses that induce locomotor hyperactivity. Conditioned preference is not induced by lower ethanol concentrations which fail to elicit behavioral changes or higher concentrations that cause sedation. Thus, flies seem to require an intoxicating, but not sedating, dose of ethanol for it to be remembered as rewarding. Remarkably, some flies will endure electric shock in order to attain the cue associated with ethanol, indicating that they are willing to endure punishment to seek the drug (or, in this case, a cue that predicts the presence of the drug) (Kaun et al., 2011). This response is reminiscent of compulsive behavior such as impaired response inhibition observed in mammalian studies of drug reward. Furthermore, flies will endure a stronger shock intensity to attain a cue associated with ethanol than a cue associated with sugar, suggesting that the preference for ethanol is distinct from a preference for food reward (Kaun et al., 2011).

**Molecular Mechanisms Underlying Ethanol-Induced Behavior in Drosophila**

As described above, an array of assays has been established to study various aspects of ethanol-induced behavior in *Drosophila*. All of these assays are relatively simple, robust, and high-throughput, allowing researchers to conduct forward genetic screens to identify the underlying mechanisms. The genes identified in these screens have led to the characterization of diverse molecular and cellular processes that mediate ethanol-induced behavior in flies.
Molecular Pathways

Several classical molecular signaling pathways have been implicated in regulating sensitivity to ethanol-induced motor impairment in flies, including the epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K)/Akt, and cyclic adenosine monophosphate (cAMP) pathways. Genetic and pharmacological evidence indicates that the EGFR and cAMP pathways promote resistance to ethanol sedation (Corl et al., 2009; Moore et al., 1998), while the PI3K/Akt pathway enhances ethanol sedation (Eddison et al., 2011). However, these pathways are likely to regulate ethanol sensitivity in more complex ways depending on factors such as the cell types in which they are expressed and the presence of multiple protein isoforms. For example, a mutation disrupting the function of the type II regulatory subunit of protein kinase A (PKA), a key effector of cAMP signaling, causes the opposite effect on ethanol sedation as expected from previous manipulations that disrupt overall PKA signaling (Park et al., 2000; Moore et al., 1998).

A genetic screen revealed that *scabrous* (*sca*), encoding a secreted protein that negatively regulates the Notch signaling pathway (Baker et al., 1990; Powell et al., 2001), is required for ethanol reward memory. Notch signaling is required for long-term memory formation in flies, suggesting that *sca* may contribute generally to long-term memory processes (Presente et al., 2004; Ge et al., 2004). However, mutation of *sca* does not affect short-term memory (LaFerriere et al., 2008). Notch signaling has been shown to regulate migration, morphology, synaptic plasticity and survival of immature and mature neurons (Ables et al., 2011). It will be interesting to examine how *sca* and the Notch pathway affect the neural plasticity underlying memory for ethanol reward.

Transcription and translation have been implicated in ethanol-induced behaviors. Two genes encoding putative transcriptional regulators, *Drosophila LIM-domain only*
(dLmo) and hangover (hang), were identified as regulators of ethanol sedation and ethanol tolerance, respectively (Lasek et al., 2011a; Scholz et al., 2005). One likely target gene whose expression is regulated by dLmo, the Drosophila homolog of anaplastic lymphoma kinase (dAlk), has been identified and shown to regulate ethanol sedation (Lasek et al., 2011b).

A mutation in krasavietz (kra), which encodes a predicted translation initiation factor that inhibits protein translation in vitro (Lee et al., 2007), causes decreased sensitivity to ethanol-induced sedation and decreased rapid and chronic ethanol tolerance (Berger et al., 2008). Although protein synthesis is required for chronic tolerance, it is not required for the development of rapid tolerance and is unlikely to occur during the brief timescale of acute ethanol intoxication (~30 min; Berger et al., 2004). However, treating flies with a protein synthesis inhibitor prior to ethanol exposure caused pronounced resistance to ethanol impairment (Berger et al., 2004), suggesting that some proteins that are constitutively synthesized in the absence of ethanol mediate naive ethanol sensitivity.

Finally, molecular pathways involved in cellular stress responses have been implicated in ethanol tolerance. hang mutants, which show decreased ethanol tolerance, also show sensitivity to oxidative stress and decreased heat-ethanol cross-tolerance (i.e. tolerance to ethanol stimulated by heat shock stress instead of ethanol exposure; Scholz et al., 2005). The gene jwa, a retinoic acid-responsive gene whose product associates with the cytoskeleton, mediates oxidative and heat stress responses and also promotes ethanol tolerance (Li et al., 2008). Since high doses of ethanol induce cellular stress, which in some ways mimics oxidative and heat stress, it may not be surprising that common molecular pathways respond to ethanol as well as other stressors (e.g. Wu and Cederbaum, 2009; Wilke et al., 1994; Piper, 1995).
Cellular Mechanisms

One of the key cellular processes that has been implicated in ethanol-induced behaviors in flies is cytoskeletal dynamics. *thousand and one (tao)*, which was identified as a critical regulator of ethanol-induced hyperactivity, implicated microtubule dynamics in the hyperactivity response (King et al., 2011). *tao* was shown to function through the conserved kinase PAR-1 (also called MARK in mammals) to regulate the microtubule-binding protein Tau during fly brain development (Matenia and Mandelkow, 2009; King et al., 2011). The mouse homolog of *jwa*, which promotes ethanol tolerance in flies (see above), is also a microtubule-associated protein (Li et al., 2008; Chen et al., 2007).

In addition to microtubule organization, the regulation of actin has been implicated in ethanol responses. *Rho GTPase activator protein 18B (RhoGAP18B)* regulates sensitivity to both ethanol-induced sedation and hyperactivity through different protein isoforms (Rothenfluh et al., 2006). RhoGAP18B is a GTPase activating protein that regulates ethanol sensitivity by functioning through Rho family GTPases, which are key regulators of actin dynamics (Rothenfluh et al., 2006). Additionally, *Kra* (described above) interacts with the crosslinking protein Short stop to regulate actin organization, suggesting that that actin regulation may underlie some of its diverse effects on ethanol-induced behavior (Lee et al., 2007; Sanchez-Soriano et al., 2009). However, it is important to note that changes in neither microtubule nor actin organization have been directly linked to altered ethanol responses in these mutants.

Finally, the integrin class of cell adhesion molecules has been implicated in ethanol-induced behaviors. Mutations in the alpha-integrin gene *scab (scb)* or the ß-integrin gene *myospheroid (mys)* cause increased ethanol sensitivity as well as increased tolerance (Bhandari et al., 2009). It will be interesting to determine the mechanisms by which disruptions in cytoskeletal organization or cell adhesion lead to altered ethanol responses.
Synaptic Function and Neuronal Excitability

Synapse number has recently been implicated in ethanol sensitivity in flies. Several genetic manipulations that lead to increased ethanol sedation sensitivity, such as mutations in *arouser (aru)* or *amnesiac (amn)* and overexpression of *PI3K* or *Ras homolog enriched in brain (Rheb)*, also increase synapse number at the larval neuromuscular junction (NMJ) and/or the adult central brain (Eddison et al., 2011). An environmental manipulation, adult social isolation, which is known to reduce the number of synapses of a specific set of fly brain neurons (Donlea et al., 2009), also reduces ethanol sensitivity (Eddison et al., 2011). Furthermore, social isolation concurrently restores normal synapse number and ethanol sensitivity to the ethanol-sensitive mutant *aru* (Eddison et al., 2011). This correlation between synapse number and ethanol sedation sensitivity using multiple independent genetic and environmental manipulations suggests that increased synapse number may directly promote increased sensitivity to ethanol sedation.

In addition to providing a novel cellular mechanism by which ethanol behaviors can be regulated, these findings suggest the hypothesis that ethanol tolerance may result from a compensatory decrease in synapse number induced by the initial ethanol exposure. While this hypothesis has not been directly tested, *hang*, which promotes ethanol tolerance (see above), negatively regulates synapse number at the larval NMJ (Schwenkert et al., 2008). For both ethanol sensitivity and tolerance, it remains to be investigated whether increased synapse number translates into increased postsynaptic excitation (or inhibition), and whether ethanol sensitivity depends on increased synapse number generally throughout the nervous system or in specific neurons.

There is abundant evidence that synaptic transmission regulates ethanol-induced behaviors in flies. Flies carrying a mutation in *Syntaxin 1A (Syx1A)* or *shibire (shi),
encoding *Drosophila* dynamin), which are respectively required for synaptic vesicle docking and recycling, show defects in ethanol sedation tolerance (Krishnan et al., 2011). Use of conditional mutations revealed that normal synaptic vesicle release is required immediately after initial ethanol exposure rather than after recovery from intoxication to promote tolerance (Krishnan et al., 2011). A different study showed that flies lacking *Synapsin* (*Syn*), encoding a presynaptic vesicle scaffolding protein, unexpectedly show increased ethanol tolerance (Godenschwege et al., 2004). This result may reflect the fact that Synapsin is involved not only in regulating neurotransmitter release, but also in neurite growth, synaptic formation and maturation, and in segregating the reserve and readily releasable pools of vesicles (Cesca et al., 2010). Finally, Homer, a protein that interacts with postsynaptic scaffolding and signaling proteins, including metabotropic glutamate receptors, regulates both initial ethanol sensitivity and ethanol tolerance (Urizar et al., 2007).

The major regulators of neuronal excitability that have been implicated in ethanol-induced behaviors in flies are the γ-Aminobutyric acid B (GABA$_B$) receptors and the large conductance calcium-activated potassium (BK) channels. GABA$_B$ receptor activity promotes sensitivity to ethanol sedation but reduces rapid ethanol tolerance (Dzitoyeva et al., 2003). As in mammals, *Drosophila* GABA$_B$ receptors are metabotropically coupled to potassium channels, thereby inhibiting neuronal excitability due to potassium efflux (Mezler et al., 2001). The BK channel encoded by the gene *slowpoke* (*slo*) has also been implicated in rapid ethanol tolerance, but in the opposite direction. Expression of the fly BK channel is upregulated by ethanol exposure and its function is required for the development of rapid ethanol tolerance; induction of BK channel expression is in fact sufficient to induce ethanol resistance, mimicking the tolerant state (Cowmeadow et al., 2005; Cowmeadow et al., 2006). The fact that GABA$_B$ receptors and BK channels likely affect neuronal excitability in the same direction, but
regulate tolerance in opposite ways, suggests that they may function in different subsets of neurons that exert opposing effects on behavior. Alternatively, it has been proposed that BK channels may in fact enhance neuronal excitability by reducing the refractory period or enhancing firing rates, allowing neurons to compensate for the depressant effect of ethanol during sedation (Atkinson, 2009).

In addition to classical neurotransmitters such as GABA, neuromodulators, including biogenic amines and neuropeptides, also regulate ethanol-induced behavior in flies. Dopamine promotes ethanol hyperactivity through the D1-like receptor DopR (Bainton et al., 2000; Kong et al., 2010b) and is also required for conditioned ethanol preference (Kaun et al., 2011). Octopamine, a biogenic amine thought to be the invertebrate analog of norepinephrine, is essential for the development of rapid but not chronic ethanol tolerance (Scholz, 2000; Berger et al., 2004). Two neuropeptides produced in the fly brain, neuropeptide F (NPF) and insulin, have been shown to regulate ethanol sedation. NPF, the fly homolog of neuropeptide Y, enhances ethanol sedation (Wen et al., 2005). Mutations in the insulin receptor (InR) cause increased sedation sensitivity, as does overexpression of the adaptor protein p60 to inhibit the coupling between the insulin receptor (InR) and PI3K, the main effector of insulin signaling (Corl et al., 2005). These results indicate that insulin acts through PI3K to promote sedation resistance. However, a different study (discussed earlier) using several more direct manipulations of the PI3K/Akt pathway demonstrated that this pathway promotes sedation sensitivity (Eddison et al., 2011). PI3K may therefore have opposing roles in regulating ethanol sedation depending on the upstream molecule to which it is coupled and the cell type in which it is expressed. In general, the mechanisms by which these neuromodulators affect postsynaptic and/or presynaptic cells have not yet been characterized. It thus remains an open question whether they directly affect postsynaptic excitability or modulate other pre- or postsynaptic properties.
**Genome-Wide Studies**

The majority of the genes discussed above were identified using genetic screens in which mutants exhibiting abnormal behavior were isolated. However, an alternative approach is to use transcriptional profiling to compare gene expression under different conditions. For example, one study identified genes differentially expressed in fly strains selected for increased versus decreased sensitivity to ethanol, and confirmed that mutations in many of these genes cause altered ethanol sensitivity (Morozova et al., 2007).

Three studies have identified genes whose expression is regulated by ethanol exposure, making them good candidates for mediating the development of tolerance. These studies used varying exposure protocols and collectively identified 1669 candidate genes, 29 of which were common to all three studies and 229 of which were common to at least two out of three studies (Morozova et al., 2006; Urizar et al., 2007; Kong et al., 2010a). Many of these genes were functionally validated using mutant analysis (Morozova et al., 2006; Kong et al., 2010a), but in most cases the molecular and cellular mechanisms by which these genes function have not been determined.

**Neural Circuits Underlying Ethanol-Induced Behavior in Drosophila**

Although the neural circuits mediating ethanol-induced behaviors in *Drosophila* have not been as extensively studied as the molecular mechanisms, new tools such as Gal4 lines to target particular neurons and transgenes to manipulate neuronal activity have made the study of circuits more accessible.

In mammals, dopamine is an important regulator of many ethanol-related behaviors (Soderpalm et al., 2009). In the fly, dopamine is expressed in several clusters of neurons that project to a variety of brain regions (Nassel and Elekes, 1992). As in
mammals, many of these dopaminergic cells have been shown to play a role in ethanol-related behaviors. The function of dopamine in regulating ethanol hyperactivity was localized to a pair of dopaminergic neurons projecting to DopR-expressing neurons in the ellipsoid body of the central complex (Kong et al., 2010b), a region known to regulate visual and locomotor behavior, arousal, and memory (Martin et al., 1999; Wu et al., 2007; Neuser et al., 2008; Ofstad et al., 2011). The ellipsoid body is also the site of Homer function in the regulation of ethanol sedation sensitivity and tolerance (Urizar et al., 2007), though it is unknown whether Homer functions via DopR signaling, or in DopR-expressing neurons.

Dopamine neurons also mediate conditioned ethanol preference. Ethanol reward memory, like other forms of memory, can be divided into three phases: acquisition (memory formation during training), consolidation (the period between training and testing), and retrieval (expression of the memory during testing) (Krashes et al., 2007). Interestingly, silencing dopaminergic neurotransmission impairs retrieval, but not acquisition or consolidation, of ethanol reward memory (Kaun et al., 2011).

While some dopaminergic neurons innervate the ellipsoid body, others terminate in the mushroom body, a brain structure implicated in olfactory processing and learning (Davis, 2011). Neurotransmission of mushroom body neurons is required for both ethanol-induced hyperactivity and conditioned ethanol preference (King et al., 2011; Kaun et al., 2011). Both behaviors are mediated by neurons in specific subregions within this structure, and distinct phases of conditioned ethanol preference are in fact localized to different mushroom body neurons (King et al., 2011; Kaun et al., 2011). Together, these studies demonstrate that different ethanol-induced behaviors can be mapped to distinct neural loci, and that some brain structures, such as the mushroom body, are important for multiple behaviors.
Mammalian Validation of Mechanisms Underlying Ethanol-Induced Behavior

Now that years of research have implicated many different molecular and cellular pathways in mediating fly responses to ethanol, it is important to ask whether these mechanisms function in mammals as well. In fact, many of the genes and molecular pathways implicated in *Drosophila* ethanol responses play a similar role in mammals (see Table 1). For example, the cAMP, EGFR, and NPF/NPY pathways all regulate ethanol sensitivity similarly in flies and rodents (Moore et al., 1998; Wand et al., 2001; Corl et al., 2009; Wen et al., 2005; Thiele et al., 1998). Furthermore, these pathways regulate not only ethanol sensitivity but also ethanol consumption in rodents (Wand et al., 2001; Corl et al., 2009; Thiele et al., 1998). Thus, simple behavioral assays that are readily used for genetic screening in flies can yield candidate genes that have homologous roles in rodent models. Moreover, an FDA-approved drug that inhibits function of EGFR, a molecule first shown to regulate ethanol-related behavior in the fly, has been shown to be effective in a preclinical rat model of ethanol addiction (Corl et al., 2009).

While most of the genes affecting ethanol-induced behavior in flies have not yet been tested for a role in humans, a few have already been associated with human ethanol-related behavior. Polymorphisms in the human *ALK* gene are correlated with multiple measures of ethanol sensitivity (Lasek et al., 2011b), and polymorphisms in one human homolog of *hang*, _ZNF699_, were found to be associated with alcohol dependence (Riley et al., 2006). Recently, a genome-wide meta-analysis revealed that polymorphisms in _autism susceptibility candidate 2_ (*AUTS2*) are associated with alcohol consumption (Schumann et al., 2011). Mice selected for high versus low alcohol consumption differ in expression of _AUTS2_, and downregulation of the fly homolog of _AUTS2_ leads to reduced ethanol sensitivity (Schumann et al., 2011). Given the significant conservation of genes affecting ethanol responses in flies and rodents, it is
likely that additional genes identified in flies will be validated in rodents and humans, and
\textit{vice versa.}

In addition to molecular pathways, some of the cellular mechanisms implicated in
\textit{Drosophila} ethanol responses have also been studied in mammals. For example, the
role of synapse function in ethanol-induced behavior is still an emerging field of study in
flies, while the effects of ethanol at the synapse have been well studied in mammals.
Ethanol acts on a variety of postsynaptic receptors, most notably GABA_{\text{A}} and N-Methyl-
D-aspartic acid (NMDA) receptors, and also exerts presynaptic effects on
neurotransmitter release (Siggins et al., 2005). Whether changes in synapse number are
associated with altered ethanol behaviors in mammals, as is the case in flies (Eddison et
al., 2011), has not yet been studied.

While at the molecular level flies and mammals share many features (Littleton
and Ganetzky, 2000; Lloyd et al., 2000), the anatomical organization of fly and
mammalian nervous systems is quite distinct. It is therefore difficult to draw parallels
between the neural circuits that regulate ethanol-induced behavior in flies and mammals.
In flies, brain structures such as the ellipsoid body and the mushroom body have been
implicated in various ethanol responses; it is unclear what the equivalent structures are
in the mammalian brain. Nevertheless, certain conserved neurochemical systems
function similarly in flies and mammals. The mammalian mesolimbic dopamine pathway,
including its target regions, is perhaps the most intensely studied neural circuit in the
context of alcohol reward and addiction (Soderpalm et al., 2009). Dopamine neurons
were similarly found to be required for ethanol hyperactivity and reward in \textit{Drosophila}
(Kong et al., 2010b; Kaun et al., 2011). Neuropeptidergic systems, such as the
NPY/NPF system, also regulate ethanol responses similarly in flies and rodents, as
discussed above. Thus, the functions of neurochemically defined neural pathways,
rather than morphologically defined brain regions, are likely to be conserved in
regulating ethanol behaviors. Overall, flies represent an ideal genetic organism for
studying the molecular, cellular, and neural pathways regulating ethanol-induced
behavior, allowing us to gain insights that can be translated into mammalian and even
human behavior.

REFERENCES


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Figure 1. Common Genetic Tools in Drosophila

(A) The Gal4/UAS system (Brand and Perrimon 1993). The transcriptional activator Gal4 is expressed in a spatially restricted pattern and activates any gene placed downstream of the upstream activating sequence (UAS).

(B) The TARGET system. (McGuire et al. 2003). At the restrictive temperature (30°C), Gal80<sup>ts</sup> is inactive, Gal4 is active and UAS-driven genes are expressed. At the permissive temperature (19°C), Gal80<sup>ts</sup> is active, Gal4 is inhibited, and UAS-driven genes are not expressed.

(C) The Shibire<sup>ts</sup> system (Kitamoto 2001). At the restrictive temperature (30°C), but not the permissive temperature (19°C), Shi<sup>ts</sup> blocks neurotransmission by disrupting endocytosis and thereby depleting synaptic vesicles.

(D) The TrpA1 system (Hamada et al. 2008; Pulver et al. 2009). At the restrictive temperature (27°C), but not the permissive temperature (19°C), cation flow through the temperature-gated cation channel TRPA1 causes neuronal depolarization.
Figure 2. Assays to Measure Ethanol-Induced Behavior in Drosophila

(A) The inebriometer measures ethanol-induced loss of postural control by measuring the time required for flies to fall down the mesh baffles from the top to the bottom of the column (Weber, 1988; Moore et al., 1998).

(B) The booz-o-mat allows for the measurement of ethanol-induced hyperactivity and sedation while streaming vaporized ethanol into horizontal tubes containing groups of flies (Wolf et al., 2002). Hyperactivity is measured by filming the flies and using tracking software to calculate their locomotor speed. Sedation is measured by recording the time required for flies to exhibit the loss-of-righting reflex.

