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The Evolution of *Drosophila melanogaster* as a Model for Alcohol Research

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Abstract

Animal models have been widely used to gain insight into the mechanisms underlying the acute and long-term effects of alcohol exposure. The fruit fly *Drosophila melanogaster* encounters ethanol in its natural habitat and possesses many adaptations that allow it to survive and thrive in ethanol-rich environments. Several assays to study ethanol-related behaviors in flies, ranging from acute intoxication to self-administration and reward, have been developed in the past 20 years. These assays have provided the basis for studying the physiological and behavioral effects of ethanol and for identifying genes mediating these effects. In this review we describe the ecological relationship between flies and ethanol, the effects of ethanol on fly development and behavior, the use of flies as a model for alcohol addiction, and the interaction between ethanol and social behavior. We discuss these advances in the context of their utility to help decipher the mechanisms underlying the diverse effects of ethanol, including those that mediate ethanol dependence and addiction in humans.

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INTRODUCTION

Alcohol is one of the most widely used and abused drugs in the world. Acute ethanol exposure produces dramatic short-term changes in behavior. These changes include both stimulant effects, such as disinhibition and euphoria, which occur at lower ethanol doses, and depressant effects, such as fatigue, cognitive and motor impairment, and loss of consciousness, which occur at higher ethanol doses (Babor et al. 1983, Pohorecky 1977). In addition to these short-term effects, long-term consumption of ethanol can lead to alcohol use disorders (AUDs), which include alcohol abuse and dependence. Alcohol abuse is characterized by continued alcohol consumption despite physically hazardous situations, legal or social problems, and a failure to fulfill obligations (APA 1994). Alcohol dependence, a more severe condition, is characterized by physical symptoms, such as tolerance and withdrawal, as well as signs of uncontrolled use, such as unsuccessful attempts to curtail alcohol consumption (APA 1994). The estimated prevalence of AUDs is 8.5% in the United States, thus affecting more than 17 million Americans (Grant et al. 2004).

Several studies have suggested a relationship between acute sensitivity to ethanol and propensity for developing an AUD. Specifically, risk of AUD has been associated with increased sensitivity to the stimulant effects of ethanol and decreased sensitivity to its depressant effects (Morean & Corbin 2010, Newlin & Thomson 1990, Schuckit 1994). Thus, studying the mechanisms underlying the short-term effects of ethanol, which are generally easier to measure, may also provide insight into the process of alcohol addiction. Genetic factors influence both acute ethanol sensitivity and susceptibility to AUDs; familial, twin, and adoption studies have estimated that the genetic contribution to AUDs is ~40–60% (Prescott & Kendler 1999, Schuckit et al. 2001). Despite these findings, investigators have identified very few genes that influence susceptibility to AUDs. Gene-expression profiling and linkage studies have identified many candidate genes (Mayfield et al. 2008), but much work remains to be done to link these genes conclusively to AUDs and to develop effective therapeutic strategies.

The difficulty and cost of human studies have led to the development of animal models to investigate the genetic, molecular, and neural mechanisms underlying both the short- and long-term effects of ethanol. Rodent models have been most widely used and have provided important insights into these mechanisms (Crabbe et al. 2006). More recently, the fruit fly *Drosophila melanogaster* has been developed as a model to study the effects of ethanol. Unless noted otherwise, the terms *Drosophila*, flies, or fruit flies are used here to refer to the

species *D. melanogaster*. The experimental and genetic accessibility of *Drosophila* makes it an ideal organism to study molecular, cellular, developmental, and neurobiological processes, most of which are conserved between flies and mammals. Flies are easy and inexpensive to maintain and breed, they have a rapid life cycle (~10 days at 25°C), and their small size allows for hundreds or thousands of genotypes to be maintained in a typical laboratory. More importantly, useful genetic tools are being developed continuously in *Drosophila*. These tools include publicly available collections of mutants and RNA interference (RNAi) lines to inactivate nearly any fly gene of interest, lines to target specific tissues or cell types, and transgenes to manipulate neuronal activity (Kaun et al. 2012, Venken et al. 2011).

In this review, we describe the use of *D. melanogaster* to study the physiological and behavioral effects of ethanol, and we examine these effects in the context of human conditions associated with excessive alcohol use. Specifically, we discuss (*a*) the ecological relationship between flies and ethanol, (*b*) the effects of acute and developmental ethanol exposure on fly behavior, (*c*) ethanol preference behaviors that may model aspects of alcohol addiction, and (*d*) the relationship between ethanol responses and social behavior. Because recent reviews have comprehensively described the molecular and neural mechanisms that mediate ethanol-related behaviors in flies, we do not examine these subjects here (Kaun et al. 2012, Rodan & Rothenfluh 2010).

ETHANOL IN THE NATURAL HABITAT OF *DROSOPHILA*

Adaptations of *D. Melanogaster* to Ethanol-Containing Environments

The relationship between *D. melanogaster* and ethanol was initially investigated from an ecological and evolutionary perspective. Fruit flies encounter ethanol in their natural environment: They inhabit and breed in ripe fruits and other plant materials that can contain up to 5% ethanol, produced by fermentation of sugars by various yeasts (Gibson et al. 1981). A long evolutionary history of ethanol exposure has allowed flies to expand to new ecological niches. For example, they frequently reside in wineries, in which wine seepages can contain up to 11% ethanol (Gibson et al. 1981).