(C) Conditioned ethanol preference is measured by training the flies in a sealed container to associate a neutral odor with the presence of an intoxicating dose of ethanol, and later testing preference for that odor in the absence of ethanol using a Y-maze (Kaun et al., 2011).
Table 1: Selected Genes Mediating Ethanol-Induced Behaviors in Flies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mechanism of action</th>
<th>Ethanol-Related Phenotype</th>
<th>Reference</th>
<th>Homolog Validated</th>
</tr>
</thead>
<tbody>
<tr>
<td>amn</td>
<td>cAMP pathway</td>
<td>increased motor impairment</td>
<td>Moore et al. 1998</td>
<td>Wand et al. 2001</td>
</tr>
<tr>
<td>hppy</td>
<td>inhibits EGFR pathway</td>
<td>decreased sedation</td>
<td>Corl et al. 2009</td>
<td></td>
</tr>
<tr>
<td>aru</td>
<td>EGFR and PI3K/Akt pathways; regulation of synapse number</td>
<td>increased sedation</td>
<td>Eddison et al. 2011</td>
<td></td>
</tr>
<tr>
<td>Rheb</td>
<td>Tor pathway; regulation of synapse number (upon overexpression)</td>
<td>increased sedation</td>
<td>Eddison et al. 2011</td>
<td></td>
</tr>
<tr>
<td>sca</td>
<td>Notch pathway?</td>
<td>decreased conditioned preference</td>
<td>Kaun et al. 2011</td>
<td></td>
</tr>
<tr>
<td>dLmo</td>
<td>transcriptional regulation of dAlk?</td>
<td>increased sedation</td>
<td>Lasek et al. 2011a</td>
<td>Lasek et al. 2011a</td>
</tr>
<tr>
<td>dAlk</td>
<td>receptor tyrosine kinase signaling</td>
<td>decreased sedation</td>
<td>Lasek et al. 2011b</td>
<td>Lasek et al. 2011b</td>
</tr>
<tr>
<td>hang</td>
<td>stress pathway; regulation of synapse number?</td>
<td>decreased tolerance</td>
<td>Scholz et al. 2005</td>
<td>Riley et al. 2006</td>
</tr>
<tr>
<td>jwa</td>
<td>stress pathway; regulation of microtubules?</td>
<td>decreased tolerance</td>
<td>Li et al. 2008</td>
<td></td>
</tr>
<tr>
<td>kra</td>
<td>regulation of translation?; actin regulation?</td>
<td>decreased sedation; decreased tolerance</td>
<td>Berger et al. 2008</td>
<td></td>
</tr>
<tr>
<td>tao</td>
<td>regulation of Tau/microtubules through par-1</td>
<td>decreased hyperactivity</td>
<td>King et al. 2011</td>
<td></td>
</tr>
<tr>
<td>RhoGAP18B</td>
<td>regulation of Rho family GTPases; actin regulation?</td>
<td>decreased sedation</td>
<td>Rothenfluh et al. 2006</td>
<td></td>
</tr>
<tr>
<td>scb</td>
<td>integrin/cell adhesion</td>
<td>increased motor impairment; increased tolerance</td>
<td>Bhandari et al. 2009</td>
<td></td>
</tr>
<tr>
<td>mys</td>
<td>integrin/cell adhesion</td>
<td>increased motor impairment; increased tolerance</td>
<td>Bhandari et al. 2009</td>
<td></td>
</tr>
<tr>
<td>Syx1A</td>
<td>synaptic transmission</td>
<td>decreased tolerance</td>
<td>Krishnan et al. 2011</td>
<td></td>
</tr>
<tr>
<td>shi</td>
<td>synaptic transmission</td>
<td>decreased tolerance</td>
<td>Krishnan et al. 2011</td>
<td></td>
</tr>
<tr>
<td>Syn</td>
<td>synaptic transmission</td>
<td>increased tolerance</td>
<td>Godenschwege et al. 2004</td>
<td></td>
</tr>
<tr>
<td>homer</td>
<td>postsynaptic signaling</td>
<td>increased sedation; decreased tolerance</td>
<td>Urizar et al. 2007</td>
<td>Szumlinski et al. 2005</td>
</tr>
</tbody>
</table>
**Gene** | **Mechanism of action** | **Ethanol-Related Phenotype** | **Reference** | **Homolog Validated**
---|---|---|---|---
GABA-B-R1 | GABA signaling | decreased sedation (RNAi and drug antagonist) | Dzitoyeva et al. 2003 | Zaleski et al. 2001
slo | calcium-activated potassium channel activity | decreased tolerance | Cowmeadow et al. 2005 | Knott et al. 2002
ple | dopamine synthesis | decreased hyperactivity (drug inhibitor) | Bainton et al. 2000 | Friedhoff and Miller 1973
DopR | dopamine signaling | decreased hyperactivity | Kong et al. 2010 | El Ghundi et al. 1998
Tbh | octopamine synthesis | decreased tolerance | Scholz et al. 2000; Berger et al. 2004 | Tabakoff and Ritzmann 1977
InR | insulin signaling | increased sedation | Corl et al. 2005 | Thiele et al. 1998
npf | NPF signaling | decreased sedation | Wen et al. 2005 | Thiele et al. 1998

*This table includes the major genes that have been functionally characterized as regulating ethanol-induced behaviors in flies, and indicates cases in which a mammalian homolog of the gene has been shown to play a similar role. Studies identifying a large number of genes with limited characterization of mechanism (e.g. Berger et al., 2008) have not been included. In cases where many genes in the same signaling pathway have been implicated, the gene initially identified is listed and the signaling pathway is described in the second column (e.g. aru, PI3K/Akt pathway). Unless otherwise specified, the ethanol-related phenotype described in the third column refers to the phenotype upon impairing the function of the gene product by mutation, RNAi, or pharmacology.*
CHAPTER 2

Preferential Ethanol Consumption in Drosophila Models Features of Addiction

Abstract
Alcohol addiction is a common affliction with a strong genetic component (Dick and Foroud, 2003). Although mammalian studies have provided significant insight into the molecular mechanisms underlying ethanol consumption (Crabbe et al., 2006), other organisms such as Drosophila melanogaster are better suited for unbiased, forward genetic approaches to identify novel genes. Behavioral responses to ethanol, such as hyperactivity, sedation, and tolerance, are conserved between flies and mammals (Wolf et al., 2002; Scholz et al., 2000), as are the underlying molecular pathways (Moore et al., 1998; Maas et al., 2005; Wen et al., 2005; Thiele et al., 1998; Corl et al., 2009). However, few studies have investigated ethanol self-administration in flies (Ja et al., 2007). Here we characterize ethanol consumption and preference in Drosophila. Flies prefer to consume ethanol-containing food over regular food, and this preference increases over time. Flies are attracted to the smell of ethanol, which partially mediates ethanol preference, but are averse to its taste. Preference for consuming ethanol is not entirely explained by attraction to either its sensory or caloric properties. We demonstrate that flies can exhibit features of alcohol addiction. First, flies self-administer ethanol to pharmacologically relevant concentrations. Second, flies will overcome an aversive stimulus in order to consume ethanol. Third, flies rapidly return to high levels of ethanol consumption after a period of imposed abstinence. Thus, ethanol preference in Drosophila provides a new model for studying aspects of addiction.

Results
Flies Prefer to Consume Food Containing Ethanol

We first characterized the basic parameters of ethanol preference in flies. We used a modified version of the two-choice Capillary Feeder (CAFE) assay to measure ethanol consumption and preference (Ja et al., 2007). Our assay is generally analogous to the two-bottle choice assay used in rodent studies of ethanol consumption. In our assay, flies consume liquid food from four capillaries placed vertically through the top of their vials, and consumption is assayed by measuring the descent of each meniscus (Figure 1A). Flies can choose to feed from two capillaries containing non-ethanol food (5% sucrose/5% yeast extract) or two with ethanol-containing food (15% ethanol in 5% sucrose/5% yeast extract). Capillaries are replaced daily. Ethanol preference was quantified by calculating a preference index (PI) defined as (ethanol consumption - non-ethanol consumption) / total consumption. PI can vary between -1 and +1, with positive values indicating preference and negative values indicating repulsion.

Flies displayed a robust preference for consuming 15% ethanol food over non-ethanol food, and this preference increased over 5 days (Figures 1B and 1C). In addition, the variability in preference decreased over the first 3 days (Figure S1A). Filming of the flies revealed that the drinking frequency from ethanol food was higher than that from non-ethanol food on day 4 of the assay but not day 1 (Figure S2A), paralleling the increase in ethanol preference over time. In contrast, the duration of drinking bouts was lower for ethanol food than non-ethanol food on both day 1 and day 4 (Figure S2B). Thus, preferential ethanol consumption occurs via an increase in frequency, but not duration, of drinking bouts associated with ethanol food.

To further analyze the changes in ethanol preference over time, we examined both shorter and longer time courses. First, we asked whether naive flies exhibit immediate ethanol preference and observed that flies displayed a positive, albeit highly variable, ethanol preference during the first 8 hours of the assay (Figure S1B). Second,
we asked whether preference continues to increase after 5 days, and found that preference stabilized after 4-5 days (Figure S1C). We also examined the dose-dependence of ethanol preference by varying the concentration of the ethanol-containing food from 5% to 25% ethanol, and observed that preference increased with increasing ethanol concentration (Figure 1D). Interestingly, this relationship was observed at the end (days 4-5) but not the beginning (days 1-2) of the preference assay (Figure 1D), indicating that the dose-dependence of preference develops over time.

Next, we investigated whether flies self-administer ethanol food to pharmacologically relevant ethanol concentrations. We first measured the concentration of ethanol present in populations of flies during day 5 of the preference assay. Flies contained an average of 5.2 mM ethanol (Figure 1E), significantly higher than background measurements of flies that did not consume ethanol (4.1 mM, p<.01). However, this value is an underestimate because flies feed sporadically and metabolize ethanol quickly (Moore et al., 1998), such that most flies were unlikely to contain significant ethanol levels at the time when measurements were conducted. Unfortunately, these measurements require many flies for a single sample, which therefore encompasses a wide range of ethanol concentrations. We hypothesized that measurements of ethanol levels might be much higher and more uniform if the feeding of flies was synchronized. To accomplish this, we starved flies for 20 hours after 4 days of drinking and then returned them to the preference assay for 10 or 60 minutes. These flies contained an average of 45 or 26 mM ethanol, respectively (Figure 1F). These concentrations are sufficiently high to produce behavioral intoxication, such as locomotor hyperactivity (∼15 mM (Wolf et al., 2002)) and loss of postural control (∼35 mM (Moore et al., 1998)). Although the starved/refed flies do not reflect the same conditions as the standard continuous access assay, it is important to note that these flies were able to
choose whether or not to consume ethanol and were clearly willing to self-administer ethanol to pharmacologically relevant concentrations.

In our preference assay, the ethanol-containing food has nearly four times the number of calories as the non-ethanol food. To determine whether attraction to calories is the primary reason flies consume ethanol, we tested whether varying the caloric ratio between the ethanol and non-ethanol food would influence ethanol preference. We varied the caloric ratio by using different concentrations of food but a fixed concentration of ethanol (Table 1). At low food concentrations ethanol provides many more calories than the food, yielding a large caloric ratio. Conversely, high food concentrations provide substantial calories on their own and yield a more modest caloric ratio. If caloric attraction were a significant factor driving ethanol preference, we would expect greater preference at higher caloric ratios. By varying the food concentration between 1% and 8%, the caloric ratio between ethanol and non-ethanol food ranged from 2.2 to 10.9. Despite this large variation, ethanol preference was not affected (Table 1). We therefore conclude that ethanol preference is unlikely to be a byproduct of caloric attraction.

**Olfactory Attraction and Gustatory Aversion Differentially Influence Ethanol Preference**

We have shown that ethanol preference is an innate, robust behavior. We next sought to characterize the sensory inputs that influence ethanol preference. To test the role of olfaction, we removed the primary olfactory organs, the third antennal segments (Vosshall and Stocker, 2007). Antennectomized flies fail to startle when ethanol vapor is presented, indicating that they cannot detect its smell (Wolf et al., 2002). Antennectomized flies exhibited decreased ethanol preference compared with controls (Figure 2A). These flies did not appear sickly and exhibited only an 8% decrease in overall consumption compared with controls (Figure S3), indicating that their decreased
preference does not reflect general impairment. To further confirm the role of olfaction, we took advantage of the finding that repeated ethanol vapor exposures can kill the olfactory receptor neurons (ORNs) in the antennae and cause antennae to turn black (French and Heberlein, 2009). Flies with black antennae had decreased ethanol preference compared with identically-treated flies with normal antennae (Figure S4). Overall, these data demonstrate that olfaction is an important mediator of ethanol preference.

To directly show that flies are attracted to the smell of ethanol, we conducted an olfactory trap assay using ethanol (Woodard et al., 1989). In this assay, flies choose between an ethanol-containing trap and a control trap on the basis of olfaction alone, since they cannot physically contact the ethanol prior to making a choice. Wild-type flies preferred olfactory traps containing 15% ethanol over control traps (Figure 2B), confirming that ethanol has attractive olfactory properties. However, the fact that both antennectomized flies and flies with ablated antennal ORNs still showed a positive preference for consuming ethanol (Figures 2A and S4) suggests that olfactory attraction is not essential for ethanol preference. To test this hypothesis, we utilized white rabbit (whir) mutant flies (Rothenfluh et al., 2006), which are strongly repulsed by the smell of ethanol (Figure 2B). Interestingly, whir flies still displayed a significant preference for consuming ethanol food (Figure 2C). whir exhibited a trend toward decreased preference compared with the control (p=.06). Although whir flies also have an ethanol sensitivity phenotype (Rothenfluh et al., 2006), they demonstrate that ethanol preference can be dissociated from olfactory attraction to ethanol and can even exist in the presence of strong olfactory repulsion.

Next, we tested the role of gustation in ethanol preference. Most ethanol-naive mammals, including rodents and humans, perceive the taste of ethanol as predominantly bitter and generally aversive (Kiefer and Dopp, 1989; Scinska et al., 2000). To determine
whether ethanol represents an attractive or repulsive gustatory stimulus in flies, we tested whether it elicits the proboscis extension reflex (PER), an appetitive response that precedes feeding (Dethier, 1976). Palatable liquids elicit PER when applied to gustatory neurons on the legs or labellum (Dethier, 1976). In contrast, unpalatable compounds fail to elicit PER on their own, and can be distinguished from tasteless compounds because they decrease the PER elicited by a palatable substance when added to the same solution (Dethier, 1976; Wang et al., 2004). We tested ethanol concentrations ranging from 0.1% to 40% and found that all concentrations failed to elicit significant PER (Figure 2D). To determine whether the lack of PER indicates that ethanol is tasteless or taste-aversive, we added the same concentrations of ethanol to 100 mM sucrose, which elicits reliable PER. When added to sucrose, ethanol caused a dose-dependent decrease in PER frequency (Figure 2E), indicating an aversive taste response. Flies therefore appear to be attracted to the smell of ethanol but averse to its taste, providing an interesting example of a single stimulus eliciting conflicting sensory responses.

Gustatory aversion to ethanol might provide an inhibitory input that actively suppresses ethanol consumption. If this were the case, taste-defective flies would have increased ethanol preference due to the absence of this gustatory repulsion. We tested this hypothesis by using pox neuro (poxn) mutant flies, in which taste bristles are transformed into mechanosensory bristles lacking gustatory receptors (Awasaki and Kimura, 1997). Surprisingly, poxn70-23 and poxnM22-85 null mutants displayed ethanol preference similar to control flies (Figure 2F). These results indicate that gustatory inputs do not play a major inhibitory role in ethanol preference, thus highlighting the difference between an initial sensory response and a long-term preference assay.

**Ethanol Preference in Flies Exhibits Features of Addiction**
Our results indicate that preference for consuming ethanol cannot be explained solely by an attraction to its caloric or sensory properties, suggesting the potential importance of its pharmacological effects. This hypothesis is supported by the fact that flies voluntarily self-administer ethanol to high internal concentrations that can alter behavior (Figure 1F). We therefore investigated whether ethanol preference in flies shares characteristics of addiction that have been modeled in rodents.

One feature of alcohol addiction is “use of alcohol despite adverse consequences” (Morse and Flavin, 1992). Rodent studies have modeled this feature by adding quinine, an aversive compound, to the ethanol solution. Ethanol-experienced rats continue to consume substantial amounts of ethanol even when quinine is added, though intake is usually decreased (Wolffgramm and Heyne, 1991). First, we tested whether naive flies would exhibit ethanol preference if quinine was added to the ethanol food. These flies did not initially prefer the quinine-laced ethanol food, but developed preference over subsequent days (Figure 3A). This preference does not simply represent habituation to quinine, since flies given a choice between quinine food and normal food exhibited quinine aversion for the entire 5-day period (Figure 3A). Second, we asked whether flies that had been drinking in the standard preference assay for 5 days would maintain ethanol preference when quinine was subsequently added to the ethanol food on day 6. Although preference was decreased compared with ethanol food lacking quinine, these flies displayed a positive preference for quinine-laced ethanol food (Figure 3B). Overall, these results indicate that flies are willing to overcome an aversive stimulus in order to consume ethanol.

A second characteristic of alcohol addiction is relapse, defined as a return to ethanol consumption levels equal to or greater than those observed previously, following a period of abstinence (Rodd et al., 2004). In rodents, relapse can be modeled by the alcohol deprivation effect (ADE), in which animals increase ethanol consumption levels
after a period of alcohol deprivation (Rodd et al., 2004). We tested whether an ADE exists in flies by depriving them of ethanol access for either 1 or 3 days after 5 days in the preference assay. During deprivation, non-ethanol food was substituted for ethanol food and the PI dropped near zero, as expected since all four capillaries contained identical non-ethanol food (Figures 3C and 3D). Following 1- or 3-day deprivation, flies rapidly returned to peak values of ethanol preference that were not significantly different from pre-deprivation values or from non-deprived controls (Figures 3C and 3D). A second 1-day deprivation yielded similar results (Figure 3C). After each 1 or 3 day deprivation, PI increased at a much greater rate than was observed with naive flies (Figures S5A and S5B). We did not detect an increase in PI following any deprivation protocol. Nevertheless, the rapid increase in preference after deprivation to peak levels rather than the levels measured early in the assay indicates a strong positive memory for ethanol and meets a criterion for relapse.

**krasavietz Exhibits Altered Ethanol Sensitivity, Tolerance, and Preference**

In order to begin identifying genes that influence ethanol preference, we tested whether mutants with known defects in other ethanol-induced behaviors might exhibit altered ethanol preference. We tested 27 mutations affecting ethanol sensitivity or tolerance (Berger et al., 2008, U.H., unpublished data; Scholz et al., 2000; Corl et al., 2009). One mutant, *krasavietz* (*kra*), exhibited decreased ethanol preference compared with the control (Figure 4A). *kra* had PI values near zero at the beginning of the assay and did not show ethanol preference until day 4 (Figure 4A). The *kra* mutation affects a gene also known as *exba*, which encodes a translation initiation factor. *kra* was previously found to exhibit decreased sensitivity to ethanol sedation (Berger et al., 2008; Figure S6A). Furthermore, *kra* is the only known mutant with defects in both rapid and chronic tolerance (Berger et al., 2008; Figures S6B and S6C), two mechanistically
distinct forms of tolerance that differ in their persistence and mode of induction (Berger et al., 2004).

Because kra has been shown to have deficits in long-term memory (Dubnau et al., 2003), we asked whether its decreased preference might be due to memory defects. We tested the ethanol preference of 4 other mutants (drujok, laska, chingis khan, and martik) with long-term memory deficits as severe as those of kra (Dubnau et al., 2003), but normal ethanol sensitivity and tolerance (Berger et al., 2008). All 4 mutants had ethanol preference similar to control flies (Figure 4B), suggesting that long-term memory may not be required for ethanol preference. Thus, the decreased preference of kra is unlikely to be due to a memory defect, and may be related to its altered ethanol sensitivity and/or tolerance.

Discussion

We have characterized voluntary ethanol consumption in Drosophila and demonstrated that flies exhibit a robust preference for ethanol-containing food. Furthermore, our assay models several features of mammalian addiction: (1) flies increase ethanol consumption and preference over time; (2) voluntary ethanol consumption leads to pharmacologically relevant ethanol concentrations; (3) caloric or sensory attraction to ethanol does not entirely account for ethanol preference; (4) flies will overcome an aversive stimulus in order to obtain ethanol; and (5) flies exhibit a relapse-like effect after ethanol deprivation. In addition, we have begun to investigate the molecular mechanisms underlying ethanol preference by identifying one mutant, kra, that exhibits deficits in preference.

In several respects, flies appear to have a stronger attraction to ethanol that that measured in most rodent assays. First, naive flies exhibit preference for 15% ethanol, while most rodent strains do not display naive preference for ethanol concentrations.
near 15% (Belknap et al., 1993; Veale and Myers, 1969). Second, flies exhibit increasing preference with increasing ethanol concentrations up to 25% (the highest concentration tested), while even high-drinking rodent lines typically consume decreasing ethanol volumes as concentration increases within this range (Rhodes et al., 2005; Lobina et al., 1997). These disparities may be partly explained by a difference in protocols: flies in our assay drink ethanol mixed with food, while rodent studies typically measure consumption of ethanol diluted in water. However, it is also likely that flies have evolved an intrinsically stronger attraction to ethanol than mammals, given that ethanol-containing fermenting plant materials are a major component of their natural diet.

A robust feature of ethanol preference in flies is its change over time. Initially, preference is low and variable, but over several days it becomes high and more consistent. These changes may reflect that flies require time to associate the pharmacological effect of ethanol with the ethanol-containing capillaries, or to reliably discriminate between the two types of capillaries. It is an open question how this discrimination is achieved: flies may utilize the olfactory and gustatory properties intrinsic to the solutions, or they may instead identify the capillaries by their location relative to other subtle cues or even leave their own cues. However, the normal ethanol preference of all 4 long-term memory mutants we tested suggests that long-term memory may not be required for either the display of ethanol preference or the increase in preference over time.

Flies display conflicting responses to the chemosensory properties of ethanol: they are attracted to its smell and averse to its taste. Other studies have reported examples of a single compound possessing both attractive and aversive qualities mediated by distinct sensory systems, such as acetic acid (Joseph et al., 2009) and carbon dioxide (Suh et al., 2004; Fischler et al., 2007). Our finding that gustatory aversion to ethanol does not actively inhibit ethanol preference fits with rodent studies.
demonstrating a dissociation between naive taste response to ethanol and ethanol consumption (Kiefer and Dopp, 1989). We speculate that olfactory attraction may be the dominating sensory input, or else flies may quickly overcome taste aversion during the preference assay.

No animal model will ever be a perfect model for alcoholism, since it is a human phenomenon comprised of social, cultural, and cognitive factors. However, animal paradigms can model particular facets of addiction, which is what we have established here for Drosophila. Although we do not claim that ethanol preference in flies and mammals are identical phenomena, our paradigm will be useful for identifying molecular mechanisms involved in ethanol consumption, which can then be tested in mammalian models. In addition to its relevance to addiction, because drugs of abuse act through neural pathways for natural rewards such as food and sex (Hyman et al., 2006), studying ethanol preference will also contribute to our understanding of general reward pathways in flies.

**Experimental Procedures**

Details of the ethanol preference assay are described here. All other methods, including statistical analyses, are described in Supplemental Information.

Our CAFE apparatus consisted of a plastic fly vial with small holes for air exchange and an opaque paper cover to eliminate external distractions, capped with a damp cotton plug. Four 5 µl capillaries (VWR) were inserted into each plug via adaptors made of truncated pipette tips. Capillaries were filled by capillary action. A small mineral oil overlay was added to reduce evaporation, and evaporation was minimal (<5% of total consumption per capillary). Capillaries were measured and replaced daily. Preference assays were conducted at 25°C and 70% relative humidity. 8 flies were allocated into each vial by brief CO₂ anesthetization. For deprivation and ethanol-experienced quinine
experiments, flies were divided into control and experimental groups after 5 days of drinking in order to equalize baseline PIs prior to manipulation.

**Supplemental Information**

Supplemental Information includes 6 figures, 1 table, and Supplemental Experimental Procedures.

**Acknowledgments**

We thank Peter Cameron for advice on PER experiments, Reza Azanchi for technical assistance, Karla Kaun for advice on statistics, and Robert Messing and Patricia Janak for comments on the manuscript. We are grateful to members of the Heberlein laboratory for helpful discussions and input on improving this manuscript. This work was supported by an NSF predoctoral fellowship (A.V.D.) and grants from NIH/NIAAA (U.H.).

**References**


Figure 1. Ethanol Preference in Drosophila

(A) Schematic of the ethanol preference assay (not to scale). Flies choose between liquid food containing 0% or 15% ethanol. Each food type is presented in 2 capillaries to increase the food supply and decrease variability.

(B) Flies consumed a greater amount of 15% ethanol food than non-ethanol food in the preference assay (**p<.01, ***p<.001, two-way repeated measures ANOVA with Bonferroni post-tests, n=16).

(C) PI calculated from consumption values (see text for formula). PI increased over time (p<.01, one-way repeated measures ANOVA, n=16).

(D) The concentration of the ethanol-containing food was varied between 5% and 25% ethanol, and PI values on days 1 and 2 and days 4 and 5 were averaged to compare preference at the beginning and end of the assay. PI increased with increasing ethanol concentration at the end (p<.05) but not the beginning (p>.05) of the assay (one-way ANOVAs, n=16).