ADH: alcohol dehydrogenase

ALDH: acetaldehyde dehydrogenase

Flies efficiently metabolize ethanol and can use it as an energy source or a substrate for lipid synthesis (Daly & Clarke 1981, Geer et al. 1985). Ethanol is first converted to acetaldehyde by the enzyme alcohol dehydrogenase (ADH), and acetaldehyde is metabolized to acetate by acetaldehyde dehydrogenase (ALDH). Both ADH and ALDH functions are essential to promote resistance to ethanol toxicity (David et al. 1976, Fry & Saweikis 2006). Natural polymorphisms in ADH have been well studied. The two major alleles that exist in natural populations are Adh^F and Adh^S. ADH^F shows higher enzymatic activity than does ADH^S, and Adh^F flies are more resistant to ethanol toxicity than are Adh^S flies (Briscoe et al. 1975, Cavener 1979, van Delden et al. 1978). Some studies have reported that Adh^F flies also show greater larval preference and adult oviposition (egg-laying) preference for ethanol compared with Adh^S flies, but other studies have disputed this relationship (Cavener 1979, Gelfand & McDonald 1983, Siegal & Hartl 1999, van Delden & Kamping 1990).

Drosophila ADH is a short-chain dehydrogenase, and its structure and enzymatic mechanism differ from those of vertebrate ADH, a medium-chain dehydrogenase (Hernández-Tobías et al. 2011, Jornvall et al. 1981). Some researchers have proposed that *Drosophila* ADH evolved as an adaptive response to detoxify environmental ethanol and colonize ethanol-containing environments (Ashburner 1998, Hernández-Tobías et al. 2011). Several findings support this hypothesis: (*a*) Flies carrying short-chain ADH evolved shortly after ethanol became available in fermenting fruits ~130–140 mya (Hernández-Tobías et al. 2011), (*b*) larval ADH is upregulated by the presence of ethanol in food (McKechnie & Geer 1984), and (*c*) ADH is critical for survival

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of flies on ethanol-containing food (see above). Additionally, flies are much more resistant to ethanol toxicity compared with toxicity induced by other primary alcohols, and this selective resistance is due to ADH (David et al. 1976). Thus, flies likely evolved greater resistance to ethanol to inhabit ethanol-containing environments, allowing flies to utilize ethanol as a food source as well as to consume other nutrients available in fermenting fruits.

In addition to providing nutritional benefits, a recent study suggests that ethanol-containing environments may also be favorable for *Drosophila* to fight infection by parasitic wasps, which lay eggs inside *Drosophila* larvae (Milan et al. 2012). In this study, environmental ethanol reduced wasp infection of *Drosophila* larvae, and ethanol consumption by infected larvae also reduced the survival of the wasp progeny. Moreover, infected larvae showed a stronger preference for ethanol-containing food compared with uninfected larvae, suggesting that flies in the wild may actively seek out ethanol-containing substrates not only for nutritional purposes, but also to combat parasitic infection.

Ethanol as a Food Source

Early studies showed that flies can use ethanol as a source of calories because providing ethanol as the sole caloric source increased survival relative to complete starvation (Van Herrewege & David 1980). Ethanol is converted to acetate by ADH and ALDH and is then converted to acetyl-CoA, which can enter the tricarboxylic acid cycle. However, some studies have suggested that the calories present in ethanol are poorly utilized by flies. For example, although providing ethanol as the sole caloric source prolonged survival compared with complete starvation, it was much less effective to prolong survival than was an isocaloric concentration of sucrose (Xu et al. 2012). Although this result may be due to differences in consumption, the large disparity in survival times suggests that flies fail to utilize some or most of the calories present in ethanol. In support of this hypothesis, flies modulate their consumption of different sucrose concentrations to maintain their caloric intake at a remarkably constant level; they do not, however, modulate their consumption of different ethanol concentrations, suggesting that these solutions provide few calories (Xu et al. 2012). A previous study also observed only a modest compensatory feeding reduction of ethanol-containing food, leading to the proposal that calories in ethanol may not be utilized effectively by flies (Ja et al. 2007).

Both the detoxification of ethanol and its utilization as a caloric source require metabolism by ADH and ALDH, suggesting that the efficiency of these two processes may be correlated. Ethanol detoxification can be quantified as the survival time upon exposure to high, toxic doses of ethanol; in contrast, caloric utilization has been measured as the increase in survival time when low ethanol concentrations (up to 5%) are provided as the sole caloric source (as compared with completely starved flies). As expected, Adh^F flies show greater utilization of the calories in ethanol compared with Adh^S flies, indicating that both ethanol detoxification and utilization are regulated by the rate of ethanol conversion to acetaldehyde (Daly & Clarke 1981). However, selective breeding studies showed that although raising fly strains on ethanol for many generations increased their resistance to ethanol toxicity, these selected lines did not always show improved caloric utilization of ethanol (Van Herrewege & David 1980). This dissociation is supported by a study that surveyed a variety of alcohols and found that detoxification and caloric utilization of these alcohols were not well correlated (Karan et al. 1999). Thus, although the detoxification and caloric utilization of ethanol both represent important adaptations that may have allowed *Drosophila* to survive in ethanol-containing environments, these processes are dissociable and may reflect partially distinct biological pathways.

Species and Population Differences

The physiological and behavioral effects of ethanol have been compared among various

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Drosophila species as well as among different *D. melanogaster* populations. Overall, these studies support the view that flies inhabiting ethanol-containing environments are more resistant to ethanol toxicity and may show greater preference for ethanol.

Several studies have compared *D. melanogaster* to *D. simulans*, a closely related species that appears to lack the adaptations to ethanol that *D. melanogaster* has evolved. *D. melanogaster* is found more frequently in ethanol-containing environments, such as wine cellars, compared with *D. simulans* (Gibson & Wilks 1988, McKenzie & McKechnie 1979, McKenzie & Parsons 1972). *D. melanogaster* also shows greater resistance to ethanol toxicity than does *D. simulans*, which is likely due to the higher ADH activity of *D. melanogaster* (David et al. 1976, McKenzie & Parsons 1972, Pipkin & Hewitt 1972). Some studies suggest that *D. melanogaster* shows greater larval preference and adult oviposition preference for ethanol compared with *D. simulans*, although conflicting results have been reported (Gelfand & McDonald 1983, McKenzie & Parsons 1972, Parsons & King 1977, Richmond & Gerking 1979).

Several studies have reported population differences within *D. melanogaster* associated with latitude as well as microenvironment. For example, resistance to ethanol toxicity in both adults and larvae tends to increase with increasing latitude (David et al. 1986, David & Bocquet 1975). Larval ethanol preference similarly increases further from the equator (Parsons 1980). These latitudinal clines correlate with a gradient of Adh^F versus Adh^S distribution: Adh^F is more frequent at higher latitudes, which generally correspond with lower temperatures (David et al. 1986, Oakeshott et al. 1982, Parkash et al. 1999). Researchers have sought to explain the correlation between the allelic and phenotypic distributions. One study found that ADH^S is more heat-resistant than ADH^F, suggesting that in warmer climates ADH^S may be superior to ADH^F in detoxifying ethanol (Sampsell & Sims 1982). However, others have failed to observe this biochemical difference and instead found that the activity of both ADH^S and ADH^F was similarly increased at higher temperatures (McElfresh & McDonald 1986). These authors proposed an alternative explanation for the genotypic and phenotypic correlation: At high temperatures, increased activity of the already more active ADH^F isoform may actually be detrimental to ethanol detoxification because the rapid conversion of ethanol to acetaldehyde, a toxic by-product, may surpass the rate at which acetaldehyde can be detoxified by ALDH. Corroborating the importance of acetaldehyde metabolism, Fry et al. (2008) showed that latitude is correlated with a polymorphism in ALDH that affects enzymatic activity. Thus, polymorphisms in ADH and ALDH likely provide a molecular explanation for the latitudinal variation in ethanol toxicity, although the evolutionary reason for this gradient is still unknown.

In addition to latitude, the microenvironment of flies strongly influences ethanol resistance. Many studies have compared populations of *D. melanogaster* found within high-ethanol-containing environments, such as wine cellars, to populations found elsewhere. The populations collected from ethanol-containing environments show greater resistance to ethanol toxicity, but the genetic factors mediating this resistance are unknown (Gibson & Wilks 1988, McKenzie & McKechnie 1978, McKenzie & Parsons 1974, Vouidibio et al. 1989). Some studies have observed a higher frequency of Adh^F in these ethanol-adapted populations (Briscoe et al. 1975, Vouidibio et al. 1989), but most studies found no difference in Adh^F or Adh^S allele frequencies (Gibson & Wilks 1988, McKenzie & McKechnie 1978, McKenzie & Parsons 1974).

Laboratory selection studies have been conducted to mimic natural selection that may have occurred as flies encountered ethanol-containing environments. In accordance with observations from natural populations, raising fly strains for many generations on ethanol-containing food typically increases their resistance to ethanol toxicity (Barbancho et al. 1987, Kerver et al. 1992, Van Herrewege

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& David 1980). One study additionally observed increased oviposition preference for ethanol in the ethanol-selected lines (van Delden & Kamping 1990). In some studies, the ethanol-selected lines showed an increase in Adh^F frequency (Cavener & Clegg 1981, van Delden et al. 1978), but other studies using lines homozygous for Adh^S or Adh^F saw no effect of selection on ADH activity (Barbancho et al. 1987, Kerver et al. 1992). Finally, a behavioral selection study selecting for greater resistance to the motor-impairing effect of ethanol also generated lines that were more resistant to ethanol toxicity, which suggests that ethanol-induced toxicity and motor impairment are mediated by overlapping sets of genes (Cohan & Hoffmann 1986).

Thus, *Drosophila*'s colonization of ethanol-containing environments is linked to the evolution of greater resistance to ethanol toxicity, allowing flies to derive multiple benefits from ethanol ingestion. Some investigators have hypothesized that the evolutionary origins of human alcoholism derive from a strong historical association between primate frugivory and ethanol consumption. Specifically, if ethanol consumption during frugivory was advantageous for our ancestors, then alcoholism could be viewed as a maladaptive consequence of modern environments in which alcohol is readily available (Dudley 2000, 2002). Given this potential shared history of ethanol consumption in flies and humans, it is interesting that many of the effects observed in flies upon acute and chronic ethanol exposure in the laboratory are remarkably similar to those observed in vertebrates, including humans. The remainder of the review focuses on these similarities and the efforts to develop *Drosophila* as a model for alcohol addiction.