(E) Ethanol concentration in flies during the preference assay was higher than that of control flies that never consumed ethanol (*p<.05, Mann-Whitney test, n=3-5 samples).
(F) Ethanol concentrations in flies that were starved and then refed for 10 or 60 min in the preference assay were higher than those of control flies that were also starved/refed but not offered ethanol (*p<.05 compared with control, Mann-Whitney tests, n=3-12 samples).

In this and all other figures, data are represented as mean ± SEM.
Figure 2. Olfactory Attraction and Gustatory Aversion Differentially Influence Ethanol Preference

(A) Flies lacking the third antennal segment had decreased ethanol preference compared with control flies (***p<.001, two-way repeated measures ANOVA with Bonferroni post-tests, n=24).

(B) Wild-type flies exhibited positive preference for ethanol in the olfactory trap assay, while whir mutants exhibited olfactory repulsion (**p<.01 for whir vs. control, Student’s unpaired t test, n=12).

(C) whir mutants exhibited positive ethanol preference. whir displayed a trend toward decreased preference compared with the control (p=.06, two-way repeated measures ANOVA, n=24).

(D) Ethanol diluted in water did not elicit significant PER (p>.05 for all concentrations). 100 mM sucrose was used as a positive control and elicited significant PER (**p<.01, one sample t tests, n=3 experiments).
(E) When added to 100 mM sucrose, ethanol caused a dose-dependent decrease in PER frequency (p<.001, one-way repeated measures ANOVA, n=3 experiments).

(F) poxn$^{\text{M22-B5}}$ and poxn$^{70-23}$ mutants exhibited ethanol preference similar to the control (p>.05, two-way repeated measures ANOVA, n=16).
Figure 3. Ethanol Preference in Flies Exhibits Features of Addiction

(A) Over time, naive flies developed ethanol preference when 300 µM quinine was added to the ethanol food throughout the assay. These flies had no preference on days 1-3 (p>.05), but had a positive preference on days 4 (p<.001) and 5 (p<.01). In the absence of ethanol, flies exhibited quinine aversion (p<.05 on all days, one sample t tests, n=16).

(B) Flies that had been drinking in the preference assay for 5 days continued to exhibit ethanol preference when 300 µM quinine was added to the ethanol food on the sixth day (p<.01, one sample t test, n=16), though this preference was decreased compared with controls lacking quinine. All 3 groups are significantly different from each other (***p<.001, one-way ANOVA with Tukey's post-test, n=16).

(C) After 5 days of drinking, flies were divided into two groups, one of which was deprived of ethanol access for two intermittent 1 day intervals (shaded). PI of the
deprived group differed from the non-deprived group only during the deprivation periods (***p<.001). Post-deprivation PI did not differ from pre-deprivation PI (p>.05 for day 7 vs. day 5 and day 9 vs. day 7) or from the non-deprived group (p>.05 for day 7 and day 9).

(D) Same as (C) using a single 3 day deprivation. PI of the deprived group differed from the non-deprived group only during deprivation (*p<.05, ***p<.001). Post-deprivation PI did not differ from pre-deprivation PI or from the non-deprived group (p>.05).

In (C) and (D), one- or two-way repeated measures ANOVAs with Bonferroni’s post-tests were used to compare values within the deprived group or between deprived and non-deprived groups, respectively. n=20 in (C) and n=10 in (D).
Figure 4. *kra* Exhibits Defects in Ethanol Preference

(A) *kra* displayed decreased ethanol preference compared with the control (p<.001, two-way repeated measures ANOVA), which was most pronounced at the beginning of the assay (*p<.05, **p<.01, Bonferroni’s post-tests, n=25).

(B) The long-term memory mutants *drujok, laska, chingis khan*, and *martik* displayed ethanol preference similar to the control (p>.05, two-way repeated measures ANOVA, n=22).
Table 1. Caloric Ratio Between Ethanol and Non-Ethanol Food Does Not Influence Ethanol Preference

In different experiments, the concentration of sucrose and yeast extract was varied between 1% and 8%, while the ethanol concentration of ethanol food was always 10%. Consequently, the caloric ratio between ethanol and non-ethanol food ranged from 2.2 to 10.9. This variation did not affect ethanol preference (p>.05, one-way ANOVA, n=24).

<table>
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<tr>
<th>Sucrose/Yeast Extract Conc., %</th>
<th>Non-Ethanol Food Calories, kcal/L</th>
<th>10% Ethanol Food Calories, kcal/L</th>
<th>Caloric Ratio Ethanol/Non-Ethanol Food</th>
<th>PI (±SEM)</th>
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<td>1</td>
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<td>3</td>
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<tr>
<td>5</td>
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<td>8</td>
<td>446</td>
<td>998</td>
<td>2.2</td>
<td>.11 (±.05)</td>
</tr>
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</table>
Figure S1. Ethanol Preference Becomes More Robust Over Time

(A) Variability in ethanol preference, quantified by the standard deviation of PI, decreased over time (p<.01, one-way repeated measures ANOVA, n=3 experiments with 20 vials each).

(B) Flies displayed positive, though highly variable, ethanol preference in the first 8 hrs (n=40). For this experiment, flies were habituated to the CAFE vial for 1 day prior to the preference assay, and 12 flies/vial were used.

(C) Ethanol preference initially increased over time but stabilized after 4-5 days (n=20).
Figure S2. Ethanol Preference Occurs by Increased Frequency, but not Duration, of Drinking Bouts

(A) Frequency of drinking from ethanol food compared with non-ethanol food was significantly higher on day 4 (*p<.05) but not day 1 (p>.05, Student’s paired t tests, n=12).

(B) The duration of each drinking bout from ethanol food was lower than that from non-ethanol food on both day 1 (*p<.05) and day 4 (**p<.01, Student’s paired t tests, n=12). The median bout duration for each vial was averaged to obtain an overall mean duration for all vials.
Figure S3. Flies Lacking the Third Antennal Segment Do Not Have Major Deficits in Feeding

Total consumption (non-ethanol food + ethanol food) during the preference assay of flies lacking the third antennal segment. Antennectomized flies exhibited slightly decreased total consumption compared with controls (p<.01, two-way repeated measures ANOVA, n=24), but on average their consumption was only 8% less than control values, indicating a relatively normal feeding rate.
Figure S4. Ablation of Antennal ORNs by Ethanol Vapor Decreases Ethanol Preference

Flies with antennal ORNs ablated by ethanol exposure displayed decreased preference compared with control flies that were similarly exposed but had normal antennae (p<.01, two-way repeated measures ANOVA, n=16).
Figure S5. Ethanol Preference Increases at a Greater Rate After Ethanol Deprivation Than in Naive Flies

(A) After each 1 day ethanol deprivation shown in Figure 3C, PI increased at a greater rate than in naive flies (**p<.01, one-way repeated measures ANOVA with Dunnett’s post-test, n=20). Rate of PI increase was calculated as the slope of a linear regression for PI vs. day, using values for days 1-5 (pre-deprivation), days 6-7 (post-deprivation 1), or days 8-9 (post-deprivation 2).

(B) After the 3 day ethanol deprivation shown in Figure 3D, PI increased at a greater rate than in naive flies (*p<.05, Student’s paired t test, n=10). Rate of PI increase was calculated as described above.
Figure S6. *kra* Exhibits Decreased Ethanol Sensitivity and Tolerance

(A) Sensitivity to ethanol-induced sedation was quantified as the time required to sedate 50% of flies during ethanol exposure (ST50). *kra* was less sensitive to ethanol sedation as revealed by its higher ST50 compared with the control (**p<.01, Student’s paired t test, n=9).

(B-C) *kra* exhibited decreased rapid (B) and chronic (C) tolerance compared with the control (*p<.05, Student’s paired t tests, n=9-10). See Supplemental Experimental Procedures for details on the assays.
<p>| Table S1. Summary of Statistics: Summary of all statistical results from this study. |
|---|---|---|---|---|---|
| Experiment | Figure | Statistical test | Test Results | Post-tests | Post-test Results |
| Two-Choice Consumption | 1B | 2-way repeated measures ANOVA | Day: F(4,120)=3.88, p&lt;.01 Food type: F(1,120)=96.75, p&lt;.001 Interaction: F(4,120)=11.26, p&lt;.001 | Bonferroni | Effect of food type: p&lt;.01 on day 1; &lt;.001 on days 2-5 |
| Preference Index | 1C | 1-way repeated measures ANOVA | F(4,60)=5.19, p&lt;.01 | Tukey | p&lt;.05 for day 1 vs. day 4; &lt;.001 for day 1 vs. day 5; &gt;.05 for all other comparisons |
| Preference Index Variability | S1A | 1-way repeated measures ANOVA | F(4,8)=13.05, p&lt;.01 | Tukey | p&lt;.01 for day 1 vs. day 3, day 1 vs. day 4, day 1 vs. day 5; &gt;.05 for all other comparisons |
| Drinking Bout Frequency | S2A | Student's paired t tests | For day 1: t(11)=1.92, p&gt;.05 For day 4: t(11)=2.64, p&lt;.05 | | |
| Drinking Bout Duration | S2B | Student's paired t tests | For day 1: t(11)=2.30, p&lt;.05 For day 4: t(11)=3.52, p&lt;.01 | | |
| Dose Response | 1D | 1-way ANOVAs | For days 1-2: F(4,75)=2.12, p&gt;.05 For days 4-5: F(4,75)=3.54, p&lt;.05 | Tukey | For days 4-5: p&lt;.05 for 5% vs. 25%; &gt;.05 for all other comparisons |
| Ethanol Levels | 1E, F | Mann-Whitney | U=0.00, p&lt;.05 (non-starved vs. ctrl) U=0.00, p&lt;.05 (each starved group vs. ctrl) | | |
| Caloric Ratio | Table 1 | 1-way ANOVA | F(3,91)=0.30, p&gt;.05 | | |
| Antennec. Preference | 2A | 2-way repeated measures ANOVA | Day: F(4,184)=8.79, p&lt;.001 Group: F(1,184)=29.11, p&lt;.001 Interaction: F(4,184)=2.05, p&gt;.05 | Bonferroni | Effect of group: p&lt;.001 on days 2,4, and 5; &gt;.05 on all other days |
| Antennec. Total Consumption | S3 | 2-way repeated measures ANOVA | Day: F(4,184)=84.47, p&lt;.001 Group: F(1,184)=8.03, p&lt;.01 Interaction: F(4,184)=1.41, p&gt;.05 | Bonferroni | Effect of group: p&lt;.01 on day 1; &gt;.05 on day 4; &gt;.05 on all other days |
| ORNs Ablated | S4 | 2-way repeated measures ANOVA | Day: F(4,120)=8.47, p&lt;.001 Group: F(1,120)=11.03, p&lt;.01 Interaction: F(4,120)=0.46, p&gt;.05 | Bonferroni | Effect of group: &gt;.05 on all days |
| Olfactory Trap | 2B | Student's unpaired t test | t(22)=5.16, p&lt;.001 | | |
| whir Preference | 2C | 2-way repeated measures ANOVA | Day: F(4,184)=14.68, p&lt;.001 Genotype: F(1,184)=3.65, p&gt;.05 Interaction: F(4,184)=0.56, p&gt;.05 | | |
| PER (in water) | 2D | One sample t tests | 100 mM sucrose: t(2)=11.89, p&lt;.01 0.1% EtOH: t(2)=1.95, p&gt;.05 1% EtOH: t(2)=2.28, p&gt;.05 5% EtOH: t(2)=2.30, p&gt;.05 10% EtOH: t(2)=1.57, p&gt;.05 15% EtOH: t(2)=1.00, p&gt;.05 40% EtOH: t(2)=1.00, p&gt;.05 | | |
| PER (in sucrose) | 2E | 1-way repeated measures ANOVA | F(6,12)=18.60, p&lt;.001 | Tukey | p&lt;.05 for 0% vs. 10%, 0.1% vs. 10%, 1% vs. 15%, 15% vs. 40%; &gt;.01 for 0% vs. 15%, 0.1% vs. 15%, 10% vs. 40%; &gt;.01 for 0% vs. 40%, 0.1% vs. 40%, 1% vs. 40%, 5% vs. 40%; &gt;.05 for all other comparisons |
| poxn Preference | 2F | 2-way repeated measures ANOVA | Day: F(4,180)=9.59, p&lt;.001 Genotype: F(2,180)=0.26, p&gt;.05 Interaction: F(6,180)=0.82, p&gt;.05 | | |</p>
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Figure</th>
<th>Statistical test</th>
<th>Test Results</th>
<th>Post-tests</th>
<th>Post-test Results</th>
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<tr>
<td>Quinine (Naive)</td>
<td>3A</td>
<td>1 sample t tests (EtOH + quinine group)</td>
<td>Day 1: ( t(15)=1.88, p&gt;0.05 ) Day 2: ( t(15)=0.42, p&gt;0.05 ) Day 3: ( t(15)=1.09, p&gt;0.05 ) Day 4: ( t(15)=7.02, p&lt;0.001 ) Day 5: ( t(15)=3.94, p&lt;0.01 )</td>
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<td>Quinine (Naive)</td>
<td>3A</td>
<td>1 sample t tests (Quinine ctrl group)</td>
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<td>Quinine ctrl vs. EtOH ctrl: ( p&lt;0.01 ) on all days; Quinine ctrl vs. EtOH + quinine: ( p&lt;0.01 ) on days 4 and 5, ( p&gt;0.05 ) on all other days; EtOH ctrl vs. EtOH + quinine: ( p&lt;0.01 ) on days 1 and 3, ( p&gt;0.01 ) on day 5, ( p&lt;0.05 ) on days 2 and 4</td>
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<td>Quinine (Naive)</td>
<td>3A</td>
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<td>Day: ( F(4,180)=14.91, p&lt;0.001 ) Group: ( F(2,180)=105.24, p&lt;0.001 ) Interaction: ( F(8,180)=1.99, p&gt;0.05 )</td>
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<td>Quinine (Ethanol-Experienced)</td>
<td>3B</td>
<td>1 sample t tests</td>
<td>Quinine ctrl: ( t(15)=4.65, p&lt;0.001 ) EtOH ctrl: ( t(15)=13.92, p&lt;0.001 ) EtOH + quinine: ( t(15)=3.75, p&lt;0.01 )</td>
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<td>1d Deprivation</td>
<td>3C</td>
<td>2-way repeated measures ANOVA</td>
<td>Day: ( F(8,304)=26.83, p&lt;0.001 ) Group: ( F(1,304)=8.17, p&lt;0.01 ) Interaction: ( F(8,304)=8.00, p&lt;0.001 )</td>
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<td>( F(8,152)=20.87, p&lt;0.001 )</td>
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<td>( p&gt;0.05 ) for day 5 vs. day 7 and day 7 vs. day 9; other days not tested</td>
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<td>3D</td>
<td>2-way repeated measures ANOVA</td>
<td>Day: ( F(8,144)=10.70, p&lt;0.001 ) Group: ( F(1,144)=16.07, p&lt;0.001 ) Interaction: ( F(8,144)=6.49, p&lt;0.001 )</td>
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<td>( p&gt;0.05 ) for day 5 vs. day 9; other days not tested</td>
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<td>1d Deprivation (Slope)</td>
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<td>( p&lt;0.01 ) for each post-deprivation group vs. ctrl</td>
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<td>3d Deprivation (Slope)</td>
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<td>4A</td>
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<td>Bonferroni</td>
<td>Effect of genotype: ( p&lt;0.01 ) on day 1; ( p&lt;0.05 ) on day 2; ( p&gt;0.05 ) on all other days</td>
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<td>( t(9)=2.61, p&lt;0.05 )</td>
<td></td>
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<td>Memory Mutants</td>
<td>4B</td>
<td>2-way repeated measures ANOVA</td>
<td>Time: ( F(1,104)=58.59, p&lt;0.001 ) Genotype: ( F(4,104)=1.94, p&gt;0.05 ) Interaction: ( F(4,104)=0.14, p&gt;0.05 )</td>
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Supplemental Experimental Procedures

Fly Stocks and Media

All flies tested were in the w Berlin background. Mutants used in this study included whir^{3} (Rothenfluh et al., 2006), poxn^{70-23} (Awasaki and Kimura, 1997), and poxn^{M22-B5} (Boll and Noll, 2002); alleles of kra and the other memory mutants are from Dubnau et al (2003). Flies were reared at 25°C and 70% relative humidity on standard cornmeal/molasses food in a 12/12 hr light/dark cycle. Flies were collected on the day of eclosion by anesthetization with CO_{2}. Behavioral experiments were performed on 3-4 day old males unless otherwise specified.

The food used in ethanol preference experiments consisted of 5% sucrose/5% yeast extract (w/v) containing either 0% or 15% ethanol (v/v). Food was autoclaved prior to adding ethanol except in caloric ratio experiments (to ensure that caloric values were not altered). Caloric values were calculated based on the following values: 4 kcal/g (sucrose), 1.58 kcal/g (yeast extract), and 7 kcal/g (ethanol).

Surgeries and Treatments

Antennectomies were performed using forceps 1 day before testing. Ethanol exposures to kill antennal ORNs consisted of four 45 min exposures over 2 days. Flies were exposed to an ethanol/air mixture of 100/50 ethanol/air (E/A) in perforated Eppendorf tubes. Flies were sorted based on antennal appearance 2 days after exposure, and testing began on the same day.

Ethanol Concentration Assay

Flies were frozen in liquid nitrogen and homogenized in 50 mM Tris-HCl (pH 7.5, 50 µL for 10 flies). Ethanol concentrations were measured on fly homogenates using the Ethanol Assay kit from Diagnostic Chemicals Limited (Charlottetown, PE, Canada). To
calculate the ethanol concentration in flies, the volume of one fly was estimated to be 2 µL.

Behavioral Assays

Filming of Ethanol Preference

Filming was conducted for ~10 hrs during the light phase of day 1 and day 4 of the assay. Drinking bouts were manually scored by observing the position of the fly's body and proboscis relative to the capillary. This scoring method led to some ambiguity between flies that were actively drinking vs. flies that were in contact with the capillary but not ingesting food. However, results were repeated in 2 independent experiments; additionally, half the vials were scored blind and their results did not differ from the other vials.

Olfactory Trap Assay

Olfactory traps consisted of two truncated 1.6 mL Eppendorf tubes with truncated p200 pipette tips inserted into the bottom to form a funnel for flies to enter. 1% agar containing 0% or 15% ethanol was added to the Eppendorf lids, and traps were placed in 100x20mm Petri dishes. 30 flies were placed in each dish for ~42 hrs in constant dark. Preference index was calculated as (# flies in ethanol trap - # flies in control trap) / total # flies in both traps.

PER Assay

1 day old flies were starved with water for 24 hrs, then immobilized on slides with myristic acid for 2 hrs before testing. Each test consisted of 2 trials in which the solution was applied to the fly's legs, and proboscis extension in at least one trial was considered a response. Individual flies were tested in only one of the two experiments conducted (ethanol in water or ethanol in sucrose), and for the given experiment each fly was tested with all concentrations of ethanol in increasing order. Flies were water-satiated
before each test. 100 mM sucrose controls were tested at the beginning and end of
testing for each fly, and the two values were averaged to obtain average sucrose PER.
Flies that did not respond to sucrose at any time during the assay were not included in
analysis. n=3 groups for each experiment, with each group consisting of at least 17 flies
(≥20 for most groups).

**Ethanol Sensitivity and Tolerance Assays**

Ethanol-induced sedation was assayed and quantified as described previously
[9] using 100/50 E/A. To assay rapid tolerance, two separate sedation assays were
conducted 4 hrs apart, with a first exposure of 30 min and a second exposure of 45 min.
Rapid tolerance was quantified as $ST50_{2nd\ exposure} - ST50_{1st\ exposure}$. To assay chronic
tolerance, flies were exposed as described [23] for 24 hrs to 15/75 E/A or air alone, and
sedation assays were conducted immediately after exposure. Chronic tolerance was
quantified as $ST50_{ethanol-exposed} - ST50_{air-exposed}$.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism, Version 4. Statistical
significance was established using Student's t-tests or 1- or 2-way ANOVAs followed by
the appropriate post-test (Bonferroni's, Tukey's, or Dunnett's). In rare cases of small and
unequal sample sizes, the non-parametric Mann-Whitney test was used. All statistical
results in this study are summarized in Table S1. All graphs represent mean ± SEM.
Unless otherwise specified, $n$ refers to the number of vials.

**Supplemental References**

behavior and fertility as revealed by a complete dissection of all enhancers.
Development 129, 5667-5681.
CHAPTER 3

The Genetic Relationships Between Ethanol Preference, Acute Ethanol Sensitivity, and Ethanol Tolerance in *Drosophila melanogaster*

ABSTRACT

The relationship between alcohol consumption, sensitivity, and tolerance is an important question that has been addressed in humans and rodent models. Studies have shown that alcohol consumption and risk of abuse may correlate with (1) increased sensitivity to the stimulant effects of alcohol, (2) decreased sensitivity to the depressant effects of alcohol, and (3) increased alcohol tolerance. However, many conflicting results have been observed. To complement these studies, we utilized a different organism and approach to analyze the relationship between ethanol consumption and other ethanol responses. Using a set of 20 *Drosophila melanogaster* mutants that were isolated for altered ethanol sensitivity, we measured ethanol-induced hyperactivity, ethanol sedation, sedation tolerance, and ethanol consumption preference. Ethanol preference showed a strong positive correlation with ethanol tolerance, consistent with some rodent and human studies, but not with ethanol hyperactivity or sedation. No pairwise correlations were observed between ethanol hyperactivity, sedation, and tolerance. The evolutionary conservation of the relationship between tolerance and ethanol consumption in flies, rodents, and humans indicates that there are fundamental biological mechanisms linking specific ethanol responses.

INTRODUCTION

Ethanol elicits diverse behavioral responses in humans as well as animal models. Acute responses to ethanol include both stimulant and depressant effects,
which in humans are typically perceived as positive and negative, respectively (Babor et al., 1983). Ethanol tolerance is defined as a decrease in any given ethanol response after previous exposure, and can develop both within and between intoxicating sessions (Kalant, 1996). Despite the large body of work on ethanol sensitivity and tolerance, an important question remains unresolved: to what extent do ethanol sensitivity and tolerance influence ethanol consumption and abuse? Understanding the relationship between these behaviors is essential for both medical and scientific reasons. These relationships may aid in predicting which individuals are at risk for alcohol use disorders (AUDs), allowing for early intervention. In addition, determining which ethanol responses are most closely associated with addictive behavior may guide future studies using animal models and highlight potential neural and molecular mechanisms underlying addiction.

Landmark studies by Schuckit and colleagues demonstrated that individuals with decreased sensitivity to the acute effects of alcohol (such as postural instability and subjective “high”) are at greater risk for developing an AUD (Schuckit, 1994; Schuckit and Smith, 1996; Trim et al., 2009). However, other studies have come to the opposite conclusion, associating risk for AUDs with increased alcohol sensitivity (Newlin and Thomson, 1990; Newlin and Renton, 2010). A potential explanation for these contradictory findings arises from the biphasic nature of alcohol intoxication. Newlin and Thomson proposed a “differentiator model”, in which individuals at risk for AUDs have increased sensitivity to the euphoric, stimulant effects of ethanol that occur earlier (as blood alcohol concentration [BAC] is rising) and decreased sensitivity to its negative, depressant effects that occur later (as BAC falls) (Newlin and Thomson, 1990; Martin et al., 1993; Babor et al., 1983). This altered balance of positive and negative effects would cause these individuals to find alcohol particularly rewarding. Several studies support
specific aspects of this model (Holdstock et al., 2000; King et al., 2002; Kaplan et al., 1988), but it remains to be tested rigorously (see Morean and Corbin, 2010 for review).

Animal studies have also addressed the relationship between ethanol sensitivity and addictive behaviors, focusing mainly on ethanol consumption preference (Crabbe et al., 2010). An association between low sensitivity to the sedative/hypnotic effects of ethanol and high ethanol preference has been observed in rodents selectively bred for high versus low ethanol preference (Kurtz et al., 1996; Froehlich and Wand, 1997) as well as in many mouse mutants (Thiele et al., 1998; Hodge et al., 1999). However, this correlation is by no means universal, as several exceptions have been reported (Colombo et al., 2000; Phillips et al., 1998; Boehm et al., 2004). In support of the differentiator model, some high-preferring lines show greater sensitivity to ethanol-induced locomotor stimulation than low-preferring lines (Waller et al., 1986; Agabio et al., 2001).

The influence of ethanol tolerance on ethanol consumption and addiction has not been extensively studied, despite the fact that tolerance is one of the DSM-IV criteria for alcohol dependence (American Psychiatric Association, 1994). Although tolerance often develops in non-alcoholics, it has been correlated with heavy drinking and alcohol abuse (Schuckit et al., 2008). Tolerance is thought to promote greater alcohol consumption by diminishing the aversive effects of alcohol as well as reducing its rewarding properties, which would require individuals to drink more alcohol to achieve the same positive effects (Kalant, 1996). Similar to studies of ethanol sensitivity, some studies of selectively bred rodent lines (Le and Kiiianmaa, 1988; Kurtz et al., 1996) and specific mutants (Bowers et al., 1999; Bowers and Wehner, 2001) point to a positive correlation between tolerance and alcohol preference, while other studies do not support this correlation (Grahame et al., 2000; Wallace et al., 2007).
In summary, we are still in the process of gaining a clear understanding of how alcohol sensitivity and tolerance contribute to alcohol consumption and AUDs. While the differentiator model may explain some of the conflicting results from human studies, some of the discrepant findings are likely to be due to the confounding factors inherent to human studies. First, cognitive and emotional factors influence ethanol intoxication and consumption in humans. Second, the previous alcohol experience of subjects cannot be precisely controlled. Third, ethanol consumption in a laboratory study is dissimilar from real-life drinking. On the other hand, studies that ask subjects to recall real-life drinking experiences are susceptible to inaccurate or biased recollections. Rodent studies also have limitations. First, lines that have been selectively bred for high versus low alcohol preference differ in many other behavioral and neurobiological measures, including anxiety, novelty-seeking, and startle responses (Murphy et al., 2002), which may produce confounding biases during assays of ethanol sensitivity and tolerance. Second, correlation studies that examine different behavioral responses in the same animals (e.g. Chappell and Weiner, 2008) face the problem that previous exposure to alcohol alters subsequent alcohol responses (i.e. naive responses cannot be obtained for more than one behavior). Finally, it is difficult to make correlations from studies of mutant mice because different mutants have been generated and tested in different laboratories, often using different protocols and genetic backgrounds (Crabbe et al., 2006).

To circumvent many of these limitations and complement the valuable studies that have been performed in humans and rodents, we have used a different organism and approach to examine the relationships between ethanol sensitivity, tolerance, and consumption preference. The fruit fly *Drosophila melanogaster* is an established model for studying acute and chronic responses to ethanol. Flies exhibit acute ethanol responses similar to those of mammals: as ethanol concentration increases, flies exhibit
locomotor stimulation (Wolf et al., 2002), loss of postural control (Moore et al., 1998), and eventually sedation (Rothenfluh et al., 2006; Corl et al., 2009). With repeated exposure, flies develop tolerance to the motor-impairing and sedating effects of ethanol (Scholz et al., 2000; Urizar et al., 2007). Flies also have an innate preference for consuming ethanol-containing solutions (Devineni and Heberlein, 2009). Furthermore, they exhibit several features of alcohol addiction, such as an increase in ethanol consumption over time, willingness to overcome an aversive stimulus in order to consume ethanol, and relapse-like behavior (Devineni and Heberlein, 2009).

We set out to test whether ethanol sensitivity, tolerance, and consumption preference are correlated in Drosophila. We utilized a set of mutants that had been previously isolated for altered ethanol responses. Our ability to test a relatively large number of mutants in the same genetic background under the same experimental conditions affords an ideal opportunity to observe meaningful correlations between ethanol-related behaviors. Based on the findings from human and rodent studies, we hypothesized that ethanol consumption preference would be positively correlated with sensitivity to the stimulant effects of ethanol and resistance to the sedative effects of ethanol. We also predicted that ethanol tolerance and preference would be positively correlated. We found that ethanol preference was indeed positively correlated with tolerance but was uncorrelated with other ethanol responses, suggesting that there are shared genetic mechanisms underlying ethanol tolerance and preference that have been conserved from flies to humans.

RESULTS

To examine the relationships between ethanol sensitivity, tolerance, and consumption, we measured these responses in 20 Drosophila mutants that we isolated for altered ethanol sensitivity (Table 1), some of which have been published (Rothenfluh
et al., 2006; Wolf et al., 2007; Corl et al., 2009; LaFerriere et al., 2008; Eddison et al., 2011). All 20 mutants contain P element insertions, and the genes located nearest to the insertion sites are listed in Table 1. These are the genes that are most likely to be responsible for the behavioral phenotypes of the mutants, though functional studies have not been conducted to confirm their role (aside from the studies listed in Table 1). We chose to test a panel of ethanol sensitivity mutants rather than wild-type strains or random mutants to ensure that a wide spectrum of ethanol responses would be represented. In the 20 mutants we assessed four different behavioral responses to ethanol: (1) consumption preference, (2) locomotor stimulation, (3) sedation, and (4) sedation tolerance.

Ethanol Consumption Preference

Flies preferentially consume ethanol-containing food over non-ethanol food in a continuous access two-choice feeding assay (Devineni and Heberlein, 2009). Ethanol preference is relatively low and variable during the first one to two days, but subsequently increases and becomes more stable (Devineni and Heberlein, 2009). We measured preference for 15% ethanol over 3 days. Because we expected that preference would be variable on days 1 and 2, we planned to primarily use the preference value on day 3 for correlation analyses. Preference was quantified by calculating a preference index (PI) defined as (volume of ethanol food consumption – volume of non-ethanol food consumption) / total consumption. PI can vary between -1 and +1, with positive values indicating preference. Control flies had a PI of 0.06 ± 0.03 on day 1 which increased to 0.26 ± 0.03 on day 3 (Fig. 1A). PI values on day 3 for the 20 mutants ranged from 0.11 to 0.31 (Fig. 1B). Three mutants (6-6, 10-110, 1514) showed PI values that were significantly different from the respective control tested in the same
experiment \((p<0.05, \text{t-test})\); one example is shown in Fig. 1A. Two additional mutants \((8-128, 8-222)\) showed a trend toward altered preference \((0.05<p<0.06, \text{t-test})\).

**Ethanol Hyperactivity**

Locomotor hyperactivity and sedation represent different aspects of ethanol sensitivity in flies. Hyperactivity occurs at moderate ethanol concentrations \((\text{Wolf et al., 2002})\) and represents a stimulant effect of ethanol, whereas sedation occurs at high ethanol concentrations \((\text{Rothenfluh et al., 2006})\) and represents a depressant effect of ethanol. We first measured ethanol hyperactivity in our set of mutants using a locomotor tracking system \((\text{Wolf et al., 2002})\). When exposed to a moderate concentration of ethanol vapor \((47\% \text{ ethanol vapor in humidified air})\), flies exhibit a gradual increase in activity as their internal ethanol concentration increases \((\text{Wolf et al., 2002}; \text{Fig. 2A})\). Maximum ethanol hyperactivity was quantified as the average of the 3 consecutive time points showing the highest locomotor speed during the 20 min assay, a measure that has been previously used \((\text{Rothenfluh et al., 2006})\). Control flies had a maximum hyperactivity of \(5.27 \pm 0.14 \text{ mm/sec}\), and hyperactivity values for the 20 mutants ranged from \(1.78\) to \(7.55 \text{ mm/sec}\) \((\text{Fig. 2B})\). Tracking profiles of a mutant exhibiting increased hyperactivity \((9-91)\) and one exhibiting decreased hyperactivity \((8-222)\) are shown in Fig. 2A \((p<0.001, \text{t-tests})\).

Ethanol hyperactivity shows a U-shaped relationship with ethanol concentration: hyperactivity initially increases as ethanol concentration increases, but decreases at high ethanol concentrations as flies become sedated \((\text{Wolf et al., 2002})\). We wondered whether mutants that showed altered hyperactivity at \(47\% \text{ ethanol}\) would also exhibit the same phenotypes at a lower concentration, given that some of those mutants may also have altered sedation sensitivity \((\text{see Fig. 3})\). Using a lower concentration of \(33\% \text{ ethanol}\), we retested the 11 mutants that showed significantly different maximum
hyperactivity from the respective control tested on the same day (p<0.05, t-test). 6 of the 11 mutants exhibited the same phenotype at both ethanol concentrations (data not shown; p<0.05, t-test), including lines 8-222 and 9-91 shown in Fig. 2A. Of the other 5 mutants, 3 mutants showed no significant phenotype at 33% ethanol (2-67, 9-220, 17-3; though 17-3 showed a trend in the same direction), and 2 mutants showed the opposite phenotype (4-12, 14-45). The majority of hyperactivity mutants therefore showed the same phenotype at multiple concentrations, although there were exceptions.

_Ethanol Sedation_

We next measured sensitivity to ethanol sedation in the 20 mutants by testing naive flies in a loss of righting reflex assay (Rothenfluh et al., 2006; Corl et al., 2009). We counted the number of non-sedated flies at 5 min intervals during exposure to a high concentration of ethanol vapor (67% ethanol; Fig. 3A) and calculated the time required for 50% of flies to sedate (ST50). Control flies had an ST50 of 20.8 ± 0.9 min. ST50 values for all mutants are shown in Fig. 3B and ranged from 11.3 to 61.7 min. Sedation curves for a mutant showing increased sedation sensitivity (5-10) and one showing decreased sensitivity (2-67) as compared with the control are shown in Fig. 3A (p<0.001 for each mutant vs. control ST50, t-tests).

_Ethanol Tolerance_

Tolerance to the sedating effects of ethanol has been previously characterized in flies (Scholz et al., 2000; Urizar et al., 2007). We measured sedation tolerance in the mutants by exposing naive flies to a sedating dose of ethanol (similar to the assays conducted above) and then re-exposing the same flies 4 hours later. The decrease in sedation sensitivity during the second exposure as compared to the first exposure reflects the development of tolerance. The duration of the first exposure was set at 30
min in order to avoid lethality that can occur with longer exposures. Even though we used a higher ethanol concentration (73% ethanol vapor) than had been used for the sedation assays described above, several vials of many genotypes failed to reach 50% sedation during the first and/or second exposure. We therefore quantified sedation in this assay as the time required for 25% of flies to sedate (ST25), and tolerance was calculated as the increase in ST25 from the first to the second exposure. Tolerance values calculated from ST25 values were very similar to those calculated from ST50 values (data not shown). Two mutants (4-12 and 20-29) consistently failed to reach even 25% sedation during the first and/or second exposure. This prevented us from calculating their ST25 and tolerance values, so they were excluded from the analysis.

Control flies had an average sedation tolerance of 13.5 ± 1.0 min, indicating that flies took an average of 13.5 minutes longer to sedate during the second exposure as compared to the first (Fig. 4A and 4B). Tolerance values for all mutants tested are shown in Fig. 4B and ranged from 4.9 to 19.9 min. Sedation curves for a mutant showing decreased tolerance (5-21) and one showing increased tolerance (2-10) relative to the control are shown in Fig. 4A. Both 5-21 and 2-10 exhibited initial sedation sensitivity similar to the control (p>0.05 for each mutant vs. control ST25, t-tests). However, during the second exposure 5-21 showed increased sensitivity relative to the control, indicating decreased tolerance, while 2-10 showed decreased sensitivity relative to the control, indicating increased tolerance (p<0.01 for each mutant vs. control ST25, t-tests).

**Ethanol Pharmacokinetics**

Since our panel of mutants displayed a wide range of ethanol responses in all four behavioral assays, we tested whether the mutants might also vary in their ethanol pharmacokinetics. We measured the internal ethanol concentration of flies that had been exposed for 15 min to a moderate concentration of 47% ethanol vapor. Control flies
contained 24.4 ± 1.6 mM ethanol (Fig. 5). The ethanol concentration in the mutants ranged from 19.3 to 38.4 mM ethanol (Fig. 5), indicating that altered ethanol absorption or metabolism may underlie the behavioral phenotypes in some of these lines.

**Correlations Between Ethanol Sensitivity, Tolerance, and Consumption Preference**

After measuring ethanol preference, hyperactivity, sedation, and tolerance in the 20 mutants, we asked whether behavioral responses in different assays were correlated. We were most interested in determining which behaviors might correlate with ethanol preference. We found that ethanol preference on day 3 of the assay correlated positively with ethanol tolerance ($r=0.664$, $p<0.01$, Pearson’s correlation; Fig. 6A). In addition, tolerance correlated slightly more strongly with the average ethanol preference across all 3 days ($r=0.707$, $p=0.001$, Pearson’s correlation; Fig. 6B).

We found no significant correlations between ethanol preference and either ethanol hyperactivity, sedation, or internal ethanol concentration (Table 2). Furthermore, there were no significant correlations for any pairwise comparisons of ethanol hyperactivity, sedation, or internal ethanol concentration (Table 2). However, the correlation between ethanol concentration and both maximum hyperactivity ($r=-0.437$, $p=.054$) and sedation ST50 ($r=-0.429$, $p=.059$) were close to significance (Pearson’s correlation). These correlations would suggest that flies that achieve higher internal ethanol levels tend to exhibit decreased hyperactivity and increased sedation.

**GAL4 Expression Patterns in the Mutant Lines**

The 20 mutant lines tested in this study are enhancer traps, in which the transcriptional activator GAL4 is expressed in cells likely to express the endogenous gene affected by each P element. We wondered whether these expression patterns might be related to the diverse behavioral phenotypes exhibited by the mutants. We
visualized the GAL4 expression pattern in each mutant line by crossing the lines to a UAS-GFP reporter (Table 3), with the exception of lines 4-12 and 13-66 which will be described in separate publications.

Of the 18 lines analyzed, 15 showed GAL4 expression in the adult brain; the remaining lines may show expression in the adult ventral nerve cord or in the developing nervous system, which were not analyzed. Most lines showed GAL4 expression in multiple brain areas. The most common site of GAL4 expression in the adult brain was the subesophageal ganglion (SOG; 13/18 lines), mainly due to projections from cells in the pars intercerebralis (PI; 12/18 lines). To determine whether this high proportion of GAL4 expression in the PI and SOG was meaningful, we analyzed the number of random enhancer trap lines from the same collection expressing GAL4 in these regions. Of 50 random lines analyzed, 38% expressed GAL4 in the PI and 60% expressed GAL4 in the SOG. In comparison to the random lines, our ethanol sensitivity mutants showed a significant over-representation of GAL4 expression in the PI but not the SOG (p<0.05, chi-square test), suggesting that this region may be important in regulating ethanol responses. Subsets of mutant lines also expressed GAL4 in the antennal lobe (9 lines), optic lobe (7 lines), central complex (5 lines), and mushroom body (5 lines). There was no clear relationship between the expression pattern and behavioral phenotypes of the mutant lines, as different lines expressing in the same brain region did not appear more likely to share the same behavioral phenotypes.

**DISCUSSION**

Correlations between ethanol consumption and both sensitivity and tolerance to ethanol have been observed in human and rodent studies, though much of the data is conflicting. Because we have recently characterized ethanol consumption preference in Drosophila and shown that this paradigm models features of addiction-like behavior
(Devineni and Heberlein, 2009), we now had the opportunity to test whether the same relationships between ethanol consumption and other ethanol responses also exist in flies. In contrast to most rodent and human studies, we were able to test many Drosophila strains under the same conditions and in the same genetic background, representing a highly systematic and controlled approach.

We measured ethanol preference, hyperactivity, sedation, and sedation tolerance in 20 mutants that had been identified as ethanol-sensitive or -resistant in various behavioral assays. Ethanol preference was the most variable behavior; this variability has been previously observed and likely reflects the complexity of ethanol preference as a choice assay in which flies must integrate chemosensory and experience-dependent information (Devineni and Heberlein, 2009). For ethanol hyperactivity, sedation, and tolerance, we identified some lines that showed an increased response and some with decreased response compared to the control. In contrast, we found mutants that had decreased ethanol consumption preference but none with increased preference.

While it was not our primary goal to find new genes modulating ethanol-induced behaviors, our use of P element mutants allowed us to easily identify the gene(s) likely to be affected in each mutant line. This lays the groundwork for future studies to confirm the role of these genes in regulating ethanol responses and uncover the molecular pathways in which they function. We have also characterized the GAL4 expression pattern in each mutant line, which represents the likely expression pattern of the affected gene, allowing future studies to more easily identify the neurons in which each gene functions. Interestingly, in our set of mutants there was an over-representation of lines expressing GAL4 in the PI, a region that contains neuropeptidergic cells, including insulin-producing cells. The PI has also been implicated in previous studies of ethanol sensitivity and may therefore represent an important locus for regulating ethanol responses (Rodan et al., 2002; Corl et al., 2005; Corl et al., 2009).
We observed a strong positive correlation between ethanol tolerance and ethanol preference. This correlation suggests that tolerance might be one reason why flies increase their ethanol consumption over time. The correlation between ethanol consumption and tolerance is consistent with human and rodent studies that have addressed this question (see Introduction). However, in humans there is also evidence that risk of AUD correlates with sensitivity to the stimulant effects of alcohol and resistance to its depressant effects (see Introduction). We did not find a correlation between ethanol consumption preference and either locomotor hyperactivity, the major stimulant effect of ethanol in flies, or sedation, the major depressant effect. These data indicate that ethanol preference is more strongly linked to tolerance, a form of ethanol-induced plasticity that develops over time, than to naive ethanol responses. In humans the relative importance of ethanol tolerance versus naive sensitivity in influencing ethanol consumption has not been determined within a single study; it will be interesting to see whether the same relationship holds.

We did not observe any pairwise correlations between ethanol sedation, hyperactivity, and tolerance. Berger et al. also did not observe a correlation between sedation and sedation tolerance in a set of 52 long-term memory mutants with widely varying ethanol sensitivities (Berger et al., 2008). Kong et al. did, however, observe correlations between sedation sensitivity and both sedation tolerance and hyperactivity, although the latter was fairly weak (Kong et al., 2010). These differences may be attributable to the different sets of mutants that were analyzed as well as the varying methods of quantifying behavior. In particular, Kong et al. conducted ethanol hyperactivity and sedation assays at the same ethanol concentration, which could account for the correlation they observed between these two behaviors, whereas we designed our experiments such that flies would not sedate during the hyperactivity assay. Kong et al. also conducted sedation assays at a lower concentration than we did.
and quantified both sedation and tolerance by the fraction of flies sedated at 26 minutes rather than the ST50. Finally, the strains analyzed by Kong et al. contained mutations in genes known to be transcriptionally regulated by ethanol, which represents a unique subset of strains different from our mutants which were chosen based on phenotype.

The lack of a strong correlation between internal ethanol concentration and any of the ethanol responses tested suggests that the primary effect of the mutants in this study is to alter the way that the nervous system reacts to ethanol rather than simply disrupting ethanol absorption or metabolism. In addition, the lack of correlation between ethanol hyperactivity, sedation, and tolerance supports the view that these assays measure distinct ways by which ethanol affects the nervous system. However, it is interesting that many mutants isolated for a phenotype in one of the assays also exhibited phenotypes in a different assay. For example, several mutants isolated for altered ethanol-induced sedation or loss of postural control (e.g. 2-10, 4-12, 10-110, 1514) also exhibited altered ethanol hyperactivity (4-12, 10-110) or tolerance (1514, 2-10). These results suggest that shared genetic pathways mediate different ethanol responses, even if the responses themselves are not correlated, which may be informative in identifying the molecular mechanisms underlying these behaviors.

In this study we measured only one of two types of tolerance characterized in flies: rapid tolerance, which is induced by a relatively brief ethanol exposure that causes intoxication (Berger et al., 2004). A second form of tolerance, chronic tolerance, is induced by prolonged (~24 hr) exposure to a low ethanol concentration that does not produce overt intoxication, and is mechanistically distinct from rapid tolerance (Berger et al., 2004). It remains to be seen whether chronic tolerance also correlates with ethanol preference in flies. Both rapid and chronic tolerance develop between discrete ethanol exposures; a third form of tolerance present in mammals, acute functional tolerance, develops within a single intoxicating session (Kalant et al., 1971). However, acute
functional tolerance has not yet been characterized in flies as it is difficult to distinguish from naive ethanol sensitivity using our current assays.

Our results indicate that shared genetic mechanisms underlie both ethanol tolerance and preference, but an open question is whether this relationship occurs at the mechanistic or behavioral level. Specifically, one possibility is that the neural or molecular pathways that are shared between ethanol tolerance and preference directly promote both of these behaviors in parallel. However, an alternative model (commonly applied to humans and rodents) posits that animals that develop greater tolerance choose to consume more ethanol because they require a greater internal ethanol concentration in order to achieve a certain desired level of behavioral intoxication. This model suggests that ethanol tolerance and preference share the same genetic mechanisms only because ethanol preference is directly modulated by tolerance. Regardless of the mechanism, the positive correlation between ethanol preference and tolerance appears to be shared by flies, rodents, and humans. Thus, there are fundamental, evolutionarily conserved biological mechanisms linking these ethanol-induced behaviors.

**MATERIALS AND METHODS**

*Fly Stocks and Maintenance*

Flies were reared at 25°C and 70% relative humidity on standard cornmeal/molasses food. Flies tested for ethanol consumption preference were kept in a 12/12 hr light/dark cycle since feeding behavior is strongly modulated by circadian rhythm. Flies tested in all other behavioral assays, which only measure short term responses, were kept in constant light in order to minimize the effect of circadian variation. All assays were performed on 3-4 day old males that were collected by anesthetization with CO₂. 19 of 20 mutants were obtained from the Heberlein Lab
P[GAL4] collection; mutant 1514 was obtained from the Japanese NP consortium (NP1514). Mutants were outcrossed for at least five generations to the w Berlin control strain to remove unlinked modifiers and homogenize the genetic background.

**Mutant Characterization**

The genomic DNA flanking the P elements was isolated by inverse PCR, allowing us to identify the insertion sites. GAL4 expression was imaged in dissected brains of GAL4/UAS-GFP adult males under a fluorescence microscope; any observable expression within a specified region (regardless of intensity) was noted in Table 3.