DEVELOPMENTAL ETHANOL EXPOSURE

Exposure to ethanol during developmental stages of the *Drosophila* life cycle leads to profound effects on development and adult behavior. Early studies showed that rearing larvae on food containing 4.5% ethanol delays development and decreases survival compared with larvae reared on control food (Geer et al. 1991, Ranganathan et al. 1987). However, the surviving adults were heavier than those grown on regular food (Geer et al. 1991), which may be due to less crowded growth conditions or utilization of ethanol as a food source. A more recent study, in which flies were exposed to at least 5% ethanol throughout embryonic, larval, and pupal development, also reported delayed development and decreased survival (McClure et al. 2011). The developmental delay was due to ethanol exposure during the larval stages (a period of extensive growth), whereas the decreased survival was due to exposure during both the larval stages as well as metamorphosis, a period of complex structural and neural remodeling. In this study, flies reared continuously on food containing 5% ethanol (or higher) were smaller, showed decreased adult weight, and had reduced larval brain size owing to decreased cell proliferation (McClure et al. 2011). The conflicting findings regarding the effect of developmental ethanol on adult weight may be explained by differences in the ethanol exposure protocol: Both Geer et al. (1991) and McClure et al. (2011) fed ethanol-containing food to larvae, but only the latter study additionally maintained the animals in an environment containing ethanol vapor, thereby avoiding ethanol evaporation from the food (which otherwise occurs within a few days) and ensuring ethanol exposure during metamorphosis, when flies do not feed.

How do we reconcile the negative consequences of developmental ethanol exposure in the laboratory with the fact that, in nature, flies frequently reside in ethanol-containing environments? First, the ethanol concentrations used in laboratory experiments may be higher than those most commonly occurring in nature (Gibson et al. 1981). Second, in their natural environment larvae can move away from highly concentrated ethanol sources and may thus experience only intermittent ethanol exposure.

Developmental ethanol exposure also affects behavioral responses to ethanol in adult flies.

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In adult flies, acute exposure to ethanol vapor causes locomotor hyperactivity (observed at low to moderate doses) and sedation (observed at higher doses; see below for detailed description of the assays). Flies reared on 5% ethanol show greater ethanol-induced locomotor hyperactivity but reduced sensitivity to ethanol sedation compared with unexposed control flies, even though ethanol-reared flies showed normal ethanol pharmacokinetics (McClure et al. 2011). The decrease in sedation sensitivity may represent a long-lasting form of tolerance (acquired resistance to ethanol caused by prior exposure; see below). In support of this model, McClure et al. (2011) showed that when ethanol-reared flies were tested for their ability to develop further tolerance after exposure to ethanol vapor during adulthood, they showed reduced tolerance compared with control flies that had been reared on normal food. These data are consistent with the idea that developmental ethanol exposure induces a form of tolerance and that the mechanisms underlying developmental ethanol tolerance and adult tolerance likely overlap.

The phenotypes of ethanol-reared flies, such as developmental delay and reduced size, are similar to phenotypes observed upon impairing insulin/insulin-like growth factor (IGF) signaling in flies. A potential connection between *Drosophila* insulin-like peptides (dILPs) and developmental ethanol exposure was therefore examined. Indeed, developmental ethanol exposure leads to reduced expression of both dILPs and the insulin/IGF receptor (InR), and mutants with reduced expression of InR are more strongly affected by developmental ethanol exposure than are wild-type flies. Moreover, the behavioral abnormalities observed in ethanol-reared flies can be reversed by increasing expression of one dILP (dILP2) in the brain (McClure et al. 2011).

Prenatal alcohol exposure in humans can cause fetal alcohol syndrome (FAS), a complex disorder characterized by multiple developmental, morphological, and neurological deficits (Jones & Smith 1973). The fly model recapitulates many FAS-related phenotypes observed in humans and rodent models, including persistent growth deficits, deficient brain growth, and altered responses to ethanol as adults (Becker et al. 1993, 1996). Perhaps most intriguing is the evolutionarily conserved effect of insulin/IGF: Developmental ethanol exposure inhibits the expression of insulin and IGF receptors in the rodent brain, and IGFs reduce ethanol toxicity in cultured neurons as well as motor coordination defects caused by developmental ethanol exposure in rats (Barclay et al. 2005, de la Monte et al. 2005, McGough et al. 2009). Thus, an unbiased genetic and molecular analysis of developmental ethanol effects in *Drosophila* should shed light on the mechanisms underlying the teratogenic effects of developmental ethanol exposure in mammals, including humans.

BEHAVIORAL EFFECTS OF ETHANOL EXPOSURE

Intoxication

Behavioral studies of acute ethanol intoxication in *Drosophila* began in the late 1980s. The inebriometer, a vertical column containing mesh baffles through which ethanol vapor is circulated, was the first apparatus used to measure ethanol-induced motor impairment, primarily in the context of selective breeding experiments (Cohan & Graf 1985, Weber 1988). Flies naturally exhibit negative geotaxis and remain at the top of the column, but ethanol (which they inhale) causes them to lose postural control and gradually fall from one baffle to the next. Ethanol sensitivity can therefore be quantified as the time required for flies to reach the bottom of the column. Another early assay for ethanol sensitivity was the line-crossing assay, in which flies were exposed to ethanol vapor in a chamber containing a grid, and locomotor activity was quantified by recording the number and pattern of gridlines crossed as a function of time (Singh & Heberlein 2000). This assay revealed that in contrast with the motor impairment induced by high doses of ethanol, lower concentrations

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of ethanol stimulate locomotor activity and cause flies to turn more frequently. Upon flies' continued ethanol exposure, their locomotor activity decreases as flies show motor incoordination and eventually sedation. Thus, even these early studies indicated that flies exhibit a biphasic response to acute ethanol exposure, similar to that in humans and other mammals (Pohorecky 1977): At lower concentrations, ethanol acts as a stimulant, inducing greater locomotor activity, whereas at higher concentrations, ethanol acts as a depressant, causing motor impairment and sedation.