**Behavioral Assays**

Ethanol consumption preference was measured as described using 8 flies per vial (Devineni and Heberlein, 2009). Briefly, flies choose between liquid food containing 0% or 15% ethanol presented in 5 µL capillary tubes placed vertically through the top of their vials. The volumes consumed are determined daily by measuring the descent of each liquid column. Ethanol hyperactivity, sedation, and sedation tolerance were measured in the booz-o-mat, an 8 chambered apparatus in which flies are exposed to a specific concentration of ethanol vapor by mixing pure ethanol vapor with humidified air at a fixed ratio (Wolf et al., 2002). Ethanol hyperactivity was measured by video tracking of flies as described (Wolf et al., 2002) using an ethanol vapor concentration of 47% or 33% as specified. Maximum hyperactivity was calculated for each vial as the average of the 3 consecutive time points with the highest speed; these points varied from vial to vial. Ethanol sedation was assayed manually as described (Corl et al., 2009) using an ethanol concentration of 67% for general sedation assays and 73% for sedation tolerance assays. 20 flies per vial were used for ethanol hyperactivity, sedation, and tolerance assays.
General sedation assays were initially conducted using a 35 min ethanol exposure. Sedation sensitivity was quantified as the time required for 50% of flies to sedate (ST50). The ST50 was calculated by linear interpolation (or linear extrapolation if the last time point was close to 50%). Four mutants (20-29, 1514, 13-66, 4-12) did not reach 50% sedation by 35 min and were therefore retested in a 60 min (20-29, 1514, 13-66) or 90 min (4-12) assay using different flies.

Tolerance assays were conducted using a 30 min ethanol exposure followed by a 45 min ethanol exposure 4 hrs later. Because many vials did not reach ST50 during either the first or second exposure, we quantified sedation in this assay as the time required for 25% of flies to sedate (ST25), and tolerance was calculated as $\text{ST25}_{2\text{nd exposure}} - \text{ST25}_{1\text{st exposure}}$. For two mutants (4-12 and 20-29), most vials did not come close to reaching 25% sedation; these lines were excluded from analysis because their tolerance could not be calculated.

Every fly strain was tested in each behavioral assay on at least two separate days (n=18 for ethanol consumption preference, n=10 for ethanol hyperactivity, n=8-13 for sedation, and n=5-7 for tolerance). Unless otherwise specified, n refers to the number of vials. The control line used was w Berlin, the genetic background for all strains tested. Mean values for the control were obtained by averaging all experiments, but when comparing specific mutants to the control (Fig. 1A-4A) we only compared samples that were tested simultaneously.

**Measurement of Ethanol Concentration**

Flies were frozen in liquid nitrogen and homogenized in 50 mM Tris-HCl (pH 7.5, 200 µL for 20 flies). Ethanol concentrations were measured in fly homogenates using the Ethanol Assay kit from Diagnostic Chemicals Limited (catalog no. 229-29). To calculate
the ethanol concentration in flies, the volume of one fly was estimated to be 2 µL as previously reported (Moore et al., 1998).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism, Version 4. Statistical significance of mutant phenotypes was established using Student’s t-test. Pearson’s correlation was used for correlation analyses. All graphs represent mean ± SEM.

Acknowledgments

We thank Liqun Luo for some GAL4 expression images, Chris Kliethermes for advice on statistical analyses, and members of the Heberlein laboratory for comments on the manuscript. This work was supported by an NSF predoctoral fellowship (A.V.D.) and grants from NIH/NIAAA (U.H.).

References


Figure 1. Ethanol Consumption Preference of 20 Drosophila Ethanol Sensitivity Mutants

(A) Ethanol preference of the control strain and mutant 6-6 in a 3 day assay. Line 6-6 showed decreased preference compared with the control on day 3 (p<0.05, t-test, n=18).

(B) Ethanol PI on day 3 for each mutant tested (n=18 for mutants, n=36 for control).
Figure 2. Sensitivity to Ethanol-Induced Hyperactivity

(A) Exposure to a moderate concentration of 47% ethanol vapor causes locomotor hyperactivity in flies. Line 8-222 exhibited decreased maximum ethanol hyperactivity and line 9-91 exhibited increased maximum hyperactivity compared with the control ($p<0.001$, t-tests, $n=10$).

(B) Maximum ethanol-induced hyperactivity of each mutant ($n=10$ for mutants, $n=20$ for control).
Figure 3. Sensitivity to Ethanol-Induced Sedation in Naive Flies

(A) Flies exhibit sedation during exposure to a high concentration of ethanol vapor (67%). Line 2-67 exhibited decreased sedation sensitivity (p<0.001) and line 5-10 exhibited increased sedation sensitivity (p<0.001) compared with the control (t-tests comparing ST50 values, n=8-9).

(B) ST50 of each mutant (n=8-13 for mutants, n=16 for control).
Figure 4. Tolerance to Ethanol-Induced Sedation

(A) Flies given two exposures to 73% ethanol vapor become less sensitive to sedation during the second exposure (dotted lines, open symbols) as compared to the first exposure (solid lines, filled symbols), reflecting tolerance. Two tolerance mutants are shown: both 5-21 and 2-10 exhibited sedation sensitivity similar to the control during the first exposure (p>0.05 for each mutant vs. control ST25, t-tests), but during the second exposure respectively showed increased sensitivity (=decreased tolerance) or decreased sensitivity (=increased tolerance) relative to the control (p<0.01 for each mutant vs. control ST25, t-tests).

(B) Ethanol sedation tolerance for each mutant (n=5-7 for mutants, n=18 for control).
Figure 5. Ethanol Pharmacokinetics of the Mutants

Flies were exposed to 47% ethanol vapor for 15 min and their internal ethanol concentration was measured (n=3).
Figure 6. Ethanol Consumption Preference Correlates Positively With Ethanol Tolerance

(A) Ethanol preference on day 3 correlated positively with ethanol tolerance (r=0.664, p<0.01, Pearson’s correlation, n=18).

(B) Average ethanol preference across all 3 days of the preference assay correlated positively with ethanol tolerance (r=0.707, p=0.001, Pearson’s correlation, n=18).
Table 1. Mutants Tested for Ethanol Responses

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Mutants were identified in genetic screens for sensitivity to ethanol hyperactivity, sedation, or loss of postural control in the inebriometer. The original phenotype identified is listed for each mutant (S=sensitive, R=resistant) as well as the reference for mutants whose ethanol sensitivity phenotype has been previously published.
Table 2. Results of Correlation Analyses Comparing Different Behavioral Responses to Ethanol. We calculated all pairwise correlations between five responses to ethanol: ethanol preference on day 3 (pref.), maximum ethanol hyperactivity (hyp.), sedation ST50 (sed.), sedation tolerance (tol.), and internal ethanol concentration (EtOH). r values for Pearson’s correlation are shown with p values in parentheses (*p<0.05, in bold). For analyses involving preference, correlations were calculated using PI values on each day of the assay as well as the average across all 3 days. n=18 mutant lines for analyses involving tolerance and n=20 mutant lines for all other analyses.
Table 3. GAL4 Expression Patterns in the Adult Brain for Each Line. Abbreviations:
OL, optic lobe; AL, antennal lobe; CC, central complex; MB, mushroom body; PI, pars intercerebralis; SOG, subesophageal ganglion.

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CHAPTER 4

Sexually Dimorphic Regulation of Ethanol-Induced Behavior by fruitless and the Novel Tumor Suppressor Homolog tank

ABSTRACT
In both mammalian and insect models of ethanol intoxication, low doses of ethanol stimulate locomotor activity while high doses induce sedation. We discovered that acute ethanol responses in Drosophila, as in humans, are sexually dimorphic: male flies exhibit both increased ethanol hyperactivity and sedation resistance compared to females. These sex differences are partially mediated by fruitless (fru). We also characterized a second gene, tank (CG15626), the homolog of the mammalian tumor suppressor EL24/PIG8, that regulates ethanol responses in a sexually dimorphic manner. fru and tank function in distinct sets of neurons that show both an anatomical and functional interaction: they form potential synaptic connections and exhibit a cooperative interaction in regulating ethanol sensitivity. Thus, fru and tank not only contribute to the generation of sexually dimorphic ethanol responses, but also define distinct sets of neurons that interact to regulate these behaviors and may form part of a larger neural circuit.

INTRODUCTION

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 (Pohorecky, 1977). 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 (Babor et al., 1983; Phillips and Shen, 1996). In contrast, the depressant effects of ethanol manifest in humans as depressed mood, fatigue, and cognitive and motor impairment (Babor et al., 1983; Miller et al., 2009); animal models similarly exhibit motor incoordination and ultimately sedation (Pohorecky, 1977).

Genetic factors influence susceptibility to alcohol use disorders (AUDs), but few specific genes have been identified (Mayfield et al., 2008). Several studies have suggested that susceptibility to AUDs is correlated with increased sensitivity to the stimulant effects of ethanol and decreased sensitivity to its depressant effects (Schuckit, 1994; Morean and Corbin, 2010). In addition, several genes regulate both acute ethanol sensitivity and ethanol consumption in rodent models (Thiele et al., 1998; Hodge et al., 1999). Characterizing the genes that mediate acute ethanol responses may therefore provide insight into alcohol addiction.

Men and women are differentially affected by both acute and long-term ethanol exposure. Men exhibit increased alcohol consumption and a higher incidence of AUDs compared to women (SAMHSA, 2011; Grant et al., 2004). However, women are more susceptible to the negative physical consequences of heavy drinking, such as organ damage and risk of death (Nolen-Hoeksema and Hilt, 2006; Ceylan-Isik et al., 2010). Women are also more strongly affected by acute ethanol intoxication. Part of this effect is pharmacological, since the same ethanol dose (adjusted for body weight) induces a higher blood alcohol content (BAC) in women due to differences in body water content (Mumenthaler et al., 1999). However, even when BAC is equalized between the sexes, women exhibit greater ethanol-induced motor impairment and subjective feelings of intoxication than men (Miller et al., 2009). 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 (Kaun et al., 2012). As in humans and rodents, lower doses of ethanol stimulate locomotor activity in flies (Wolf et al., 2002), while higher doses induce motor incoordination and sedation (Moore et al., 1998; Rothenfluh et al., 2006). Many evolutionarily conserved genes regulate acute ethanol responses in both flies and mammals, such as *adenylate cyclase* and *protein kinase A* (Moore et al., 1998; Maas et al., 2005; Thiele et al., 2000), *epidermal growth factor receptor (EGFR)* (Corl et al., 2009), and *neuropeptide F/Y* (Wen et al., 2005; Thiele et al., 1998). Flies have also been used to study the neural circuits underlying ethanol-related behaviors (Kaun et al., 2012). For example, studies have identified a pair of dopaminergic neurons and their dopamine receptor target neurons that mediate ethanol-induced hyperactivity in flies (Kong et al., 2010), which may be analogous to the role of the mesolimbic dopamine circuit in mediating ethanol reinforcement in mammals (Gonzales et al., 2004). Thus, Drosophila offers powerful tools for dissecting both 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 observe clear sexual dimorphisms in Drosophila ethanol responses. Males exhibit increased ethanol-induced hyperactivity and ethanol sedation resistance compared to females. These sex differences are partially mediated by *fruitless (fru)*, a neural sex determination gene that encodes the male-specific protein FruM. We also characterize a novel tumor suppressor homolog, *tank*, that regulates ethanol responses in a sexually dimorphic manner. The activity of both *fru-* and *tank*-expressing neurons regulates ethanol sensitivity. Furthermore, we provide anatomical and behavioral evidence that these two sets of neurons interact to regulate ethanol sensitivity and may function as part of a larger circuit.
RESULTS

Ethanol Responses in Flies Are Sexually Dimorphic

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 (Wolf et al., 2002), and ethanol-induced sedation can be measured using a loss-of-righting assay (Rothenfluh et al., 2006). These two responses are uncorrelated and measure distinct aspects of ethanol intoxication (Devineni et al., 2011).

We asked whether wild-type male and female flies show differences in these ethanol responses, starting with ethanol hyperactivity. Upon exposure to a moderate concentration of ethanol vapor (47%), flies exhibit two phases of locomotor activation: an immediate, transient startle response mediated by olfaction, and a more gradual, sustained hyperactivity response mediated by ethanol intoxication (Wolf et al., 2002; Figure 1A). We observed that males showed a more rapid onset of ethanol hyperactivity as compared to females, as well as an increase in peak hyperactivity (Figures 1A, 1B, and 1C). Males also exhibited a higher 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 exhibited startle responses of a similar magnitude (Figure 1D), and females achieved a similar speed as males exhibit during peak ethanol hyperactivity (compare to Figure 1C). The decreased hyperactivity of females therefore cannot be attributed to a difference in general locomotion. We next tested the sedation sensitivity of males and females exposed to a high ethanol vapor concentration (67%), which can be quantified as the time required for 50% of flies to sedate (ST50). Females sedated more quickly than males, exhibiting a lower ST50 (Figures 1E and 1F).
To determine whether these sexually dimorphic ethanol responses could be due to a difference in ethanol absorption or metabolism, we measured the internal ethanol concentration in males and females during ethanol exposure. Under all exposure protocols tested, females contained approximately 20-30% more ethanol than males (Figure 1G). This increased ethanol concentration in females may at least partially explain their increased sedation sensitivity. In contrast, the difference in ethanol levels is not likely to explain the sex difference in ethanol hyperactivity since 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.

We wondered whether the sex differences in ethanol hyperactivity and sedation are specific to our strain (w Berlin) or are general within Drosophila melanogaster. We therefore tested the behavior of several other “wild-type” strains (strains commonly used as background controls, though some carry eye color mutations). All strains tested showed a sex difference in either ethanol hyperactivity or sedation, and in all cases the direction of the difference was the same as observed in w Berlin, with males showing increased hyperactivity and sedation resistance compared to females (Figures S1A and S1C). Sex differences in ethanol hyperactivity and the ethanol startle response were not correlated (Figure S1A and S1B), further indicating that the dimorphism in ethanol hyperactivity is not due to a general locomotor difference. Most strains showing a sex difference in ethanol sedation sensitivity also showed a sex difference in internal ethanol concentration, similar to w Berlin (Figure S1D). However, in one strain (Oregon R), males and females contained a similar ethanol concentration despite showing a robust difference in sedation sensitivity (Figure S1D). These results indicate that sexually dimorphic sedation sensitivity can occur independently of a difference in ethanol levels. Taken together, our data show that flies exhibit sex differences in ethanol hyperactivity
and sedation that are general within *Drosophila melanogaster* but can be modified or suppressed by genetic background.

**fru Regulates Ethanol Sensitivity**

Many sexually dimorphic behaviors in Drosophila are mediated by the gene *fruitless* (*fru*). *fru* encodes a set of putative male-specific transcription factors collectively termed FruM; sex-specific splicing of *fru* prevents the production of FruM in females (Ryner et al., 1996). FruM is largely necessary and sufficient for the production of male-typical courtship and aggression behaviors (Demir and Dickson, 2005; Manoli et al., 2005; Vrontou et al., 2006). Because FruM functions in the nervous system and does not regulate sexual differentiation of external morphology, it is considered a neural sex determination factor. We hypothesized that FruM might normally act in males to promote ethanol hyperactivity and sedation resistance, thereby generating the sexually dimorphic responses we observe.

We provide several lines of evidence indicating that FruM promotes ethanol sedation resistance in males. First, males carrying various loss-of-function mutations in *fru* showed increased sedation sensitivity, with ST50 values similar to those of wild-type females (Figure 2A; Table S1). Second, males expressing TraF (*elav^{Gal4}/UAS-traF*), a female-specific splicing factor that prevents production of FruM (Ryner et al., 1996), in all neurons also showed increased sedation sensitivity (Figure 2B). Third, females expressing FruM due to expression of an RNA interference (RNAi) transgene against *tra2* (*fru^{Gal4}/UAS-tra2^{RNAi}*), which normally acts along with *traF* to prevent FruM production (Ryner et al., 1996), showed increased sedation resistance (Figure 2C). Interestingly, none of these phenotypes could be explained by altered internal ethanol concentration (Figures S2A-S2C). Thus, while different internal ethanol levels may
partially account for sexually dimorphic sedation sensitivity in wild-type flies, FruM promotes sedation resistance in males without affecting ethanol pharmacokinetics.

The role of FruM in regulating ethanol hyperactivity was less clear. Most genotypes of fru mutant males (excluding lines with general locomotor defects) showed a slower onset of ethanol hyperactivity and one line also showed decreased peak hyperactivity, both of which represent female-like phenotypes (Figures 2D-G; Table S1). However, manipulating FruM expression by expressing traF in males or downregulating tra2 in females yielded conflicting results (Figures S2D-S2F). Some of these results may be explained by the fact that traF and tra2 also regulate expression of doublesex, which has traditionally been thought to regulate only somatic sexual differentiation but is also expressed in the nervous system (Robinett et al., 2010). Thus, while some evidence suggests that FruM promotes the onset of ethanol hyperactivity in males, its role in regulating this behavior is likely to be more complex, and the sexual dimorphisms in hyperactivity may be attributable to other factors. In contrast, the data strongly indicate that FruM promotes ethanol sedation resistance in males, and is probably at least partially responsible for the sex difference observed in wild-type flies.

4-12 Mutants Exhibit Sexually Dimorphic Ethanol-Related Phenotypes

Our results suggest that some of the sex differences in ethanol responses are mediated by fru. We wondered what additional genes might act either in combination or in parallel with fru to generate sexually dimorphic ethanol responses. By chance, we observed that 4-12, a mutation previously isolated from a genetic screen for ethanol sensitivity (Corl et al., 2009; Devineni et al., 2011), induced sexually dimorphic ethanol-related phenotypes. 4-12 caused increased ethanol hyperactivity in males, but not females (Figures 3A and 3B). In contrast, 4-12 increased ethanol sedation resistance in both sexes, though the phenotype was stronger in males (Figure 3C).
Both 4-12 mutant males and females exposed to ethanol contained a lower internal ethanol concentration than controls (Figure S3A), which likely contributes to their sedation resistance. To test whether other factors also contribute to their sedation resistance, we conducted dose response experiments. We found that control males tested at 73% ethanol sedated at the same rate as 4-12 males tested at 100% ethanol (Figure S3B), but under these conditions 4-12 males contained more ethanol than control males (Figure S3C). 4-12 males therefore require a higher internal ethanol concentration in order to exhibit the same sedation sensitivity as control males, indicating that the 4-12 mutation induces adaptations promoting sedation resistance that are independent of its effect on regulating ethanol levels. We therefore conclude that the increased sedation resistance induced by 4-12 has both a pharmacokinetic component (resistance due to altered internal ethanol concentration) and a pharmacodynamic component (resistance independent of ethanol concentration resulting from alterations in the physiological effects of ethanol).

4-12 Disrupts Expression of the Novel Tumor Suppressor Homolog tank

The 4-12 mutant carries a P element insertion that we mapped to a novel gene, CG15626. We decided to name this gene tank because its mutation allows flies to imbibe much more alcohol before becoming sedated. The 4-12 mutant will henceforth be referred to as tank^{4-12}. tank encodes two transcripts, tank-RA and tank-RB, which are predicted to encode a single protein (Figure 3D). The mammalian homolog of tank, EI24 (also known as PIG8), encodes a tumor suppressor that regulates apoptosis and has been linked to human cancer (Gu et al., 2000; Gentile et al., 2001; Zhao et al., 2005). tank bears significant homology to human EI24 in the central region of the protein (43% identity and 65% similarity; Blast e value = 2e-77). Like EI24, tank is a predicted six-transmembrane domain protein but contains no additional identifiable protein domains.
To confirm that tank^{4-12} disrupts tank expression, we measured tank transcript levels by quantitative RT-PCR (qPCR). tank^{4-12} males and females showed a strong reduction in levels of both tank transcripts compared to controls (Figure 3E). The level of tank reduction in tank^{4-12} males and females was not significantly different, indicating that the stronger behavioral phenotypes observed in tank^{4-12} males are not due to a difference in tank knockdown. tank expression in wild-type flies was not sexually dimorphic (Figure 3E).

Precise excision of the P element in tank^{4-12} flies restored normal ethanol hyperactivity and sedation sensitivity (Figure S3E and S3F), confirming that the behavioral phenotypes are due to the P element insertion. We also tested males containing additional transposon insertions in or near the tank locus: two alleles increased ethanol hyperactivity (Figure S3G), and two alleles increased sedation resistance (Figure S3H), in accordance with the tank^{4-12} phenotypes. tank^{4-12} mutant males show an abnormally high ethanol startle response (Figure 3A), which may contribute to their increased hyperactivity. However, the two additional alleles of tank that showed increased hyperactivity both exhibited a normal startle response (Figure S3G). In addition, removal of the third antennal segment, which abolishes most olfactory input (Vosshall and Stocker, 2007), eliminated the increased startle of tank^{4-12} males but did not eliminate their increased hyperactivity phenotype, though hyperactivity was reduced (data not shown). These data suggest that tank can regulate ethanol hyperactivity independently of the ethanol startle response. Taken together, our results indicate that tank inhibits ethanol hyperactivity in males and promotes ethanol sedation sensitivity in both sexes, though more strongly in males.

**tank Functions in Adult 4-12^{Gal4}-Expressing Cells to Promote Ethanol Sensitivity**
The P element inserted in $tank^{4-12}$ flies is a GawB enhancer trap, in which the transcriptional activator Gal4 is expressed in cells likely to express endogenous $tank$. We visualized this expression pattern using $UAS-GFP$. Within the central nervous system, we observed GFP expression in a limited number of cells. GFP expression was strongest in the pars intercerebralis (PI), which contains neurosecretory cells that project to the subesophageal ganglion (SOG) (Figure 4A). Expression was also observed in the mushroom body, antennal lobe, and cell bodies in the SOG and lateral brain (Figure 4A). Males and females did not exhibit any obvious differences in $4-12^{Gal4}$ expression (data not shown).

We tested whether $tank$ function in $4-12^{Gal4}$-expressing cells is necessary and sufficient to regulate behavior. For these basic characterization experiments we focused on ethanol sedation in males, which represents the most robust phenotype. RNAi downregulation of $tank$ in $4-12^{Gal4}$-expressing cells increased sedation resistance in males, as did pan-neuronal downregulation of $tank$ (Figures 4B, 4C, and S4A). We confirmed these results using an additional $tank^{RNAi}$ line targeted to a non-overlapping region of the gene (Figures S4B and S4C). Thus, $tank$ function is required in $4-12^{Gal4}$ cells and specifically in neurons to promote ethanol sedation. Pan-neuronal downregulation of $tank$ did not affect internal ethanol concentration during ethanol exposure (Figure S4D), confirming that $tank$ can regulate sedation sensitivity independently of its effect on ethanol pharmacokinetics.

Expression of $tank-RA$ in $4-12^{Gal4}$-expressing cells ($tank^{4-12}; UAS-tank^{RA/+}$) restored normal sedation sensitivity to $tank^{4-12}$ mutant males, conclusively demonstrating that the mutant phenotype is attributable to decreased $tank$ expression in these cells (Figure 4D). To determine whether $tank$ functions during adulthood to regulate ethanol sensitivity, we expressed $tank-RA$ in the $tank^{4-12}$ mutant exclusively during adulthood by using Gal80$^{ts}$, a temperature-sensitive Gal4 repressor that inhibits Gal4 function at 18°
but not at 29\(^\circ\) (McGuire et al., 2003). Adult-specific expression of \textit{tank-RA} in 4-12\textsuperscript{Gal4} expressing cells restored normal sedation sensitivity to \textit{tank}\textsuperscript{A-12} males (Figure 4E). Adult \textit{tank} function is therefore sufficient to induce normal ethanol sedation sensitivity, suggesting that \textit{tank} regulates some aspect of neuronal function rather than affecting neuronal development. Taken together, our results indicate that \textit{tank} function in 4-12\textsuperscript{Gal4} expressing cells is both necessary and sufficient to regulate ethanol sensitivity, strongly suggesting that some or all of these cells express endogenous \textit{tank}.