Investigators have subsequently developed more sophisticated automated systems to measure the effects of ethanol on locomotor activity (Parr et al. 2001, Wolf et al. 2002). For example, one method relies on filming the flies during ethanol exposure and using image-processing software to calculate their locomotor velocity (Wolf et al. 2002). This approach enabled precise quantification of the kinetics of ethanol-induced hyperactivity and revealed that ethanol increases the length, but not the frequency, of activity bouts to produce an overall increase in activity (Wolf et al. 2002). Several groups of flies can be tested and analyzed simultaneously using these automated methods, thereby making it possible to conduct forward genetic screens to identify molecules that regulate ethanol responses (Kaun et al. 2012, Rodan & Rothenfluh 2010).

New assays to quantify the sedative phase of ethanol intoxication have also been developed. The ethanol rapid iterative negative geotaxis (eRING) assay measures the loss of negative geotaxis during ethanol exposure. In this assay, the experimenter knocks the flies to the bottom of a vial and then measures the vertical distance that they climb (Bhandari et al. 2009). The loss-of-righting reflex (LORR) assay measures ethanol-induced sedation (Corl et al. 2009, Rothenfluh et al. 2006). To quantify LORR, the experimenter knocks over the flies by mechanically agitating the horizontally positioned vials and then counts the fraction of flies that are unable to regain upright posture. Flies' recovery from ethanol sedation has also been used as a measure of ethanol sensitivity (Berger et al. 2004). The eRING, LORR, and sedation recovery assays all have the advantage that they generally require a smaller number of flies per experiment than does the inebriometer. These assays may also be more specific in the behavioral effect that they measure because sensitivity in the inebriometer reflects a combination of several factors, including propensity for negative geotaxis, loss of postural control, and ability to cling to the baffles. Overall, these assays have demonstrated that acute ethanol exposure produces specific behavioral alterations in *Drosophila*, including both stimulant and depressant effects, that bear strong resemblance to those observed in mammals, including humans.

The relationship between the stimulant and depressant effects of ethanol has been analyzed in flies by measuring both types of responses in various sets of mutants. One study observed a small correlation between ethanol-induced hyperactivity and sedation (Kong et al. 2010). However, in this study the two measures were not entirely independent because ethanol hyperactivity was quantified as the total distance traveled during ethanol exposure, including the end of the assay when many flies are sedated. In contrast, a different study quantified peak hyperactivity during exposure to a nonsedating concentration of ethanol and found no significant correlation between ethanol hyperactivity and sedation (Devineni et al. 2011). These results suggest that ethanol-induced hyperactivity and sedation reflect ethanol's distinct effects on the nervous system and may be largely influenced by different genes.

Acute ethanol responses in larvae have also been studied, although not as extensively as in adults. Low doses of ethanol (producing an internal ethanol concentration of ~7 mM) impair associative olfactory learning in larvae without affecting general olfactory or locomotor behavior (Robinson et al. 2012b). This effect may model the ethanol-induced impairment observed in humans and rodents on various short-term memory tasks (Acheson et al. 1998, Gibson 1985, Givens & McMahon 1997, Lister et al. 1991). It remains to be

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investigated whether higher ethanol levels also produce stimulant and/or sedative effects in larvae, as they do in adult flies.

Tolerance, Sensitization, and Withdrawal

Ethanol tolerance is defined as acquired resistance to the behavioral effects of ethanol due to repeated ethanol exposure. Tolerance to the sedative effects of ethanol is generally quantified as the decrease in ethanol sensitivity after ethanol preexposure and can be measured using any of the ethanol sensitivity assays described above (Berger et al. 2004, Bhandari et al. 2009, Scholz et al. 2000). Although tolerance studies often vary in the timing and concentration of ethanol exposure, two broad classes of ethanol tolerance have been characterized in flies, termed rapid and chronic (Berger et al. 2004). Rapid tolerance is induced by relatively brief exposure (~30 min) to a high, sedating concentration of ethanol, whereas chronic tolerance is induced by a longer exposure (~24 h) to a low ethanol concentration that does not produce overt signs of intoxication. These two types of tolerance are mediated by partially distinct molecular mechanisms (Berger et al. 2004), although some genes appear to affect both (Berger et al. 2008). A third type of tolerance present in mammals, acute functional tolerance, develops within a single intoxicating session (Kalant et al. 1971). This form of tolerance has not yet been characterized in flies because it is difficult to distinguish from naive ethanol sensitivity using existing assays.

Most studies examining the effect of multiple ethanol exposures have focused on the sedative effects of ethanol. However, one study tested the effect of repeated ethanol exposure on ethanol hyperactivity. Flies that had been previously exposed to ethanol showed an earlier onset and overall increase in ethanol hyperactivity compared with naive flies (Kong et al. 2010). Thus, flies appear to show sensitization (increased response after preexposure) to the stimulant effect of ethanol, which contrasts with the tolerance that they show to its sedative effects. These findings closely match observations in rodents, which also tend to show sensitization to ethanol's stimulant effects and tolerance to its sedative effects (Crabbe et al. 1994, Phillips et al. 1997).