\textbf{\textit{tank} Functions in PI Neurons Projecting to the SOG}

To more precisely define the neurons in which \textit{tank} functions to regulate ethanol sensitivity, we downregulated \textit{tank} in various neuronal subsets in males by combining \textit{UAS-tank}\textsuperscript{RNAi} with 19 selected \textit{Gal4} lines. We selected \textit{Gal4} lines that label well-defined brain regions or neuronal subtypes that could potentially overlap with the 4-12\textsuperscript{Gal4} expression pattern, which is already fairly limited. For our initial screen we tested ethanol sedation; a subset of lines were subsequently tested for ethanol hyperactivity. We found that several \textit{Gal4} lines whose expression was largely restricted to the PI (Figures 5A, 5B, and S5A-S5D) increased ethanol hyperactivity and/or sedation resistance when driving \textit{UAS-tank}\textsuperscript{RNAi} (Figure 5C and 5D; Table S2). Interestingly, most of the PI-expressing \textit{Gal4} lines induced either a hyperactivity phenotype or a sedation phenotype, but not both (Table S2). These results indicate that \textit{tank} functions in the PI to regulate both ethanol hyperactivity and sedation, and may act in different subsets of PI neurons to affect the two behaviors.

\textbf{Activity of \textit{fru-} and \textit{tank}-Expressing Neurons Regulates Ethanol Sedation Sensitivity}
We have identified two genes, fru and tank, that regulate ethanol responses in a sexually dimorphic manner and are each expressed in a limited subset of neurons. We next asked whether the activity of either fru- or tank-expressing neurons directly regulates ethanol responses. To acutely activate these neurons, we used the heat-activated cation channel TrpA1, which causes neuronal depolarization (Hamada et al., 2008). To silence these neurons, we used ShiTs, a temperature-sensitive dynamin allele that depletes synaptic vesicles (Kitamoto, 2001), or adult-specific expression of Kir2.1, a potassium channel that induces neuronal hyperpolarization (Johns et al., 1999). To manipulate tank neurons we used flies heterozygous for 4-12\textsuperscript{Gal4}, which retain one functional copy of tank. For all of these experiments we focused on ethanol sedation instead of ethanol-induced hyperactivity since most manipulations of fru or 4-12\textsuperscript{Gal4} neuron activity affected general locomotion, such as by causing locomotor sluggishness (fru\textsuperscript{Gal4}/UAS-ShiTs, data not shown) or locomotor hyperactivity (4-12\textsuperscript{Gal4}/UAS-TrpA1; Figures S6A and S6B).

We first tested whether the activity of fru neurons regulates ethanol sedation in males. TrpA1 activation of fru neurons increased ethanol sedation sensitivity (Figure 6A), while silencing fru neurons using ShiTs had no effect (see Figures 8A and 8B). Thus, while fru neuron activity can directly affect ethanol sedation, these neurons may play a modulatory role rather than being essential for normal sedation behavior.

We next tested the role of tank neuron activity in regulating ethanol sedation in males. TrpA1 activation of 4-12\textsuperscript{Gal4} neurons increased sedation sensitivity, similar to activating fru neurons (Figure 6B). Surprisingly, silencing 4-12\textsuperscript{Gal4} neurons using either ShiTs or Kir2.1 affected sedation sensitivity in the same direction as neuronal activation, causing increased sensitivity (Figures 8A, 8B, and S6C). These results suggest that activity of 4-12\textsuperscript{Gal4} neurons does not regulate ethanol sedation in a straightforward manner. There are several possible explanations for these results, such as an inverted-
U model in which a certain level of $4-12^{\text{Gal4}}$ neuron activation might be necessary to mediate normal ethanol sedation sensitivity.

Given the role of both $tank$ and $tank$-expressing neurons in regulating ethanol sedation as well as the fact that $tank$ regulates sedation by functioning in adult neurons (Figure 4), we wondered whether $tank$ might regulate sedation by affecting neuronal activity. To test this hypothesis, we asked whether the behavioral effects of activating $4-12^{\text{Gal4}}$ neurons would be suppressed in the absence of $tank$ function. Indeed, we found that TrpA1 activation of $4-12^{\text{Gal4}}$ neurons in male $tank^{4-12}$ heterozygotes, but not homozygotes, increased sedation sensitivity (Figures S6D and S6E). Loss of $tank$ also suppressed the general locomotor hyperactivity induced by activating $4-12^{\text{Gal4}}$ neurons (Figures S6A and S6B). These data suggest that $tank$ function may be required in order for depolarization of $4-12^{\text{Gal4}}$ neurons to affect behavior. Overall, our results indicate that the activity of both $fru$ and $tank$ neurons affects ethanol sedation in males, and $tank$ may directly or indirectly regulate neuronal activity.

$tank$-Expressing Neurons Regulate Ethanol Sedation in a Sexually Dimorphic Manner

Our initial activation and silencing experiments were conducted in males, so we next tested whether similar phenotypes occur in females. The majority of $fru$-expressing neurons are present in both males and females, though many exhibit sexually dimorphic arborizations (Cachero et al., 2010; Yu et al., 2010). TrpA1 activation of $fru$ neurons in females increased ethanol sedation sensitivity (Fig 6C), similar to the effect observed in males, indicating that $fru$ neurons have a similar role in regulating ethanol sedation in both sexes. In contrast, while activation of $4-12^{\text{Gal4}}$ neurons in males increased sedation sensitivity, this manipulation had no effect in females (Figure 6D). Thus, the function of $tank$-expressing neurons, like $tank$ function, is sexually dimorphic.
To test whether the behavioral effects of $4-12^{\text{Gal4}}$ neuron activation might be attributable to neurons in the PI, where tank function is required, we specifically activated PI neurons using a subset of the PI-expressing Gal4 lines employed for our UAS-tank$^{\text{RNAi}}$ screen (Table S2). TrpA1 activation using two of these Gal4 lines increased sedation sensitivity in males, but not females, mimicking $4-12^{\text{Gal4}}$ neuron activation (Figures S6F-I). Taken together, these results indicate that both tank and tank-expressing neurons regulate ethanol sedation in a sexually dimorphic manner, and both of these functions can be localized to the PI.

**fru and tank Function in Distinct Neurons Likely to be Synaptically Connected**

We have shown that both fru- and tank-expressing neurons regulate ethanol sedation, and activating either set of neurons in males induces the same phenotype, increased sedation sensitivity (Figures 6A and 6B). These results suggest that fru and tank might be expressed in overlapping sets of neurons, or that fru and tank neurons might function within the same neural circuit. To address the former possibility, we asked whether fru and tank were co-expressed. Expression of FruM has been observed in approximately 1% of neurons, including regions that express $4-12^{\text{Gal4}}$ such as the PI and the median bundle, which contains projections from the PI to the SOG (Manoli et al., 2005). Interestingly, we did not observe any co-expression between markers for fru ($fru^{\text{Jax}}$) and tank ($4-12^{\text{Gal4}}$) in the adult male brain (Figures 7A-7C). Both markers were strongly expressed in the PI and the median bundle, but these two markers appeared to segregate to different fibers. We corroborated this lack of co-expression using an intersectional FLP-out method (Figures S7A and S7B). In addition, downregulation of tank in fru-expressing cells ($fru^{\text{Gal4}}/\text{UAS-tank}^{\text{RNAi}}$) did not affect ethanol hyperactivity or sedation (Figures S7C and S7D), providing behavioral evidence that tank does not
function in *fru*-expressing cells. Thus, *fru* and *tank* regulate ethanol responses by functioning in distinct sets of neurons.

Although *fru* and *tank* markers were expressed in different cells, we noticed that the projections of *fru* and *tank* neurons appeared highly intermingled in the SOG, suggesting that they might form synaptic connections (Figures 7D and 7E). To test this possibility, we utilized GRASP (GFP Reconstitution Across Synaptic Partners; Feinberg et al., 2008; Gordon and Scott, 2009). This method relies on expression of two halves of GFP on the membrane of distinct sets of neurons, and full-length GFP is reconstituted at sites of cell-cell contact, including synapses. GRASP between *fru* and 4-12*Gal4*-expressing neurons revealed a large number of GFP puncta that were almost exclusively localized to the SOG (Figures 7F and 7G). These puncta were not present in control flies expressing either of the individual halves of GFP (Figures 7H and 7I). While we cannot rule out the possibility that the GFP signal represents non-synaptic cell-cell contact, our results suggest that subsets of *fru* and *tank*-expressing neurons may form synaptic connections in the SOG. The fact that we have localized *tank* function in regulating ethanol responses to PI neurons projecting to the SOG (Figure 5) suggests that these PI cells may represent the *tank* neurons that anatomically interact with *fru* neurons.

*fru*- and *tank*-Expressing Neurons Interact to Regulate Ethanol Sensitivity

The GRASP experiment provides anatomical evidence of an interaction between *fru* and *tank* neurons, suggesting that they may function within the same neural circuit to regulate ethanol sedation. We next asked whether *fru* and *tank* neurons also show a functional interaction. As stated earlier, silencing 4-12*Gal4*-expressing neurons increased ethanol sedation sensitivity, whereas silencing *fru* neurons had no effect on this behavior (Figures 8A and 8B). Interestingly, silencing *fru* neurons in addition to 4-12*Gal4* neurons
further enhanced sedation sensitivity compared to silencing $4-12^{Gal4}$ neurons alone (Figures 8A, 8B, and S8). Thus, *fru* and $4-12^{Gal4}$ neurons show a synergistic interaction in regulating sedation sensitivity. We favor the possibility that the subsets of *fru* and $4-12^{Gal4}$ neurons that interact at the behavioral level are the same neurons that form synaptic connections in the SOG.

**DISCUSSION**

In this study we examine sexually dimorphic regulation of acute ethanol responses in Drosophila. We first describe general sex differences in ethanol hyperactivity and sedation: wild-type males exhibit increased hyperactivity and sedation resistance compared to females. These differences are at least partially mediated by male-specific *fru* function. The novel tumor suppressor homolog *tank* also exhibits sexually dimorphic regulation of ethanol responses. *tank* suppresses ethanol hyperactivity in males and promotes sedation sensitivity in both sexes, though more strongly in males. Unlike *fru*, the sexually dimorphic function of *tank* is not likely to be due to dimorphic expression, and is therefore attributable to dimorphisms in the function of *tank* itself or of downstream elements.

Overall, our data are consistent with a model (Figure 8C) in which *tank* functions in adult PI neurons to regulate ethanol hyperactivity and sedation, and *fru* functions in a non-overlapping, unknown set of neurons to regulate ethanol sedation. The activity of both *fru* and *tank* neurons regulates sedation sensitivity in a non-linear manner, and *tank* neurons exhibit a sexually dimorphic function. Furthermore, *fru* and *tank* neurons show both an anatomical and functional interaction: they form potential synaptic connections in the SOG, and they exhibit a cooperative interaction in regulating sedation sensitivity. We are attracted to the possibility that the same *fru* and *tank* neurons are responsible for
both the anatomical and functional interaction, an idea that is supported by the fact that \textit{tank} function is required in PI neurons projecting to the SOG. We suggest that \textit{tank} and \textit{fru} may affect ethanol sedation by either directly or indirectly regulating neuronal activity.

In summary, we have characterized two genes that contribute to the generation of sexually dimorphic ethanol responses, and we have used these genes to define distinct sets of neurons that interact to regulate these behaviors.

\textbf{Sexually Dimorphic Ethanol Responses in Flies and Humans}

Human females are more sensitive than males to ethanol-induced motor impairment. This difference is partly due to differences in BAC, but females also exhibit greater impairment when BACs are equalized (Miller et al., 2009). 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 \textit{fru}, which promotes sedation resistance in males without affecting ethanol levels. A previous study did not observe sex differences in Drosophila ethanol sensitivity using a negative geotaxis assay (Bhandari et al., 2009). This discrepancy may be due to differences in either the fly strains used or the behavioral measure examined, as negative geotaxis is not a direct correlate of either ethanol-induced hyperactivity or sedation and may be affected by both the stimulant and depressant effects of ethanol.

The neural factors that generate the human sexual dimorphism in ethanol sensitivity are unknown, although differences in dopaminergic transmission and GABA receptor function have been proposed to play a role (Ceylan-Isik et al., 2010; Grobin et al., 1998). A mammalian homolog of \textit{fru} has not been identified, so the sex differences in fly and human ethanol sensitivity may be produced by different mechanisms. However, it
is possible that a different human gene may play a functional role similar to \textit{fru} in regulating this behavior, and that the mechanisms downstream of \textit{fru} are conserved.

The question of why sexual dimorphism in ethanol sensitivity should exist at all, let alone show similarities in flies and humans, is an interesting one. An evolutionary explanation has been proposed in humans (Nolen-Hoeksema and Hilt, 2006). 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. A similar argument might apply for Drosophila, since flies encounter ethanol in fermenting fruit within their natural environment.

**Regulation of Ethanol Sedation Sensitivity by \textit{fru}- and \textit{tank}-Expressing Neurons**

The activity of \textit{fru} and \textit{tank} neurons seems to regulate ethanol sedation sensitivity in a non-linear manner, since in some cases opposite manipulations of neuronal activity caused similar behavioral effects. In particular, both activating and silencing \textit{4-12}^{Gald} neurons increased ethanol sedation sensitivity. Similarly, activating \textit{fru} neurons increased sedation sensitivity, whereas silencing \textit{fru} neurons, which had no effect on its own, enhanced the sedation sensitivity caused by silencing \textit{4-12}^{Gald} neurons. Several explanations could account for these counterintuitive results. For example, our results are consistent with an inverted-U model in which a certain level of \textit{4-12}^{Gald} neuron activation is required to mediate normal sedation sensitivity, or these neurons might exhibit temporally patterned activity. In both cases, artificial activation and inhibition could similarly disrupt \textit{4-12}^{Gald} neuronal function and produce the same behavioral effect. Alternatively, some \textit{4-12}^{Gald} neurons might promote sedation sensitivity
while others promote sedation resistance, so manipulating all of these neurons simultaneously might cause a non-linear effect depending on the relationships between these neurons.

Interestingly, tank neuron activity regulates ethanol sedation in a sexually dimorphic manner: activation of $4-12^{Gal4}$ neurons promotes sedation sensitivity in males, but not females. This difference parallels the sexual dimorphism in tank function, as tank promotes sedation sensitivity more strongly in males than in females. The dimorphic effect of $4-12^{Gal4}$ neuron activity might at least partially account for the dimorphism in tank function. Because we observed no obvious differences in the number or morphology of $4-12^{Gal4}$-expressing neurons in males and females, the behavioral difference caused by activating these neurons could be due to differences in the physiological properties of $4-12^{Gal4}$ neurons or to anatomical or functional dimorphisms in the downstream circuit, potentially in fru neurons.

**Role of PI Neurons in the Regulation of Ethanol Responses**

We showed that downregulating tank in PI neurons affects both ethanol hyperactivity and sedation, and the activity of PI neurons directly regulates sedation sensitivity. The PI is a major locus of peptidergic neurons, including insulin-producing cells that have been previously implicated in regulating ethanol sensitivity (Corl et al., 2005). At least 35 neuropeptides have been identified in Drosophila (Nassel and Winther, 2010), several of which are expressed in the PI (Park et al., 2008). Interestingly, downregulation of tank using some PI-expressing Gal4 lines affected only ethanol hyperactivity, while others affected only sedation. These results suggest that tank may function in different sets of PI neurons to regulate ethanol hyperactivity and sedation, raising the intriguing possibility that these distinct sets of neurons could be defined by expression of different neuropeptides. fru is also expressed in the PI, and it is
possible that \textit{fru} function in this region regulates ethanol responses. Expression of \textit{traF}, which negatively controls FruM expression, in a cluster of PI neurons has been shown to regulate sexually dimorphic locomotor patterns (Gatti et al., 2000), which might be linked to the potential function of \textit{fru} in regulating ethanol hyperactivity.

**Mechanisms of \textit{tank} and \textit{fru} Function**

Given that \textit{tank} and \textit{fru} regulate ethanol responses, as does the activity of the neurons in which they are expressed, how does the function of these genes relate to the function of the neurons? In the case of \textit{tank}, we have linked gene function to neuronal activity in two ways. First, we localized the function of both the gene and neuronal activity in regulating ethanol sedation to neurons within the PI. Second, we showed that activating 4-12\textsuperscript{Gal4} neurons affects locomotion and ethanol sensitivity in \textit{tank}\textsuperscript{4-12} heterozygotes but not homozygotes. These results suggest that \textit{tank} might affect behavior by either directly or indirectly regulating a process downstream of neuronal depolarization, such as action potential propagation or synaptic transmission. Alternatively, \textit{tank} might regulate neuronal connectivity and thereby disrupt activation of the proper downstream neurons, but this possibility seems less likely since adult \textit{tank} function is sufficient to regulate ethanol sensitivity, and neuronal connectivity in Drosophila is generally established during development.

The mammalian homolog of \textit{tank}, \textit{El24}, encodes a transcriptional target of p53 that induces apoptosis in response to DNA damage (Gu et al., 2000; Mork et al., 2007). \textit{El24} is a clinically important tumor suppressor, as its inactivation has been associated with breast and cervical cancers (Gentile et al., 2001; Zhao et al., 2005; Mazumder et al., 2011). The \textit{C. elegans} homolog of \textit{El24}, \textit{epg-4}, is required for autophagy, a catabolic process involving lysosomal degradation of the cytosol (Tian et al., 2010). It will be interesting to determine whether \textit{tank} also regulates apoptosis or autophagy, and, if so,
whether these processes affect ethanol responses. It is not clear how these potential functions of *tank* would be related to the regulation of neuronal activity, but it is possible that *tank* performs multiple cellular functions, perhaps depending on developmental stage or cell type.

*fru* is well-known for its developmental role in regulating neuronal survival and differentiation (Kimura, 2011), leading to the generation of sex-specific neurons and neurons with sexually dimorphic morphology (Cachero et al., 2010; Yu et al., 2010). Whether *fru* also regulates adult neuronal function is not well understood. Indirect evidence suggests that *fru* may affect neuronal excitability: artificial activation of *fru* neurons in the ventral nerve cord was shown to elicit male-typical courtship song in both males and females, but a higher level of neuronal activation was required to stimulate this behavior in normal females as compared to males or FruM-expressing females (Clyne and Miesenbock, 2008). In our study, artificial activation of *fru* neurons affected ethanol sedation in both males and females, suggesting that male-specific regulation of ethanol sedation by *fru* is not due to the presence of male-specific *fru* neurons and may be due to a role of *fru* in regulating neuronal function or activity. While the molecular and cellular mechanisms of *fru* and *tank* function remain to be elucidated, in this work we have characterized the role of *fru* and *tank* in regulating sexually dimorphic ethanol responses, and used these genes to define distinct sets of neurons that interact to regulate ethanol sensitivity.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks and Maintenance**

Flies were generally reared at 25°C and 70% relative humidity on standard cornmeal/molasses food. Flies for TrpA1 or Shi<sup>ts</sup> experiments were reared at 22°C and
flies for Gal80ts experiments were reared at 18°C and shifted to 29°C during adulthood. w Berlin was used as the control strain. All stocks were outcrossed into this background for at least 5 generations except for lines lacking phenotypic markers (e.g. fru mutants and fruGal4), which were therefore crossed into w Berlin using balancers. All assays were performed on 3-5 day old males or females that were generally non-virgin. For more information about fly stocks, see Supplemental Experimental Procedures.

Behavioral Assays

Ethanol hyperactivity and sedation were assayed in the booz-o-mat, an eight-chambered apparatus that delivers ethanol vapor. Twenty flies per vial were assayed, and n refers to the number of vials tested. Assays were generally conducted at ~25°C; TrpA1 and ShiIs experiments were conducted at various temperatures specified in the figure legends. The temperature used to activate TrpA1 varied between 26°C and 30°C depending on the Gal4 line, since we observed that TrpA1 activation using some Gal4 lines induced general behavioral defects when the temperature was raised too high.

Ethanol hyperactivity was measured by video tracking of flies as previously described (Wolf et al., 2002) using 47% ethanol vapor. Peak hyperactivity was calculated for each vial as the average of the three consecutive time points with the highest speed (excluding the startle response). Rate of hyperactivity onset was calculated as the slope of the line defined by linear interpolation for the three time points following the ethanol startle. Ethanol sedation was assayed manually as described in Corl et al. (2009) using 67% ethanol vapor, unless otherwise specified. See Supplemental Experimental Procedures for details regarding these assays.

Measurement of Internal Ethanol Concentration
Internal ethanol concentration was assayed as described in Devineni et al. (2011). For experiments comparing males and females or genotypes that appeared to differ in size, their size was first measured in order to accurately calculate ethanol concentration. See Supplemental Experimental Procedures for details.

**Immunohistochemistry**

Immunohistochemistry was carried out essentially as previously described (Joseph et al., 2009); see Supplemental Experimental Procedures. For characterization of Gal4 expression patterns with UAS-GFP, brains were stained with anti-GFP and nc82 (anti-Bruchpilot). fru^{boxy}4-12Gal4 colocalization and GRASP experiments were performed in unstained brains.

**Molecular Biology**

The genomic DNA flanking the 4-12 insertion was isolated using inverse PCR and sequenced. UAS-tank^{RA} was generated by cloning EST RE16861, encoding tank-RA, into the pUAST vector. The transgene was verified by DNA sequencing and injected into w Berlin flies. qPCR was performed as previously described (Tsai et al., 2004) except that we used the SYBR green method (see Supplemental Experimental Procedures).

**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,
statistical significance was attributed only to experimental lines that differed from both the *Gal4/+* and *UAS/+* controls (not including experimental values that were in between the two controls).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes eight figures, two tables, and Supplemental Experimental Procedures.

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Figure 1. Ethanol Hyperactivity and Sedation Are Sexually Dimorphic

(A) Tracking profiles of male and female flies exposed to 47% ethanol vapor. The black bar represents the duration of ethanol exposure. t=0 min 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 to 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, unpaired t-test, n=11).

(E) Sedation curves of males and females exposed to 67% ethanol vapor.

(F) Females had a lower ST50 than males, representing increased sensitivity to ethanol sedation (n=8).

(G) Females contained a higher internal ethanol concentration than males using various ethanol exposure protocols (n=4).

*p<0.05, **p<0.01, ***p<0.001, unpaired t-tests.
Figure 2: fru Regulates Acute Ethanol Responses

(A) Males of several fru mutant genotypes showed increased sedation sensitivity compared to control males (n=8). In (A) and (D-G), fru^3 and fru^4 are P element mutations, fru^{lex} is a targeted gene replacement with a lex insert, and fru^{4-40} and fru^{sat15} are deficiencies that remove fru as well as a number of adjacent genes (Anand et al., 2001; Mellert et al., 2010).

(B) Males expressing TraF pan-neuronally (elav^{Gal4}/UAS-traF) showed increased sedation sensitivity compared to control males (n=7).

(C) Females expressing Tra2^{RNAl} (fru^{Gal4}/UAS-tra2^{RNAl}) showed increased sedation resistance compared to control females (n=16).

(D and E) Males of several fru mutant genotypes showed a slower onset of ethanol hyperactivity compared to control males (n=6-8). Examples of tracking curves from three mutant lines are shown in (D), and rate of hyperactivity onset for each fru mutant (excluding those with general locomotor defects) is quantified in (E).
(F and G) Males carrying one fru mutation, fru\textsuperscript{4}/fru\textsuperscript{sat15}, showed decreased peak ethanol hyperactivity (n=8).
*p<0.05, **p<0.01, ***p<0.001.

(A and D-G) Unpaired t-tests comparing mutants to control.
(B and C) One-way ANOVA followed by Newman-Keuls post-tests.
Figure 3: tank Regulates Ethanol Responses in a Sexually Dimorphic Manner

(A and B) tank^{4-12} males showed greater peak ethanol hyperactivity than control males, as well as an increased startle response. tank^{4-12} females did not exhibit a significant difference in peak ethanol hyperactivity compared to control females (p>0.05, n=10-12).

(C) tank^{4-12} males and females showed increased sedation resistance compared to controls (n=8-12). The effect of tank^{4-12} on sedation sensitivity was stronger in males than in females (p<0.001, genotype by sex interaction).

(D) Diagram of the tank transcription unit. Black boxes represent coding regions and grey boxes represent untranslated regions. The insertion sites of the tank mutants tested are indicated, as well as the regions targeted by RNAi. RNAi line 1 was primarily used throughout this study; results using line 2 are shown in Supplemental Figure 4.

(E) Expression of both tank transcripts was reduced in tank^{4-12} males and females as compared to their respective controls. The reduction in each tank transcript level was
similar for both sexes (p>0.05, genotype by sex interaction). Control males and females did not differ in expression of either tank transcript (p>0.05). n=4 replicates.

***p<0.001.

(A and B) Unpaired t-tests comparing mutant to control.

(C and E) Two-way ANOVA followed by Bonferroni post-tests.
Figure 4: *tank* Functions in Adult 4-12\textsuperscript{Gal4}-Expressing Neurons

(A) Visualization of the 4-12\textsuperscript{Gal4} expression pattern in the adult male brain using UAS-GFP (green), along with nc82 counterstaining of neuropil (magenta).

(B) Downregulation of *tank* in 4-12\textsuperscript{Gal4}-expressing cells (4-12\textsuperscript{Gal4}/UAS-\textsuperscript{tank\textsuperscript{RNAi}}) increased sedation resistance in males (n=9).

(C) Pan-neuronal downregulation of *tank* (elav\textsuperscript{Gal4}/UAS-\textsuperscript{tank\textsuperscript{RNAi}}) increased sedation resistance in males (n=12).

(D) The sedation resistance of *tank*\textsuperscript{4-12} males was rescued by expression of *tank*-RA in 4-12\textsuperscript{Gal4}-expressing cells. *tank*\textsuperscript{4-12} homozygotes significantly differed from the background control, whereas no significant difference was observed between *tank*\textsuperscript{4-12}; UAS-\textsuperscript{tank\textsuperscript{RA}}/+ rescue flies and UAS-\textsuperscript{tank\textsuperscript{RA}}/+ controls (p>0.05, n=8).

(E) The sedation resistance of *tank*\textsuperscript{4-12} males was rescued by adult-specific expression of *tank*-RA in 4-12\textsuperscript{Gal4}-expressing cells. Flies were reared at 18\degree and shifted to 29\degree during adulthood to induce adult-specific *tank*-RA expression. A significant difference was observed between *tank*\textsuperscript{4-12}; Gal80\textsuperscript{ts}/+ and the background control, but not between *tank*\textsuperscript{4-12}; Gal80\textsuperscript{ts}/UAS-\textsuperscript{tank\textsuperscript{RA}} rescue flies and Gal80\textsuperscript{ts}/UAS-\textsuperscript{tank\textsuperscript{RA}} controls (p>0.05, n=8).
**p<0.01, ***p<0.001, one-way ANOVAs followed by Newman-Keuls post-tests.  
Repeated measures ANOVAs were used in panels (B) and (E).
Figure 5: tank Functions in PI Neurons Projecting to the SOG

(A and B) Expression patterns for two of the PI-expressing Gal4 lines used to drive UAS-tank\textsuperscript{RNAi} were visualized in the adult male brain with UAS-GFP (green), along with nc82 counterstaining (magenta). See Supplemental Figure S5 for expression patterns of additional lines.

(C and D) Expression of UAS-tank\textsuperscript{RNAi} using Gal4 lines primarily expressed in the PI increased ethanol hyperactivity (C) and sedation resistance (D) in males (n=8-12). Examples of three Gal4 lines inducing phenotypes are shown for each behavior; see Supplemental Table S2 for all phenotypic data. Each experimental line was compared to both UAS-tank\textsuperscript{RNAi}/+ and its respective Gal4/+ control.

*p<0.05, **p<0.01, ***p<0.001, one-way ANOVAs followed by Newman-Keuls post-tests.
Figure 6: Activity of fru- and tank-Expressing Neurons Regulates Ethanol Sedation

(A) Activation of fru neurons in males (fruGal4/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) Activation of 4-12Gal4 neurons in males (4-12Gal4/UAS-TrpA1) at 30° increased sedation sensitivity (n=13); no effect was observed at 22° when TrpA1 was inactive (p>0.05, n=8).

(C) Activation of fru neurons in females (fruGal4/UAS-TrpA1) at 26° increased sedation sensitivity (n=6); no effect was observed at 22° (ST50 of experimental flies was in between the two control values; n=8).

(D) Activation of 4-12Gal4 neurons in females (4-12Gal4/UAS-Trp) at 30° did not affect sedation sensitivity, nor was an effect observed at 22° (p>0.05, n=8).
*p<0.05, **p<0.01, ***p<0.001, one-way repeated measures ANOVAs followed by Newman-Keuls post-tests.

For experiments conducted at 30° shown in panels (B) and (D), the ethanol concentration was decreased to 60% instead of 67% because flies sedated more quickly at this temperature.
Figure 7: *fru* and *tank* Are Expressed in Distinct Neurons Likely to be Synaptically Connected 

(A-C) Expression of 4-12\textsuperscript{Gal4} (magenta) and *fru\textsuperscript{lex*} (green) was simultaneously visualized in the adult male brain using UAS-RFP and lexAop-GFP, respectively. No co-expression was observed in the anterior (A) or posterior (B) brain. A magnified view of the median bundle is depicted in (C), showing 4-12\textsuperscript{Gal4} and *fru\textsuperscript{lex*} markers segregating to different fibers.
(D and E) Magnified views of the SOG show that projections of 4-12Gal4- and fruLex-
expressing neurons were highly intermingled in the anterior (D) and posterior (E) SOG.
(F and G) GRASP experiment using male 4-12Gal4; fruLex/ UAS-GFP1-10; lexAop-GFP11
flies. Many GFP puncta were observed, almost all of which were localized to the SOG
(magnified in G).
(H and I) GFP puncta were not observed in negative control flies expressing either of the
individual GFP halves (4-12Gal4/+; UAS-GFP1-10; lexAop-GFP11 [H] or fruLex/ UAS-GFP1-
10; lexAop-GFP11 [I]). Brains were imaged at equal or greater gain as used in panels (F)
and (G).
Figure 8: fru- and tank-Expressing Neurons Interact to Regulate Ethanol Sensitivity

(A and B) Silencing fru neurons in males had no effect on its own, but enhanced the sedation sensitivity induced by silencing 4-12Gal4 neurons. (A) 4-12Gal4 + fruGal4/UAS-ShiTs flies show increased sedation sensitivity at several time points compared to 4-12Gal4/UAS-ShiTs flies (n=8).

(B) The ST50 values for 4-12Gal4/UAS-ShiTs and 4-12Gal4 + fruGal4/UAS-ShiTs flies were significantly lower than all controls and showed a non-significant trend toward differing from each other. Silencing fru neurons alone (fruGal4/UAS-ShiTs) had no effect (p>.05). n=8.

(C) Model for regulation of acute ethanol responses by fru and tank (see Discussion).

*p<0.05, **p<0.01, ***p<.001.

(A) Two-way repeated measures ANOVA followed by Bonferroni post-tests.

(B) One-way ANOVA followed by Newman-Keuls post-tests.
Figure S1, related to Figure 1. Several Wild-Type Drosophila Strains Exhibit Sexually Dimorphic Ethanol Responses

(A) In 3 of 6 wild-type strains tested for ethanol hyperactivity, peak hyperactivity was greater in males than in females (n=8-12).

(B) Ethanol startle responses of males and females of wild-type 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 3 of 4 wild-type strains, males showed increased ethanol sedation resistance compared to females (n=8-12).

(D) Internal ethanol concentration after 12 min exposure to 47% ethanol (n=4-8). 2202U and Canton S males contained lower ethanol levels than 2202U and Canton S females, respectively, potentially accounting for their differences in ethanol sedation sensitivity. Oregon R males and females did not differ in ethanol concentration, so their difference in sedation sensitivity is due to other factors.

*p<0.05, **p<0.01, ***p<0.001, paired or unpaired t-tests.
Figure S2, related to Figure 2. fru Regulates Acute Ethanol Responses

(A) Internal ethanol concentration of fru mutant males that showed increased sedation sensitivity (Figure 2A), measured after 10 min exposure to 47% ethanol (*p<0.05, **p<0.001, unpaired t-tests comparing each mutant to control, n=4). None of the mutants showed an increased ethanol concentration compared to the control, indicating that the effect of fru on ethanol sensitivity is not due to altered ethanol pharmacokinetics. Two mutants (fru^3 and fru^3/fru^4-40) unexpectedly showed a decreased ethanol concentration.

(B and C) After 12 min exposure to 47% ethanol, males expressing TraF (elav^Gal4/UAS-traF) (B) and females expressing tra2^RNAi (fru^Gal4/UAS-tra2^RNAi) (C) did not show altered internal ethanol levels compared to their respective controls (p>0.05, n=4).

(D) Males expressing TraF pan-neuronally (elav^Gal4/UAS-traF) showed a slower onset of ethanol hyperactivity (p<0.01), though some of these flies exhibited chaining, which may decrease their locomotor speed. Peak hyperactivity was unaffected (p>0.05, n=8).
(E) Males expressing TraF in fru-expressing neurons ($fru^{Gal4}/UAS-traF$) showed a more rapid onset of ethanol hyperactivity ($p<0.001$) and greater peak hyperactivity ($p<0.05$, $n=8$), opposite of the phenotypes generally exhibited by fru mutant males.

(F) Females expressing $tra2^{RNAi}$ in fru-expressing neurons ($fru^{Gal4}/UAS-tra2^{RNAi}$) did not display phenotypes in either rate of ethanol hyperactivity onset or peak hyperactivity ($p>0.05$, $n=4$).

(B-F) One-way ANOVAs followed by Newman-Keuls post-tests.
Figure S3, related to Figure 3. tank Regulates Acute Ethanol Responses

(A) Internal ethanol concentration of flies exposed to 47% ethanol for 12 min. 4-12 males and females contained less ethanol than their respective controls (two-way ANOVA followed by Bonferroni post-tests). Ethanol concentration was similarly decreased in both sexes (p>0.05, genotype by sex interaction).

(B) Control males tested at 73% ethanol sedated at the same rate as 4-12 males tested at 100% ethanol (p>0.05, unpaired t-test, n=8), with both groups exhibiting an ST50 of approximately 21 min.

(C) Internal ethanol concentration measured under the conditions shown in (B), with control males exposed to 73% ethanol and 4-12 males exposed to 100% ethanol, each for 21 min. 4-12 flies contained more ethanol than controls (unpaired t-test, n=4) despite showing similar sedation sensitivity under these conditions.

(D-E) Precise excision of tank^{4-12} restored normal peak ethanol hyperactivity (D) and ethanol sedation sensitivity (E) in males (p>0.05, one-way ANOVA followed by Newman-Keuls post-test, n=8).
(F-G) Ethanol hyperactivity (F) and sedation sensitivity (G) of males carrying various transposon alleles of tank. tank$^{03581/+}$ was tested as a heterozygote because homozygotes were not viable. (F) Two alleles, tank$^{03581/+}$ and tank$^{EY12458}$, showed increased peak ethanol hyperactivity (p<0.05) without a significant difference in the startle response (p>0.05, unpaired t-tests, n=8-12).

(G) Two alleles, tank$^{03581/+}$ and tank$^{DG03609}$, showed increased sedation resistance (unpaired t-tests, n=7-8).

**p<0.01, ***p<0.001.
Figure S4, related to Figure 4. Downregulation of *tank* Promotes Ethanol Sedation Resistance

(A) Adult expression of *tank* RNAi (line 1) using the ubiquitous driver tub^Gal4^ (along with Gal80^K; flies grown at 18° and shifted to 29° during adulthood) caused a significant downregulation of both *tank* transcripts, indicating that the RNAi is effective (n=4 replicates).

(B-C) Expression of *tank* RNAi line 2 either in 4-12^Gal4^-expressing cells (B) or in all neurons (C) of male flies (n=12-13). Both manipulations increased sedation resistance, similar to the effect of *tank* RNAi line 1.

(D) Pan-neuronal downregulation of *tank* (elav^Gal4^/UAS-tank^RNAi^) in males did not affect internal ethanol concentration after 15 min exposure to 47% ethanol (p>0.05, n=4).

*p<0.05, **p<0.01, ***p<0.001, one-way ANOVAs followed by Newman-Keuls post-tests. Repeated measures ANOVA was used in (B).
Figure S5, related to Figure 5. Expression Patterns of *Gal4* Lines Used to Downregulate *tank* in the PI

(A-D) Expression patterns of PI-expressing *Gal4* lines that caused phenotypes with UAS-tank^{RNAi} were visualized in the adult male brain using UAS-GFP (green), along with nc82 counterstaining (magenta). These *Gal4* lines were primarily expressed in the PI with additional expression in a small number of other cells.
Figure S6, related to Figure 6. Activity of tank Neurons Regulates Behavior in a tank-Dependent Manner

(A) TrpA1 expression in 4-12Gal4-expressing neurons of males had no effect on locomotor activity at 22º when TrpA1 was inactive (p>0.05, n=8).

(B) TrpA1 activation of 4-12Gal4-expressing neurons at 30º in tank4-12 heterozygous males (red bar) stimulated locomotor activity in the absence of ethanol. This effect was not observed by activating 4-12Gal4-expressing cells in tank4-12 homozygotes (blue bar;
tank$^{4-12}$ homozygotes without TrpA1 (green bar) showed normal locomotor activity (p>.05) and were not significantly different from tank$^{4-12}$ homozygotes expressing TrpA1 (p>.05). n=12.

(C) Silencing 4-12Gal4-expressing neurons in males using adult-specific expression of Kir2.1 (4-12Gal4/Gal80TS; UAS-Kir2.1/+ flies reared at 18° and shifted to 29° during adulthood) increased sedation sensitivity (n=8).

(D) TrpA1 expression in 4-12Gal4 neurons had no effect on ethanol sedation sensitivity at 22° (p>.05, n=8).

(E) TrpA1 activation of 4-12Gal4-expressing neurons at 30° increased ethanol sedation sensitivity in tank$^{4-12}$ heterozygous males (red bar), but not in tank$^{4-12}$ homozygotes (blue bar; p>.05). tank$^{4-12}$ homozygotes without TrpA1 (green bar) exhibit a weaker sedation resistance phenotype when grown at 22°, which was necessary for TrpA1 experiments, as compared to 25° (the usual rearing condition). n=15.

(F-I) Ethanol sedation sensitivity of males (F and G) or females (H and I) expressing TrpA1 using two PI-expressing Gal4 lines. Each experimental line was compared to both UAS-TrpA1/+ and its respective Gal4/+ control.

(F) Expression of TrpA1 in PI neurons of males did not affect sedation sensitivity at 22° (ST50s of experimental flies were in between the two control values, n=8).

(G) TrpA1 activation of PI neurons in males at 27° increased sedation sensitivity (n=4-6).

(H and I) Expression of TrpA1 in PI neurons of females did not affect sedation sensitivity at 22° (H) or 27° (I) (ST50s for all experimental lines were in between the two control values, n=8).

*p<0.05, **p<0.01, ***p<0.001, one-way ANOVAs followed by Newman-Keuls post-tests. Repeated measures ANOVAs were used in panels (D)-(F) and (H). Flies tested at 27° or 30° (panels [D], [E], [G], and [I]) were tested at 60% ethanol instead of 67% ethanol because flies sometimes sedated too quickly at higher temperatures.
Figure S7, related to Figure 7. fru and tank Function in Distinct Neurons

(A) An intersectional method was used to identify cells co-expressing fru and 4-12\textsubscript{Gal4} (as a marker for tank). We utilized a UAS>STOP>GFP transgene, which is a GFP reporter containing an FRT-flanked transcriptional stop cassette which can be excised by FLP recombinase. We generated males carrying 4-12\textsubscript{Gal4}, fru\textsubscript{FLP}, and UAS>STOP>GFP transgenes. In these flies GFP is expressed only in cells expressing both fru and 4-12\textsubscript{Gal4}, because the stop cassette is excised by FLP recombinase only in fru-expressing cells, and the resulting UAS-GFP is expressed only in 4-12\textsubscript{Gal4}-expressing cells. In these males we observed no GFP expression (green) within the central nervous system (the green signal in the right optic lobe represents autofluorescence from residual retinal
tissue that was not entirely dissected away). Brains were counterstained with nc82 (magenta).

(B) GFP expression (green) was present in positive control flies expressing $fru^{FLP}$ and $UAS>STOP>GFP$ along with the pan-neuronal driver $elav^{Gal4}$, confirming that the lack of GFP using $4-12^{Gal4}$ in (A) is not due to loss or inactivity of the $fru^{FLP}$ or $UAS>STOP>GFP$ transgenes. Brains were counterstained with nc82 (magenta).

(C-D) Downregulation of tank in $fru$-expressing cells ($fru^{Gal4}/UAS$-$tank^{RNAi}$) did not affect ethanol hyperactivity (C) or sedation (D) ($p>0.05$, one-way ANOVAs followed by Newman-Keuls post-tests, n=8-11). Repeated measures ANOVA was used in (C).
Figure S8, related to Figure 8. Permissive Temperature Control for Simultaneous Silencing of fru and tank Neurons

At the permissive temperature (22º), Shi136 did not affect ethanol sedation sensitivity when expressed in either fru or tank neurons, or in both fru and tank neurons simultaneously (p>0.05, one-way repeated measures ANOVA followed by Newman-Keuls post-tests, n=8).
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Table S1, related to Figure 2. Phenotypes of fru Mutant Males

Ethanol hyperactivity and sedation sensitivity were assayed in males of 10 different loss-of-function fru genotypes. Phenotypes noted in the table indicate a significant difference from control males (p<0.05, unpaired t-tests, n=6-12). Four genotypes exhibited an abnormally low ethanol startle response (p<0.01, n=8) as well as decreased response to a mechanical stimulus (data not shown); these alleles were considered sluggish and were excluded from ethanol hyperactivity analysis. Because even one copy of the fru<sup>sat15</sup> deficiency caused strong sedation resistance (p<0.001, n=8), presumably due to loss of one of the multiple genes affected by the deficiency, we excluded genotypes carrying this allele from our sedation analysis.
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### Table S2, related to Figure 5. RNAi Downregulation of tank in Specific Neuronal Subsets

Selected Gal4 lines were combined with UAS-tankRNAi in males and screened for altered ethanol sedation sensitivity; lines of interest were subsequently tested for ethanol hyperactivity. Phenotypes noted in the table signify experiments where Gal4/UAS-tankRNAi flies significantly differed from both Gal4/+ and UAS-tankRNAi/+ controls (p<0.05, one-way ANOVAs followed by Newman-Keuls post-tests, n=8-12).
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Fly Stocks

UAS-traF, UAS-tra2\textsuperscript{RNAi}, fru\textsuperscript{irx}, and all fru mutants tested were obtained from Devanand Manoli. fru\textsuperscript{FLP} and UAS>STOP>GFP lines were obtained from Barry Dickson. Two fru\textsuperscript{Gal4} strains were used in this study: (1) a targeted insertion of Gal4 into the fru locus (Manoli et al., 2005) and (2) the enhancer trap NP21 (Kimura et al., 2005). The fru\textsuperscript{Gal4} from Manoli et al. 2005 was used for most experiments, but because this line showed low viability we later switched to using the NP21 fru\textsuperscript{Gal4} line because it did not have these issues. The only experiments shown in this study using the NP21 fru\textsuperscript{Gal4} are the UAS-tra2\textsuperscript{RNAi} experiments in Figure 2 and Figure S2. The expression patterns of both Gal4 lines have been validated (Manoli et al., 2005, Kimura et al., 2005).

The tank\textsuperscript{4-12} mutant (carrying the GawB element) was generated in our laboratory and was originally identified in a genetic screen for ethanol sensitivity described in Corl et al. (2009). The three other alleles of tank, tank\textsuperscript{603581} (insertion of PBac[WH]), tank\textsuperscript{EY12458} (insertion of P[EPgy2]), and tank\textsuperscript{DG03609} (insertion of P[wHy]), were obtained from the Bloomington Stock Center. Precise excision lines of tank\textsuperscript{4-12} were generated by remobilization of the P element using delta2-3 transposase (Robertson et al., 1988). Precise excision lines were verified by DNA sequencing. The tank RNAi lines were obtained from the Vienna Drosophila RNAi Center: line 1 represents GD7844 and line 2 represents KK106205. The elav\textsuperscript{Gal4} line used represents the C155 insertion. The UAS-GFP line used throughout this study contained two insertions, a membrane-targeted UAS-CD8-GFP and a nuclear-targeted UAS-T2-GFP.

Behavioral Assays
Ethanol hyperactivity and sedation assays were performed in the booz-o-mat, an apparatus that delivers a specific concentration of ethanol vapor by mixing pure ethanol vapor with humidified air at a specified ratio (Wolf et al., 2002). Each experiment was carried out on at least two different days using flies from independent bottles, and the results were pooled.

Hyperactivity assays were conducted as described previously (Wolf et al., 2002). Flies were introduced into the booz-o-mat, then allowed to acclimate to the chamber for 10 minutes before filming commenced. The flies were recorded for 2 minutes in humidified air (to assay baseline locomotion), followed by 21 minutes in 47% ethanol vapor (70 units ethanol / 80 units air). Films were analyzed with DIAS software using a custom script that calculated average locomotor speed for each vial of flies during 20 second time windows at 13 time points throughout the assay.

Sedation assays were conducted as described previously (Corl et al., 2009). Flies were exposed to 67% ethanol vapor (100 units ethanol / 50 units air), unless otherwise specified. Flies were assayed for sedation at 5 minute intervals by applying a mechanical stimulus (twirling each vial within the booz-o-mat chamber), causing flies to lose balance, and flies that were unable to right themselves were scored as sedated. The experimenter was blinded to the identity of each genotype. ST50 values were determined by linear interpolation.