Ethanol withdrawal, like tolerance and sensitization, reflects long-term neural changes that occur in response to ethanol exposure. In humans, the presence of withdrawal symptoms upon alcohol abstinence, which can include dysphoria, anxiety, cognitive impairment, and seizures, is considered a sign of alcohol dependence (Kosten & O'Connor 2003, Stavro et al. 2012). Withdrawal seizures occur because the depressant effects of ethanol in the central nervous system induce homeostatic adaptations promoting neural excitability, which can lead to seizures when ethanol is removed (Littleton 1998). A recent study described a model for ethanol withdrawal seizures in flies (Ghezzi et al. 2012). In this study, seizure susceptibility was measured in an electrophysiological preparation by electrically stimulating the brain and recording seizure-like activity in a flight muscle. The stimulation threshold for inducing seizures was lowered in flies that were preexposed to a sedating dose of ethanol. The mechanisms underlying withdrawal and tolerance in flies at least partially overlap because both processes rely on the large conductance (BK) calcium-activated potassium channel encoded by the gene *slo* (Cowmeadow et al. 2005, Ghezzi et al. 2012).

In addition to the physiological model of ethanol withdrawal described above, a behavioral model for withdrawal has been recently characterized in *Drosophila* larvae (Robinson et al. 2012a). This study first showed that although acute ethanol exposure impairs associative learning in larvae, larvae that were chronically exposed to ethanol for six days showed normal learning, likely owing to neural adaptations related to tolerance. However, chronically exposed larvae that were removed from ethanol for 6 h showed reduced learning, representing a withdrawal-induced learning deficit, and this effect was reversed upon reexposure to ethanol for 1 h. Ethanol-withdrawn larvae also showed

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enhanced nervous system excitability, similar to the findings in adult flies: Ethanol-withdrawn larvae were more sensitive than control larvae to seizures induced by picrotoxin, a GABA receptor antagonist that increases neural excitability, and this effect was partially reversed by ethanol reinstatement. Thus, flies have now been established as a model for both physiological and behavioral signs of withdrawal.

In summary, *Drosophila* shows both acute and chronic behavioral responses to ethanol that are similar to those observed in rodents as well as in humans. During acute ethanol exposure, adult flies exhibit locomotor hyperactivity followed by motor incoordination and sedation, whereas larvae show learning deficits. Repeated ethanol exposures can induce either tolerance or sensitization in adult flies, depending on the behavioral response being measured, and chronic ethanol exposure induces alcohol dependence in larvae, which show withdrawal signs when ethanol is removed. All these behaviors are amenable to genetic analyses to help decipher the underlying molecular and neural mechanisms.

ETHANOL SELF-ADMINISTRATION AND REWARD

Ethanol Consumption

As discussed above, initial studies investigated larval positional and adult ovipositional preference for ethanol, but they did not assess whether flies choose to consume ethanol. Assays to measure ethanol consumption (or feeding in general) were fairly limited until recently. One early study measured the duration of proboscis (the feeding organ of the fly) extension on ethanol-containing food as a proxy for ethanol intake (Cadieu et al. 1999) because this action is necessary for food consumption. When given a choice between ethanol-containing food and regular food, naive flies showed a slight preference for the ethanol-containing food, and ethanol preexposure increased this preference.

The development of the capillary feeder (CAFE) assay has allowed for more precise quantification of ethanol consumption (Ja et al. 2007). In this assay, flies consume liquid food from microcapillary tubes placed vertically through the top of a vial, and the volume consumed is quantified by measuring the descent of the liquid column. When flies are offered a choice between food with or without ethanol, they show a robust, dose-dependent preference for food containing 5–25% ethanol, and this preference increases over several days (Devineni & Heberlein 2009). The increase in ethanol preference over time may represent a positional learning effect because altering the position of the ethanol-containing capillary on a daily basis resulted in a constant, rather than increasing, level of preference (Xu et al. 2012). Preference for consuming ethanol is not dependent entirely on chemosensory attraction to ethanol because it persists in flies with olfactory or gustatory defects (Devineni & Heberlein 2009, Xu et al. 2012).

Role of Calories in Ethanol Preference

The question of whether flies consume ethanol for its caloric value or its pharmacological properties is a topic of ongoing debate. Although the calories in ethanol may be poorly utilized by flies, as discussed above, the caloric value of ethanol may nevertheless represent an attractive stimulus. Initial studies suggested that preference for consuming ethanol was not strongly dependent on its caloric value because it was not significantly affected by varying the caloric ratio between the ethanol and nonethanol food (Devineni & Heberlein 2009). In support of this idea, ethanol preference can be dissociated from general food consumption, which likely reflects attraction to calories. For example, (*a*) sexual experience modulates ethanol preference (see below) without affecting general food consumption (Shohat-Ophir et al. 2012), and (*b*) mutation of the gene *rutabaga* decreased both general feeding and ethanol preference; however, restoring *rutabaga* function in adult neurons reinstated normal ethanol preference but not normal overall feeding (Xu et al. 2012). Thus, manipulations that alter attraction

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to calories do not necessarily affect ethanol preference, and vice versa.

The role of calories in ethanol preference was more directly tested by using a variety of nutritive carbohydrates to equalize the caloric content of the ethanol-containing and nonethanol food (Pohl et al. 2012). Of several carbohydrates tested, three were believed to have neither an attractive nor aversive taste based on the proboscis extension assay. Equalizing the caloric content of food with and without ethanol by using two of these three carbohydrates caused flies to lose their preference for ethanol; in the third case, flies showed relatively normal ethanol preference. These results suggest that the caloric value of ethanol likely represents a significant attractive force driving ethanol consumption in flies but that other factors may also contribute. One caveat of these experiments is that the published caloric values of each compound are estimated for humans, not flies, so the number of calories available to a fly is not precisely known. In fact, if flies cannot utilize all the calories in ethanol, as discussed above, then these caloric-balancing experiments do not actually equalize the number of utilizable calories but instead add excess calories to the nonethanol food. Overall, calories likely contribute to ethanol preference in *Drosophila*, but their relative importance has yet to be conclusively determined.

Role of Hedonic Effects in Ethanol Preference

In addition to the caloric value of ethanol, the hedonic effects of ethanol likely contribute to ethanol consumption and preference. In support of this notion, flies will consume ethanol and develop ethanol preference even when it is laced with the aversive, bitter-tasting compound quinine (Devineni & Heberlein 2009). In addition, flies rapidly return to peak levels of ethanol consumption after a period of ethanol abstinence, suggesting a relapse-like effect (Devineni & Heberlein 2009). These properties are at least outwardly reminiscent of addiction-like behavior, as both humans and rodent models consume ethanol despite its initially aversive taste and other negative consequences, and they rapidly return to high levels of ethanol consumption after a period of abstinence (Morse & Flavin 1992, Rodd et al. 2004, Wolffgramm & Heyne 1991).

Some have argued that because flies contain a low internal ethanol concentration after each drinking bout ($\leq$4 mM), they are not likely to reach intoxication during the ethanol preference assay, and the pharmacological effects of ethanol are therefore unlikely to contribute to ethanol consumption (Pohl et al. 2012). However, ethanol concentration in flies may reach higher levels after a cluster of multiple drinking bouts, which is important because the frequency of drinking bouts increases over time (Devineni & Heberlein 2009). Even if this is not the case, low internal ethanol levels (in the range of 5 mM) may still have behavioral relevance in flies; they can induce locomotor hyperactivity (Kaun et al. 2011), tolerance (Berger et al. 2004), and conditioned preference (see below). Further experiments will be required to determine whether the ethanol consumed during the preference assay induces either immediate or long-term behavioral changes related to its intoxicating or hedonic effects.

Conditioned Ethanol Preference

A recently developed assay for conditioned ethanol preference directly addresses the question of whether flies find intoxicating doses of ethanol to be rewarding or aversive (Kaun et al. 2011). Similar to conditioned place preference in mammals, in this assay flies are exposed to two neutral odor cues, one of which is paired with ethanol vapor exposure. Flies are later offered a choice between the two odors. Preference for the ethanol-associated odor is thought to represent the rewarding effect of ethanol.

At early time points following ethanol exposure (~30 min), flies show aversion to the ethanol-associated cue; however, within

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24 h this aversion transforms into long-lasting preference (Kaun et al. 2011). Flies show conditioned preference when exposed to moderate concentrations of ethanol that induce locomotor hyperactivity (Kaun et al. 2011), but not when exposed to lower concentrations that fail to produce behavioral effects or higher concentrations that induce sedation (K. Kaun and U. Heberlein, unpublished data). Flies therefore form a positive association with the stimulant effects of ethanol but not with its sedative effects (although it is possible that sedation impairs flies' ability to remember the association). These findings bear resemblance to humans: The stimulant effects of ethanol are typically perceived as positive, whereas its sedative effects are perceived negatively (Babor et al. 1983). These results are also supported by an earlier finding showing that although ethanol preexposure increases subsequent attraction to ethanol-containing food, ethanol preexposure along with an ADH inhibitor instead induces aversion (Cadieu et al. 1999). Thus, the high internal levels of ethanol that accumulate in the absence of ADH function may be aversive to flies, whereas flies form a positive association with lower internal ethanol levels. In the conditioned preference assay, some flies will endure electric shock to attain the ethanol-associated cue, representing a strong attraction to ethanol reminiscent of addiction-like behavior.

In summary, flies show not only strong attraction to ethanol in multiple types of assays, but also some traits associated with addictive behavior. Flies prefer to consume ethanol-containing solutions, they return to peak levels of ethanol consumption after a period of abstinence, and they form a positive association with an intoxicating dose of ethanol. Furthermore, flies continue to show both ethanol consumption and conditioned ethanol preference in the presence of aversive cues (quinine or electric shock, respectively). Taken together with the recent results describing ethanol withdrawal in flies (see above), *Drosophila* has now become a fairly comprehensive model to study multiple facets of alcohol addiction.

ETHANOL AND SOCIAL BEHAVIOR

For the remainder of this review, we focus on an emerging area of study in *Drosophila*: the relationship between ethanol and social behavior. Recent studies have shown not only that ethanol can alter social behavior, such as courtship, but also that social experience can affect ethanol responses.