Measurement of Ethanol Concentration

Ethanol concentration in flies was measured as described previously (Devineni et al., 2011). Flies were frozen in dry ice and homogenized in 50 mM Tris-HCl (pH 7.5). Ethanol concentrations were measured in fly homogenates using the Ethanol Assay kit from Diagnostic Chemicals Limited. Because sedated flies show an increased rate of ethanol absorption compared to non-sedated flies (U.H., unpublished data), ethanol
concentration was measured in flies that were exposed to a non-sedating dose of ethanol, generally 10-15 minutes at 47% ethanol (except for the experiment in Figure S3 in which sedation times were equalized between groups).

To calculate the ethanol concentration in flies of different sizes, such as males and females, we attempted to accurately measure fly volume using liquid displacement. The volume of a male fly was previously reported to be 2 µL (Moore et al., 1998), but we found this value to be an overestimate. The average volume that we measured for wild-type male flies was 1.5 µL and for female flies was 2.0 µL, but these measurements were highly variable due to the technical difficulty of accurately measuring small volumes, even when up to 100 flies were pooled for each measurement. In contrast, our measurements of the wet weight of flies were extremely consistent. We therefore decided to use the relative weight of male and female flies to estimate the relative difference in volume, normalizing all volumes to our estimate of 2 µL for one wild-type female. This method assumes that the flies are a similar density; we have no reason to believe otherwise. We carried out a similar procedure to compare different genotypes that appeared to differ in size.

**Immunohistochemistry**

Immunohistochemistry was performed essentially as previously described (Joseph et al., 2009). Brains were dissected in phosphate buffered saline (PBS) and fixed for 20 min in 4% paraformaldehyde in PBS. Brains that did not undergo antibody staining were washed in PBS after fixation and imaged on the same day as dissection. Brains undergoing antibody staining were washed in PBT (PBS with 0.3% Triton X-100) after fixation, blocked for 1 hour in 5% normal goat serum, incubated with primary antibody for 24-48 hours at 4°C, washed in PBT, incubated with secondary antibody overnight at 4°C, washed in PBT, and mounted on slides in Vectashield mounting solution.
(Vector Laboratories). All wash steps included three brief washes followed by three 15 minute washes. Primary antibodies used were rabbit anti-GFP (1:200) and mouse nc82 (1:50). Secondary antibodies used were Cy3-coupled goat anti-mouse antibody (1:500) and FITC-coupled goat anti-rabbit antibody (1:500). Brains were imaged with a Leica confocal microscope and images were analyzed using ImageJ.

**qPCR**

RNA was extracted from fly heads, which were separated from bodies by repeatedly vortexing and freezing in liquid nitrogen. cDNA was prepared using Reverse Transcription reagents and was analyzed by qPCR using a standard SYBR green protocol (Applied Biosystems). tank transcript levels were normalized to levels of the control transcript rp49. Relative RNA abundance was calculated using the standard curve method. The tank primers were designed using Primer Express, were targeted to exon junctions to avoid amplifying genomic DNA, and were verified by BLAST to be specific to tank. The primer sequences were as follows:

*tank* RA: 5’-ATCAGCAAGCACTGGCACAG-3’ and 5’-CGGGTGGACAGACCTTAGTTCA-3’

*tank* RB: 5’-CCCCCGATCAGGAACTAAGG-3’ and 5’-TTCTTTATCGCCGACCATTCTC-3’

**SUPPLEMENTAL REFERENCES**


Drosophila as a Model for Studying Addiction-Like Behavior

For many years flies have been used as a model to study acute ethanol responses, and the mechanisms underlying these behaviors have turned out to be remarkably conserved from flies to mammals. New assays, such as voluntary ethanol consumption described in Chapter 2 as well as the recently developed conditioned ethanol preference assay (Kaun et al., 2011), have suggested that flies may exhibit addiction-like behavior. Flies appear to fulfill several of the criteria proposed for an animal model of alcohol addiction (Cicero, 1979; McBride and Li, 1998). The ethanol consumption assay reveals that 1) flies voluntarily consume ethanol; 2) their consumption is not entirely dependent on sensory properties of ethanol; and 3) they exhibit a relapse-like effect. The conditioned preference assay further demonstrates that flies find ethanol intoxication rewarding, since flies subsequently seek out an odor cue that was associated with ethanol intoxication (Kaun et al., 2011). In both assays flies were willing to overcome negative stimuli (bitter-tasting compound or electric shock, respectively) in order to obtain ethanol or the ethanol-associated cue, suggesting compulsive-like behavior toward ethanol.

Ethanol Consumption in Flies: Calories or Pharmacological Effects?

Despite the evidence suggesting that ethanol consumption in flies models features of addiction, it is difficult to truly determine an animal’s motivation for exhibiting a certain behavior, and to assess whether there is a similarity to human behavior. In
particular, the question of whether flies consume ethanol due to its caloric value rather than its pharmacological properties has been recently debated. My work described in Chapter 2 did not identify a role for calories in ethanol preference, since varying the caloric ratio between the ethanol and non-ethanol food had no significant effect on the preference index.

A recent study presented additional evidence suggesting that calories do not strongly contribute to ethanol preference (Xu et al., 2012). These authors first showed that the calories in ethanol are poorly utilized by flies, since providing ethanol as the sole caloric source prolonged survival only slightly compared to providing no calories at all, and was much less effective in prolonging survival than providing an isocaloric concentration of sucrose. However, the authors did not verify that flies consumed equal amounts of ethanol and sucrose, so decreased consumption of ethanol as compared to sucrose might account for the difference in survival time, especially since ethanol in the absence of food is not appetitive to flies (Ja et al., 2007). However, the low concentrations of ethanol and sucrose used (< 1%) (which are less likely to produce gustatory responses), along with the large difference in survival times, suggest that flies indeed fail to utilize some or most of the calories present in ethanol.

Xu et al. (2012) also showed that while flies modulate their consumption of different sucrose concentrations in order to maintain their caloric intake at a remarkably constant level, they do not modulate their consumption of various ethanol concentrations. One interpretation of this result is that flies do not show compensatory feeding toward ethanol because few calories in ethanol are effectively utilized, in accordance with the survival experiment described above.

However, these results do not entirely rule out a role for calories in the ethanol preference assay, and in fact I attempted to undertake more precise experiments to balance the caloric content of the ethanol and non-ethanol food without affecting their
gustatory attractiveness, such as by (1) adding a tasteless, nutritive compound to the non-ethanol food, or (2) adding sucrose to increase the calories of the non-ethanol food, while compensating for its sweetness by adding a non-nutritive artificial sweetener to the ethanol food or by using sugar-blind taste mutants. Unfortunately, I was not able to find compounds or mutants that met these criteria to my satisfaction. In particular, I did not come across any nutritive compounds to which flies did not show a gustatory response (as judged by a short term consumption preference assay).

In a recent study, Pohl et al. (2012) took a similar (but more successful) approach in attempting to identify a role for calories in ethanol preference. These authors used a variety of nutritive carbohydrates to equalize the caloric content of the ethanol and non-ethanol food, three of which were believed to be neither gustatorily attractive nor aversive based on the proboscis extension assay. Equalizing the caloric content using two of these three carbohydrates caused flies to lose their preference for ethanol; in the third case the flies showed approximately normal ethanol preference. These results suggest that the caloric value of ethanol represents an attractive force in driving ethanol consumption in flies, but that other factors also contribute.

The experiments conducted by Pohl et al. (2012) are elegant in their direct manipulation of caloric content, but they also have some caveats: (1) the proboscis extension response may not entirely represent the gustatory attractiveness of a compound, especially since each compound was applied only to the labellum and not to the many other taste organs of the fly (e.g. legs, wings), and (2) the caloric content of each compound is based on human data, so the number of calories available to a fly is not precisely known. In fact, if flies cannot utilize all of the calories in ethanol, as suggested by Xu et al. (2012), then the caloric balancing experiments conducted by Pohl et al. do not actually equalize the number of utilizable calories but instead add excess
calories to the non-ethanol food. Clearly the role of calories in ethanol preference is still a controversial issue that remains to be resolved.

Pohl et al. (2012) have also argued that because flies contain a very low internal ethanol concentration after each drinking bout (≤ 4 mM), they are not likely to reach intoxication during the preference assay. However, it is possible that the ethanol concentration in flies reaches higher levels after a cluster of multiple drinking bouts as compared to a single drinking bout, a possibility that is especially relevant since the frequency of drinking bouts increases over time (see Chapter 2). Even if this is not the case, Pohl et al. (2012) have not ruled out the possibility that the low levels of ethanol that were measured may still have biological relevance. For example, long-term exposure (24-48 hrs) to a low dose of ethanol that produces an internal ethanol concentration of less than 5 mM has been shown to induce ethanol tolerance, indicating that even low internal ethanol concentrations can induce neurobiological and subsequent behavioral changes (Berger et al., 2004). Furthermore, an internal ethanol concentration of only 6-8 mM (which was calculated without subtracting the baseline level of ~3 mM in naive flies, and may in fact be lower) produces a robust increase in the locomotor activity of flies (Kaun et al., 2011). Further experiments are required to determine whether the ethanol consumed during the preference assay is sufficient to induce either immediate or long-term behavioral changes related to the intoxicating or hedonic effects of ethanol.

A recent study may help shed light on the relationship between ethanol consumption and its rewarding properties. Ophir et al. (2012) showed that social experience altered ethanol consumption and preference of male flies, and that these effects were mediated by neuropeptide F (NPF). Specifically, mating increased NPF levels in males, which reduced their ethanol preference; in contrast, rejection by females decreased NPF levels in males, which increased their ethanol preference. Two
observations suggest that these effects are not due to altered attraction toward the caloric value of ethanol. First, mated and rejected males did not differ in food consumption in a non-choice assay, suggesting that they show a similar attraction to calories. Second, artificial activation of NPF neurons not only decreased ethanol preference, but also diminished the rewarding properties of ethanol, as assessed by the conditioned ethanol preference assay. Thus, the NPF-mediated effect of social experience on ethanol preference is likely to be due to the rewarding properties of ethanol rather than its calories, suggesting that the rewarding effect of ethanol may indeed play a role in the ethanol preference assay.

Ophir et al. (2012) have proposed a “reward homeostat” model to explain the relationship between sexual experience and ethanol consumption. In this model, NPF signaling may serve as an internal representation of the state of the reward system; experiences that decrease NPF signaling (such as sexual rejection) stimulate reward-seeking behavior in the fly (such as increased ethanol consumption), and vice versa. In support of this model, the authors use the conditioned preference assay to show that both mating and ethanol intoxication are rewarding, as is activation of the NPF pathway. Interestingly, the mammalian homolog of NPF, neuropeptide Y (NPY), regulates ethanol sensitivity and intake in mice (Thiele et al., 1998). However, NPY has not been shown to link social experience to ethanol consumption, and it will be interesting to determine whether this function is conserved from flies to mammals.

**Studying Addiction in Flies: Future Outlook**

There is still much work to be done in establishing Drosophila as model for studying addiction-like behavior. Certain important criteria for addiction (Cicero, 1979) have not yet been met in flies. For example, it has not been shown that voluntary ethanol consumption leads to ethanol tolerance, that ethanol removal causes withdrawal
symptoms, or that flies are willing to “work” in order to obtain ethanol. The latter criterion could be demonstrated by showing that flies exhibit operant responding for ethanol, a paradigm which has not yet been developed in Drosophila.

While more complex assays improve the validity of Drosophila as a model system to study drug addiction, these assays are necessarily more cumbersome and time-consuming, thus making large-scale genetic screening difficult. It is therefore important to understand how the simple and more complex assays for drug-induced behavior are related. In Chapter 3 I analyzed the relationships between initial ethanol sensitivity, ethanol tolerance, and voluntary ethanol consumption in flies. In our set of mutants, ethanol consumption was positively correlated with the development of tolerance, but not with naive sensitivity to the sedating or hyperactivating effects of ethanol. These results suggest that complex behaviors such as voluntary ethanol consumption are not simply readouts of acute responses; they may incorporate acute sensitivity and the development of tolerance in addition to other factors, such as experience and learning, and perhaps the sensory and caloric properties of ethanol.

Some genes have been found to regulate multiple ethanol-induced behaviors: for example, krasavietz (kra) regulates sedation sensitivity, tolerance, and voluntary ethanol consumption (Berger et al., 2008; Devineni and Heberlein, 2009). In contrast, other genes regulate individual ethanol-induced behaviors: for instance, scabrous mediates conditioned ethanol preference, but not acute ethanol sensitivity (Kaun et al., 2011; LaFerriere et al., 2008). Thus, while the simpler assays are ideal for rapidly identifying ethanol-related genes and have some predictive value for more complex behaviors, genetic screening using the more complex assays will also be necessary to identify genes that specifically affect these behaviors.
Molecular and Neural Mechanisms Underlying Ethanol Consumption Preference

The genes and molecular pathways mediating ethanol consumption preference remain largely unknown. I observed that mutation of kra decreased ethanol preference (see Chapter 2), but we have not examined the molecular mechanisms by which kra regulates this behavior. A recent study showed that mutation of rutabaga (rut), a type I calcium-activated adenylyl cyclase, decreased ethanol preference, implicating the cyclic AMP pathway in this behavior (Xu et al., 2012). Both kra and rut also regulate acute ethanol sensitivity, suggesting that common pathways may regulate acute intoxication and ethanol preference (Berger et al., 2008; Moore et al., 1998).

However, of the ~50 candidate lines that I tested for ethanol preference (primarily mutants with ethanol sensitivity phenotypes), only kra showed a robust phenotype, suggesting that most of the genes regulating acute ethanol sensitivity are not required for normal ethanol preference (data not shown). Thus, there are likely to be genes affecting ethanol preference but not acute intoxication. Unbiased genetic screens will help uncover these genes and shed light on the molecular pathways mediating ethanol preference.

In order to identify neurons affecting ethanol preference, I conducted a neural circuitry screen in which I used Shibire ts to silence neurotransmission of 27 different sets of neurons defining specific brain regions or neurochemical systems (e.g. dopaminergic cells). Surprisingly, none of these manipulations produced a strong effect on ethanol preference (data not shown). These data suggest that ethanol preference is a fairly robust behavior that may not be dependent on a single brain region or any of the well-known neurochemical systems that I tested. However, Ophir et al. (2012) showed that artificially activating NPF signaling reduced ethanol preference to near-zero levels, indicating that it is indeed possible to abolish ethanol preference in flies. In fact, copious mating had a similar effect on severely reducing ethanol preference (in my experiments
the mating status of males was not carefully controlled, but most males were probably unmated). Thus, identifying the downstream neurons that mediate the effect of mating and NPF signaling may represent a promising new strategy for characterizing the neural circuits underlying ethanol preference.

Xu et al. (2012) showed that *rut* promotes ethanol preference by functioning in the mushroom body, suggesting that this brain region may represent part of the neural circuitry underlying ethanol consumption. The mushroom body is also required for conditioned ethanol preference (Kaun et al., 2012), suggesting that shared neural circuits may underlie these two ethanol preference behaviors. This strategy of identifying genes regulating ethanol preference and mapping gene function within the brain represents an alternative method for identifying neurons that regulate this behavior.

Overall, my hope is that future work from our lab as well as others will characterize both the molecular and neural pathways that mediate ethanol preference. It will be interesting to determine to what extent these pathways overlap with the pathways that mediate general feeding behavior as well as other ethanol responses. The mechanisms underlying ethanol preference in *Drosophila* can then be tested for a similar function in mammalian models of ethanol consumption and addiction.

**Sexually Dimorphic Regulation of Ethanol-Induced Behavior**

The first half of my graduate work focused on establishing ethanol consumption preference as a model for studying addiction in flies, but I encountered many roadblocks in identifying the molecular and neural mechanisms underlying this behavior, as described above. However, in the course of studying the relationship between ethanol preference and other ethanol responses (Chapter 3), I rediscovered some interesting mutants affecting acute ethanol sensitivity that had been isolated from previous genetic screens but were never characterized-- the most interesting of which was the mutant 4-
12. I therefore decided to devote the rest of my graduate work to understanding the role of the gene affected by this mutation, tank, in regulating ethanol-induced behavior. This project took me in several unexpected directions. First, my discovery that tank has a sexually dimorphic function led me to study general sexual dimorphisms in ethanol responses, and the role of the fruitless (fru). Second, I became more interested in the function of tank neurons than in tank itself, and gradually I shifted from a molecular project to a neural circuitry project.

In studying general sex differences in ethanol responses I discovered that female flies show decreased ethanol-induced hyperactivity and increased ethanol sedation sensitivity compared to males (Chapter 4). The latter difference parallels the sex difference that has been observed in humans, in which women show greater ethanol-induced motor impairment compared to men (Miller et al., 2009). The sex difference in Drosophila sedation sensitivity is at least partially attributable to the neural sex determination gene fru. fru encodes a putative transcription factor, so it will be interesting to identify its downstream targets that regulate ethanol sensitivity. A mammalian homolog of fru has not been identified, but it is possible that the downstream targets of fru are conserved and play a similar role in flies and mammals.

Other genes may also contribute to the sex differences in Drosophila ethanol responses. For example, doublesex (dsx) is sex-specifically spliced by traF and tra2 in a similar manner as fru. In fact, the experiments in which I manipulated these splicing factors in order to express FruM in females or prevent FruM production in males also affect dsx, and therefore cannot conclusively distinguish a role for fru as opposed to dsx. dsx was traditionally thought to regulate only somatic sexual differentiation, but it is also expressed in the nervous system (Robinett et al., 2010) and has been shown to regulate sexual behavior (Shirangi et al., 2006). Future studies can determine whether dsx in fact contributes to the sex differences in ethanol responses.
Regulation of Ethanol Responses by *tank*

The novel gene *tank* has an important role in promoting ethanol sedation sensitivity, since mutation of the gene produces robust sedation resistance (Chapter 4). However, the molecular function of *tank* still remains a mystery. The mammalian homolog of *tank*, *EI24*, encodes a tumor suppressor that is transcriptionally activated by p53 and induces apoptosis in response to DNA damage (Gu et al., 2000; Mork et al., 2007). Sequence analysis indicates that Tank and EI24 show high homology in the central protein region. Both proteins are predicted to have 6 transmembrane domains, and EI24 has been localized to the endoplasmic reticulum, where it inhibits the anti-apoptotic factor Bcl-2 (Zhao et al., 2005).

It will be interesting to determine whether *tank* plays a similar role in regulating apoptosis in flies as EI24 does in mammals. While we did not directly test the role of *tank* in apoptosis, we noticed that *tank*<sup>4-12</sup> mutants are resistant to antennal blackening induced by ethanol toxicity, a process which involves apoptosis of olfactory neurons (French and Heberlein, 2009), and this resistance was not dependent on altered ethanol pharmacokinetics (data not shown). These data suggest that *tank* promotes ethanol-induced apoptosis and may have a more general pro-apoptotic function as well. The function of p53 in regulating apoptosis is conserved in flies and mammals (Sutcliffe and Brehm, 2004). Intriguingly, sequence analysis identified a putative p53 binding site in a non-coding region near the 5’ end of *tank*, suggesting that it may be transcriptionally activated by p53 in a similar manner as EI24.

I conducted several preliminary experiments that suggest a potential role for p53 in regulating Drosophila ethanol responses. Most notably, I found that pan-neuronal expression of three different dominant negative p53 constructs increased ethanol sedation resistance (data not shown), suggesting that p53 functions in neurons to
promote sedation sensitivity. Mutations that eliminated p53 expression in all tissues did not affect sedation behavior, but, interestingly, one of these mutations partially suppressed the sedation resistance of the tank^{4-12} mutant (data not shown). This genetic interaction suggests that p53 may function in the same molecular pathway as Tank. More work needs to be done to conclusively determine whether p53 regulates ethanol sedation, and, if so, to determine whether this effect is attributable to p53 function in neurons, whether it occurs during development or in adulthood, and whether p53 and Tank act in the same molecular pathway.

In addition to its robust ethanol-related phenotypes, the tank^{4-12} mutant also exhibits other phenotypes, most notably in stress responses. I found that tank^{4-12} mutant flies were more sensitive than wild-type flies to a variety of stressors including heat, starvation, and oxidative stress, and that they had a shorter lifespan (data not shown). These phenotypes are particularly interesting since ethanol is often considered a stressor, especially at high toxic doses, and while tank^{4-12} mutant flies are sensitive to many other stressors, they are resistant to ethanol-induced sedation as well as developmental ethanol toxicity and ethanol-induced apoptosis of olfactory neurons (data not shown). Thus, tank appears to have a complex role in regulating stress responses and fly behavior in general. It will be interesting to identify the molecular and cellular mechanisms by which tank produces these phenotypes and to determine whether common or separate pathways are responsible for its diverse functions, especially functions that appear contradictory (promoting sensitivity to ethanol toxicity but resistance to other stressors).

**Regulation of Ethanol Responses by fru- and tank-Expressing Neurons**

The activity of both fru- and tank-expressing neurons regulates ethanol sedation sensitivity (Chapter 4). These two distinct sets of neurons show both an anatomical and
a functional interaction in regulating ethanol sedation. However, there are many remaining questions regarding the functions and interactions of these neurons. First, one would ideally like to pinpoint the specific fru- and tank-expressing neurons that are responsible for regulating ethanol sedation. In the simplest scenario, these would represent the same neurons in which fru and tank, respectively, function to regulate ethanol sensitivity. I have begun to address this question by localizing tank function to neurons within the pars intercerebralis (PI) and showing that PI neurons directly regulate ethanol sedation, but even this small brain region is a heterogenous structure containing many cells releasing various types of neuropeptides.

Second, although I have provided evidence for an anatomical interaction between fru and tank neurons in the SOG and demonstrated that they synergistically regulate ethanol sedation, I have not confirmed a direct functional connection between these two sets of neurons. This would require identification of the specific fru and tank neurons that appear to be connected and using either electrical or transgenic methods of paired stimulation and recording of neural activity. These methods can also help determine which neurons are presynaptic and which are postsynaptic, a question that the GRASP method does not address. Anatomical experiments have thus far been unhelpful in answering this question, as both fru neurons and tank neurons appear to have both dendritic and axonal arborizations within the SOG (Yu et al., 2010; data not shown).

Finally, all of the experiments described in this project have focused on manipulating neuronal activity rather than recording endogenous activity. It would be interesting to use calcium indicators to determine whether the activity of fru or tank neurons is altered by ethanol. This experiment could also provide candidates for specific fru or tank neurons that are responsible for regulating ethanol-induced behavior. We could then determine whether either baseline neuronal activity or neuronal
activation/inhibition by ethanol is altered in the \textit{fru} or \textit{tank} mutants, thus linking the function of the genes to the function of the neurons. We could additionally test whether males and females show differences in the activity of \textit{fru} or \textit{tank} neurons, which might contribute to the sexually dimorphic effects of ethanol. Thus, future work may help shed light on the role of \textit{fru} and \textit{tank} in regulating neuronal function, the role of \textit{fru}- and \textit{tank}-expressing neurons in regulating behavior, and the role of these neurons as part of a larger neural circuit regulating ethanol-induced behavior in Drosophila.

\textbf{References}


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