One study examined the acute and long-term effects of ethanol on one type of social behavior, courtship, which males typically display toward virgin females but not toward other males (Lee et al. 2008). When males are exposed to an intoxicating dose of ethanol for the first time, they rarely court either females or other males while intoxicated. However, repeated daily ethanol exposures altered their behavior dramatically: Males that had been previously exposed to ethanol showed a strong increase in courtship toward both females and other males while intoxicated. This increase is not likely explained by tolerance to the motor-impairing effects of ethanol, which developed from the first to second ethanol exposure and was not enhanced by subsequent exposures, whereas the level of male-male courtship continued to increase from the second to the fourth ethanol exposure. In addition, the effect of ethanol preexposure on male-male courtship could not be explained by an increase in the attractiveness of ethanol-exposed males because preexposed males also courted ethanol-naive males. Ethanol-preexposed males did not show male-male courtship in the absence of ethanol. Thus, although an acute effect of ethanol is necessary to stimulate male-male courtship, it does so only in males that have been repeatedly exposed to ethanol and have likely experienced some form of molecular or neural plasticity. The mechanisms underlying this effect are largely unknown, although both dopamine and the sex-determination gene *fruitless* seem to be required (Lee et al. 2008).

Whereas this courtship study characterized the effect of ethanol on social behavior, two other studies have demonstrated an effect of

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social experience on ethanol responses. The first study examined the effect of adult social isolation on sensitivity to ethanol sedation. Male flies that were isolated for 6 days showed a strong decrease in ethanol sensitivity compared with males that were housed in groups of 20 (Eddison et al. 2011). Adult isolation was previously shown to reduce the number of synapses of a subset of brain neurons (Donlea et al. 2009), suggesting that decreased synapse number may account for decreased ethanol sensitivity observed in isolated flies. In support of this model, the mutant *arouser* shows both increased ethanol sensitivity and increased synapse number, and adult isolation rescues both of these phenotypes (Eddison et al. 2011). Evidence from rodent models also indicates that social isolation reduces sensitivity to ethanol sedation (Jones et al. 1990). In addition, isolation increases ethanol consumption and operant responding for ethanol in rodents (McCool & Chappell 2009, Sanna et al. 2011). Social isolation is thought to represent a form of stress in rodents and induces many behavioral deficits (Fone & Porkess 2008). It will be interesting to determine whether isolation is also a stressor in flies and whether other social stressors may similarly affect ethanol sensitivity.

A second study examined the relationship between social experience and ethanol consumption (Shohat-Ophir et al. 2012). In this study, the sexual experience of male flies was varied, taking advantage of the fact that virgin females will readily mate with males, but previously mated females will actively reject the courtship advances of males. This study found that mated males show decreased ethanol consumption and preference compared with rejected males. This effect is due primarily to successful mating, rather than the experience of rejection, because mated males also showed lower ethanol preference than did naive males that had not interacted with females. In addition, mated males showed lower ethanol preference than did males allowed to interact with decapitated virgin females, which retain chemosensory cues typical of normal virgin females and elicit male courtship but not copulation. Successful mating therefore appears to be the key factor in reducing ethanol preference.

Several lines of evidence suggest that the effect of sexual experience on ethanol preference is mediated by neuropeptide F (NPF), the fly homolog of neuropeptide Y (NPY) in mammals (Shohat-Ophir et al. 2012). First, NPF levels in males are increased by mating and reduced by rejection. Second, downregulation of NPF signaling increases ethanol preference in mated males, mimicking the effect of rejection. Third, activation of NPF neurons to stimulate NPF release decreases ethanol preference in virgin males, mimicking the effect of mating. 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.

One possible explanation for the relationship between sexual experience, ethanol consumption, and NPF is a reward homeostat model (Shohat-Ophir et al. 2012). 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 (such as increased ethanol consumption), and vice versa. In support of this model, the conditioned preference assay demonstrates that both mating and ethanol intoxication are perceived as rewarding, as is artificial activation of the NPF pathway (Kaun et al. 2011, Shohat-Ophir et al. 2012). In rodents, NPY regulates ethanol intake (Thiele et al. 1998) but has not been shown to link social experience to ethanol consumption, so it will be interesting to determine whether this function is conserved from flies to mammals.

CONCLUSIONS

Drosophila and its ancestors have reproduced and grown on ethanol-containing substrates for many millions of years (Ashburner 1998, Dudley 2002). Flies have evolved mechanisms to metabolize ethanol for the purposes of both

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detoxification and nutrition (although it is uncertain exactly how many of the available calories in ethanol flies utilize), and flies likely use their ability to smell ethanol to locate ripe fruit in the wild. It is therefore not surprising that in a laboratory setting flies develop tolerance when chronically exposed to low levels of ethanol, show preference for ethanol-containing food, and find exposure to low levels of ethanol to be rewarding. What at first glance appears surprising, however, is the observation that behaviors induced by exposure to moderate-to-high ethanol doses (which flies would never have encountered in their natural habitat) are remarkably similar in flies and humans. As mentioned in this review, with increasing ethanol doses flies become hyperactive, no longer walk in straight lines, display incoordination and loss of postural control, and eventually succumb to sedation. Because the majority of the known targets of ethanol are present in flies (Heberlein et al. 2004), these similarities are perhaps not that surprising after all; the molecular and cellular actions of ethanol and the ensuing physiological changes may in fact be the same, whether in a fly, rodent, or human. We would argue, then, that using the powerful behavioral and molecular genetic approaches that are feasible in *Drosophila* should continue to provide novel insights into the mechanisms by which ethanol affects behavior. In particular, investigators can exploit recently developed assays that model aspects of addiction, such as withdrawal, voluntary consumption, relapse, and use despite adverse consequences, although more laborious than the simpler assays for acute intoxication, to reveal the underlying molecular mechanisms.

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